Nanosized Selenium: A Novel Platform Technology to Prevent Bacterial Infections

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ABSTRACT

As an important category of bacterial infections, healthcare-associated infections (HAIs) are considered as an increasing threat to the safety and health of patients worldwide. HAIs lead to extended hospital stays, contribute to increased medical costs, and are a significant cause of morbidity and mortality. In the United States, infections encountered in the hospital or a health care facility affect more than 1.7 million patients, cost $35.7 billion to $45 billion, and contribute to 88,000 deaths in hospitals annually.

The most conventional and widely accepted method to fight against bacterial infections is using antibiotics. However, because of the widespread and sometimes inappropriate use of antibiotics, many strains of bacteria have rapidly developed antibiotic resistance. Those new, stronger bacteria pose serious, worldwide threats to public health and welfare. In 2014, the World Health Organization (WHO) reported antibiotic resistance as a global serious threat that is no longer a prediction for the future and it has the potential to affect anyone, of any age, in any country.

The most effective strategy to prevent antibiotic resistance is
minimizing the use of antibiotics. In recent years, nanomaterials have been investigated as one of the potential substitutes of antibiotics. As a result of their vastly increased ratio of surface area to volume, nanomaterials will likely exert a stronger interaction with bacteria which may affect bacterial growth and propagation. A major concern of most existing antibacterial nanomaterials, like silver nanoparticles, is their potential toxicity. But selenium is a non-metallic material and a required nutrition for the human body, which is recommended by the FDA at a 53 to 60 µg daily intake. Nanosized selenium is considered to be healthier and less toxic compared with many metal-based nanomaterials due to the generation of reactive oxygen species from metals, especially heavy metals.

Therefore, the objectives of this dissertation were to synthesize selenium nanoparticles, characterize nanosized selenium coatings on various materials, test the effectiveness of selenium coated materials at inhibiting bacteria growth and biofilm formation and investigate the mechanisms of how selenium nanoparticles inhibit bacteria growth. The nanosized selenium coated materials showed significant and continuous inhibitions to bacteria growth by up to 92.5% without using any
antibiotics. The work performed in this dissertation presents a novel platform technology based on nanosized selenium to inhibit bacterial infections on various materials, which demonstrates the strong potential applications of nanosized selenium as an antibacterial agent in hospital environments and healthcare settings.
VITA

Qi Wang was born on September 24, 1988 in Loudi, Hunan Province, a newly developing city in the central south of China. Before coming to the United States for graduate studies, he received his Bachelor’s degree in Chemical Physics at the University of Science and Technology of China (USTC) in 2010. In his first two years in United States, he completed his Master of Science degree in Chemistry at Brown University. Then and now he has been working on nanomaterials, especially selenium nanoparticles, for antibacterial applications in Prof. Thomas Webster's Nanomedicine Lab. He has published 5 journal papers, 1 book chapter and more than 6 conference proceedings as first author. He has presented his research at over 20 national and international conferences. He has also mentored 5 undergraduates and master students in their research projects. His research is now being commercialized by a company called Senstruct. After finish his PhD, he plans to work as a professional engineer in the area of biomedical research and development.
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# TABLE OF CONTENTS

Abstract ....................................................................................................................... ii

Vita .............................................................................................................................. v

Acknowledgements ..................................................................................................... vi

Table of Contents ........................................................................................................ viii

List of Tables ............................................................................................................... xiv

List of Illustrations ..................................................................................................... xv

Chapter 1

**Introduction** ......................................................................................................... 1

1.1 Bacterial and Biofilm Infections ........................................................................... 1

1.2 Antibacterial and Anti-infection Strategies ......................................................... 5

1.3 Nanomaterials as a Novel Antibacterial Approach ............................................ 12

1.4 Selenium and Nanosized Selenium .................................................................. 16

1.5 Objectives ............................................................................................................ 20

1.6 References .......................................................................................................... 21

Chapter 2

**Selenium Nanoparticle Coatings for Preventing Biofilm Formation**
on Polycarbonate Medical Devices.....................................................38
  2.1 Introduction..................................................................................38
  2.2 Materials and Methods.................................................................42
  2.3 Results..........................................................................................46
    2.3.1 SEM Images, AFM Images and AAS Results.........................46
    2.3.2 Bacterial Assays.................................................................51
  2.4 Discussions..................................................................................56
  2.5 Conclusions..................................................................................58
  2.6 References..................................................................................59

Chapter 3

Inhibition of Various Gram-positive and Gram-negative Bacteria

Growth on Paper Towels by Selenium Nanoparticles.........................64
  3.1 Introduction..................................................................................64
  3.2 Materials and Methods.................................................................70
    3.2.1 Synthesis of Selenium Nanoparticle Coatings.......................70
    3.2.2 Characterization of Coated Paper Towels...............................72
    3.2.3 Mechanical Tests.....................................................................73
    3.2.4 Bacterial Inhibitory Tests.......................................................74
    3.2.5 Mechanism of Action: Protein Adsorption Assays..............76
3.2.6 Release of Selenium...............................................................77
3.2.7 Statistics..............................................................................78
3.3 Results and Discussion.............................................................79
  3.3.1 Synthesis and Characterization of Selenium Nanoparticles.................................79
  3.3.2 Mechanical Tests....................................................................84
  3.3.3 Bacterial Inhibitory Tests.........................................................86
  3.3.4 Mechanism of Action: Protein Adsorption Assays......................94
  3.3.5 Release of Selenium.................................................................95
3.4 Conclusions...............................................................................97
3.5 References...............................................................................98

Chapter 4

Red Selenium Nanoparticles and Gray Selenium Nanorods as Antibacterial Agents for PEEK Medical Devices.................................106
  4.1 Introduction..............................................................................106
  4.2 Materials and Methods.............................................................112
    4.2.1 Preparation of Selenium Coated PEEK..............................112
    4.2.2 SEM and EDS of PEEK with Selenium Coatings...............114
    4.2.3 Contact Angle Measurements.............................................115
4.2.4 Bacterial Inhibitory Tests..................................................116
4.2.5 Statistics.............................................................................118
4.3 Results and Discussion............................................................119
  4.3.1 PEEK Coated with Selenium Nanoparticles.......................119
  4.3.2 SEM and EDS of PEEK with Selenium Coatings..............120
  4.3.3 Contact Angles................................................................125
  4.4.4 Bacterial Inhibitory Tests..................................................127
4.4 Conclusions............................................................................131
4.5 References.............................................................................132

Chapter 5

Antibacterial Selenium Quantum Dots and Nanoparticles Produced
by UV/VIS/NIR Pulsed Nanosecond Laser Ablation.........................138

  5.1 Introduction............................................................................138
  5.2 Experiments and Results.......................................................140
    5.2.1 Preparation of Selenium Nanoparticles by Ablation...140
    5.2.2 Characterization of Selenium Nanoparticles.................142
    5.2.3 Generation of Selenium Quantum Dots.........................150
    5.2.4 Antibacterial Tests........................................................153
  5.3 Conclusions............................................................................155
Chapter 6

Inhibited Growth of *Pseudomonas aeruginosa* by Dextran- and Polyacrylic Acid-coated Ceria Nanoparticles

6.1 Introduction

6.2 Materials and Methods

6.2.1 Synthesis and Characterization of Ceria Nanoparticles

6.2.2 Bacterial Assays

6.2.3 Statistics

6.3 Results

6.3.1 Characterization of Ceria Nanoparticles

6.3.2 Bacterial Assays

6.4 Conclusions

6.5 References

Chapter 7

Investigation of Antibacterial Properties of ZTA and Silicon Nitride Implants Compared with Titanium

7.1 Introduction
7.2 Materials and Methods.................................................................184

7.2.1 Materials Characterization....................................................184

7.2.2 Bacterial Assays.................................................................185

7.3 Results.......................................................................................187

7.3.1 Materials Characterization....................................................187

7.3.2 Bacterial Assays.................................................................189

7.4 Conclusions..............................................................................193

7.5 References................................................................................193

Chapter 8

Conclusions and Future Outlook...................................................199

Appendix..........................................................................................204
LIST OF TABLES

Table 2-1. Amount of selenium coated on polycarbonate surfaces determined using AAS.................................................................48

Table 2-2. Difference in percentage of viable bacteria numbers on nanosized selenium coated polycarbonate compared with control groups (uncoated polycarbonate)..................................................52

Table 2-3. Log difference of viable bacteria numbers on nanosized selenium coated polycarbonate compared with control groups (uncoated polycarbonate) .................................................................53

Table 3-1. Maximum load (the load at the moment of complete breakage) of paper towels in tension tests.................................................86

Table 3-2. The release of selenium from the selenium coated paper towels......................................................................................97

Table 4-1. Contact angles on three types of PEEK samples coated with and without selenium.................................................................127

Table 5-1. Summary of the laser parameters used, the corresponding size and zeta potential of the selenium nanoparticles obtained...........147
LIST OF FIGURES

Figure 1-1. The biofilm life cycle.................................................................4
Figure 1-2. Common sites of biofilm infection...........................................5
Figure 1-3. Mean frequency of healthcare worker contact for 28 surfaces in an intensive care unit.................................................................9
Figure 1-4. Mechanisms of nano-scaled silver interacting with bacteria.14
Figure 1-5. Major mechanistic injury responses induced by common nanomaterials.................................................................16
Figure 1-6. Black/gray, glassy amorphous and red amorphous selenium.................................................................17
Figure 2-1. SEM images of selenium coated polycarbonate samples before and after a tape test.................................................................48
Figure 2-2. AFM images and RMS roughness of polycarbonate surface.........................................................................................50
Figure 2-3. Bacteria (S. aureus) growth on the surface of polycarbonate. Polycarbonate samples were treated with bacteria in 0.03% TSB (Tryptic Soy Broth) and were incubated for 24, 48 or 72 hours……52
Figure 2-4. S. aureus densities on various amounts of coated selenium on
the surface of polycarbonate.................................................................54

Figure 3-1. Light image of selenium coated and uncoated paper towels..............................................................80

Figure 3-2. SEM images of selenium coated (image a) and uncoated paper (image b) towel samples at a magnification of 8.0 K............81

Figure 3-3. Scanning electron microscopy images of paper towels........82

Figure 3-4. AFM images and RMS roughness of paper towel surfaces...83

Figure 3-5. Tension tests for paper towels in various conditions.........85

Figure 3-6. The growth of *Staphylococcus aureus* on the surface of paper towels.................................................................88

Figure 3-7. The growth of *Staphylococcus epidermidis* on the surface of paper towels.................................................................89

Figure 3-8. The growth of *Pseudomonas aeruginosa* on the surface of paper towels.................................................................91

Figure 3-9. The growth of *E. coli* on the surface of paper towels........93

Figure 3-10. The amount of adsorbed total proteins on selenium coated and uncoated paper towels.......................................................95

Figure 4-1. Light images of (a) uncoated PEEK, (b) PEEK coated with selenium nanoparticles (red coated PEEK), and (c) PEEK coated with
selenium nanoparticles and with a heating treatment (gray coated PEEK).

Figure 4-2. Scanning electron microscopy images of: (a) uncoated PEEK, (b) PEEK coated with selenium nanoparticles, (c) PEEK coated with selenium nanoparticles and with a heating treatment.

Figure 4-3. Energy-dispersive X-ray spectroscopic analyses of (a) a spot of selenium nanoparticles on PEEK, (b) a blank area without nanoparticles on PEEK, (c) a spot of selenium nanorods on PEEK, and (d) a blank area without nanorods on PEEK.

Figure 4-4. Contact angle measurements on (a) uncoated PEEK, (b) red selenium nanoparticles coated PEEK, and (c) gray selenium nanorods coated PEEK.

Figure 4-5. The growth of P. aeruginosa biofilm on red selenium coated PEEK, gray selenium coated PEEK and uncoated PEEK.

Figure 5-1. Diagram of generating selenium nanoparticles by ablation.

Figure 5-2. TEM images and diffraction pattern of selenium nanoparticles after the first set of irradiation at 532 nm in de-ionized water, before any centrifugation and any filtration.

Figure 5-3. DLS spectra of the centrifuged and filtrated colloidal solution.
of selenium nanoparticles after the first and second set of irradiations (time=15 min, f=10Hz) in de-ionized water and ethanol

Figure 5-4. TEM images of selenium nanoparticles obtained in a) de-ionized water and b) ethanol.

Figure 5-5. HRTEM images of selenium quantum dots synthesized during the second set of irradiation by a) UV irradiation in de-ionized water, b) visible irradiation in de-ionized water, c) UV irradiation in ethanol and d) visible irradiation in ethanol.

Figure 5-6. The growth of *E. coli* with a treatment of selenium nanoparticles.

Figure 6-1. Characterization of dextran-coated ceria nanoparticles.

Figure 6-2. Inhibited *P. aeruginosa* growth in the presence of ceria nanoparticles at three time points (1, 6, and 24 hours). The volume ratio of bacterial solution and ceria nanoparticles is 10:1.

Figure 6-3. Inhibited *P. aeruginosa* growth in the presence of ceria nanoparticles at three time points (1, 6, and 24 hours). The volume ratio of bacterial solution and ceria nanoparticles is 5:1.

Figure 7-1. SEM images of (a) titanium and (b) as-fired silicon nitride at a magnification of 10k.
Figure 7-2. AFM images and RMS (root mean square, scan area = 10um×10um) roughness of (a) titanium and (b) ZTA..................189

Figure 7-3. The growth of *Pseudomonas aeruginosa* on silicon nitride, ZTA and titanium, measured by the method of colony forming units.................................................................190

Figure 7-4. The growth of *Staphylococcus aureus* on silicon nitride, ZTA and titanium, measured by the method of crystal violet staining....192

Figure A1-1. SEM images of selenium nanoparticle coated isoplast before (a) and after (b) the tape test.................................................................205

Figure A1-2. SEM images of selenium nanoparticle coated PVC (a) and uncoated PVC (b).................................................................205

Figure A1-3. SEM images of selenium nanoparticle coated on the surface of a commercially available keyboard (a) and the uncoated surface of the keyboard (b).................................................................206

Figure A1-4. SEM images of selenium nanoparticle coated on the surface of the back side of a “Windows Surface” tablet (a) and the uncoated surface of the table (b).................................................................206

Figure A1-5. SEM images of selenium nanoparticle coated glass (a) and uncoated glass (b).................................................................207
Figure A1-6. SEM images of stainless steel..........................209

Figure A1-7. SEM images of (a) uncoated polyurethane and (b) selenium coated polyurethane by spray coating.................................210

Figure A1-8. SEM images of selenium nanoparticle coated paper towels after 5 minutes of sonication treatments.................................211

Figure A2-1. SEM images of flat and porous titanium......................212

Figure A2-2. SEM images of flat titanium after being treated with acid..........................................................................................213

Figure A2-3. SEM images of porous titanium after being treated with acid.......................................................................................214
Chapter 1

Introduction

1.1 Bacterial and Biofilm Infections

Bacteria are single-celled prokaryotic microorganisms. There are thousands of different types of bacteria and they present in every conceivable environment all over the earth.¹ Bacteria have a number of shapes, ranging from spheres to rods and spirals. They are broadly classified by their color after a Gram stain is applied. Gram-positive bacteria stain blue, while Gram-negative bacteria stain pink. Gram-positive bacteria possess a thick cell wall containing many layers of peptidoglycan and teichoic acids. Gram-negative bacteria have thinner cell walls but surrounded by a second lipid membrane containing a substance known as lipopolysaccharide (LPS), a highly inflammatory chemical that is responsible for much of the toxicity of Gram-negative bacteria and provokes an immune response in the human body.²

The human body normally contains trillions of individual bacteria, which outnumber the mammalian cells of the body by approximately 10 to 1.³
The vast majority of the bacteria in the human body are harmless and some are beneficial, such as helping break down food in the intestine. Only a few types of bacteria are pathogenic and cause infectious diseases by producing harmful toxins or invading tissues. Examples of bacteria that cause infections include *Streptococcus*, *Pseudomonas*, *Staphylococcus*, and *Escherichia coli*. These bacteria cause bacterial infections such as pneumonia, ear infections, diarrhea, urinary tract infections, skin disorders and so on. Bacterial infections can result in mild to life-threatening illnesses that require immediate intervention or treatment.

As an important category of bacterial infections, healthcare-associated infections (HAIs) are considered an increasing threat to safety and health of patient in the United States. HAIs are infections that patients develop during the course of receiving healthcare treatment for other conditions. HAIs lead to extended hospital stays, contribute to increased medical costs, and are a significant cause of morbidity and mortality. In the United States, infections encountered in the hospital or a health care facility affect more than 1.7 million patients, cost $35.7 billion to $45 billion, and
contribute to 88,000 deaths in hospitals annually.\textsuperscript{5-7} Catheter-associated urinary tract infections are the most common HAIs, followed by surgical site infections, bloodstream infections and pneumonia. Many cases of HAIs result from the contamination of medical devices in the healthcare setting, which have been associated with biofilm growth. It has been reported by the National Institutes of Health that 80\% of all chronic infections are due to biofilms.\textsuperscript{8,9}

A biofilm is an aggregate of bacteria in which bacterial cells adhere to each other on a wet or moist surface. Biofilms may form on living or non-living surfaces and can be prevalent in natural, industrial and hospital settings.\textsuperscript{10,11} The formation of a biofilm (as shown in Figure 1-1) begins with the attachment of free-floating bacteria to the surface. Along with the generation of exopolysaccharide, the attachment of bacteria becomes irreversible. As the bacteria propagate quickly, the biofilm structure develops and becomes more complicated. At the last stage of biofilm growth, the bacteria release into the environment and contaminate other surfaces.
Figure 1-1. The biofilm life cycle. 1: individual cells populate the surface. 2: EPS (exopolysaccharide) is produced and attachment becomes irreversible. 3 & 4: biofilm architecture develops and matures. 5: single cells are released from the biofilm.

Biofilms are considered easy to form but hard to treat, which can cause wide-spread infections in the human body; for example, through catheter infections, infections on inert surfaces of artificial implants and the formation of dental plaques (Figure 1-2). Statistics show that biofilms are involved in an estimated 80% of all infections. These biofilm infections can be serious and hard to treat because the development of the biofilm structure may allow for bacteria to be increasingly antibiotic resistant, because the bacteria in the biofilm is held together and protected by a matrix of EPS (extracellular polymeric...
substance or exopolysaccharide). This matrix protects bacteria cells within it and facilitates communication among them through biochemical signals, resulting in their increased resistance to detergents and antibiotics. In some cases, the effect of antibiotic resistance can be increased a thousand-fold. Thus, bacteria biofilms are frequently related with persistent infections in the human body.

![Image of biofilm infection](image)

**Figure 1-2.** Common sites of biofilm infection. Once bacteria enter the circulatory system, they can spread to any moist surface of the human body.

1.2 **Antibacterial and Anti-infection Strategies**

The most conventional and widely accepted method to fight against bacterial infections is using antibiotics. After their developments in the
1940s, antibiotics offered physicians a powerful tool to fight against bacterial infections which has saved millions of human lives. There are generally two types of antibiotics, bactericidal antibiotics and bacteriostatic antibiotics. Bactericidals kill bacteria while bacteriostatics just prevent bacteria from dividing. They are also classified based on their mechanism of action when targeting bacteria functions or growth processes. For example, penicillins and polymyxins target the bacterial cell wall or cell membrane; lipiarmycins and sulfonamides interfere with essential bacterial enzymes; and lincosamides and macrolides target protein synthesis, which are usually a mechanism for bacteriostatics.\textsuperscript{20,21}

In the recent years, more and more antibiotics have been identified and are being used to treat patients. However, because of the widespread and sometimes inappropriate use of antibiotics, many strains of bacteria have rapidly developed antibiotic resistance.\textsuperscript{22} Antibiotic resistance is developed in bacteria strains through a process of natural selection. New, stronger bacteria not killed by antibiotics pose serious, worldwide threat to public health and welfare. It has also become a significant challenge to researchers. The WHO (World Health Organization) recently reported
antibiotic resistance as a global serious threat that is no longer a prediction for the future but is now reality. It has the potential to affect anyone, of any age, in any country. The most typical example of antibiotic resistance is MRSA (Methicillin-resistance *Staphylococcus aureus*) which means any strain of *Staphylococcus aureus* that is resistant to a group of antibiotics including penicillin, methicillin, dicloxacillin, and oxacillin. Other examples include *Escherichia coli* with resistance to third-generation cephalosporins and to fluoroquinolones, *Enterococcus* with resistance to penicillin and vancomycin, and so on. One of the most important action recommended by the WHO to fight against antibiotic resistant bacteria is preventing infections from happening in the first place, which could be achieved through better hygiene, access to clean water, infection control in health-care facilities, and vaccination to reduce the need for antibiotics. Another approach is to discover potential alternatives to antibiotics, such as bacteriophages. Bacteriophages are tiny viruses that infect bacteria. They can replicate in a bacterium and make enzymes that dissolves the wall of the bacteria, which enables them to leave the bacterium and infect other bacteria. Bacteriophage have been considered as a possible therapy against
antibiotic-resistant strains for many bacteria. Moreover, with significant advances in nanotechnology, nanomaterials have also been widely investigated for their potential ability to kill bacteria under some circumstances. This will be further discussed in the next part of this dissertation.

In a nosocomial environment, preventing infections often starts with sterilization of the patient’s skin or surgeon’s hands which are reservoirs for bacterial contamination. The immediate vicinity of patients has been considered to play an important role in the transmission of nosocomial pathogens. Studies have showed that “high-touch” (i.e., frequently touched) surfaces in the immediate vicinity of patients may be a reservoir for nosocomial bacteria causing wide-spread infections. Figure 1-3 shows surfaces that are frequently touched in nosocomial environments. Some nosocomial pathogens, such as methicillin-resistant Staphylococcus aureus, vancomycin-resistant Enterococci, Acinetobacter baumannii and Escherichia coli, have been shown to persist on those surfaces for several days to several months. Regarding environmental infection control in healthcare facilities, it is also recommended by the Healthcare Infection
Control Practices Advisory Committee and the Centers for Disease Control, to clean and disinfect high-touch surfaces (such as bed rails, doorknobs and light switches, and surfaces around toilets in patients’ rooms) on a more frequent basis than minimal-touch surfaces.\textsuperscript{31}

\textbf{Figure 1-3.} Mean frequency of healthcare worker contact for 28 surfaces in an intensive care unit.\textsuperscript{32} ABHR = alcohol based hand rub, SCD = sequential compression device.

The antimicrobial agents used to destroy pathogenic microorganisms on those surfaces are called disinfectants. The two types of disinfectants that
are most frequently used are alcohols and oxidizing agents. In the healthcare setting, alcohols, usually ethanol or isopropanol, have generally underrated germicidal characteristics.\textsuperscript{33} The optimum bactericidal concentration is 60\%-90\% alcohol in water and their activity drops sharply when diluted below a 50\% concentration.\textsuperscript{34} As disinfectants, they are not corrosive and easy to prepare, but can be a fire hazard and have no or limited residual activity making extended exposure difficult to achieve due to evaporation. They also have limited activity in the presence of an organic material. In addition, alcohols cannot destroy bacterial spores so they are considered as an intermediate level disinfectant. The FDA (Food and Drug Administration) has not cleared any liquid chemical sterilant or high-level disinfectant with alcohol as the main active ingredient. Alcohols are not recommended for sterilizing medical and surgical materials principally because they lack sporicidal action and they cannot penetrate protein-rich materials.\textsuperscript{35} Fatal postoperative wound infections with \textit{Clostridium} have occurred when alcohols were used to sterilize surgical instruments contaminated with bacterial spores.\textsuperscript{36}
Oxidizing agents, such as hypochlorites and hydrogen peroxide, are also widely used as disinfectants. The most prevalent hypochlorite products are aqueous solutions of 5.25%-6.15% sodium hypochlorite, usually called household bleach. It works as a disinfectant by oxidizing the cell membrane of microorganisms, which results in a loss of structure and leads to cell lysis and death. The advantages of sodium hypochlorite include that it has a broad spectrum of antimicrobial activity, does not leave toxic residues, is unaffected by water hardness, is inexpensive and fast acting. However, there are also some limitations where using sodium hypochlorite as a disinfectant. At the concentration used in household bleach (5.25-6.15%), sodium hypochlorite can produce ocular irritation or oropharyngeal, esophageal, and gastric burns. Other disadvantages of hypochlorites include corrosiveness to some metals, inactivation by organic matter, discoloring of fabrics, and the release of toxic chlorine gas when mixed with ammonia or acid that is used in household cleaning agents. Therefore, due to the limitations of conventional disinfection strategies, it is necessary and significant to develop other antimicrobial methods used in nosocomial environments.
1.3 Nanomaterials as a Novel Antibacterial Approach

An nanomaterial is usually defined as having at least one dimension with morphological features in the nanoscale, specifically between 1 nm and 100 nm. In recent years, nanomaterials have drawn increasing attention from many researchers because these materials exhibit special properties stemming from their nanoscale dimensions, which are different compared with conventional micron or bulk materials. These special properties enable nanomaterials to be used for various applications including drug delivery\textsuperscript{42-44}, tissue engineering\textsuperscript{45,46}, diagnostics\textsuperscript{47-49}, energy storage\textsuperscript{50,51}, novel semiconductor\textsuperscript{52}, catalysis\textsuperscript{53-55}, construction\textsuperscript{56} and so on.

An important feature of nanomaterials or nanoparticles is their vastly increased ratio of surface area to volume, which allows for potentially increased interactions between nanomaterials and biological targets, such as mammalian cells and bacteria.\textsuperscript{46} As a result, nanomaterials will likely exert a stronger interaction with bacteria which may affect their growth and propagation. Thus, various nanomaterials have been developed and studied for their potential antibacterial applications.
The most widely investigated antibacterial nanomaterials to date are silver nanoparticles and silver-based materials.\textsuperscript{58} Silver nanoparticles have proved to be an effective antimicrobial agent against bacteria, viruses and other eukaryotic micro-organisms.\textsuperscript{58,59} Silver nanoparticles of relatively small sizes (1~10 nm) can interact with the bacteria cells directly.\textsuperscript{60} They interact with and kill bacteria through two possible mechanisms.\textsuperscript{61} As shown in Figure 1-4, one mechanism is that silver nanoparticles binds to the bacterial cell wall and cell membrane and interacts with thiol group compounds found in the respiratory enzymes of bacteria, thus inhibiting the respiration process.\textsuperscript{62} Silver forms stable S-Ag bonds with thiol group compounds and participate in catalytic oxidation reactions resulting in the formation of disulfide bonds (R-S-S-R). In another mechanism, silver nanoparticles penetrate inside the bacteria and interact with DNA molecules because they release reactive oxygen species (ROS) and silver ions. DNA molecules turn into a condensed form (only when DNA molecules are in a relaxed state, do they replicate effectively) and lose their replication ability leading to cell death.\textsuperscript{63,64} Besides silver nanoparticles, many other nanostructured materials, such as copper containing materials\textsuperscript{65,66}, zinc oxide
nanoparticles\textsuperscript{67}, gold nanoparticles\textsuperscript{68}, carbon nanotubes\textsuperscript{69}, polymer films\textsuperscript{70} and so on, have also been studied for antibacterial applications.

\textbf{Figure 1-4.} Mechanisms of nano-scaled silver interacting with bacteria.\textsuperscript{57}

The biggest concern for using nanomaterials for biomedical applications is their potential toxicity. As summarized in Figure1-5, nanomaterials can result in injury responses through various mechanisms or pathways, such as producing reactive oxygen species (ROS) that can interact and destroy the structure of many proteins and DNA in healthy cells.\textsuperscript{72,73} In a clinical study, using silver nanoparticle coated dressings for the treatment of
burns has led to abnormal elevation of blood liver levels and argyria (blue or gray discoloration of the skin).\textsuperscript{74} Silver nanoparticles and some other nanomaterials have also been shown to be cytotoxic to alveolar macrophage cells as well as epithelial lung cells.\textsuperscript{75} The toxicity of nanomaterials is usually affected by their composition, however, other physicochemical properties, such as size, surface chemistry, shape, protein absorption gradient and surface roughness, also play a crucial role in determining the toxicity of nanomaterials.\textsuperscript{76} But, for most nanomaterials, their interactions with bacteria or mammalian cells remain largely unknown and there is not a material that is well-established and widely accepted for antibacterial purposes. In this dissertation, a novel nanomaterial, selenium nanoparticles, are investigated for their potential antibacterial applications.
Figure 1-5. Major mechanistic injury responses induced by common nanomaterials. A) Oxidative stress; B) dissolution and release of toxic metal ions; C) cationic injury to surface membrane and organelles; D) pro-fibrogenic responses to CNT; E) inflammasome activation by long-aspect-ratio materials; F) photoactivation and influence of bandgap; G) Zebrafish embryo hatching interference; H) cell membrane lysis by surface reactivity.

1.4 Selenium and Nanosized Selenium
Selenium belongs to the group of metalloids from the chalcogen family in the periodic table of elements. It exists in a range of oxidation states from
+6 to -2. The most common selenium compounds are selenide, selenite and selenate with oxidation states of -2, +4, and +6, respectively. Selenium has various allotropic forms (as shown in Figure 1-6), such as the red amorphous form, black vitreous form, three (α, β, γ) of red crystalline monoclinic forms and the gray/black crystalline hexagonal form. \(^{77-80}\) Commercially, selenium is produced as a byproduct of copper refining. It is used in electronics, glass, ceramics, steel and pigment manufacturing.\(^{81}\) Selenium salts are toxic in large amounts, but trace amounts are necessary for cellular function in many organisms. Selenium is naturally found in humans and animals as a part of selenoproteins, which play an important role in antioxidant defense systems, thyroid hormone metabolism and redox control of cell reactions.\(^{82}\)

Figure 1-6. Black/gray, glassy amorphous and red amorphous selenium.\(^{83}\)
Previously, selenium and its compounds were studied for reducing or preventing cancer. It has been shown that high levels of selenium in the blood (~154 μg/ml) are correlated with reduced numbers of cancers including pancreatic, gastric, lung, nasopharyngeal, breast, uterine, respiratory, digestive and gynecological and people in areas of low soil selenium (lower than 0.05 ppm) and people with decreased plasma selenium levels (below 128 ng/ml) have higher cancer incidence and/or cancer mortality. Many in vitro studies also demonstrated the inhibitory effects of selenium on the growth of many cancerous cell lines. However, the mechanisms of selenium-related chemoprevention are complex and remain largely unknown.

What is more, other researchers provided evidence of the antibacterial properties of many selenium compounds. For example, selenium-enriched probiotics have been shown to strongly inhibit the growth of pathogenic E. coli in vivo and in vitro. The synthesized organoselenium compounds were shown to be as effective as penicillin in inhibiting S. aureus growth in solution in vitro. One of the possible
mechanisms of antibacterial properties of selenium is due to the ability of selenium to generate superoxide radicals and to catalyze oxidation of intracellular thiol resulting in thiol depletion that leads to cell death.\textsuperscript{93}

Recently, as the organic forms of selenium have been studied for its biological effects, elemental selenium nanoparticles have also drawn some attention.\textsuperscript{94} Various methods of synthesizing selenium nanoparticles have been reported.\textsuperscript{95-101} As selenium compounds, selenium nanoparticles have also drawn a great deal of attention as promising cancer therapeutic agents and drugs carriers.\textsuperscript{102,103} In addition, because of the small size and higher ratio of surface area to volume compared to conventional selenium materials, selenium nanoparticles are believed to have different possible mechanisms against bacterial growth and biofilm formation, such as a change in hydrophobicity of a surface preventing bacteria from attachment.\textsuperscript{104} Importantly, abundant evidence also supports that nanosized selenium has better biocompatibility, bioefficacy and lower toxicity compared with inorganic and organic selenocompounds.\textsuperscript{94,103,105}
1.5 Objectives

The objectives of this thesis are to synthesize selenium nanoparticles, characterize nanosized selenium coatings, test the effectiveness of selenium coated materials at inhibiting various bacteria growth and biofilm formation, and investigate the mechanism(s) of how selenium nanoparticles inhibit bacteria growth. Chapter 2 describes introducing antibacterial properties to polycarbonate medical devices using selenium nanoparticles. Chapter 3 describes studies concerning how to inhibit several types of bacterial growth and biofilm formation on normal paper towels. Chapter 4 explains how red selenium nanoparticles and gray selenium nanorods work as antibacterial agents for PEEK (polyether ether ketone) medical device. Chapter 5 evaluates the antibacterial effects of selenium quantum dots and nanoparticles produced by UV/VIS/NIR pulsed nanosecond laser ablation. Chapter 6 describes the inhibited growth of *Pseudomonas aeruginosa* by dextran- and polyacrylic acid-coated ceria nanoparticles. Chapter 7 evaluates the antibacterial properties of an implant material, silicon nitride. Lastly, based on our studies, the conclusions of this chapter presents the overall promise of nanosized selenium for preventing bacterial infection with some future
outlook for this research area to advance.

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Chapter 2
Nanosized Selenium for Preventing Biofilm Formation on
Polycarbonate Medical Devices

2.1 Introduction
An implanted medical device provides a surface for bacteria to attach to and multiply in a patient's body, resulting in the formation of a biofilm. A bacterial biofilm is an aggregate of one or more types of bacteria in a hydrated polymeric-like matrix.\textsuperscript{1} Once formed, this polymeric matrix works as a shield to prevent drugs from penetrating the biofilm. Thus, bacteria in biofilms are more tolerant to antibiotic therapies than planktonic bacteria, making these pharmaceutical treatments less effective or even completely ineffective.\textsuperscript{2-5} Moreover, outer bacteria that break off from a biofilm can enter and attack the rest of the body, causing widespread infection. Clearly, preventing biofilm formation is critical in the fight against healthcare-associated infections (or HAIs)\textsuperscript{6} and currently no consistent solution exists.
*S. aureus* (*Staphylococcus aureus*) is one of the bacterium commonly found in numerous infections. These infections can be serious when they occur on surgical wounds, in the bloodstream, or in the lungs. Each year, there are 11 million outpatient/emergency room visits and 464,000 hospital admissions in the U.S. alone due to *S. aureus* infections. S. *aureus* biofilms have been found on a wide range of medical devices including prosthetic heart valves, central venous catheters, urinary catheters, orthopedic prostheses, penile prostheses, contact lenses, endocarditis, otitis media, osteomyelitis, and sinusitis. These biofilms are easy to form but hard to treat. Therefore, it is significant to develop a method to prevent bacteria from attaching on the surface of today's medical devices.

One of the most widely publicized antibacterial coatings has been with silver to prevent biofilm formation (such as using a pure silver coating, silver-palladium alloys, and polymers coated with silver). Besides silver coatings, copper and its alloys have also been considered as a promising material for biofilm inhibition. However, when it comes to medical devices, one major problem with both silver and copper coatings
is their potential toxicity to mammalian cells due to the generation of reactive oxygen species since neither of these elements are part of our recommended daily nutrition.\textsuperscript{16,17}

Unlike other antibacterial materials used in the healthcare industry, selenium is a naturally occurring micronutrient needed for a healthy life and is recommended for daily intake by the FDA. In human beings, nutrition from selenium is achieved from 25 selenoproteins or enzymes with selenocysteine at their active center,\textsuperscript{18} which are fundamental for a human’s antioxidant defense system and other processes. Importantly, a low selenium intake (less than 40 $\mu$g per day) has been associated with an increased risk of mortality, poor immune function, and cognitive decline.\textsuperscript{19}

In previous studies, selenium has been investigated for various medical applications such as anticancer applications\textsuperscript{20,21} and as a potential orthopedic implant material.\textsuperscript{22} There are also studies showing that particulate selenium and its compounds can inhibit bacteria growth (specifically, \textit{S. aureus})\textsuperscript{23} and biofilm formation.\textsuperscript{24} Specifically, selenium
nanoparticles demonstrated an effectiveness towards inhibiting the growth of planktonic *S. aureus* in solution by up to 60 times compared with no treatment,\textsuperscript{23} however, as mentioned, inhibiting bacteria in biofilms is even harder. Along this line, inhibition of *P. aeruginosa* and *S. aureus* biofilms was recently reported when using an organo-selenium compound,\textsuperscript{24} but the coating only contained 0.2% selenium and the substrate coated was cellulose, which has a rough surface and fibrous structure not commonly used in medical devices. In addition, the process used to coat the organo-selenium compound on cellulose discs was complicated including polymerization, curing, and treating with glutathione (GSH) before use; processes that would be difficult to implement for numerous medical devices, especially catheters.

Therefore, the objective of this study was for the first time to coat selenium nanoparticles on polycarbonate medical devices using a novel, easy and quick precipitation reaction. Further, such materials were tested for their effectiveness to kill bacteria and/or prevent bacteria from attaching. In doing so, this study revealed a novel, easy to use, and cost-effective nano-selenium based coating method to prevent *S. aureus*
biofilm formation.

2.2 Materials and Methods

2.2.1 Materials

Selenium nanoparticles were synthesized through a simple reaction between glutathione (reduced form, GSH) (97%, TCI America, Portland, OR) and sodium selenite (99%, Alfa Aesar, Ward Hill, MA) (glutathione:sodium selenite = 4:1 molar mixture) and at the same time were coated on the surface of one popular medical device polymer (polycarbonate films, McMaster-CARR, 85585k82, cut into round films 7.01mm in diameter and 0.13mm in thickness). Sodium hydroxide (NaOH) was added to bring the pH of the solution to the alkaline regimen to start the reaction. As the size of selenium nanoparticles on the surface is influenced by the concentration of NaOH and the coating time, two coating conditions were used in this study. One was using a 0.5M NaOH solution for 30 seconds and the other one was using a 1.0M NaOH solution for 60 seconds. Selenium nanoparticles were formed immediately following the addition of NaOH as visualized by a color change of the reactant solution form clear white to clear red. The coated
substrates were rinsed in deionized water three times to stop the coating reaction and to remove the free, non-adherent, selenium nanoparticles.

2.2.2 Materials Characterization

After coating, tape tests (D3359-09e2: Standard Test Methods for Measuring Adhesion by Tape Test) were used to test the strength of adhesion of the selenium nanoparticles on the substrate surfaces. SEM (Scanning Electron Microscope, HITACHI 2700) images of the substrate surfaces were taken before and after the adhesion tests to determine coating strength and the distribution of selenium nanoparticles. Before scanning the surface of polycarbonate under SEM, the films were coated with a 2nm gold layer using a Sputter Coater (EMITECH K550, Emitech Ltd.) to make them conductive. The coverage of selenium nanoparticles on the polycarbonate surface was analyzed and calculated based on SEM images using ImageJ (Wayne Rasband). An AFM (Atomic Force Microscope, MFP3D, Asylum Research, sharp tipped cantilever, K = 0.06N/M, Contact Mode) was used to demonstrate that the coated selenium nanoparticles increased the surface area and surface roughness of polycarbonate. Lastly, the coated samples before and after a tape test
were treated in 1mL aqua regia for 30 minutes to dissolve all the selenium into solution. After treatment, the solutions were collected in glass vials separately and then boiled to remove all the liquid. 5 mL of 2% nitric acid was added into each vial to dissolve the residue. After about 24 hours, the solutions were measured by AAS (Atomic Absorption Spectroscopy, Furnace, AA600) to determine the amount of selenium in each solution. SEM images of the treated films were taken to confirm that all the coated selenium was removed by aqua regia. Then, the amount of selenium on every sample was calculated using AAS. Measurements were completed in triplicate for each of the coating conditions.

**2.2.3 Bacterial Assays**

For the bacteria experiments, a bacteria cell line of *S. aureus* was obtained in freeze-dried form from the American Type Culture Collection (catalog number 25923). The cells were propagated in 30 mg/mL of tryptic soy broth (TSB). A bacteria solution was prepared at a concentration of $10^6$ bacteria/ml, which was assessed by measuring the optical density of the bacterial solution using a standard curve correlating optical densities and bacterial concentrations. The optical densities were
measured at 562nm using a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA). Selenium coated samples were rinsed with 75% ethanol for 20 minutes for sterilization purposes and were left in the sterile petri dishes for 30 minutes to completely dry. Then, the samples were transferred into a 24-well plate and treated with the prepared bacterial solutions (10^6 bacteria/ml) and cultured for either 24, 48 or 72 hours in an incubator (37°, humidified, 5% CO₂). For those samples that were cultured for 48 and 72 hours, the media was changed with 1mL sterile and fresh TSB (0.3mg/mL) every 24 hours. After the treatment, the samples were rinsed with a PBS (phosphate buffered saline) solution twice and placed into 1.5ml microfuge tubes with 1ml of PBS. These tubes were shaken at 3000 rpm for 15 minutes on a vortex mixer to release the bacteria attached on the surface into the solution. Solutions with bacteria were spread on agar plates and bacteria colonies were counted after 18 hours of incubation.

2.2.4 Statistics
Bacterial tests were conducted in triplicate and repeated three times. Data were collected and the significant differences were assessed with the
probability associated with a one-tailed Student's t-test. Statistical analyses were performed using Microsoft Excel (Redmond, WA).

2.3 Results

2.3.1 SEM Images, AFM Images and AAS Results

SEM images of the selenium coated polycarbonate surfaces showed that the selenium nanoparticles were spherical and were well distributed on the polymer surface (Figure 2-1). Most of the nanoparticles were approximately 50-100nm in diameter. Nanoparticles coated with a condition of 1.0M NaOH for 60s were larger than those with the condition of 0.5M NaOH for 30s, since greater amounts of NaOH increased the rate of the reaction resulting in larger selenium nanoparticles. The concentration of selenium nanoparticles on the polycarbonate surfaces were 19.34g/m² and 20.95g/m² for the 0.5M NaOH for 30s condition and for the 1.0M NaOH for 60s condition, respectively. After the tape test, some of the selenium nanoparticles came off and parts of the surface were not covered by selenium. Tape tests removed about 50% and 75% of the selenium nanoparticles for the 0.5M NaOH for 30s condition and for the 1.0M NaOH for 60s condition,
respectively, according to AAS results (Table 2-1), which was also demonstrated by the coverage analysis from the four SEM images (see the caption for Figure 2-1). This result indicated that selenium nanoparticles with larger sizes might have less adhesion to an underlying substrate than smaller selenium nanoparticles. However, the selenium coated surfaces provided a desirable large surface area, which is important for increasing the exposure of selenium to bacteria to maximize inhibition.
Figure 2-1. SEM images of selenium coated polycarbonate samples before and after a tape test. (a) 0.5M NaOH, coating for 30 seconds and before a tape test. (b) 0.5M NaOH, coating for 30 seconds and after a tape test. (c) 1.0M NaOH, coating for 60 seconds and before a tape test. (d) 1.0M NaOH, coating for 60 seconds and after a tape test. The coverage of selenium nanoparticles on the surface for image (a), (b), (c), (d) are 11.74%, 6.94%, 11.38%, 5.88% respectively.

Table 2-1. Amount of selenium coated on polycarbonate surfaces determined using AAS.

<table>
<thead>
<tr>
<th>Amount of selenium</th>
<th>0.5M NaOH, 30s</th>
<th>1.0M NaOH, 60s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before tape test</td>
<td>19.34 g/m²</td>
<td>20.95 g/m²</td>
</tr>
<tr>
<td>After tape test</td>
<td>9.22 g/m²</td>
<td>5.09 g/m²</td>
</tr>
</tbody>
</table>

From the AFM images (Figure 2-2), the RMS (root mean square, scan area = 10 μm×10 μm) roughness for the selenium coated surface at the condition of 0.5M NaOH for 30s (image a) and with the condition of 1.0M NaOH for 60s (image c) were 45.997nm and 53.084nm,
respectively; after the tape test, the RMS roughness decreased to 21.731nm (image b) and 34.925nm (image d) for those two coating conditions. The results were consistent with the SEM images and AAS data because parts of the coated selenium nanoparticles were removed from the surface by the tape test, resulting in a decrease in roughness. The RMS roughness of the uncoated surface (image e) was 14.898nm, which was much smaller than the selenium coated polycarbonate samples. So, there was a significant increase in roughness, therefore surface area, after coating with selenium nanoparticles.
Figure 2-2. AFM images and RMS (root mean square, scan area = 10um×10um) roughness of polycarbonate surface. (a) Selenium coated with 0.5M NaOH for 30s, RMS roughness = 45.997nm. (b) Selenium coated with 0.5M NaOH for 30s, after tape test, RMS roughness = 21.731nm. (c) Selenium coated with 1.0M NaOH for 60s, RMS
roughness = 53.084nm. (d) Selenium coated with 1.0M NaOH for 60s, after tape test, RMS roughness = 34.925nm. (e) Uncoated sample, RMS roughness = 14.898nm.

2.3.2 Bacterial Assays

Most strikingly, all of the selenium coated samples showed a higher effectiveness at inhibiting bacteria growth on the polycarbonate surface than the uncoated surface (Figure 2-3). Compared to uncoated samples, more than 91%, 15% and 73% of the bacteria (compared with uncoated polycarbonate) died or had been removed after 24, 48 and 72 hours on the selenium coated polycarbonate (Table 2-2). The logarithmic difference between control groups and selenium coated samples were over 1.05, 0.07 and 0.60 for incubation after 24, 48 and 72 hours, respectively (Table 2-3).
Figure 2-3. Bacteria (*S. aureus*) growth on the surface of polycarbonate. Polycarbonate samples were treated with bacteria (*S. aureus*) in 0.03% TSB (Tryptic Soy Broth) and were incubated for 24, 48 or 72 hours. The media was changed with 0.03% TSB every 24 hours for those samples incubated with 48 hours or 72 hours. The control group is uncoated polycarbonate. bf = before tape test; aft = after tape test. Data=Mean ± standard deviation by mean, n=3; *p<0.05 compared with control group (uncoated polycarbonate) after 24 hours; **p<0.004 compared with control group after 48 hours; ***p<0.02 compared with control group after 72 hours.

Table 2-2. Difference in percentage of viable bacteria numbers on nanosized selenium coated polycarbonate compared with control groups (uncoated polycarbonate). bf = before tape test; aft = after tape test.
Table 2-3. Log difference of viable bacteria numbers on nanosized selenium coated polycarbonate compared with control groups (uncoated polycarbonate). Log difference = log(numbers of viable bacteria for control group) - log(numbers of viable bacteria for coated samples). bf = before tape test; aft = after tape test.

<table>
<thead>
<tr>
<th>% Difference</th>
<th>0.5M, 30s, bf</th>
<th>0.5M, 30s, aft</th>
<th>1.0M, 60s, bf</th>
<th>1.0M, 60s, aft</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hours</td>
<td>5.75</td>
<td>7.49</td>
<td>8.88</td>
<td>7.36</td>
</tr>
<tr>
<td>48 hours</td>
<td>48.50</td>
<td>63.43</td>
<td>29.87</td>
<td>85.00</td>
</tr>
<tr>
<td>72 hours</td>
<td>23.47</td>
<td>26.96</td>
<td>14.45</td>
<td>13.70</td>
</tr>
</tbody>
</table>

However, results did not show any significant difference at inhibiting bacteria growth between samples coated with the various amounts of
selenium when the samples were incubated for 24 hours (Figure 2-4). The reason might be that a 24 hour bacteria culture time was not long enough to show such differences between the coated samples or even the lowest concentration (5.09 g/m²) of selenium was not significant enough to stop bacteria growth.

**Figure 2-4.** *S. aureus* densities on various amounts of coated selenium on the surface of polycarbonate. The polycarbonate films were cultured in a bacteria solution (10⁶ bacteria/ml) for either 24 hours, 48 hours or 72 hours. The media was changed with 0.03% TSB every 24 hours.

But when the selenium coated samples were cultured in bacteria solutions for 48 hours, the inhibition of bacteria growth was consistent with the amount of selenium on the polycarbonate surface (Figures 2-3 and 2-4).
Thus, after 48 hours of bacteria culture time, the polycarbonate surface with more selenium had a stronger ability to prevent bacteria from growing on the surface.

Lastly, the difference in bacteria growth among the samples with various amounts of coated selenium disappeared after the selenium coated samples were cultured with bacteria for 72 hours (Figures 2-3 and 2-4). However, the selenium coated samples still significantly inhibited bacteria growth compared to the control. After 24 hours of treatment, the bacteria density on the uncoated polycarbonate was almost constant, around 12000 cells/mL, which meant that the uncoated polycarbonate surface was saturated by the attached bacteria and most likely, bacteria started forming a biofilm. But for selenium coated samples, the results after 24 hours of treatment showed that they highly inhibited biofilm formation. Although the bacteria numbers increased after 48 hours, the selenium coated samples finally inhibited biofilm formation by at least 73% compared to the uncoated polycarbonate after 72 hours of culture. Clearly, to fully inhibit bacteria, the concentration, size, and coverage of selenium on polycarbonate needs to be optimized.
2.4 Discussions

Nanoparticles have been widely investigated for various medical applications because of their high surface-to-volume ratios and their smaller size compared with conventional micron-size particles. The high surface area of nanoparticles provides more sites for interacting with biological entities and for functionalization with other bioactive molecules, such as anticancer and antibacterial drugs. As for the present studies, the nanostructured selenium increased the surface area available to interact with and kill bacteria, while also changing the surface morphology to ultimately inhibit the attachment of bacteria.

Although a wide range of nanoparticles have been created and investigated for various applications, such as iron oxide nanoparticles, carbon nanotubes, silver and gold nanoparticles, only a few studies have reported the effectiveness of nanoparticles at inhibiting biofilm formation, in particular S. aureus. Nanostructured selenium as a novel nano-scale natural material remains largely unexplored in this respect. However, there have been studies reporting the synthesis of selenium nanoparticles
and their biological effects toward mammalian cells *in vitro*.\textsuperscript{27,28} It was demonstrated in those studies that nanostructured selenium had a 7-fold lower acute toxicity than sodium selenite in mice showing less pro-oxidative effects than selenite, as measured by cell growth.\textsuperscript{28} Selenium nanoparticles have also been shown effective at preventing the growth of planktonic *S. aureus*\textsuperscript{23} rather than the formation of biofilms.

In a possible mechanism towards inhibiting bacteria, nanostructured selenium may serve as a catalyst, oxidizing thiol groups, and reducing oxygen to superoxide.\textsuperscript{29} As thiol is an essential substance for bacteria cell function, selenium can inhibit bacteria by depleting their thiol levels. This intracellular thiol depletion mechanism is significant because healthy cells are more resilient to this effect than bacteria cells. Moreover, the nano features of the selenium coating and the change in hydrophobicity that may have resulted from coating polycarbonate with selenium nanoparticles\textsuperscript{30} may also serve as an important role in inhibiting biofilm formation. However, the mechanism of selenium inhibited bacteria growth in biofilms is likely complicated and further studies are certainly required.
In summary, the potential ability of nanostructured selenium to inhibit biofilm formation was investigated in this study. Based on the results of bacteria assays after 24, 48 and 72 hours, the selenium coating did significantly decrease the number of bacteria attached on the surface, thus, inhibiting biofilm formation on polycarbonate. Importantly, all this was accomplished without using antibiotics.

2.5 Conclusions
Overall, it was demonstrated that selenium nanoparticles were precipitated on polycarbonate medical devices using a simple fast reaction (occurring within 60 seconds) which strongly inhibited the growth of bacteria (S. aureus) on the surface after 24 and 72 hours by at least 91% and 73%, respectively, compared to uncoated polycarbonate surfaces. This result suggests that nanostructured selenium maybe an effective way to prevent S. aureus infections and biofilm formation. More in-depth and long-term studies with different kinds of bacteria and substrates should be implemented to achieve a better understanding of such novel antibacterial properties of selenium.
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Chapter 3

Inhibition of Various Gram-positive and Gram-negative Bacteria

Growth on Paper Towels by Selenium Nanoparticles

3.1 Introduction

Bacterial contaminations are frequently found on paper products worldwide, involving various strains from the genera *Bacillus*, *Staphylococcus*, *Pseudomonas* and *Enterobacter*.1-3 These bacteria contribute to the formation of biofilm in machinery, resulting in contamination or even corrosion on paper products. In the hospital environment, hand washing has been identified as the most significant manners towards preventing the spread of microbial infections,4,5 with hand drying as the critical last stage of the hand washing process. Among the three most frequently used methods to dry hands (hot air dryers, cloth towels and paper towels), paper towels have been recognized as the most hygienic method.6-8 However, in some circumstances, such as for paper towels hanging in the splashing zone or those used for cleaning surfaces, they have been considered as potential sources of bacteria contaminations,9,10 especially in a nosocomial environment.
Previously, studies evaluated the potential bacterial contamination of unused paper towels.\textsuperscript{11-13} In a hand wash experiment, participants who washed their hands with water, regular or antibacterial soap followed by drying with paper towels, surprisingly had more bacteria on their hands after washing than before, which indicated a possible bacterial transmission from paper towels.\textsuperscript{12} It was further demonstrated that a zig-zag transfer of bacteria between paper towel dispensers and hands could take place if either one is contaminated.\textsuperscript{13} In addition, there are also concerns for bacterial contamination on numerous paper products, for example, filter paper in water and air purifying systems, and wrapping paper used in the food industry.\textsuperscript{14,15} Filter papers are commonly covered by a thick layer of bacterial biofilm and need frequent replacement. Microbial contamination is one of the major reasons causing food spoilage during food storage, especially when the foods are packaged and stored for a long period of time. Actually, developing effective, antimicrobial packaging materials for controlling microbial growth in/on food is now an active area of research.\textsuperscript{16,17}
Due to the porous structure of fibers in all paper products, such materials are prone to bacteria growth and, thus, are sources for continual contamination. Besides, this porous structure provides an environment that favors the attachment of bacteria and makes it more difficult to kill bacteria once forming a biofilm. One of the most promising approaches towards preventing infections is coating paper products with antimicrobial materials. For example, Wenbing Hu and his colleagues reported that introducing antibacterial properties to filter paper by coating the paper with graphene oxide, showed about a 70% inhibition to \textit{Escherichia coli} growth after 2 hours. However, the graphene-based paper had mild cytotoxicity resulting in 20% of healthy mammalian A945 cell death after 2 hours.\textsuperscript{18} Kalyani Chule et al. studied the antibacterial activities of ZnO nanoparticle coated paper\textsuperscript{19} and results showed a significant decrease in bacteria counts after 24 hours. Besides ZnO nanoparticles, silver nanoparticles have also been loaded on filter paper for antibacterial purposes, processing strong antibacterial properties.\textsuperscript{20} But one major problem for ZnO, silver nanoparticles and other metal-based materials is their toxicity to healthy cells due to the generation of reactive oxygen species.\textsuperscript{21,22} Those materials may result in
severe health problems when such coated paper products are used for food wrapping or clinical applications.

Selenium and its compounds have been widely used in electronics, glasses, ceramics, steel and pigment manufacturing.\textsuperscript{23} But due to toxicity concerns, not until recently has selenium been considered as material for biomedical applications. Selenium is found naturally in humans and animals as a critical element in selenoproteins, which play an important role in antioxidant defense systems, thyroid hormone metabolism and redox control of cell reactions.\textsuperscript{24} Because of this, selenium and selenium nanoparticles have been receiving dramatically increasing attention in several biomedical applications. For example, it has been shown that high levels of selenium in the blood (~154 μg/ml) correlated with a reduced number of cancer occurrence including pancreatic, gastric, lung, nasopharyngeal, breast, uterine, respiratory, digestive and gynecological cancer\textsuperscript{25} and many \textit{in vitro} and \textit{in vivo} studies have demonstrated the inhibitory effects of selenium on the activity of various cancer cells.\textsuperscript{25-29} Selenium nanoparticles have also been considered as a biocompatible
anticancer orthopedic implant material due to the increased adhesion of healthy osteoblasts on nanostructured selenium.$^{30}$

However, in terms of potential antibacterial applications, selenium is a novel material that has not been widely explored. There are a few studies concerning the antibacterial properties of selenium or its compounds. For example, selenium-enriched probiotics have been shown to strongly inhibit the growth of pathogenic \textit{E. coli} \textit{in vivo} and \textit{in vitro}.$^{31}$ The synthesized organoselenium compounds were shown to be as effective as penicillin at inhibiting \textit{Staphylococcus aureus} growth in solution \textit{in vitro}.$^{32}$ In addition, the growth of planktonic \textit{Staphylococcus aureus} was strongly inhibited in the presence of selenium nanoparticles.$^{33}$

Actually, many kinds of other nanomaterials, including silver nanoparticles, zinc oxide nanoparticles, copper nanoparticles and so on, have been studied to provide a novel strategy to eradicate bacterial infections, especially for various medical device applications.$^{34-37}$ But a major problem for these metal-based nanoparticles has been their toxicity to healthy mammalian cells due to their generation of reactive oxygen
species,\textsuperscript{21,22,38} which could result in severe health problems when used as medical devices or clinical applications. Compared with the above mentioned materials, selenium is expected to be healthier and less toxic to humans and the environment. For an adult, selenium is recommended by the FDA to be consumed at 53-60 $\mu g$ per day and is the nutrition for 25 selenoproteins with selenocysteine at their active center.\textsuperscript{39} Moreover, studies have demonstrated that selenium and selenium nanoparticles can improve healthy human cell growth.\textsuperscript{30,40}

Therefore, in this chapter, selenium nanoparticles were used as a coating material to introduce antibacterial properties to paper towels. Selenium nanoparticles were coated on paper towel surfaces through a quick precipitation reaction (happening within 60 seconds). In addition, their effectiveness at preventing biofilm formation was tested in bacterial assays involving four diverse types of bacteria. The results showed that the selenium coatings significantly inhibited all bacterial activities on paper towels. Coating with nanosized selenium successfully introduced antibacterial properties to paper towels, revealing a promising
selenium-based method to decrease the presence and spreading of numerous human infections.

3.2 Materials and Methods

3.2.1 Synthesis of Selenium Nanoparticle Coatings

Selenium nanoparticles were synthesized and coated on paper towels (MB550A Hand Towel, Tork Advanced, cut into round chips, 7.00mm in diameter) through a simple and quick precipitation reaction. The reaction of the synthesis was:

$$4GSH + 2\text{SeO}_3^{2-} \rightarrow 2\text{GSSG} + \text{Se} + \text{H}_2\text{O} + 2\text{OH}^-$$

which involves glutathione (reduced form, GSH) (97%, TCI America, Portland, OR) and sodium selenite (99%, Alfa Aesar, Ward Hill, MA) mixed at a 4:1 molar ratio.

The paper towel samples were first washed with 75% ethanol followed by rinsing with deionized water five times and then transferred into a flask containing a mixed solution of glutathione and sodium selenite. Sodium hydroxide was added into the mixture of glutathione and sodium selenite to bring the pH of the solution into the alkaline regimen to initiate the
reaction at room temperature. Selenium nanoparticles were formed and precipitated on the surface of paper towels immediately following the addition of NaOH as visualized by a color change of the reactant solution from colorless to clear red. The paper towel samples were coated for 30s under a 200 rpm (round per minute) agitation to ensure a uniform coating. The coated substrates were rinsed in deionized water five times and soaked in water for 24 hours to remove the free, non-adherent, selenium nanoparticles and remaining reactants. Then, the selenium coated paper towels were left in petri dishes for several days to completely dry before being used in any further experiments or tests.

The uncoated samples that were used as a control group in the following experiments were prepared by treating paper towels with the same procedure for selenium coated paper towel samples described above except that sodium hydroxide was not added so that the reaction did not occur.
3.2.2 Characterization of Coated Paper Towels

SEM (Scanning Electron Microscope, HITACHI S-4800) images of the paper towel surfaces were taken to determine the size, coverage and distribution of selenium nanoparticles. Before scanning the surface of a paper towel under SEM, the samples were coated with a 5 nm platinum layer using a sputter coater (Cressington 208 HR, Cressington Scientific Instruments Ltd.) to make the samples conductive. The coverage of selenium nanoparticles on the paper towel surface was analyzed and calculated based on the SEM images using ImageJ (Wayne Rasband). An AFM (Atomic Force Microscope, MFP3D, Asylum Research, sharp tipped cantilever, K = 0.06N/M, Contact Mode) was used to demonstrate that there was an increase in surface roughness on paper towel samples after being coated with selenium nanoparticles.

The coated samples were treated in 1mL aqua regia for 30 minutes to dissolve all the selenium into solution. After treatment, the solutions were collected in glass vials separately and then boiled to remove all the liquid. 5 mL of 2% nitric acid was added into each vial to dissolve the residue, which contained all the selenium from the solution. After about 24 hours,
the solutions were measured with AAS (Atomic Absorption Spectroscopy, Furnace, AA600) to determine the concentration of selenium in each solution. SEM images of the treated films were taken to confirm that all the coated selenium was removed by aqua regia. Then, the amount of selenium on every sample was calculated based on the AAS results. Measurements were completed in triplicate for both blank control samples (uncoated paper towels) and selenium coated paper towel samples.

3.2.3 Mechanical Tests

A MTESTQuattro materials testing system (ADMET, Norwood, MA) was developed according to ASTM and ISO standards. The system was used to measure the mechanical properties of selenium coated and uncoated paper towels. The samples were prepared by cutting the paper towels into 18.00mm × 40.00mm rectangle pieces and then coating them with selenium nanoparticles using the procedure described above. Tension tests were performed by stretching the paper towel until complete breakage occurred. The size of tension loaded area was 18.00mm width by 30.00mm length (the stretching direction). The tension tests were
performed for both completely dry and wet (saturated by DI water) paper towels separately. Load and stress data were recorded automatically by the ADMET software. The values of maximum stress were calculated based on the data and the loaded area (18.00 mm × 30.00 mm) of the tested samples.

3.2.4 Bacterial Inhibitory Tests

Four bacterial cell lines were involved in the tests to evaluate the growth of various types of bacteria on selenium coated and uncoated paper towels. The bacterial cell lines, *Staphylococcus aureus* (catalog number 25923), *Pseudomonas aeruginosa* (catalog number 27853), *Escherichia coli* (catalog number 25922) and *Staphylococcus epidermidis* (catalog number 35984), were obtained in freeze-dried form from the American Type Culture Collection (Manassas, VA). Each strain of bacteria was propagated in 30 mg/mL tryptic soy broth (TSB) for 20 hours. A bacteria solution was prepared using 0.3 mg/mL TSB at a concentration of 10^6 bacteria/ml, which was assessed by measuring the optical density of the bacterial solution using a standard curve correlating optical densities and bacterial concentrations. The optical densities were
measured at 562nm using a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA).

Selenium coated and uncoated paper towel samples were rinsed in separate petri dishes with 75% ethanol for 20 minutes for sterilization purposes and were left in the sterile petri dishes for 30 minutes to completely dry. Then, the samples were transferred into a 48-well plate and rinsed with sterile 0.3 mg/mL TSB twice to remove any possible ethanol residue. Each sample was treated separately with 1 mL of the prepared bacterial solutions (10^6 bacteria/ml) and cultured for either 24, 48 or 72 hours in an incubator (37°C, humidified, 5% CO₂). For those samples that were cultured for 48 and 72 hours, the media was changed with 1 mL of sterile and fresh TSB every 24 hours. After the treatment, the samples were rinsed with a PBS (phosphate buffered saline) solution twice and placed into 1.5ml microfuge tubes with 1ml of PBS. These microfuge tubes were shaken at 3000 rpm for 15 minutes on a vortex mixer to completely release the bacteria attached on the surface into the solution. Solutions with bacteria from each sample were spread on agar plates and bacteria colonies were counted after about 20 hours of
incubation. The bacterial inhibitory tests on the four types of bacteria strains were conducted individually and identically.

3.2.5 Mechanism of Action: Protein Adsorption Assays

The total protein adsorption on the selenium coated and uncoated paper towel samples was measured using a Piece BCA Protein Assay Kit (catalog number 23227, Piece Biotechnology, Thermo Scientific, Rockford, IL). The paper towel samples were rinsed with 75% ethanol for 20 minutes for sterilization purposes. After being left in sterile petri dishes for 30 minutes to completely dry, the samples were transferred into a 48-well plate and rinsed with sterile PBS twice. Each sample was treated separately with 1 mL of sterile TSB and then cultured for 24, 48 or 72 hours in an incubator (37°C, humidified, 5% CO₂). For those samples that were cultured for 48 and 72 hours, the media was changed with 1 mL of sterile and fresh TSB every 24 hours. After the treatment, the samples were transferred to a new 48-well plate and washed with a cold (~4°C) PBS solution twice to remove non-adsorbed proteins in the media. Then, each sample was treated with 0.5 mL RIPA buffer (catalog number 89900, Piece Biotechnology, Thermo Scientific, Rockford, IL)
for 10 minutes, swirling the plate occasionally at the same time. After the treatment, the solution in each well was transferred to a microcentrifuge tube and stored in a -80°C freezer for further analysis. The concentration of proteins in solution gathered after 24, 48 and 72 hours were measured following the manufacturer’s instructions for the Pierce BCA Protein Assay Kit, in which the sample to work reagent ratio and incubation time were modified compared with the standard protocol to achieve a lower minimum detection level. BSA (bovine serum albumin) standards for plotting a standard curve were also treated in the same way as the samples. The concentration of protein adsorbed on each sample was calculated based on the standard curve.

3.2.6 Release of Selenium

The release of selenium from the surface of coated paper towels were measured using ICP-MS (inductively coupled plasma mass spectrometry, Bruker Aurora M90). To measure the total amount of Se on the surface of coated paper towels, 1 mL of 10 M NaOH was added with each sample (7 mm in diameter, as described in 3.2.1) into a 10 mL vial. The vial was placed standing for 3 days to allow all coated selenium to be completely
dissolved by NaOH. Then, the solution was diluted 100 times using DI water before ICP measurements. Paper towel samples without Se coating were also treated with the same procedure and the solution was also collected for ICP measurements. In order to measure the release of the selenium from the paper towel samples, both coated and uncoated samples were treated individually with 1 mL sterile TSB in a 37 °C incubator for 24, 48 and 72 hours, which are the same time scales as the treatments for the bacterial assays. The media for each sample was collected every 24 hours and replaced with 1 mL of fresh TSB. The collected solutions were diluted 10 times using DI water before ICP measurements.

3.2.7 Statistics

All bacterial inhibitory tests and protein adsorption assays were conducted in triplicate and repeated three times. Data were collected and the significant differences were assessed with the probability associated with a one-tailed Student's t-test. Statistical analyses were performed using Microsoft Excel (Redmond, WA).
3.3 Results and Discussion

3.3.1 Synthesis and Characterization of Selenium Nanoparticles

After coated with selenium nanoparticles, the color of the paper towel surfaces changed from white to light red (Figure 3-1). The red color indicated the formation of selenium nanoparticles as one of the selenium allotropes is red\textsuperscript{41}. The coated paper towels can maintain its red appearance for at least 12 months at room temperature, although the most stable form of selenium is considered as a gray color crystal and this gray selenium can be formed by the mild heating of other allotropes. The nano-sized red selenium has a high biological activity in terms of cell proliferation, enzyme induction and protection of human cells against damage from free radicals. It also has a much lower acute toxicity compared with sodium selenite\textsuperscript{42}. 
Figure 3-1. Light image of selenium coated and uncoated paper towels. The three pieces on the top are paper towels without selenium coating. The three on the bottom are paper towels coated with selenium nanoparticles.

The SEM images of the selenium coated paper towels (image a) and uncoated paper towels (image b) are shown in Figure 3-2 and Figure 3-3. As shown in image a, the selenium nanoparticles were well distributed on the fibrous structure of the paper towels and covered almost all parts of the surface. Image b showed a paper towel surface without coatings, onto which no particles were observed. From image c in Figure 3-3, which is a selenium coated sample, observed under higher magnification, the
diameters of the selenium particles were measured and the size
distribution was found to be between 50 and 100 nm. The surface
coverage of selenium nanoparticles was 4.36% according to
measurements by ImageJ.

Figure 3-2. SEM images of selenium coated (image a) and uncoated
paper (image b) towel samples at a magnification of 8.0 K.
Figure 3-3. Scanning electron microscopy images of: (a) a paper towel with a nano-selenium coating at a 3.5 k magnification, (b) a paper towel
without a selenium coating at a 3.5 k magnification, and (c) the paper towel in image a at a 10.0 k magnification.

The AFM images in Figure 3-4 showed that the RMS (root mean square, scan area = 10 μm×10 μm) roughness of the paper towel surface increased from 15.89nm (Figure 3-4, image a) to 31.14nm (Figure 3-4, image b) after coated with selenium nanoparticles. The surface roughness was significantly increased after coated the paper towels with selenium nanoparticles.

Figure 3-4. AFM images and RMS (root mean square, area = 10 μm×10 μm) roughness of paper towel surfaces. Image (a) is the surface of selenium coated paper towel with RMS roughness = 31.14 nm. Image (b) is the surface of uncoated paper towel with RMS roughness =15.89 nm.
According to AAS results, the concentration of the selenium nanoparticles on the coated paper towel surface was 69.00 g/m². This concentration is about 4 times larger than concentration of selenium on the coated polycarbonate surfaces under the same coating conditions as mentioned in chapter 2. The reason was that the fibrous structure of the paper towel significantly increased surface area to allow for more selenium nanoparticle deposition. Compared with the flat surfaces of most other materials, the surface of paper towels with a fibrous structure had a larger surface area, resulting in increased deposition and adhesion of selenium nanoparticles. Overall, the selenium nanoparticles were successfully synthesized and coated on the paper towels using a simple quick reaction (within a minute), and the selenium coatings can retain its red appearance for a long time period (over a year) at room temperature.

3.3.2 Mechanical Tests

In the mechanical tension tests, the maximum stress of the uncoated and selenium coated paper towels were 27.87 kN/m² and 27.79 kN/m² (or 2046 g and 2040 g stretching loads), respectively, as shown in Figure 3.
and Table 1. After the samples were saturated with DI water, the maximum stresses are 2.969 kN/m² and 2.901 kN/m² (or 218 g and 213 g loads) for the wet paper towel samples with and without selenium coatings, respectively. Therefore, in both dry and wet conditions, there was no significant change in terms of the mechanical properties for the paper towels after coated with selenium nanoparticles.

**Figure 3-5.** Tension tests for paper towels in various conditions: a. dry and uncoated; b. dry and selenium nanoparticle coated; c. wet and uncoated; d. wet and selenium nanoparticle coated.
Table 3-1. Values of maximum stress (the stress at the moment of complete breakage) for each paper towel sample in the tension tests.

<table>
<thead>
<tr>
<th>Maximum stress (kN/m²)</th>
<th>Without coating</th>
<th>Selenium coated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry</td>
<td>27.87</td>
<td>27.79</td>
</tr>
<tr>
<td>Wet</td>
<td>2.901</td>
<td>2.969</td>
</tr>
</tbody>
</table>

3.3.3 Bacterial Inhibitory Tests

The results of bacteria assays involving *Staphylococcus aureus* (*S. aureus*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Escherichia coli* (*E. coli*) and *Staphylococcus epidermidis* (*S. epidermidis*) showed a high effectiveness for the selenium coated paper towels at inhibiting bacteria growth. As seen in Figure 3-6, the selenium coated paper towels had 88.6%, 88.9% and 88.8% less bacteria (*S. aureus*) attached than the uncoated paper towels after 24, 48 and 72 hours, respectively. Moreover, from the 24 hour culture time to the 48 hour culture time, there was an increase in bacteria numbers on uncoated paper towel samples, but was constant to the 72 hours culture time, implying that the uncoated paper
towel was saturated by bacteria after 48 hours of treatment. In contrast, the bacteria numbers on the selenium coated paper towels remained at a low level not increasing from 24 to 48 to 72 hours, indicating successful inhibition to the growth of *S. aureus*. In the bacterial assays involving *S. aureus* on polycarbonate samples in chapter 2, there were distinct increases in bacteria numbers from 24 hours to 48 hours on all the coated samples, which indicated that after 48 hours the bacteria growth was not completely inhibited by the selenium coatings on polycarbonate. However, Figure 3-6 showed that the selenium coatings on paper towel samples almost completely inhibited bacterial growth after 48 treatment. The reason was that the amount of coated selenium nanoparticles on polycarbonate samples was much smaller than the amount on paper towel samples. Thus, the surface attached with more selenium nanoparticles revealed a stronger ability to prevent the formation of *S. aureus* biofilms, especially when the bacteria in the biofilm propagating quickly.
Figure 3-6. The growth of *Staphylococcus aureus* on the surface of paper towels. The control group is the uncoated paper towels. Data=Mean ± standard deviation, n=3; *p<0.02 compared with the control group (uncoated paper) after 24 hours; **p<0.002 compared with control group after 48 hours; ***p<0.002 compared with the control group after 72 hours.

A similar inhibitory activity was observed in the bacterial assays involving another gram-positive bacterium, *S. epidermidis*. As shown in Figure 3-7, the selenium coated paper towels had 90.66%, 92.50% and 92.16% less bacteria attached compared with the uncoated paper towels after 24, 48 and 72 hours, respectively. From 24 hours to 48 hours, there was a large increase in the numbers of bacteria, which means the bacteria propagated quickly during this period on paper towels without a selenium
coating. However, on the surface of selenium coated paper towels, there was not an obvious increase in the bacteria numbers from 24 hours to 48 hours. In addition, after incubation for 48 hours and 72 hours, the uncoated paper towel was saturated by bacteria (*S. epidermidis*), indicating the formation of a matured biofilm, while the selenium coated paper towels still had a significantly reduced number of bacteria after 72 hours. Overall, the numbers of *S. epidermidis* on the selenium coated paper towels remained at a low level not increasing from 24 to 48 to 72 hours, indicating successful inhibition to the growth and biofilm formation of *S. epidermidis*.

**Figure 3-7.** The growth of *Staphylococcus epidermidis* on the surface of paper towels. There was more than a 90% inhibition of bacteria growth compared with control groups. Data=mean ± standard deviation, n=3; *, **, ***p<0.05 compared with the control group (uncoated paper) after either 24, 48 or 72 hours, respectively.
Results also showed a significant inhibition of *P. aeruginosa* on selenium coated paper towels. Specifically, the results in Figure 3-8 showed a successful inhibition of the growth of *P. aeruginosa* by 55% and 84% after 48 or 72 hours, respectively, onto the surface of selenium coated paper towels. But after 24 hours of incubation, there was no significant decrease in bacteria numbers observed on selenium coated paper towels compared with uncoated paper towels. The reason may be that *P. aeruginosa* are gram-negative bacteria that have an extra layer of bacterial outer membrane (made of lipopolysaccharide and protein) as a part of their cell wall. Thus, compared with *S. aureus* and *S. epidermidis*, which are gram-positive bacteria that only have the plasma membrane in their cell wall but don’t have the bacterial outer membrane, it took longer for selenium to penetrate into and interact with *P. aeruginosa*; thus, it took a longer time for the gram-negative bacteria to be killed. However, the total amount of *P. aeruginosa* increased from 24 to 48 to 72 hours on paper towels without selenium coatings, while on the surface of selenium coated paper towels, the amount of *P. aeruginosa* decreased from 24 hours to 48 hours and further decreased dramatically from 48 hours to 72
hours. Therefore, the activity of *P. aeruginosa* was successfully inhibited on selenium coated paper towels and the inhibition increased as the time of treatment increased. Future studies will be helpful to investigate higher concentrations of selenium to decrease *P. aeruginosa* growth over long time periods.

**Figure 3-8.** The growth of *Pseudomonas aeruginosa* on the surface of paper towels. Data=mean ± standard deviation, n=3; *, **p<0.05 compared with the control group (uncoated paper) after either 48 or 72 hours, respectively.

The results of the bacterial inhibitory assays with *E. coli* are shown in Figure 3-9. The selenium coated paper towels inhibited *E. coli* growth by about 50%~60% compared with the uncoated paper towels after treatment for 24 hours, 48 hours and 72 hours. As shown in Figure 3-6 and Figure 3-7, a saturation of *S. epidermidis* and *P. aeruginosa* growth
on the uncoated paper towel surfaces was observed after incubation for 48 hours. But for *E. coli*, after 24 hours of incubation, the bacteria saturated the surface of paper towels not coated with selenium and formed a mature biofilm. The reason for this could be that *E. coli* propagates much faster than the other two bacteria as the re-generation time of *E. coli* is only 15~20 minutes under optimal conditions in the laboratory. Moreover, although the growth of *E. coli* was significantly inhibited, the percentage of bacteria population reduction was not as much as the reduction for *S. aureus* and *S. epidermidis*. Because *E. coli* is also a gram-negative bacterium, its outer membrane can prevent the interaction between the selenium nanoparticles or the selenium element and the bacteria cells. Overall, compared with the uncoated paper towels, the selenium coated paper towels showed a significant inhibition to the growth of *E. coli* continuously from 24 to 72 hours.
Figure 3-9. The growth of *E. coli* on the surface of paper towels. Data=mean ± standard deviation, n=3; *, **, ***p<0.05 compared with the control group (uncoated paper) after either 24, 48 or 72 hours, respectively.

In conclusion, the growth of *S. aureus, S. epidermidis, P. aeruginosa* and *E. coli* on paper towels was successfully inhibited after coated with selenium nanoparticles. The selenium particles when coated on paper towels were extremely effective and continuously inhibited (about a 90% reduction) the growth of gram-positive bacteria including *S. aureus* and *S. epidermidis* after 24, 48 or 72 hours. From gram-negative bacteria like *P. aeruginosa* and *E. coli*, the selenium coated paper towels also significantly inhibited bacterial growth after either 48 or 72 hours of incubation. However, it took a longer time for *P. aeruginosa* and *E. coli*
to be influenced by selenium nanoparticles and the overall reduction of the bacteria population was less than that observed for the gram-positive bacteria, which means that the activities of gram-negative bacteria were more difficult to slow by selenium nanoparticle coatings on the surface of paper towels. However, further studies will investigate higher concentrations or altered selenium nanoparticle sizes to maximally decrease gram-negative bacteria growth.

### 3.3.4 Mechanism of Action: Protein Adsorption Assays

Based on the results from protein adsorption assays as shown in Figure 3-10, the selenium nanoparticle coated paper towels significantly increased protein adsorption over uncoated paper towels after a treatment in TSB for either 24, 48 or 72 hours. After coating the paper towels with selenium nanoparticles, there was an increase in the surface area and nanoscale roughness, which allowed more proteins to adsorb to the surface of the paper towels. The increased protein adsorption might play an important role in inhibiting bacteria growth on the selenium coated paper towels, because those proteins could interact with bacteria cell membranes and prevent bacteria cells from attaching to the surface. This
could eventually prevent bacteria from forming biofilms, thus, inhibiting bacteria growth on the surface. Further mechanistic studies are necessary to achieve a better understanding of how selenium nanoparticle coatings inhibit the growth of various bacteria on the surface of paper towels.

**Figure 3-10.** The amount of adsorbed total proteins on selenium coated and uncoated paper towels. Data=mean ± standard deviation, n=3; *, **, ***p<0.05 compared with the paper towels without coating for a treatment after either 24, 48 or 72 hours.

### 3.3.5 Release of Selenium

The table 3-2 below shows the release of selenium from the selenium nanoparticle coated paper towels, which was measured by ICP-MS.
About 0.94% of coated selenium was dissolved into media after first 24 hours. Another 0.51% was dissolved in the following 24 hours and an even lower amount (0.45%) was released into solution during the third day of treatment. As observed from the antibacterial tests, the paper towels coated with selenium nanoparticles significantly inhibited bacteria growth from 24 hours to 72 hours (except the test with *E. coli* for 24 hours). During this 3-day period, there was only 2 percent of selenium released from the surface, which means that low selenium concentrations between 15 ppb to 35 ppb were effective enough to inhibit bacterial growth. As less than 1% of the selenium coatings were released during the first 24 hours, the selenium coatings could remain in an aqueous environment for more than 100 days as a minimum (although most likely longer since selenium release decreased with time). Thus, the nanosized selenium coatings on the paper towels surface could retain its potential antibacterial properties for at least several months.
Table 3-2. The release of selenium from the selenium coated paper towels. Results are the average of three samples.

<table>
<thead>
<tr>
<th>Release of Se</th>
<th>Total</th>
<th>1st day</th>
<th>2nd day</th>
<th>3rd day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Se concentration (ppb)</td>
<td>3663.8±151.8</td>
<td>34.47±3.45</td>
<td>18.68 ± 1.93</td>
<td>16.31 ± 1.52</td>
</tr>
<tr>
<td>Percentage</td>
<td>N/A</td>
<td>0.94%</td>
<td>0.51%</td>
<td>0.45%</td>
</tr>
</tbody>
</table>

3.4 Conclusions

Selenium nanoparticles were synthesized and coated on the surface of paper towels through a simple and quick precipitation process which happened within one minute. Impressively, compared with the paper towels without selenium coatings, the selenium coated paper towels significantly inhibited the growth of *S. aureus*, *S. epidermidis*, *P. aeruginosa* and *E. coli* after 24, 48 or 72 hours. There was significant and continuous bacteria inhibition with about a 90% reduction from 24 to 72 hours in the growth of gram-positive bacteria including *S. aureus* and *S. epidermidis*. The selenium coated paper towels showed a relatively lower effectiveness at inhibiting gram-negative bacteria like *P. aeruginosa* and *E. coli* with about a 57% and 84% reduction, respectively, after 72 hours.
of treatment. In addition, there were larger amounts of proteins adsorbed on the surface of selenium coated paper towels compared with uncoated paper towels, which might be an important reason why selenium nanoparticle coatings can inhibit diverse bacterial growth on the surface of paper towels. Therefore, this study suggested that coating paper products with selenium nanoparticles may be an effective way to decrease various gram-positive and gram-negative bacteria growth on paper products, which might be used for potentially important applications for antimicrobial purposes in the food packaging industry and in clinical environments.

3.5 REFERENCES


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Chapter 4

Red Selenium Nanoparticles and Gray Selenium Nanorods as Antibacterial Agents for PEEK Medical Devices

4.1 Introduction

Polyetheretherketone (PEEK) is a polymer that has been used, or at least intended to, in many different biomedical applications such as spinal cages\(^1\), craniomaxillofacial implants\(^2\), artificial heart valves\(^3\), dental applications\(^4\), bone implants\(^5\), and so on. The major concern about this material, and many other polymeric materials used for medical implantation, is that they can be infected by bacteria. These infections are usually complicated and hard to treat due to many factors such as various bacteria species attaching to PEEK, quick growth of bacteria, the immediate presence of serum proteins adsorbed to PEEK surfaces mediating such bacteria adhesion, fluid flow around the implant which provides for constant nutrients to bacteria, host immune system variables, and many other complications\(^6\).

Clearly, device associated infections are very complicated because when
an implant becomes colonized by bacteria, significant patient morbidity can follow\textsuperscript{7}. The reason is that adherent bacteria are resistant to the host immune system and systemic antibiotic therapy, since the implant provides a niche for bacteria. Thus, the bacteria can grow on an implant and form a biofilm over its surface, causing oral infections, inflammatory reactions, destruction of the adjacent tissue, and implant loosening or even detachment. Moreover, these bacterial contaminations can lead to poor tissue (such as bone) healing.\textsuperscript{7}

The treatment of such implant-related infections requires extensive repeated surgery, implant extrication, extended duration of systemic antibiotic therapy, tissue loss, substantial cost, suffering, and disability\textsuperscript{2,6}. In the case of spinal instrumentation, these load-bearing implants cannot always be removed, and so the bacterial infection, which occurs in 6\% of these patients, cannot frequently be fully eliminated.\textsuperscript{8} For hip replacements, deep infections occur at 1.63\% within 2 years of surgery.\textsuperscript{9} Moreover, the percentage of deep prosthetic infections for total knee arthroplasty (TKA) and total hip arthroplasty (THA) procedures have been reported to be up to 18\%.\textsuperscript{10} This infection is also the most common
reason for repeated surgery after an otherwise successful prosthetic joint replacement\textsuperscript{2}. Finally, treatment costs related to infected orthopedic implants can range from 1.52 to 1.76 times the cost of the original surgery.\textsuperscript{11}

Because of all the reasons mentioned above, many strategies to modify PEEK implant surfaces have been developed and evaluated to prevent or limit implant-associated infections. There are two different approaches in these strategies. First, anti-adhesive surfaces are created to prevent or limit the initial adhesion of bacteria to the surface of the implants. The second approach is the incorporation of antimicrobial agents into the polymeric implant materials.

Examples of anti-adhesive surfaces are material surfaces treated by oxygen plasma. Oxygen plasma has been investigated to prevent the initial adhesion of bacteria by increasing the surface energy of PEEK when adding functional groups onto the polymer surface. However, Rochford et al. showed that it does not affect bacterial adhesion in a
pre-operative model and there is still a need for further investigation in a post-operative model including determining the effect of the oxygen plasma treatment on the immune response.\textsuperscript{2} The surface of PEEK has also been modified by photoinduction and self-initiated graft polymerization with 2-methacryloyloxyethyl phosphorylcholine (MPC; PMPC-grafted PEEK). Previous research by Tateishi et al. reported that bacteria (specifically, \textit{E. coli}) adhered to the surface of PMPC-grafted PEEK at a significantly reduced number, less than 1%, however, this result is limited because of a short period of treatment time (1 hour) in the tests.\textsuperscript{3,12} Thus, it has not been possible to capture an accurate \textit{in vivo} assessment of this antibacterial strategy.

Another method to improve the antibacterial properties of PEEK is by coating or incorporating PEEK with hydroxyapatite (HA). Wang et al. have tried to modify smooth and rough PEEK surfaces with nano-fluorohydroxyapatite (nano-FHA) since nano-FHA has been used successfully for bone regeneration.\textsuperscript{13} The results of their study showed that a few bacteria cells (\textit{S. mutans}) were detected on the PEEK/nano-FHA compared to the pristine PEEK after 4 hours of bacteria
exposure. After 24 hours, there was a reduction of *S. mutans* by 86.5% on the surface of PEEK/nano-FHA compared to the bare PEEK. However, the growth of *S. mutans* on PEEK was not detected after 24 hours and *S. mutans* is a gram-positive bacterium that is commonly found in oral infections but not found in many other infections which occur on PEEK medical devices.

Silver additives have become ideal candidates for the development of infection-resistant PEEK composites. However, initial investigations performed by Jaekel showed that incorporation of up to 5 weight percent of bactericidal silver agents in the PEEK matrix caused no significant reduction in adherent bacteria with both injection-molded and machined surfaces. The experiment was repeated and after 24 hours of bacterial incubation, no significant differences could be observed between Ag-composites and unfilled PEEK controls. It was concluded in that study that silver was not effective at decreasing bacteria growth at the concentrations tested and the method by which the silver was incorporated. Therefore, the present Ag incorporation techniques need to be optimized and other approaches need to be developed to improve
the antimicrobial properties of PEEK implants. Such studies also clearly highlight the sensitivity of introducing Ag into PEEK to obtain antibacterial properties.

Compared with many other nanoparticles used to modify the surfaces of medical devices (such as silver nanoparticles, zinc oxide nanoparticles, and copper oxide nanoparticles, etc.\textsuperscript{15-17}), selenium nanoparticles, as a nonmetal material, have been considered as a healthier and less toxic biomaterial for surface coatings.\textsuperscript{18,19} Previously, nanosized selenium has also been studied for anticancer applications\textsuperscript{20-22} and investigated for preventing various bacterial infections.\textsuperscript{23-26} However, nanosized selenium has never been used with PEEK to impose antibacterial properties.

In addition, red selenium and gray selenium are two elemental forms of selenium. The red form of selenium can be transformed into the gray form with mild heating as the gray selenium is thermodynamically more stable.\textsuperscript{27} A few studies have reported that red selenium is biological active,\textsuperscript{28,29} while almost no studies have investigated the potential bioactivity of gray selenium. Therefore, in this study, selenium
nanoparticles were tested for the first time as a coating material for PEEK medical devices to inhibit bacterial growth. Two types of nanosized selenium, red selenium nanoparticles and gray selenium nanorods, were successfully coated on PEEK medical devices. The results of bacterial tests showed that both red and gray nanosized selenium coatings inhibited the growth and biofilm formation of P. aeruginosa (a bacterium responsible for numerous medical device infections) on the surface of PEEK medical devices. Therefore, this study demonstrated that coating PEEK with selenium nanoparticles could be a viable approach to improve the antibacterial properties of PEEK medical devices.

4.2 Materials and Methods

4.2.1 Preparation of Selenium Coated PEEK
Selenium nanoparticles were synthesized through a simple and quick precipitation reaction and were precipitated on the surface of PEEK (PEEK Optima, Invibio, Lancashire, UK). The synthesis of selenium nanoparticles involved a reaction between glutathione (reduced form, GSH) (97%, TCI America, Portland, OR) and sodium selenite (99%, Alfa Aesar, Ward Hill, MA) that were mixed at a 4:1 molar ratio. The PEEK
samples were first washed with 75% ethanol followed by rinsing with deionized water three times and then transferred into a flask containing a mixed solution of glutathione and sodium selenite. Sodium hydroxide was added into the mixture of glutathione and sodium selenite to bring the pH of the solution into the alkaline regimen to initiate the reaction at room temperature. Selenium nanoparticles were formed and precipitated on the surface of PEEK immediately following the addition of NaOH as visualized by a color change of the reactant solution from colorless to clear red. The PEEK samples were treated in the reactants for 60 seconds under 200 rpm (rounds per minute) of agitation to ensure a uniform coating. The coated PEEK samples were rinsed in deionized water five times and soaked in deionized water for 24 hours to remove the free, non-adherent, selenium nanoparticles and possible remaining reactants. Then, the selenium nanoparticle coated PEEK samples were left in petri dishes for several days to dry before being used in any further experiments.

A group of coated PEEK samples were further heated in a vacuum oven (Lindberg Blue M, Thermo Scientific) for 6 days at a temperature of
100 °C to create gray selenium coated PEEK. The samples were turned over after 3 days to ensure uniform heating on both sides. Both gray and red selenium coated PEEK medical devices were left at room temperature for more than six months to see whether there was any color change. The uncoated PEEK samples that were used as controls in the following experiments or tests were prepared by soaking PEEK in the mixture of glutathione and sodium selenite, but sodium hydroxide was not added into the mixture so that the reaction did not occur. Then, the uncoated PEEK samples were treated using the same procedure as described above for the selenium coated PEEK.

4.2.2 SEM and EDS of PEEK with Selenium Coatings

In order to determine the size, coverage and distribution of the coated selenium nanoparticles, the surfaces of selenium coated PEEK were characterized by scanning electron microscope (SEM) using a HITACHI S-4800 field emission scanning electron microscope (Hitachi High Technologies America, Inc., Schaumburg, IL). Before scanning the surface of PEEK using SEM, the samples were coated with a 5 nm platinum layer using a sputter coater (Cressington 208 HR, Cressington
Scientific Instruments Ltd., Watford, United Kingdom) to make the PEEK samples conductive.

Energy-dispersive X-ray spectroscopy (EDS) was used to measure the elemental composition on the surface of the PEEK samples after coating. The EDS instrument was operated based on the SEM system mentioned above and an acceleration voltage of 20 kV was used in the measurements. The measurements were taken at various spots on the PEEK surface (both with and without nanosized selenium coverage).

4.2.3 Contact Angle Measurement

Water contact angles on the PEEK surfaces before and after being coated with selenium nanoparticles were measured using a Pioneer 800 Contact Angle Measurement System (Surface Electro Optics Co., Ltd., South Korea). The pictures were taken and the contact angles were analyzed automatically by the Surfaceware 8 program. The height between the needle and the surface was 1.7 mm and the volume of the water drop was 15 μL. The contact angle was measured 5 seconds after the drop was
placed on the surface and the tests were conducted at a temperature of 20 °C.

4.2.4 Bacterial Inhibitory Tests

Bacterial inhibition tests using crystal violet assays were conducted to evaluate the growth of bacteria on selenium coated and uncoated PEEK medical devices. The bacteria cell line, *Pseudomonas aeruginosa* (catalog number 27853), was obtained in freeze-dried form from the American Type Culture Collection (Manassas, VA). The bacteria strain was propagated in 30 mg/mL tryptic soy broth (TSB) for 20 hours. A bacteria solution of *P. aeruginosa* was prepared using 0.3 mg/mL TSB at a concentration of $10^6$ bacteria/ml, which was assessed by measuring the optical density of the bacterial solution using a standard curve correlating optical densities and bacterial concentrations. The optical densities were measured at 562nm using a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA).

The PEEK samples with or without selenium coatings were rinsed in separate Petri dishes with 75% ethanol for 20 minutes for sterilization
purposes and were left in the sterile petri dishes for 30 minutes to dry them completely. Then, the samples were transferred to a 24-well plate and rinsed with sterile TSB (30 mg/mL) to remove any possible ethanol residue. Each sample was treated separately with 1 mL of the prepared bacterial solutions (10⁶ bacteria/ml) and they were cultured for either 24, 48 or 72 hours in an incubator (37°C, humidified, 5% CO₂). For those samples that were cultured for 48 and 72 hours, the media was changed with 1 mL of sterile and fresh TSB every 24 hours. After the treatment, the solution in each well was removed and each sample was rinsed with a PBS (phosphate buffered saline) solution three times, then the samples were transferred into a new 24-well plate.

After turning off the light in the laminar flow hood, 0.5 mL of a 0.1% crystal violet solution was added into each well and incubated for 15 minutes to stain the bacterial biofilm formed on the PEEK samples. The remaining staining solution was removed and each sample was washed with sterile PBS extensively (rinsing with 1 mL PBS three times) to remove any access crystal violet. The plate was air dried at room temperature for one day to let the stained samples dry completely. Then, l
mL of 100% ethanol was added into each well to dissolve the crystal violet dye. After letting the well plate sit for 2 minutes, the solution from each well was transferred into a 96 well plate and the optical density was measured at 562 nm using a SpectraMax M5 plate reader.

In the bacterial inhibition tests, the treatment of each sample was conducted individually and identically. Another group of coated and uncoated PEEK samples were included as blank controls. They were treated with a sterile TSB solution without bacteria and were also stained using the same process as described above. The optical density of the blank control group was deducted from the optical density of bacteria treated PEEK samples to calculate the absorbency from the bacterial biofilm formed on PEEK surfaces. The growth of bacterial biofilm was quantified based on the absorbency.

4.2.5 Statistics

All bacterial inhibitory tests were conducted in triplicate and repeated three times. Data were collected and the significant differences were assessed with the probability associated with a one-tailed Student's t-test.
Statistical analyses were performed using Microsoft Excel (Redmond, WA).

4.3 Results and Discussion

4.3.1 PEEK Coated with Selenium Nanoparticles

After the coating process as described in 4.2.1, the color of the PEEK samples changed from light tan (Figure 4-1, image a) to light red (Figure 4-1, image b). The red color indicated the formation of selenium nanoparticles on the surfaces of PEEK. The selenium coated PEEK could maintain its red appearance for several months at room temperature. After heated at 100 °C for 6 days, the red PEEK in image b turned into the gray PEEK as shown in image c in Figure 4-1. During the heat treatment, a gray color gradually appeared as the red color decayed on the PEEK surface. The color changed from red to gray indicating the formation of gray selenium on the PEEK surfaces, because the gray selenium allotrope is the most stable form of selenium and can be formed by the mild heating of the red allotrope. Both red and gray PEEK could retain its color for at least six months at room temperature.
Figure 4-1. Light images of (a) uncoated PEEK, (b) PEEK coated with selenium nanoparticles (red coated PEEK), and (c) PEEK coated with selenium nanoparticles and with heating treatment (gray coated PEEK).

4.3.2 SEM and EDS of PEEK

As observed form the SEM image (Figure 4-2, image b) of red coated PEEK, the PEEK surface was coated with a large amount of selenium nanoparticles. These nanoparticles were well distributed on the PEEK surface and covered almost all parts of the polymer surface. The diameters of the coated nanoparticles were measured and the size
distribution was found to be between 50 and 100 nm. Image a in Figure 4-2 was the surface of a PEEK sample without coatings, onto which no nanoparticle was observed. As shown the SEM image (image c in Figure 4-2) of gray coated PEEK (formed by heating red coated PEEK at 100 °C for 6 days), there were rod-shaped selenium clusters observed on the surface and all of the selenium nanoparticles from the red coated PEEK (image b) disappeared. The length of these nanorods was from 1000 nm to 3000 nm and the width was from 100 nm to 200 nm.
Figure 4-2. Scanning electron microscopy images of: (a) uncoated PEEK,
(b) PEEK coated with selenium nanoparticles, (c) PEEK coated with selenium nanoparticles and with a heating treatment. Magnification = 10.0 k and acceleration voltage = 5.0 kV.

According to the light images (Figure 4-1) and SEM images (Figure 4-2) of red selenium coated PEEK before and after the heat treatment, it is likely that the red selenium nanoparticles were transferred into gray selenium nanorods during the heat treatment. In order to confirm that both attached nanoparticles and nanorods were consist of selenium element, EDS analyses were taken on various spots on the PEEK surfaces. As shown in Figure 4-3 (a), a strong peak in the spectra demonstrated the existence of selenium element on the spot with a nanoparticle attached. But on a blank spot in Figure 4-3 (b), no selenium element was detected. Thus, the nanoparticles attached on the PEEK surfaces were made of selenium element. Similar peaks were also observed in Figure 4-3 (c) and (d) and the only difference from spectra (c) to spectra (d) is the disappearance of the selenium peak, which indicated that the gray nanorodes formed by heating the red selenium nanoparticles
Figure 4-3. Energy-dispersive X-ray spectroscopic analyses of (a) a spot with visualization of nanoparticle on red PEEK, (b) a blank area without nanoparticle on red PEEK, (c) a spot with visualization of nanorodes on gray PEEK, and (d) a blank area without nanorodes on gray PEEK.

4.3.3 Contact Angles

The results of water contact angle measurements of on the three types of PEEK surfaces were shown in Figure 4-4 and Table 4-1 below. The water contact angle on the uncoated PEEK was 68.47°. The red nanoparticles coated and gray nanorodes coated PEEK had a contact angle of 78.14° and 76.98°, respectively. Both were larger than the uncoated PEEK, which indicated that the PEEK surface became more hydrophobic after coated with selenium nanoparticles or nanorodes. The contact angle of red selenium coated PEEK and gray selenium coated PEEK was similar. Thus, there was not a significant change in the hydrophilicity of the selenium coated PEEK after the coated red selenium nanoparticles was heated and transferred into gray selenium nanorodes.
Figure 4-4. Contact angle measurements of (a) uncoated PEEK, (b) red selenium nanoparticles coated PEEK, and (c) gray selenium nanorodes coated PEEK.
Table 4-1. Contact angles of three types of PEEK samples.

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Uncoated PEEK</th>
<th>Red PEEK</th>
<th>Gray PEEK</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>68.72°</td>
<td>76.78°</td>
<td>77.26°</td>
</tr>
<tr>
<td>2</td>
<td>67.10°</td>
<td>78.78°</td>
<td>77.78°</td>
</tr>
<tr>
<td>3</td>
<td>69.59°</td>
<td>78.87°</td>
<td>75.91°</td>
</tr>
<tr>
<td>Average</td>
<td>68.47°±1.26°</td>
<td>78.14°±1.18°</td>
<td>76.98±0.97°</td>
</tr>
</tbody>
</table>

4.3.3 Bacterial Inhibitory Tests

As seen in Figure 4-4, the results of bacteria assays involving *Pseudomonas aeruginosa* (*P. aeruginosa*) showed that either red selenium nanoparticles coated paper towels or gray selenium nanorodes coated paper towels had a high effectiveness at inhibiting bacterial biofilm growth from 1 day to 3 days. The crystal violet absorbance in the graph was the optical density of crystal violet from the bacteria treated PEEK sample deducted by the optical density of crystal violet from the blank control group treated with sterile tryptic soy broth. In first day of the treatment, the biofilm was at the stage of initial attachment and it took time for the bacteria to adapt to the microenvironment on the surfaces.
There was not too much biofilm attached on all 3 types of PEEK surfaces, but a larger biofilm mass was still detected on the uncoated PEEK than red nanoparticles or gray nanorodes coated PEEK. From 1 day to 2 days, the amount of the biofilm increased quickly for all the PEEK samples, which indicated the biofilm began to mature and bacteria propagated rapidly in the biofilm. After the second day, there was significantly more biofilm attached on the uncoated PEEK than the red selenium or gray selenium coated PEEK. From 2 days to 3 days, the biofilm was almost saturated by bacteria and there were not large increases in the total amount of the biofilm. However, in the third day of the treatment, the total amount of biofilm increased more on the surface of uncoated PEEK than the two selenium coated PEEK, so after the third day, the red and gray PEEK revealed more significant inhibitions to the growth of *P. aeruginosa* biofilm than the inhibition after the second day. Besides, no significant difference in the biofilm growth was observed between the red selenium nanoparticles coated PEEK and the gray selenium nanorodes coated PEEK at the first day, while after three days, the red PEEK showed a little higher inhibition to the biofilm growth. The reason could be that there were more nano-scale features on the red PEEK than the
gray PEEK, which might result in a more active interaction between the coated nanoparticles and the bacterial cells. Overall, both types of nanosized selenium coated PEEK successfully inhibited the formation of *P. aeruginosa* biofilm compared with PEEK without selenium coating.

![Graph](image.png)

**Figure 4-5.** The growth of *P. aeruginosa* biofilm on red selenium coated PEEK, gray selenium coated PEEK and uncoated PEEK. Data=mean ± standard deviation, n=3; *p<0.05 compared with red PEEK after treatment of the same time period. **p<0.05 compared with gray PEEK after treatment of the same time period.

As mentioned previously, *P. aeruginosa* are gram-negative bacteria that have an extra layer of bacterial outer membrane as a part of their cell wall,
which results in that it might take longer for selenium element or selenium nanoclusters to penetrate into and interact with *P. aeruginosa*. Thus, it may be expected that the red nanoparticles or gray nanorodes coatings on the surface of the PEEK will show a stronger inhibition to gram-positive bacteria, like *S. aureus*. Further bacterial inhibitory tests on PEEK medical devices involving other bacteria stains will be helpful to explore more of the antibacterial properties of red or gray selenium coated PEEK.

In addition to preventing bacterial infections on PEEK, red selenium nanoparticle coatings demonstrated improved healthy bone cell (osteoblast) growth while inhibiting cancerous bone cell (osteosarcoma) growth.\(^{19,30}\) Selenium is also an important component for selenoproteins in the human body that were found to be fundamental for the human antioxidant defense system\(^{31}\) and were recently proven to be an essential transporter for generating healthy bone.\(^{32}\) Therefore, coating PEEK with red selenium nanoparticles may potentially improve its antibacterial without being toxic to healthy cells (especially bone cells), which are both of vital importance for PEEK, especially PEEK medical devices.
used in the design for orthopedic and spinal devices.

### 4.4 Conclusions

In conclusion, red selenium nanoparticles were synthesized and coated on the surface of PEEK medical devices here. By heating the coated PEEK samples, the red selenium nanoparticles were successfully transferred to gray selenium nanorods, which were shown to have antibacterial properties for the first time here. Both red and gray coatings on PEEK medical devices were stable for at least six months at room temperature. Importantly, compared with the PEEK without selenium coatings, the red or gray selenium coated PEEK significantly inhibited the growth of *P. aeruginosa* after 1, 2 and 3 days. The inhibition of bacterial biofilm growth was more and more significant from 1 to 3 days. In addition, the red or gray selenium coatings on the PEEK surfaces decreased the hydrophilicity of the PEEK medical devices, which might play an important role in inhibiting the growth of a bacterial biofilm. Therefore, this study suggested that coating PEEK medical devices with red selenium nanoparticles or gray selenium nanorods might be an effective way to decrease the growth of bacterial biofilm on those medical devices.
which could have potential applications for preventing infections on various medical devices used in healthcare environments. Moreover, coupled with previous studies that have demonstrated that red selenium increases healthy bone cell growth, the present results demonstrate the promise of red selenium nanoparticle coatings on PEEK for numerous orthopedic and spinal applications.

4.5 REFERENCES


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28. Zhang JS, Gao XY, Zhang LD, Bao YP. Biological effects of a


Chapter 5
Antibacterial Selenium Quantum Dots and Nanoparticles Produced by UV/VIS/NIR Pulsed Nanosecond Laser Ablation

5.1 Introduction
Selenium has been defined by the Materials Research Society and the American Physical Society as an energy critical element meaning that this element is required for emerging sustainable energy sources that might encounter supply disruptions\(^1,2\). Selenium belongs to the oxygen family (group 16 in the periodic table), it is then not oxidized in air and insoluble in water. It is a p-type semiconductor having a direct energy bandgap around 1.7 eV, it is therefore used in CuInSe\(_2\) (CIS) solar cells\(^3\) and quantum dot (CdSe) solar cells\(^4\). Moreover, selenium has also very interesting biological assets, as anti-cancer\(^5,6\) and anti-bacterial properties\(^7,8\). The goal of this chapter is to report the synthesis of selenium by pulsed laser ablation in liquids (PLAL) and characterization of very pure selenium nanoparticles and quantum dots that can be use either for photovoltaic or biological applications.
The ability to produce nanoparticles free of any surface contamination is very challenging especially for bio-medical applications. The advantages of PLAL synthesis compared to the other conventional methods are the simplicity of the set-up and the surface purity of the synthesized nanoparticles (absence of unnecessary adducts and byproducts). Indeed, selenium nanoparticles are generally synthesized by chemical methods. Only three papers in the literature report the synthesis of selenium nanoparticles by pulsed laser ablation in liquids. In 2010, Singh et al. used a Nd:YAG laser at 1064 nm, 10 ns pulse duration, 10 Hz repetition rate. In 2012, Kuzmin et al. used a copper vapor laser at 511 nm and 578 nm, 15 ns pulse duration, 15 kHz repetition rate. In 2013, Van Overschelde et al. used a KrF excimer laser at 248 nm, 6 ns pulse duration, 50 Hz repetition rate. They all used water as solvent and irradiated selenium powder, ingot and pellet respectively.

In this chapter, selenium powders were irradiated using three different wavelengths, a near-infra-red wavelength at 1064 nm (fundamental beam, $\lambda$), a visible wavelength at 532 nm (second harmonic beam, $\lambda/2$) and an ultra-violet wavelength at 355 nm (third harmonic beam, $\lambda/3$), all issued
from the same Nd:YAG laser source. Their effects on the particle size distribution were studied by dynamic light scattering and transmission electronic microscopy, revealing then the production of selenium quantum dots (size < 4 nm) by photo-fragmentation. It was found that the crystallinity of the nanoparticles depends on their size. The zeta-potential measurement revealed that the colloidal solutions produced in de-ionized water were stable while the ones synthesized in ethanol agglomerate. The concentration of selenium was measured using ICP-MS. The antibacterial effect of selenium nanostructures were analyzed on *E. Coli* bacteria. Finally, selenium quantum dots produced by this method could also be useful for quantum dots solar cells.

**5.2 Experiments and Results**

**5.2.1 Preparation of Selenium Nanoparticles by Ablation**

Selenium is known to have several different allotropic structures: trigonal, monoclinic (α-, β-), cubic (α-, β-), rhombohedral, orthorhombic and amorphous\(^\text{16}\); however, the most common allotropic phases of selenium are trigonal, α-monoclinic and amorphous. Raman spectroscopy and X-Ray Diffraction were performed on a Horiba-Yvon Jobin spectrometer
(iHR 320) using a red laser at 785 nm, and on a Rigaku Ultima IV
diffractometer working at 40 kV, 44 mA, respectively. Results of both the
Raman spectroscopy and X-Ray Diffraction on the pure selenium powder
(99.999%, ~200 mesh) purchased from Alfa Aesar reveals the trigonal
(hexagonal) structure of selenium.

The experimental setting for ablation is shown in Figure 5-1. Pure
selenium powder has been placed at the bottom of the glass cuvette and
then filled with the liquid (de-ionized water or ethanol 99.5%) until the
top of the cuvette (~4 ml). As the selenium’s density (4.28 g/cm$^3$) is
higher than the one of water (1g/cm$^3$) and the one of ethanol (0.79 g/cm$^3$),
selenium is then found at the bottom of the cuvette, requiring a magnetic
stirrer to mix selenium with the solvent. A magnetic stirrer is used at 1000
rpm/minute to assure a uniform dispersion of selenium powder into the
solution during the irradiation. The laser beam is then focused in the
middle of the solution. The laser used in this experiment is a Nd:YAG
laser from EKSPLA NT342B with a pulse duration of 3.6 ns and a
repetition rate of 10 Hz, each pulse having a top hat profile. Energy of the
laser was monitored during all the duration of the experiment and kept
constant at ~23±1 mJ/pulse. Upon focusing this pulse energy corresponds to a fluence of ~2 J/cm².

**Figure 5-1.** Diagram of generating selenium nanoparticles by ablation.

During the first set of irradiations, the solution was irradiated at a given wavelength over a 15 minute interval at a 10Hz repetition rate. A red-brick color solution was produced either in water or ethanol, indicating the presence of amorphous nanoparticles as already noted by Ref.15; while the solution becomes colorless after centrifugation at 10000 rpm for 10 minutes leaving in suspension only the smallest nanoparticles.

**5.2.2 Characterization of Selenium Nanoparticles**

From Transmission Electronic Microscope (TEM) observations, carried
out on a JEOL 2010F microscope operating at 200 kV, the diffraction pattern of the biggest nanoparticles shows an amorphous structure while the smallest ones exhibit a crystalline structure, as shown in Figure 5-2. This means that the nanoparticles of selenium grow so quickly after the irradiation pulse that the vast majority of them do not have time to crystallize due to the rapid quenching led by the surrounding liquid media. Then, most of these big particles settled down progressively by gravity after the stop of the magnetic stirrer and for sure after the centrifugation treatment.

Figure 5-2. TEM images and diffraction pattern of selenium
nanoparticles after the first set of irradiation at 532 nm in de-ionized water, before any centrifugation and any filtration. a) Spherical particle around 300 nm, b) Spherical particle around ~150 nm, c) Spherical particle around 80 nm, d), e) and f) are the diffraction pattern of image a), b) and c) respectively.

To refine the size distribution of the selenium nanoparticles produced, the solutions were also filtrated through a 200 nm pore size filter to be easily measurable by Dynamic Light Scattering (Zetasizer Nano ZS from Malvern Instruments). The particle size distributions of the selenium colloidal solutions measured by DLS in de-ionized water and ethanol are shown on Figures 5-2a and 5-2b after the first set of irradiations. The particles sizes synthesized in de-ionized water are all around ~100 nm whatever the wavelength used, except for the infra-red irradiation where smaller nanoparticles (~20 nm) are also produced. In ethanol, the situation is quite different, very small particles (<10 nm) and big nanoparticles (~ 200nm) are produced at the same time. The polydispersity is then much higher in ethanol compared to de-ionized water.
Figure 5-3. DLS spectra of the centrifuged and filtrated colloidal solution of selenium nanoparticles after the first and second set of irradiations (time=15 min, f=10Hz) in de-ionized water and ethanol. a) first set of irradiations in DI water; b) first set of irradiations in ethanol; c) second set of irradiations in DI water; d) second set of irradiations in ethanol. The solution was centrifuged at 10000 rpm during 10 minutes after each set of irradiations (time=15 min, f=10Hz) and then filtrated through a 200 nm pore size filter. Then, the solution was analyzed by DLS. The insets indicate the zeta potential of each sample.
The stability of the colloidal solutions was evaluated by the measurement of the zeta potential, which is the electrical potential surrounding the particle. It is clear from the insets of Figure 5-3 that the particles synthesized in de-ionized water are stable while the ones in ethanol are not and tend to flocculate. Indeed, the zeta potential indicates the degree of repulsion between adjacent particles. If the zeta potential is higher than 30 mV, then the solution is considered as stable which is mostly the case in de-ionized water except for the solutions synthesized with the infra-red wavelength. At the contrary, if the zeta potential has a value below 30 mV, the solution is unstable and the particles flocculate which is the case in ethanol. Due to flocculation, the particle size distributions obtained in ethanol are more poly-dispersed compared to the ones obtained in de-ionized water (Figure 5-3). From TEM images carried out at low magnification on a JEOL 1230 microscope operating at 120 kV (as shown in Figure 5-4), we clearly see the difference between the two solvents, nanoparticles are quite dispersed in water while they flocculate in ethanol and form a cluster of particles which is detected by the DLS as the size distribution around 200 nm (Figure 5-3b). The instability of the nanoparticles synthesized in ethanol has already been noted by Werner et
who investigated the synthesis of silver nanoparticles in different primary alcohols.

Figure 5-4. TEM images of selenium nanoparticles obtained in a) de-ionized water and b) ethanol.

Table 5-1. Summary of the laser parameters used, the corresponding size and zeta potential of selenium nanoparticles obtained. The energy at all wavelengths has been fixed to \( \sim 23 \pm 1 \text{ mJ/pulse} \) corresponding to a fluence of \( \sim 2 \text{ J/cm}^2 \) and the laser repetition rate was 10 Hz.

<p>| Liquid | Samples* | Wavelength (nm) | Size determined by DLS (nm) | Zeta potential determined by DLS |</p>
<table>
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<tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td>(mV)</td>
<td></td>
</tr>
<tr>
<td>DI water 1a</td>
<td>355</td>
<td>98±13</td>
<td>-40±12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2a</td>
<td>532</td>
<td>118±15</td>
<td>-30±10</td>
</tr>
<tr>
<td></td>
<td>3a</td>
<td>1064</td>
<td>148±32</td>
<td>-12±5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(83%)</td>
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<td></td>
<td></td>
<td></td>
<td>22±3 (17%)</td>
<td></td>
</tr>
<tr>
<td>DI water 1b</td>
<td>355</td>
<td>64±9</td>
<td>-24±6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2b</td>
<td>532</td>
<td>62±12</td>
<td>-26±7</td>
</tr>
<tr>
<td></td>
<td>3b</td>
<td>1064</td>
<td>107±3</td>
<td>-16±7</td>
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<tr>
<td>Ethanol 4a</td>
<td>355</td>
<td>183±16</td>
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<td></td>
<td></td>
<td></td>
<td>(51%)</td>
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<td></td>
<td></td>
<td></td>
<td>2.4±0.3</td>
<td>(27%)</td>
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<td>0.6±0.1</td>
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<td>6a</td>
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<td>209±29</td>
<td>42%</td>
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<td></td>
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<td>3.2±0.5</td>
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<td>0.7±0.1</td>
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<tr>
<td>Ethanol</td>
<td>4b</td>
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<td>178±13</td>
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<td></td>
<td></td>
<td></td>
<td>83%</td>
<td></td>
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<td></td>
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<td>2±17 (17%)</td>
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<td>532</td>
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<td></td>
<td>94%</td>
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<td></td>
<td>0.7±0.1 (6%)</td>
<td></td>
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<tr>
<td></td>
<td>6b</td>
<td>1064</td>
<td>196±38</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>-2±15</td>
<td></td>
</tr>
</tbody>
</table>

*“a” means first set of irradiation i.e. these solutions have been irradiated during 15 minutes and then centrifuged during 10 minutes at 10000 rpm and finally filtrated through a 200 nm pore size filter, “b” means
secondary set of irradiations i.e. these solutions have been irradiated a first time during 15 minutes and then centrifuged during 10 minutes at 10000 rpm and irradiated again during 15 minutes and centrifuged a last time during 10 minutes at 10000 rpm and finally filtrated through a 200 nm pore size filter.

5.2.3 Generation of Selenium Quantum Dots

To reduce the polydispersity, the solutions were irradiated a second time for 15 minutes at 10 Hz and then centrifuged again at 10000 rpm for 10 minutes and filtrated through a 200 nm pore size filter. From Figure 5-3c, the smallest nanoparticles (~60 nm) synthesized in de-ionized water were obtained with the UV and visible wavelengths. This can be explained by photo-fragmentation, selenium absorbs below ~730 nm, i.e. the UV and visible wavelengths used in our experiment, and is therefore photo-fragmented; while the synthesis using the NIR wavelength only produced nanoparticles by thermal ablation. Therefore, the particle size decreases with decreasing wavelength. From Figure 5-3d, the particles synthesized in ethanol are poly-dispersed whatever the wavelength used. Furthermore, they are also bigger compared to the ones synthesized in
water except for the UV and visible wavelength where a significant percentage (~17% and 6%, respectively) of particles below 3 nm are produced. These nanoparticles are quantum dots of selenium; indeed, the exact Bohr radius of selenium has been calculate to be around 1.16 nm\textsuperscript{18} and experimentally the quantum confinement limit has been determined around 3.75 ± 0.15 nm\textsuperscript{13} by the shift of the surface plasmon resonance peak. Actually, a strong blue shift occurs on this peak below the confinement limit. This is the first time in the literature that selenium quantum dots have been synthesized using ultra-violet and visible wavelengths. The synthesis of selenium quantum dots by infra-red wavelength was already reported in 2010 by Singh \textit{et al.}\textsuperscript{13}.

High Resolution Transmission Electron Microscopy (HRTEM) experiments were also carried out on a JEOL 2010F microscope operating at 200 kV. Quantum dots are efficiently synthesized in water and ethanol by using the UV and visible wavelength (Figure 5-5). Indeed, the laser mechanism inducing size reduction can be either thermal ablation or photo-fragmentation depending on the irradiation wavelength. For UV and visible wavelengths, the size reduction of selenium particles
is due to photo-fragmentation while for NIR wavelength, it is due to thermal ablation. From the diffraction patterns shown in Figure 5-5 as insets, the quantum dots are crystalline and adopt the trigonal (hexagonal) structure of selenium. Therefore, the crystal structure of the initial powder is conserved even after the irradiation.

Figure 5-5. HRTEM images of selenium quantum dots synthesized
during the second set of irradiation by a) UV irradiation in de-ionized water, b) visible irradiation in de-ionized water, c) UV irradiation in ethanol and d) visible irradiation in ethanol. In the right upper corner, as an inset, the FFT pattern of the quantum dots is shown.

5.2.4 Antibacterial Tests
The antibacterial test was conducted on *E. Coli* bacteria by using the most stable and less dispersed selenium nanoparticles, obtained after the second irradiation in de-ionized water (samples 1b, 2b, 3b). The samples used the antibacterial test had to be the same concentration. To measure the selenium concentrations of the prepared samples, 1 mL of each solution was dissolved using 10 M NaOH and then diluted 100 times before ICP (Bruker Aurora M90) measurements. The concentrations of samples 1b, 2b are 2.1 ppm and 1.35 ppm, respectively, while the concentration of sample 3b was undetectable. Therefore, sample 3b was not considered for the antibacterial test and sample 1b was diluted to the same concentration as sample 2b. A red selenium nanoparticle sample that previously showed significant inhibition to bacterial growth\textsuperscript{19,20} was also included in the bacterial tests as a comparison. These samples have
been sterilized by UV light irradiation. Then, 0.02 ml of each solution was mixed with 0.18 ml of bacteria solution (E. coli, $10^6$ bacteria/ml) in 96-well plates. Incubation was running for either 4, 8 or 24 hours. The bacteria numbers were calculated by measuring the absorbency curves of the samples registered at 562 nm and compared with the standard absorbency curve of E. coli. As shown in Figure 5-6 below, all selenium samples show significant inhibition to the growth of E. coli after 8 hours and 24 hours. The percentages of inhibited bacterial growth were not very high, and the reason could be that the samples prepared and used in the bacterial treatment had a very low concentration (only 0.135 ppm), which is much lower than the concentrations of selenium used in the bacterial assays in the chapter 3. Even though, the selenium nanoparticles and quantum dots produced by laser ablation showed promising properties to prevent bacterial growth.
Figure 5-6. The growth of *E. coli* with a treatment of selenium nanoparticles. Data=Mean ± standard deviation, n=3; *p<0.05 compared with the control group (DI water) after either 4, 8 or 24 hours.

5.3 Conclusions

In conclusion, nanosecond laser irradiation provides enough energy density (~2 J/cm²) to melt selenium powder whatever the wavelength used. Size-reduction efficiency in PLAL increases by decreasing wavelength because of the increase in photon energy. Amorphization or crystallization of the molten selenium will depend on the size of the particle. Nanoparticles with size above ~300 nm are amorphous while
below ~ 80 nm they are crystalline. Thus, contrary to what is generally believed, the nanoparticles produced by laser ablation in liquids are not only amorphous but exhibit a size-dependent crystallinity behavior. The centrifugation process and the second set of irradiations are an essential part of the synthesis process to obtain a well-defined mono-dispersed size distribution. In terms of stability, de-ionized water has to be preferred as solvent compared to ethanol due to agglomeration. Quantum dots have also been obtained by irradiating selenium powder in de-ionized water and ethanol using the ultra-violet or visible wavelength. Furthermore, quantum dots adopt the trigonal structure of selenium. To conclude, selenium nanoparticles can be used for various biomedical applications, including antibacterial applications, while selenium quantum dots can be used for optoelectronics applications like for example in quantum dot solar cells.

5.3 References


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Chapter 6

Inhibited Growth of *Pseudomonas aeruginosa* by Dextran and Polyacrylic acid Coated Ceria Nanoparticles

6.1 Introduction

Among rare earth elements (the fifteen lanthanides along with scandium and yttrium), cerium is the most abundant element at 66.5 ppm in the Earth’s crust compared to that of copper (60 ppm) or tin (2.3 ppm).\(^1,2\) As a highly abundant material, ceria (or cerium oxide, \(\text{CeO}_2\)) is technologically important due to its wide-range of applications, for example in catalysts for the elimination of toxic auto-exhaust gases,\(^3,4\) oxygen sensors,\(^5,6\) fuel cells,\(^7,9\) electrochromic thin-films\(^10,11\) and so on. Upon the transition from a macro-sized particle into a nanocrystalline particle, ceria significantly changes its physicochemical properties to usually possess a high density of nanocrystalline ceria interfaces.\(^12\) Thus, nanostructured ceria has attracted extensive attention in such applications due to improvements in redox properties, transport properties, and high surface to volume ratios.
However, although ceria nanoparticles have been studied for numerous applications in traditional science and engineering, there have been almost no studies regarding the potential biomedical applications of ceria until recently when researchers demonstrated that ceria nanoparticles possess antioxidant properties at physiological pH values. The ability of these nanoparticles to act as an antioxidant lies in their ability to reversibly switch from Ce$^{3+}$ to Ce$^{4+}$. In recent studies, this antioxidative ability enables the application of ceria nanoparticles to protect against radiation damage, oxidative stress and inflammation. For example, Tsai et al. reported that free radical scavenging can be achieved in murine insulinoma βTC-tet cells by the introduction of crystalline ceria nanoparticles.

Moreover, in previous studies, due to safety concerns for biomedical applications, the toxicity of ZnO, CeO$_2$ (ceria) and TiO$_2$ was compared based on dissolution and oxidative stress properties. The results showed that ceria nanoparticles can suppress the generation of ROS (reactive oxygen species) production and induce cellular resistance to an
exogenous source of oxidative stress to protect cells from oxidant injury. However, to further minimize the potential toxicity of ceria nanoparticles, the synthesis of biocompatible dextran-coated ceria nanoparticles and its enhanced stability in aqueous solution was also reported.\textsuperscript{15}

To build off of these exciting medical applications of ceria nanoparticles, the present study investigated for the first time the influence of ceria nanoparticles on bacteria growth. \textit{Pseudomonas aeruginosa} (\textit{P. aeruginosa}) is an aerobic Gram-negative bacterium that is an important cause of various infections especially in hospitals. Hospitalized patients may be colonized with \textit{P. aeruginosa} on admission or during their hospital stay and \textit{P. aeruginosa} can be isolated from nearly any conceivable source within hospitals.\textsuperscript{20,21} It counts for 11-13.8\% of all nosocomial infections when a microbiological isolate is identifiable.\textsuperscript{22} Infections caused by \textit{P. aeruginosa} are not only common,\textsuperscript{23,24} but are also associated with high morbidity and mortality when compared with other bacterial pathogens.\textsuperscript{25,26} An additional concern involves the increased antimicrobial resistance of nosocomial \textit{P. aeruginosa} isolates.\textsuperscript{27} Even more troublesome, \textit{P. aeruginosa} attaches to biotic and abiotic surfaces
forming a biofilm. Once formed, the *P. aeruginosa* infections are much more difficult to treat due to the formation of exopolysaccharide (EPS). Biofilms of *P. aeruginosa* have been found on numerous medical devices including central venous catheters, contact lenses, mechanical heart valves, intrauterine devices, and indwelling urinary catheters.\(^{28,29}\) Thus, it is significant to develop active and biocompatible molecules (that do not rely on antibiotics that bacteria may develop a resistance towards) that kill bacteria at early stages of infections before biofilm formation.

Therefore, in this chapter, for the first time, we investigated the effectiveness of ceria nanoparticles on influencing/inhibiting bacterial growth (specifically, *P. aeruginosa*). The results showed that the ceria nanoparticles significantly inhibited the growth of *P. aeruginosa*, revealing a promising application of ceria nanoparticles to prevent bacterial infections that should be further explored.

### 6.2 Materials and Methods

#### 6.2.1 Synthesis and Characterization of Ceria Nanoparticles
The corresponding dextran or polyacrylic acid coated ceria nanoparticles were synthesized according to previously described procedures. Briefly, a solution of cerium (III) nitrate (1.0 M, Aldrich, 99%) in water (5.0 mL) was mixed with an aqueous solution of either polyacrylic acid (PAA, 0.5 M, Sigma) or dextran (10 kDa, 1.0 M, Sigma). The resulting mixtures were then added to an ammonium hydroxide solution (30.0 mL, 30 %, Sigma Aldrich) under continuous stirring for 24-h at room temperature. The preparation was then centrifuged at 4000 rpm for two 30-minute cycles to settle down any debris and large agglomerates. The supernatant solution was then purified from free polymers and other reagents and then concentrated using a 30K Amicon cell (Millipore Inc.). The final dextran and polyacrylic acid coated nanoceria preparation were stored at a 1.5 mg Ce/mL concentration in DI water until further used.

The various polymer-coated nanoceria preparations were characterized by dynamic light scattering to determine the nanoparticles size and zeta potential to determine nanoparticle's surface charge (Malvern DLS/Zetasizer). FT-IR experiments were performed on vacuum dried
samples to verify the surface functionalities on nanoparticles (Perkin Elmer Spectrum 100 FT-IR spectrometer).

6.2.2 Bacterial Assays

A bacteria cell line of *Pseudomonas aeruginosa* (*P. aeruginosa*) was obtained in freeze-dried form from the American Type Culture Collection (catalog number 27853; Manassas, VA, USA). The bacterial cells were propagated in 30 mg/mL tryptic soy broth (TSB) (catalog number 22092, Sigma-Aldrich, St. Louis, MO, USA) for 24 hours in an incubator (37°C, humidified, 5% carbon dioxide). When the second passage of bacteria reached its stationary phase, the second passage was frozen in one part TSB and one part 40% sterile glycerol. Before bacterial seeding, a sterile 10 μL loop was used to withdraw bacteria from the prepared frozen stock and streaked onto a TSB agar plate, and the TSB agar plate was incubated for 20 hours to form single bacterial colonies. Then, a single colony was collected using a sterile loop and was incubated in a 15 mL sterile test tube containing 3 mL of TSB for 20 hours. The bacterial solution was diluted to a concentration of $10^7$ bacteria/mL, which was assessed by measuring the optical density of the bacterial solution using a standard
curve correlating optical densities and bacterial concentrations. The optical densities were measured at 562 nm using a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA).

Two concentrations of either dextran or polyacrylic acid coated ceria nanoparticles were tested against bacterial growth. The solutions of ceria nanoparticles were mixed with bacterial solutions (10^7 bacteria/mL) at a 1:10 or 1:5 volume ratio and cultured for either 1, 6 or 24 hours in the incubator. The solutions of sterile TSB were mixed with bacterial solutions as blank controls. After the treatment, the solutions with bacteria were diluted with a PBS (catalog number P4417, Sigma-Aldrich, St. Louis, MO, USA) solution and were spread on agar plates and bacteria colonies were counted after 18 hours of incubation.

6.2.3 Statistics

Bacterial assays were conducted in triplicate and repeated three times. Data were collected and differences were assessed with the probability associated with a one-tailed Student's t-test. Statistical analyses were performed using Microsoft Excel 2010 (Microsoft Corp., Redmond, WA).
6.3 Results

6.3.1 Characterization of Ceria Nanoparticles

The ceria nanoparticle preparations used in this study were aqueous suspension of nanoparticles coated with either polyacrylic acid or dextran. The synthetic procedure used to make these nanoparticles is highly reproducible, yielding nanoparticles with a cerium oxide core of 3-4 nm by TEM as shown in Figure 6-1. Overall hydrodynamic diameter of the polyacrylic acid coated nanoceria was 5 nm while the size for dextran coated cerium oxide nanoparticles was 14 nm. XPS analysis showed the presence of Ce$^{3+}$ and Ce$^{4+}$ in the nanoceria preparation (Figure 6-1D). Confirmation of polymer coating was accessed by FT-IR as previously described$^{15,30,31}$ and by zeta potential, confirming the presence of a highly negative charged polymer coating for the polyacrylic acid coated ceria nanoparticles ($\zeta = -45$ mV) and a lower negative charge (close to neutral) for the dextran coated ceria nanoparticles ($\zeta = -2$ mV).
**Figure 6-1.** Characterization of dextran-coated ceria nanoparticles. A) TEM image with corresponding SAED pattern (inset). B) Representative HRTEM image of a single crystal. C) XRD image showing the presence of (111), (220), (311), and (331) planes, typical of an fcc crystal. D) XPS analysis showing the presence of Ce$^{+3}$ and Ce$^{+4}$ in the nanoceria preparation. E) DLS analysis showing a monodisperse nanoparticle preparation with an average hydrodynamic diameter of 10 nm. F) Photograph showing a 1) 10.0, 2) 20.0, 3) 30.0, and 4) 40.0mM aqueous solutions of dextran-coated nanoparticles.\textsuperscript{15}
6.3.2 Bacterial Assays

As shown in Figures 6-2 and 6-3, the ceria nanoparticles did not show significant inhibition to *P. aeruginosa* growth compared with TSB (tryptic soy broth) controls after 1 hour of treatment. However, after 6 hours of treatment, both concentrations of the dextran coated ceria nanoparticles significantly inhibited the growth of *P. aeruginosa* by about 20% compared with TSB controls (p < 0.05). The polyacrylic acid coated ceria nanoparticles showed significant inhibition of *P. aeruginosa* growth only for the higher concentration (bacterial solution:ceria nanoparticles = 5:1 volume ratio).
Figure 6-2. Inhibited *P. aeruginosa* growth in the presence of ceria nanoparticles at three time points (1, 6, and 24 hours). The volume ratio of bacterial solution (10^7 bacteria/mL) and ceria nanoparticles is 10:1. Data is represented as mean ± standard deviation, n=3; *p < 0.01 compared with the TSB (tryptic soy broth) control group after 24 hours of treatment. **p < 0.05 compared with the TSB (tryptic soy broth) control group after 6 hours of treatment. Dex = dextran and PAA = polyacrylic acid.

![Graph showing bacterial growth inhibition](image)

**Figure 6-3.** Inhibited *P. aeruginosa* growth in the presence of ceria nanoparticles at three time points (1, 6, and 24 hours). The volume ratio...
of bacterial solution (10⁷ bacteria/mL) and ceria nanoparticles is 5:1. Data is represented as mean ± standard deviation, n=3; *p < 0.01 compared with the TSB (tryptic soy broth) control group after 24 hours of treatment. **p < 0.05 compared with the TSB (tryptic soy broth) control group after 6 hours of treatment. Dex = dextran and PAA = polyacrylic acid.

Most importantly, the growth of *P. aeruginosa* was strongly inhibited in the presence of dextran or polyacrylic acid coated ceria nanoparticles after 24 hours compared with TSB controls (p < 0.01). When the volume ratio of bacterial solution to ceria nanoparticles was 10:1, *P. aeruginosa* growth was inhibited by 40.12% and 26.88% in the presence of dextran and polyacrylic acid coated ceria nanoparticles, respectively, compared with the TSB controls. When the concentration of ceria nanoparticles doubled, the inhibition of *P. aeruginosa* growth reached 55.41% and 36.44% for dextran and polyacrylic acid coated ceria nanoparticles, respectively. Critically, all of this inhibition occurred without the use of antibiotics, only when using the ceria nanoparticles themselves.

Based on the results of bacterial assays, the inhibition to *P. aeruginosa*
growth by ceria nanoparticles was more significant as treatment time increased to 24 hours. In addition, the dextran coated ceria nanoparticles showed stronger inhibition to bacterial growth than that of polyacrylic acid coated ceria nanoparticles, especially after treatment for 6 and 24 hours. One possible reason for dextran coating and PAA coating could affect the bacterial growth differently is that the size of dextran coated ceria nanoparticles is 14 nm and the size of PAA coated ceria nanoparticles is 5 nm, which results in that they interact with bacterial cells differently. Moreover, as expected, from 6 hours to 24 hours, the bacteria propagated quickly in the TSB control, but the bacteria numbers decreased even more during this time period for the treatment with both kinds of ceria nanoparticles.

Importantly, the mechanism of how ceria nanoparticles prevent bacterial growth remains unexplored. Since ceria nanoparticles have been shown to suppress the generation of ROS (reactive oxygen species)\textsuperscript{19}, its antibacterial properties demonstrated here for the first time could be different from many other nanoparticles (such as zinc oxide nanoparticles, silver nanoparticles and so on) that are believed to kill bacterial mainly
through the generation of ROS.

Although requiring additional investigation, the possible mechanism for the antibacterial action of the coated CeO$_2$ nanoparticles formulated here could be that the dextran and PAA coatings interacted with specific proteins on the bacterial cell membranes to alter the permeability of the cell membranes, thus, killing the bacterial cells. Moreover, it is possible that the dextran or PAA coated CeO$_2$ nanoparticles studied here may have promoted the adsorption, from the TSB, of proteins that are known to inhibit bacteria growth (such as fibronectin, mucin, vitronectin, etc.).

Future studies should also investigate how universal such bacterial inhibition is, by exposing such coated CeO$_2$ nanoparticles to other bacteria (such as Staphylococcus aureus and Escherichia coli).

### 6.4 Conclusions

In conclusion, the growth of *P. aeruginosa* was significantly inhibited in the presence of either dextran or polyacrylic acid coated ceria nanoparticles after 24 hours. The effectiveness of bacterial growth inhibition reached 55.41% for dextran coated ceria nanoparticles
compared with TSB control. This study revealed for the first time that the characterized ceria nanoparticles could potentially serve as a novel antibacterial agent for biomedical applications. Further studies, such as longer term treatment and elucidating the mechanism of action, are important to achieve a better understanding and application of ceria in numerous antibacterial applications.

6.5 References


Chapter 7
Investigation of Antibacterial Properties of ZTA and Silicon Nitride Implants Compared with Titanium

7.1 Introduction
As is well known, bacteria can easily form biofilms when they attach to a artificial implant surfaces. A bacterial biofilm is an aggregate of one or more types of bacteria in a hydrated polymeric matrix.\(^1\) An implanted medical device provides a surface for bacteria to attach and multiply in the patient's body, resulting in the formation of biofilms. Once formed, the polymeric matrix works as a shield to prevent drugs from penetrating inside the biofilm. Moreover, the outer cells that break off from the biofilm can enter and attack the body, causing wide spread infections. Preventing biofilm formation is critical in the fight against healthcare associated infections (or HAIs).\(^2\)

*Pseudomonas aeruginosa* is one of the bacterium commonly found in numerous infections. It is an effective and common opportunistic pathogen of humans, causing serious infections in cystic fibrosis,
intensive care, burn and immunocompromised patients.\(^3\)\(^4\) *Pseudomonas aeruginosa* have been implicated during chronic infection of cystic fibrosis patients.\(^5\)\(^6\) In addition to chronic infection, *P. aeruginosa* biofilms contribute to morbidity of patients with medical implants including catheters\(^7\), prosthetics\(^8\) and stainless steel implants\(^9\). Therefore, it is significant to develop a material to prevent bacterial growth on the surface of implant medical devices.

Titanium is the most established implant material in recent years. About one million patients worldwide are treated every year for replacement of arthritic or damaged hips and knees.\(^10\)\(^11\) Although it is considered as one of the few materials that naturally meet every requirement for implantation in the human body, bacterial infections is a severe problem for titanium being used as implant materials.

As novel ceramics materials for biomedical applications, both ZTA (zirconia toughened alumina) and Si\(_3\)N\(_4\) (silicon nitride) have been recently investigated as potential implant materials.\(^12\)\(^-\)\(^19\) ZTA is a ceramic material comprising alumina and zirconia. ZTA components are
comprised of an alumina-rich composition (about 20% zirconia and 80% alumina) where zirconia is evenly dispersed in the alumina matrix. These ceramics exhibit superior strength and toughness compared to conventional alumina and zirconia. These ceramic properties have made ZTA a potential advancement of current clinically available orthopedic biomaterials. Studies have showed the promise of ZTA being used as an orthopedic implant material.\textsuperscript{21-23}

Silicon nitride is a chemical compound of the elements silicon and nitrogen and has been used in many industrial applications. Due to its hydrophilic and microtextured surface, silicon nitride has better materials strength, durability and reliability than conventional implant materials, such as PEEK and titanium.\textsuperscript{24,25} Polished and porous implants made of silicon nitride have shown encouraging outcomes in spine and maxillofacial surgery.\textsuperscript{26,27} However, the antibacterial properties of either ZTA or silicon nitride have not been widely studied, and the resistance to bacterial infection is an important and desirable property for implant materials.
Thus, the objective of this chapter is to investigate the antibacterial properties of the novel implant materials, silicon nitride and ZTA, compared with titanium. The results of this study showed that ZTA significantly inhibited the growth of *Pseudomonas aeruginosa* and *Staphylococcus aureus* compared with the widely used implant material, titanium, which suggested that ZTA could be a potential implant material with antibacterial functions and could be used as an alternative to titanium. Moreover, silicon nitride also successfully inhibited the growth of *Staphylococcus aureus* biofilm compared with titanium.

### 7.2 Materials and Methods

#### 7.2.1 Materials Characterization

The ceramics used in this study included medical grades of silicon nitride and ZTA, and both are provided by Amedica Corporation, Salt Lake City, UT. The silicon nitride an “as-fired” surface that has nanostructured features. Titanium samples used in this study are ASTM grade 4 titanium from Fisher Scientific, Continental Steel & Tube Co. Fort Lauderdale, FL. The morphology of all the surfaces was characterized using a SEM (Scanning Electron Microscope, HITACHI 2700). Before scanning the
surface of polycarbonate under SEM, the films were coated with a 2 nm gold layer using a Sputter Coater (EMITECH K550, Emitech Ltd.) to make them conductive. An AFM (Atomic Force Microscope, MFP3D, Asylum Research, sharp tipped cantilever, $K = 0.06\text{N/M}$, Tapping Mode) was used to measure the surface roughness of the materials.

### 7.2.2 Bacterial Assays

For sterilization purpose, all samples were exposed under ultraviolet light for 24 hours on all sides. In the bacteria experiments, bacteria cell lines of *Pseudomonas aeruginosa* and *Staphylococcus aureus* were obtained in freeze-dried form from the American Type Culture Collection (catalog number 27853). The cells were propagated in 30 mg/ml of Tryptic soy broth (TSB). A bacteria solution was prepared at a concentration of $10^6$ bacteria/ml, which was assessed by measuring the optical density of the bacterial solution at 562 nm using a standard curve correlating optical densities and bacterial concentrations. The ZTA, silicon nitride and titanium samples were treated with bacterial solutions in a 24-well plate and cultured for either 24, 48 or 72 hours in an incubator ($37^\circ$, humidified, 5% CO$_2$). The media was changed with fresh and sterile 0.03% TSB.
every 24 hours for those samples incubated with 48 hours or 72 hours. Then, the samples were put in 15ml microfuge tubes with 3ml of PBS. These tubes were sonicated for 10 minutes to release the bacteria attached on the surface into the solution. Solutions with bacteria were spread on agar plates and bacteria colonies were counted after 18 hours of incubation.

Besides the colony counting method mentioned above, the crystal violet assays were also used to test the growth of bacteria. After the ZTA, silicon nitride and titanium samples were treated with bacteria solution for 24, 48 or 72 hours, for each sample, 1 mL bacterial solution with both planktonic and attached bacteria will be stained with 0.5 mL 0.1% crystal violet solution for 15 minutes in a 1.5 mL centrifuge tube. Then the centrifuge tubes will be centrifuged with 10000 g for 5 minutes. The supernatant solution was removed leaving only the bacteria pellet in the tube. The pellet was washed with PBS (phosphate buffered saline) once followed with a centrifuge for 5 minutes under 10000 g. After removing the supernatant solution again, 1 mL 100% ethanol was added into each tube to dissolve the crystal violet stain in the bacteria. Then the
absorbency of solutions in each tube was measured at 562nm using a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA).

Bacterial tests were conducted in triplicate and repeated three times. Data were collected and the significant differences were assessed with the probability associated with a one-tailed Student's t-test. Statistical analyses were performed using Microsoft Excel (Redmond, WA).

7.3 Results

7.3.1 Materials Characterization

SEM images of the surfaces of titanium and as-fired silicon nitride were illustrated in Figure 7-1. The SEM image of titanium showed a typical micron-rough surface of machined materials. The as-fired silicon nitride had nanostructured surface features with larger total surface area compared with titanium. The as-fired silicon nitride surface morphology reflected the natural condition of this material subsequent to densification by sintering and hot-isostatic pressing. The surface was composed of randomly oriented acicular protruding grains typically less than 1μm in cross-section, yielding a unique nanotexture. Individual hexagonal grains
of silicon nitride have definitive linear facets (in cross-section) and sharp corners at the termination of the acicular grains, which are typically less than 100 nm. These unique nano-features provided a desirable large surface area, which might play a role in interaction with bacteria.

**Figure 7-1.** SEM images of (a) titanium and (b) as-fired silicon nitride at a magnification of 10k.

The AFM images of titanium and ZTA are shown in the Figure 7-2 and the surface roughness measurements were derived from the AFM images. The titanium surface had randomly microsized features with a RMS (root mean square, scan area = 10 μm×10 μm) roughness of 393.9 nm. The ZTA surface showed more uniform nanoscale patterns with a much higher RMS roughness, which is 490.8 nm. These results showed that Ti and
ZTA have markedly different surface topographies that might affect bacterial adhesion.

Figure 7-2. AFM images and RMS (root mean square, scan area = 10um×10um) roughness of (a) titanium and (b) ZTA. RMS roughness of titanium = 393.9 nm. RMS roughness of ZTA = 490.8 nm.

7.3.2 Bacterial Assays

As shown in Figure 7-3, the ZTA samples showed significant inhibition to the growth of *Pseudomonas aeruginosa* from 24 hours to 72 hours compared with titanium samples. There are about 50% less bacteria adhesion on the surface of ZTA than titanium after 24 hours. After 48 hours, the ZTA samples inhibited the growth of *Pseudomonas aeruginosa* by 46% compared with the titanium samples. After 72 hours, the ZTA
samples still showed a significantly decreased bacterial growth by 45% compared with titanium samples. However, there was no significant difference in the growth of *Pseudomonas aeruginosa* on the surface of silicon nitride and titanium, although there are relatively less bacteria attached on silicon nitride than titanium. Thus, the results demonstrated that there is a continuous and significant prevention to *Pseudomonas aeruginosa* growth on the surface of ZTA from 24 hours to 72 hours.

**Figure 7-3.** The growth of *Pseudomonas aeruginosa* on silicon nitride, ZTA and titanium, measured by the method of colony forming units. Data=Mean ± standard deviation, n=3; *p<0.05 compared with titanium samples after 24 hours; **p<0.05 compared with titanium samples after 48 hours; ***p<0.05 compared with titanium samples after 72 hours. SN = silicon nitride.
In another bacterial inhibitory test, the growth of *Staphylococcus aureus* biofilm after either 24, 48 or 72 hours on silicon nitride, ZTA and titanium was also measured using crystal violet assays. As shown in Figure 7-4, the titanium samples had more bacteria attached compared with silicon nitride or ZTA samples. Particularly, there was some decrease in bacteria numbers for silicon nitride compared with titanium after 24 hours, but the differences between these two were not significant. However, the silicon nitride surface showed significantly less bacterial growth than titanium surface after 48 and 72 hours, which means silicon nitride inhibited the growth of *Staphylococcus aureus* biofilm compared with titanium. Moreover, less crystal violet absorbance was observed for ZTA compared with titanium after incubation of 24, 48 and 72 hours. From 24 hours to 72 hours (Figure 7-4), ZTA surface showed a continuously significant inhibition to the growth of *Staphylococcus aureus* biofilm compared with titanium surface. Overall, less bacterial activity of *Staphylococcus aureus* was manifested on both silicon nitride and ZTA than on titanium, probably because of the surface chemistry and nanostructure differences between these materials. The results of the
bacterial tests showed that ZTA effectively inhibited the growth of both gram-positive (*Staphylococcus aureus*) and gram-negative (*Pseudomonas aeruginosa*) bacteria after 1, 2 and 3 days compared with titanium, which demonstrated that ZTA might be considered as a potential alternative of titanium for implant material.

**Figure 7-4.** The growth of *Staphylococcus aureus* on silicon nitride, ZTA and titanium, measured by the method of crystal violet staining. Data=Mean ± standard deviation, n=3; *p<0.05 compared with titanium samples after 24 hours; **p<0.05 compared with titanium samples after 48 hours; ***p<0.05 compared with titanium samples after 72 hours. SN = silicon nitride.
7.4 Conclusions
In this study, it was showed that ZTA and silicon nitride as novel ceramic materials strongly inhibited the growth of gram-positive bacteria (*Staphylococcus aureus*) on the surface compared with titanium. ZTA surface also showed a significant inhibition to the growth of gram-negative bacteria (*Pseudomonas aeruginosa*) compared with titanium after 24, 48 and 72 hours. Because the substantially lower loads of *Staphylococcus aureus* and *Pseudomonas aeruginosa* were measured on ZTA surfaces than titanium, for up to 72 hours of incubation, using ZTA as an implant material might be an effective way to prevent infections on implant medical devices. Therefore, this study suggested that ZTA with advantages of preventing infections might serve as a promising alternative of titanium for biomedical applications.

7.5 References
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Chapter 8
Conclusions and Future Outlook

The studies performed in this dissertation presented an approach based on nanosized selenium to inhibit bacterial infections on several types of materials, which demonstrated the potential applications of nanosized selenium as an antibacterial agent in surface coatings.

Specifically, nanosized selenium was successfully synthesized and coated on various substrates including polymeric medical devices, paper towels, and metallic materials through a simple fast precipitation method. The properties of nanosized selenium coatings, such as size, distribution, coverage, composition, and change of surface geography, were fully characterized using techniques such as a standard tape test, scanning electron microscopy, energy-dispersive X-ray spectroscopy, atomic force microscopy, contact angle measurement, and so on. The concentrations of the selenium coatings were measured using atomic absorption spectroscopy and inductively coupled plasma mass spectrometry. Most importantly, the nanosized selenium coatings significantly decreased the
growth of various gram-positive and gram-negative bacteria, thus inhibiting bacterial biofilm formation on these substrates. These antibacterial effects with inhibitory percentages of up to 91% were achieved without using any antibiotics. This dissertation suggests that nanosized selenium maybe used as an effective antibacterial agent for helping medical devices and healthcare facilities fighting against infections.

Several aspects in this work need to be further investigated in the future to achieve a better understanding of the antibacterial properties of nanosized selenium and to expand a wider range of applications of nanosized selenium as an antibacterial agent.

Particularly, for different materials, the reaction to synthesize and coat nanosized selenium needs to be modified. Several parameters, such as the concentrations of each reactant, temperature for the reaction, speed of agitation during the reaction, and time for the coating process, should be optimized to prepare selenium coatings that are more uniform, having stronger adhesion and more desired concentration. Another way to think
about conducting the reaction in practice is doing a spray coating on the surface of the targeted material. A few primary results were included in the Appendix of this dissertation. With a precise control and balanced spray, this could be a more efficient way, compared with the existing aqueous phase reaction, to coat the surfaces of some materials with nanosized selenium and introduce antibacterial properties to these materials. The spray coating method could also potentially use the two reactants more effectively, which can save the costs of synthesizing selenium nanoparticles and is a very important aspect for commercial applications.

Moreover, this nanosized selenium based antibacterial approach should be applied to other materials that are also widely used for various medical devices and healthcare products, such as polyvinyl chloride, co-polyesters, titanium, silicone, stainless steel and so on. The surface properties, such as roughness, hydrophilicity, chemical composition, and surface charges, could be remarkably different between each kind of these materials, thus, the interaction between bacteria and the nanosized selenium coated material surface might be dramatically varied. These factors may cause
difficulties to use the nanosized selenium based antibacterial technology on various materials.

In addition, the anti-infective properties should be further tested on other bacterial species and even other microorganisms. Because compared with *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa* and *Escherichia coli* used in this project, other bacterial species or other microorganisms such as fungus and virus, may have significantly different sizes, surface proteins, cell membranes and other properties, which may reveal different interactions with nanosized selenium. Further studies, such as gene expression tests, mammalian cell assays, selenium uptake measurements, should be implemented to achieve a better understanding of the antibacterial effects of nanosized selenium and access its potential toxicity to human and environment.

Lastly, but not the least, *in vivo* studies in animals such as mice, should be implemented to further investigate the antibacterial properties and safety concentrations of nanosized selenium for biomedical applications. Specifically, the released amount and distribution of selenium
nanoparticles in the animals should be measured. The lifetime of released selenium nanoparticles or how long it takes for the clearance of selenium nanoparticles in body of the animals should be accessed. It is also of vital importance to determine the effectiveness of the nanosized selenium at inhibiting various infections in the \textit{in vivo} situation.

Overall, the work concerning nanosized selenium described in this dissertation established a novel platform technology to prevent bacteria and biofilm infections. This technology may be a significant contribution to the field of nanomedicine, especially improving the strategies of fighting against infections in various hospital environments and healthcare settings.
Appendix

A1. Selenium Coatings on Surfaces of Various Materials

In addition to the materials that were coated with nanosized selenium and studied on their antibacterial properties, various other material substrates were successfully coated with selenium nanoparticles. The SEM images of these substrates were included below to demonstrate the nanosized selenium coatings on these materials. Besides polycarbonate and PEEK mentioned in the previous chapters, selenium nanoparticles were also coated on other polymeric materials that were used for medical devices, such as isoplast and PVC, as shown in Figure A1-1 and Figure A1-2. In order to introduce antibacterial properties to the “high-touch” (frequently touched) surfaces around us, nanosized selenium was coated on the surfaces of computer keyboard and tablet, which were both real commercial products that people use almost every day. Figure A1-3 and Figure A1-4 showed that the surfaces of computer keyboard and tablet were fully covered with selenium nanoparticles compared with the uncoated ones. In Figure A1-5, glass, as other frequently used material, was also coated with selenium nanoparticles. The glass surface was fully and evenly covered by
selenium nanoparticles.

**Figure A1-1.** SEM images of selenium nanoparticle coated isoplast before (a) and after (b) the tape test as mentioned in Chapter 2.

**Figure A1-2.** SEM images of selenium nanoparticle coated PVC (a) and uncoated PVC (b).
Figure A1-3. SEM images of selenium nanoparticle coated on the surface of a commercially available keyboard (a) and the uncoated surface of the keyboard (b).

Figure A1-4. SEM images of selenium nanoparticle coated on the surface of the back side of a “Windows Surface” tablet (a) and the uncoated surface of the table (b).
Moreover, nanosized selenium was also coated on stainless steel as shown in Figure A1-6(a), because stainless steel is a metallic material that widely used and often touched in healthcare environment, such as being used for medical supply carts and bed rails in the patient’s room. Besides the normal aqueous phase reaction to synthesize and coat nanosized selenium on the surface of materials, another spray coating method was used to coat selenium nanoparticles on stainless steel. In this method, the two reactants, sodium selenite and glutathione, were put in two spray bottles separately and then the reactants were sprayed one by one to the surface of stainless steel. The result of spray coating on stainless steel was shown in Figure A1-6(b). The formation of nanosized selenium was
observed after the coating process. However, this coating method was not working quite successful for polyurethane as shown in Figure A1-7. No much particle-like feature was observed after spray coating. The reason might be the hydrophobicity of polyurethane that prevented the reactants from evenly distributed on the surface. Further studies are important to modify this coating process so that it can work for more types of materials. In many circumstances, the spray coating method could be an easier approach to coat nanosized selenium and introduce antibacterial property to material surfaces than the conventional aqueous phase reaction.
Figure A1-6. SEM images of stainless steel that were (a) coated with nanosized selenium by the aqueous phase reaction; (b) coated with nanosized selenium by spray coating; (c) without selenium coatings.
Figure A1-7. SEM images of (a) uncoated polyurethane and (b) selenium coated polyurethane by spray coating.

In order to test the adhesion of selenium nanoparticle coatings on paper towels, the selenium coated paper towels were sonicated for 5 minutes in DI water. Then, SEM images (Figure A1-8) of the sonicated paper towels were taken under two magnifications. It was observed from the SEM images that there were still a lot of selenium nanoparticles attached on the surface of paper towels after the sonication, which means that the most of the nanosized selenium coatings were strongly attached to the paper towels and hard to be removed by washing process, like sonication.
Figure A1-8. SEM images of selenium nanoparticle coated paper towels after 5 minutes of sonication treatments. (a) magnification = 3k; (b) magnification = 20k.

A2. Surface Treatment of Flat and Porous Titanium Implants

Commercially available bone implant materials, flat and porous titanium from Stryker Corporation, Kalamazoo, Michigan, were treated with nitric acid to modify the surface features. Both flat and porous titanium were treated with 1 M, 5 M or 10 M nitric acid separately. In each concentration of nitric acid, both flat and porous titanium were treated for 5, 30 or 60 minutes. SEM images of titanium samples were taken before and after acid treatments using a Scanning Electron Microscope, HITACHI 2700. The SEM images of the flat and porous titanium without acid treatment
are in Figure A2-1. The SEM images of flat titanium after being treated with acid are in Figure A2-2. The SEM images of porous titanium after being treated with acid are in Figure A2-3.

**Figure A2-1.** SEM images of (a) untreated flat titanium at a magnification of 50; (b) untreated flat titanium at a magnification of 10k; (c) untreated porous titanium at a magnification of 50; (d) untreated porous titanium at a magnification of 10k.
Figure A2-2. SEM images of flat titanium after being treated with acid. (a) 1M nitric acid, treat for 5 minutes; (b) 1M nitric acid, treat for 30 minutes; (c) 1M nitric acid, treat for 60 minutes; (d) 5M nitric acid, treat for 5 minutes; (e) 5M nitric acid, treat for 30 minutes; (f) 1M nitric acid, treat for 60 minutes; (g) 10M nitric acid, treat for 5 minutes; (h) 10M nitric acid, treat for 30 minutes; (i) 10M nitric acid, treat for 60 minutes.
Figure A2-3. SEM images of porous titanium after being treated with acid. (a) 1M nitric acid, treat for 5 minutes; (b) 1M nitric acid, treat for 30 minutes; (c) 1M nitric acid, treat for 60 minutes; (d) 5M nitric acid, treat for 5 minutes; (e) 5M nitric acid, treat for 30 minutes; (f) 1M nitric acid, treat for 60 minutes; (g) 10M nitric acid, treat for 5 minutes; (h) 10M nitric acid, treat for 30 minutes; (i) 10M nitric acid, treat for 60 minutes.