A Thesis

entitled

Development of Titania Nanofibers and Films for the Mitigation of Wound Infection

by

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Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

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Disease-carrying pathogens in the body not only destroy healthy tissue but can eventually multiply and spread throughout the blood stream causing infection. Infections can be reduced and healing accelerated by using a nanotechnological approach with photoactive antimicrobial materials. In order to achieve this, two different approaches exploiting the known photoactive antimicrobial attributes of titanium dioxide (TiO$_2$, also known as titania) were conceived. These are: (i) the development of non-woven nanofibers of titanium dioxide (TiO$_2$), either in pristine or in doped form; this could be used as disinfectant gauze for wound healing upon activation by a pocket IR flashlight, and, (ii) the development of a simple and benign procedure for creating nanotubular or nanofibrillar structure of photoactive TiO$_2$ on the surface of the implants (made from Ti or Ti6Al4V alloys).

In the first case, the technique of electrospinning was used for fabricating non-woven TiO$_2$ mesh, using titanyl nitrate as a benign and inexpensive precursor. In the second case, nanoscale TiO$_2$ film formation on Ti-implants was achieved either by
hydrothermal processing under very mild and benign experiment conditions or by anodization in the presence of dilute mineral acids and under mild voltages.

Systematic and thorough structural and microstructural characterization was performed on the as-spun and the fired nanomats as well as on the films grown on the Ti coupons. Results pertaining to the biocidal activities of the self-standing nanofibers and the films on the Ti coupons showed significant decrease in the population of the *E. coli* cell colonies upon exposure of their aqueous broths to IR radiation for 3-12 seconds.

Consequently, by interposing an effective procedure based on nanotechnology, the use of orthopedic and spinal implants can be made safer allowing bone healing to occur even faster, eliminating the probability of wound infection during and after surgical procedures.
Acknowledgments

This thesis would not have been possible without the support and love of my family and friends. They helped me grow and learn what it takes to survive in today’s world. I deeply appreciate them for all their encouragement and support to allow me to be in school for this many years.

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Glossary of Symbols and Abbreviations

2-θ .......... Degree
A ............ Ampere
ACS .......... American Chemical Society.
aq .......... Aqueous
At .......... Atomic
a.u .......... Arbitrary Unit
c ........... Speed of Light
CA .......... California
CDC .......... Centers for Disease Control and Prevention
cfu/ml ....... Colony Forming Units per Milliliter
CHF₃ .......... Fluoroform Gas
cm .......... Centimeter
CO₂ .......... Carbon Dioxide
cp .......... Commercially Pure
cps .......... Counts per Second
Da .......... Dalton
°C .......... Degree Celsius
DI .......... Deionized
DIC .......... Differential Interference Contrast
e⁻ .......... Electron
E .......... Energy
ECM .......... Extracellular Matrix
E. coli ....... Escherichia coli
EDS .......... Energy Dispersive Spectroscopy
e-spinning .. Electrospinning
EtOH .......... Ethanol
f ............ Frequency
Fe .......... Iron
FeCl₂ ......... Ferrous Chloride
FeCl₃·6H₂O ... Ferric Chloride
Fe(NO₃)₃·9H₂O Ferric Nitrate
Fe₃O₄ .......... Iron Oxide (Magnetite)
FESEM ........... Field Emission Scanning Electron Microscopy
FR ............... Mass Fraction of Rutile in the Sample

g ............... Gram
γ-Fe₂O₃ ........... Gamma Iron Oxide (Maghemite)

h ................ Hour
h ................ Plank’s constant
HCl ............... Hydrochloric Acid
HF ............... Hydrofluoric Acid
HNO₃ ............ Nitric Acid
H₂O .............. Water
H₂O₂ ............ Hydrogen Peroxide
H₃PO₄ ........... Phosphoric acid

IA ................ Integrated Intensities of the <101> Plane for Anatase Phase
ICDD .......... International Center for Diffraction Data
IL ............... Illinois
in. ............. Inch
IR ............. Infrared
Ir ............ Integrated Intensities of the<110> Plane for Rutile Phase

J ............... Joule

kV ............. Kilovolt

λ ............... Wavelength

M ............... Molar
MA ............ Massachusetts
MI ............ Michigan
μl ............ Microliter
μW ............ Microwatt
min ........... Minute
ml ............ Milliliter
mm ............ Millimeter
MP ........... Multiphoton
MRSA ......... Methicillin-Resistant Staphylococcus aureus
mW ........... Milliwatt

NaF ............ Sodium Fluoride
NaOH ........ Sodium Hydroxide
NH₃ ............ Ammonia
NH₄OH ......... Ammonium Hydroxide
(NH₄)₂SO₄ .... Ammonium Sulfate
NJ ............. New Jersey
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
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<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>NY</td>
<td>New York</td>
</tr>
<tr>
<td>O₂</td>
<td>Oxygen</td>
</tr>
<tr>
<td>O₂⁻</td>
<td>Singly Charged Molecular Oxygen Anion</td>
</tr>
<tr>
<td>OD₆₀₀</td>
<td>Optical Density at 600 nm</td>
</tr>
<tr>
<td>OH</td>
<td>Ohio</td>
</tr>
<tr>
<td>OH⁻</td>
<td>Hydroxyl Radical</td>
</tr>
<tr>
<td>PED</td>
<td>Plasma Electro-Deposition</td>
</tr>
<tr>
<td>%</td>
<td>Percent</td>
</tr>
<tr>
<td>PR</td>
<td>Photoresist</td>
</tr>
<tr>
<td>psi</td>
<td>Pounds per Square Inch</td>
</tr>
<tr>
<td>PVP</td>
<td>Polyvinyl Pyrrolidone</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per Minute</td>
</tr>
<tr>
<td>s</td>
<td>Second</td>
</tr>
<tr>
<td>S. aureus</td>
<td>Staphylococcus aureus</td>
</tr>
<tr>
<td>S-cm</td>
<td>Siemen-Centimeter</td>
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<tr>
<td>SEM</td>
<td>Scanning Electron Microscopy</td>
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<td>S. epidermidis</td>
<td>Staphylococcus epidermidis</td>
</tr>
<tr>
<td>SiC</td>
<td>Silicon Carbide</td>
</tr>
<tr>
<td>SPION</td>
<td>Supermagnetic Iron Oxide Nanoparticles</td>
</tr>
<tr>
<td>sq. in.</td>
<td>Square Inch</td>
</tr>
<tr>
<td>SSI</td>
<td>Surgical Site Infection</td>
</tr>
<tr>
<td>STEM</td>
<td>Scanning Transmission Electron Microscope</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
</tr>
<tr>
<td>Ti</td>
<td>Titanium</td>
</tr>
<tr>
<td>Ti⁴⁺</td>
<td>Titanium (IV) Ion</td>
</tr>
<tr>
<td>Ti₆Al₄V</td>
<td>Titanium Alloy with 6 wt. % Aluminum and 4 wt. % Vanadium</td>
</tr>
<tr>
<td>TiCl₄</td>
<td>Titanium (IV) Chloride</td>
</tr>
<tr>
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<td>Titanium (IV) Fluoride</td>
</tr>
<tr>
<td>TiO₂</td>
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<tr>
<td>Ti(OH)₄</td>
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<tr>
<td>(TiO(NO₃)₂)</td>
<td>Titanyl Nitrate (TN)</td>
</tr>
<tr>
<td>TMAOH</td>
<td>Tetramethylammonium Hydroxide</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>UVA</td>
<td>Ultraviolet A</td>
</tr>
<tr>
<td>V</td>
<td>Volt</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume by Volume Ratio</td>
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<tr>
<td>W</td>
<td>Watt</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
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<td>--------------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>Wt.</td>
<td>Weigh</td>
</tr>
<tr>
<td>w/w</td>
<td>Weight by Weight Ratio</td>
</tr>
<tr>
<td>XRD</td>
<td>X-ray Diffraction</td>
</tr>
<tr>
<td>ZnO</td>
<td>Zinc Oxide</td>
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Chapter 1

Introduction

Disease-carrying pathogens in the body not only destroy healthy tissue but can eventually multiply and spread throughout the blood stream causing infection. Infections can be reduced and healing accelerated by using a nanotechnological approach with photoactive antimicrobial materials. In order to achieve this, two different approaches exploiting the known photoactive antimicrobial attributes of titanium dioxide (TiO$_2$, also known as titania) were conceived. These are: (i) the development of non-woven nanofibers of titanium dioxide (TiO$_2$), either in pristine or in doped form; this could be used as disinfectant gauze for wound healing upon activation by a pocket IR flashlight, and, (ii) the development of a simple and benign procedure for creating nanotubular or nanofibrillar structure of photoactive TiO$_2$ on the surface of commercially pure (cp) Ti or Ti implants (Ti6Al4V alloys).

In the first case, the technique of electrospinning was used for fabricating non-woven TiO$_2$ mesh, using titanyl nitrate as a benign and inexpensive precursor. In the second case, nanoscale TiO$_2$ film formation on Ti plates, wires and implants was achieved either by hydrothermal processing under very mild and benign experiment conditions or by anodization in the presence of dilute mineral acids.
1.1 Infections
Infections contracted at health clinics and from the hospital, rank among the 10 leading causes of death in the United States $^{[1]}$. In 2002, about 1.7 million patients were infected from hospital-related diseases and 99,000 died as a result of these diseases or infections. Extensive research has been conducted in an attempt to prevent and eliminate these infections. Among the most potent infections, the surgical site infections (SSI) are most common and occur in about 2% of surgical procedures. According to Reichman, a surgical site infection (SSI) is an infection at or near the surgical incisions that occurred within 30 days of the operation $^{[2]}$. The SSIs can have local symptoms such as erythema, warmth, tenderness and possible loss of function or more systemic symptoms such as fever, rigors and even a possibility of systemic shock $^{[3]}$. Therefore, measures need to be taken to ensure that the outcome of surgical procedures is improved for the health and safety of individuals. Prevention is the most important precaution that can be taken in order to ensure that SSIs do not occur to begin with.

1.2 Surgical Site Infections
According to the Center for Disease Control and Prevention (CDC), three different types of SSIs have been defined: (i) surgical incisional SSI, (ii) deep incisional SSI and (iii) organ/space SSI $^{[3]}$, as shown in figure 1.1.
Figure 1.1: Classification of surgical-site infections [3]

The relevance on our work using photoactivated implants pertains to organ/space SSI. The organ/space SSI is an infection that occurs within 30 days after the surgery if no implant is left in place or within 1 year if the implant is in place and the infection appears to be related to the surgery [2].

The pathogen source that causes the majority of the SSI cases is the native flora of the patient’s skin, mucous membranes or hollow viscera [4]. Of these, the most common is aerobic gram-positive cocci such as *Staphylococcus aureus* and the gram-negative bacilla such as *Escherichia coli* (*E. coli*) [2]. The results reported in this thesis pertain to *E. coli*. Future studies will concentrate on the bactericidal characteristics of the nanostructured self-standing fibrillar mat and coating on the implants against *Staphylococcus aureus*. 
1.3 Tissue Engineering

In recent years tissue engineering has received extensive attention as an emerging and rapidly growing field. The ultimate goal of tissue engineering as a treatment concept is to replace or restore the anatomic structure and the function of damaged, injured or missing tissues or organs following any injury or pathological process. Treatment is facilitated by combining biomaterials, cells, tissue, biologically active molecules, and/or stimulating mechanical forces of the tissue microenvironment. The scaffolds should be hierarchical, porous structures to allow seeding of cells at high densities upon implantation into the body. This assists in the infiltration and formation of large numbers of blood vessels supplying nutrients to the transplanted cells and removal of waste products.

In this context, metal-containing nanoparticles (mostly quantum dots) have been exploited extensively. These particles still remain attractive merely as chemical, pharmacological and medical toolboxes, though not as a clinical solution for diagnosis or therapy. Recent reviews have provided instructive examples of nanotechnology application in basic neurosciences and its use in addressing interesting biological questions [5-12].

1.4 Photocatalytic Property of Titanium Dioxide

Systematic research has been initiated to evaluate the prophylactic/therapeutic antimicrobial efficacy of nanostructured titanium dioxide (TiO$_2$, commonly known as titania) as an adjunctive treatment for wounds. One of the unique physical properties of TiO$_2$ is its antimicrobial/ disinfective activity upon activation by incident light.
Titania exists in three crystallographic forms, viz., anatase (3.18 eV), rutile (3.03 eV) and brookite (2.96 eV), but the first two are the most commonly known and widely used structures, in addition to being photocatalytic (brookite has not photocatalytic attributes). In addition to photocatalysis, titania is used in multiple applications such as paints, food coloring, anti-biofouling, dye-sensitized photovoltaic solar cells and sunscreens. When irradiated with light of proper wavelength, excitation by free electrons in the conduction band and holes in the valence band, give rise to reactive oxygen and hydroxyl free radicals which then oxidize organic matter (including live microorganism), as shown in figure 1.2.

**Figure 1.2: Free radical creation mechanism on TiO₂**

This property makes titania an ideal candidate for numerous medical applications where infection control is needed via bactericidal action. Construction of non-woven TiO₂ nanofibers and nanomats possessing a three-dimensional scaffold structure and optimal porosity, in conjunction with photo-activated antimicrobial activity, could provide a significant improvement in the management of infection. A photoactive TiO₂ nanomat
can be used as ultralight disinfectant gauze for wound healing with brief activation from a pocket UV/IR flashlight. Titania can also be created on implants and mitigate infection in post-surgery scenarios, upon photoactivation by suitable incident beam. Photoactivation of titania by ultraviolet light (300nm ≤ λ ≤ 420nm) has been reported\textsuperscript{[16-18]}. Thus, TiO\textsubscript{2} could provide a significant improvement in the management of wound infection and segmental bone defects. In the presence of infections, the interposed disinfectant gauze is energized via activation by light.

According to Maness\textsuperscript{[19]}, the underlying mechanism of E. coli necrosis via titania irradiation with UV light (λ = 356 nm; intensity ~8 W/m\textsuperscript{2}) is by the lipid peroxidation reaction; TiO\textsubscript{2} photocatalysis promoted peroxidation of the polyunsaturated phospholipid component of the lipid membrane and caused irregularities in the E. coli cell membrane. Consequently, functions such as respiratory activity were lost due to disorder in the cell membrane and causing the E. coli to die.

1.5 Electrospinning

Electrospinning (e-spinning) is a promising technique of fabricating biodegradable and/or physiologically benign and biosorbable scaffolds\textsuperscript{[20-21]}. E-spun fibers are found to possess features that bear morphological similarity to the ECM (extracellular matrix) of natural tissue, such as high porosity and effective mechanical properties. Since the natural scaffold consists of a multilayered fibrous and porous architecture, utilizing e-spinning as a novel nanomanufacturing technique is sensible and applicable to tissue engineering. During e-spinning, external electrical forces produce fibers of nearly uniform diameters
ranging around 10s of nanometers and from several micrometers to a few millimeters in length. Potential application of this technology in the bioengineering field includes membranes for immobilized enzymes, artificial blood vessels and anti-septic wound dressing materials just to name a few\textsuperscript{[22-24]}. It is possible to assemble truly porous and non-woven polymeric or ceramic mats with nanofeatures using this technique. The possibility of extending the concept of nanoscale materials and systems to the biological systems in the area of spinal infection tissue engineering and orthopaedic infection mitigation is relevant to future research. Due to the great similarities between the make-up, morphology and structure of the natural extracellular matrix (ECM) in the human body and the synthetically fabricated nanofibers, a new era has opened in the application of nanosystems to the treatment of infections and traumatic injuries\textsuperscript{[25]}. An overall setup of the e-spinning is shown in figure 1.3.

E-spinning works when an adequately high voltage (5-25 kV) is applied to a droplet of liquid, causing the liquid to become positively charged. When the electrostatic repulsion counteracts the surface tension at the liquid droplet, the droplet stretches until surface tension reaches a critical point and a stream of liquid erupts from the surface at the liquid droplet. When the liquid erupts towards the ground plate, the liquid turns into a solid ceramic fiber. The fibers start to form a Taylor cone expanding from the liquid droplet to the ground plate\textsuperscript{[26]}. 
1.6 Photoactive Films on Implants

A second aspect of this research centers on creating a coating of photoactive TiO$_2$ on titanium (Ti) and Ti alloy implants in order to prevent infection. Even though commercially pure (cp) Ti has acceptable mechanical and medical properties to be used in orthopaedic implants; the titanium alloy mixed with 6 weight (wt.) percent aluminum and 4 weight percent vanadium typically is better suited for the implant application. This alloy is usually abbreviated as Ti6Al4V. This grade of Ti alloy is used in Aerospace, medical, marine and chemical processing. Titanium is used in these applications because of its combination of high strength to weight ratio and corrosion resistant properties. Some typical titanium structures routinely used are implants or fixtures in orthopaedic and/or spinal corrective surgery. Some of these are shown in figure 1.4.
Figure 1.4: Typical titanium implants and fixtures
Chapter 2

Research Significance

It is evident from the literature that development of photoactive antimicrobial materials is needed for the mitigation of bacteria for wound healing. Development of new products would reduce infection and accelerate healing. This objective is the motivation of the research activity, which is to develop:

1. Titania nanofibers interlaced to produce a disinfectant gauze
2. Titania nanotubular or nanofibrillar coating on Ti or Ti6Al4V implants.

2.1 Relevance of Titania Nanofiber Development

The basic structure of the intended nanofibers is expected to possess photoactive antimicrobial attributes – either in pristine/doped form or after antibacterial agents are incorporated. The non-woven nanofibers possessing photoactive antimicrobial attributes were created via e-spinning which allows the fabrication of physiologically benign and biosorbable scaffolds compatible with natural ECM tissue. To improve the performance and accelerate the bacterial death, small amount of Fe (≤ 5 wt. %.) was added. The as spun and processed nanofibers were characterized using XRD, SEM and TEM, and tested for their efficacy against \textit{E. coli} by activation with IR and UV light. The use of a MP
confocal microscope provided visual evidence of bacterial death, making it useful in determining the optimum conditions for bactericidal activities.

2.2 Relevance of Titania Nanocoating Development on cp Ti or Ti6Al4V Implants

There is an urgent need to prevent infection during and after orthopaedic surgeries that invariably involve implants. Therefore the development of nanoscale photoactive disinfectant films in the form of a tubular or fibrillar structure on the surface of implants could be used to help thwart SSI’s. A nanoscale coating of TiO$_2$ on Ti-implants was accomplished by: (i) hydrothermal processing under mild and benign experimental conditions or (ii) anodization at low voltage in dilute acids. Systematic and thorough structural and microstructural characterization was performed to ensure the creation of nanofibrillar and nanotubular coatings. Their biocidal activities were evaluated in terms of significant decrease in the population of *E. coli* bacteria upon exposure to the multiphoton laser, UV or IR beams.
Chapter 3

Materials and Methods

3.1 Nanofibers

3.1.1 Electrospinning

Pure and doped titania nanofibers were electrospun as ceramic-polymer (cermer) composite nanofibers. Previously the starting inorganic titanium precursor used for electrospinning titania was titanium (IV) fluoride (TiF₄, Alfa-Aesar, MA, purity 98%), described in detail in reference number [27]. Briefly, in that process, titanium (IV) fluoride was dissolved in deionized water followed by precipitation as titanium hydroxide [Ti(OH)₄]. The hydrated Ti(OH)₄ was dissolved in concentrated nitric acid (HNO₃, Fisher Scientific, Waltham, MA, purity 69.5% w/w) to form titanyl nitrate (TiO(NO₃)₂) as a clear solution, which was then used as Ti⁴⁺ source.

Though this technique is benign, it is, however, laborious and time-consuming. Therefore, in the present work, titanyl nitrate precursor was made by a slightly modified version of the procedure reported by Lee and Choi [28]. 5 g of titanium powder (-200 mesh, Alfa-Aesar, MA, purity 99.5%) was added slowly to a mixture of 250 ml of 30 % H₂O₂ (Fisher Scientific, Waltham, MA, purity 30%) and 167 ml of concentrated HNO₃.
with constant stirring to avoid any boil over, violent reaction or explosion (if added all at once). The reaction vessel was covered and the solution was left to react overnight under constant stirring, at the end of which an aq solution of titanyl nitrate (TiO(NO$_3$)$_2$; 0.4 M) was formed. For abbreviation, hereafter titanyl nitrate will be referred to as TN.

Granular polyvinyl pyrrolidone (PVP, Alfa-Aesar, MA, average molecular weight $\sim$1.3×10$^6$ Da) was used as the polymeric component. A 15 wt.% PVP solution was made by dissolving 11.84 g of PVP powder in 78.9 g of reagent grade ethanol (Pharmco-AAPER, Brookfield, CT, purity 95%) under constant and vigorous stirring. Due to the pronounced volatility of ethanol during and after the preparation as well as the tendency of the solution to dry out plus leave a stiff gel in the container upon prolonged storage, the PVP solution was prepared in small batches and only when electrospinning was to be carried out.

Altogether four different formulations were used. The main purpose of this iteration was to examine fibers arising from which particular mixture performed most optimally against the bacteria. The first solution contained TN and PVP solutions in the volume by volume (v/v) ratio of 1:2; it was labeled solution B.

The remaining three solutions also contained, in addition to TN and PVP, precursors for iron oxide (Fe$_3$O$_4$). It has been shown that superparamagnetic iron oxide nanoparticles have the propensity of preventing biofilm formation $^{[29]}$; therefore, iron-doped titania nanomats were also fabricated from solutions designated as C, D and E.
The solution C is essentially solution B referred to above (containing titanyl nitrate and PVP solutions in the ratio of 1:2 v/v), to a 1 wt. % ferric nitrate solution was added. First, 1 wt. % ferric nitrate solution was made by dissolving 100 mg of ferric nitrate (Fe(NO$_3$)$_3$·9H$_2$O, Fisher Scientific, Waltham, MA,) in 100 ml of DI water. To make composition C, 50 µl of this solution was added to 5 ml of aq TN solution.

For solution D, 0.069 g of FeCl$_2$ (Alfa-Aesar, MA) and 0.146 g of FeCl$_3$·6H$_2$O (Alfa-Aesar, MA) were first added to 24.786 g of TN solution and thoroughly mixed. This was added to PVP solution in the 1:3 v/v ratio and homogenized. For example, for a typical run, 28 ml of solution D was mixed with 84 ml of the ethanolic PVP solution.

Solution E was made in the following way. First a 1:1 molar mixture of ferrous and ferric iron was prepared. 0.069 g of ammonium iron (III) sulfate dodecahydrate (NH$_4$Fe(SO$_4$)$_2$; 12H$_2$O) and 0.028 g of ammonium iron (III) sulfate hexahydrate ((NH$_4$)$_2$Fe(SO$_4$)$_2$.6H$_2$O) both from Alfa-Aesar, MA, were mixed and added to 81.65 ml of the TN solution. The solution was thoroughly homogenized by mixing on a magnetic stirrer for 2h and eventually combined with PVP solution on the v/v ratio of 1:3. This would yield 5 wt. % Fe$_3$O$_4$ doped-TiO$_2$ nanomats in the fired sample.

3.1.2 Electrospinning Protocol

For the e-spinning experiments, the precursors were mixed in different ratios found to be optimum through the process of trial and error. The optimal solution of the TN and PVP was drawn into an array of ten 5-ml capacity clinical syringes. Precision-tip 25 gauge
stainless steel needles were attached to each syringe, and the entire array was mounted on a programmable syringe pump (model 230, KD Scientific, Holliston, MA). The preferred orientation of the syringe pump, for this work, was horizontal. A custom-made direct current power supply with a high voltage system (30 kV maximum) described previously [27, 30-31] was used for e-spinning. One terminal of the power supply was connected to a metallic wire looped through all the needles to keep them at identical voltage, while the other terminal was connected to a grounded metallic plate kept at a distance of 2-3 inches from the array of syringes.

For the ease of sample handling and subsequent thermal processing, ceramic plates instead of metal were used as the collector terminal in this work. In order to enhance the fiber collection area, a modified collection set-up was devised using two or four high density alumina containers (3 in. long x 2 in. wide x 0.25 in. deep). The containers were placed next to each other and short lengths of electrical wire were attached to the back of each plate at their center by 1-sq. inch blocks of aluminum foil with electrical tape. The other ends of the electrical leads were twisted into a common junction for the collector as seen from figure 3.1.
The syringe pump was interfaced with the high-voltage DC power supply (shown in figure 3.2) and was turned on. A flow rate of 0.03 ml/h was chosen and an electrical pulse was applied between the needle and the collectors in order to initiate the e-spinning.

Figure 3.2: Syringe pump (left) and high-voltage DC power supply (right) used in this work
The voltage was tweaked precisely until the fibers began to form steadily and collect on the plates placed ~ 3 in. away from the tip of the needles; the optimized voltage in this case was found to be 18 kV. The cermer fibers were spun continuously with short intermittent interruptions for periodic cleaning of the clogged needle tip from time to time. Figure 3.3 shows the experimental set-up and the progress of e-spinning; the non-woven matt of the e-spun cermer composite deposited on the collector plates can be clearly seen.

![Figure 3.3: Progress of electrospinning of ceramic-polymer (cermer) composite in real-time (left) and as-spun non-woven cermer composite collected on the ceramic plates (right)](image)

3.2. Film Fabrication on Titanium and Implant Coupons

The desired coating on Ti substrates was prepared by using three methods: hydrothermal processing of cp Ti coupons (Alfa-Aesar, MA and United Titanium Inc, Wooster, OH), hydrothermal processing of TiO$_2$-coated Ti coupons (Henkel Corporation, Madison Heights, MI) and anodization of cp Ti wires and cp Ti coupons (Alfa-Aesar, MA). The goal was to optimize fabrication conditions which could be mimicked on the real-life Ti and Ti6AlV4 implants. Another goal was to examine which of the two morphologies
(nanofibrillar growth via hydrothermal processing versus nanotubular growth via anodization) has higher bactericidal propensity.

3.2.1 Hydrothermal Processing of cp Ti Plates

Hydrothermal processing is a homogeneous (for nanoparticles) or heterogeneous (for bulk materials) reaction in the presence of mineralizers under high pressure and mild temperature conditions in a closed system to dissolve and recrystallize materials that are slightly insoluble under normal conditions. Invariably, autoclaves are used for this purpose with suitable construction materials that could withstand temperature up to ~250-300°C and pressures up to 2000 psi. The technique offers the advantage of upscaled production without much difficulty or compromising the consistency and reproducibility.

The hydrothermal synthesis of titania films on Ti substrates was carried out in a bench-top 1-L capacity autoclave (Autoclave Engineers, Erie, PA) shown in figure 3.4a. Initial experiments were carried out on Ti coupons and then repeated on the Ti6Al4V alloy. For this purpose, 2 mm thick cp Ti plate was cut into about ½ x ½ in.² coupons and polished unidirectionally with 400 grid SiC abrasive papers. This helped in creating some surface defects which are likely to assist in nucleation and growth of fibers during autoclaving. Coupons were rinsed in deionized (DI) water and dried in air. Small bucket-shaped reaction vessel (figure 3.4b) designed and fabricated in-house was used to conduct the hydrothermal reactions. The Ti coupons were placed in the bucket containing 20 ml of hydrogen peroxide (30% aqueous H₂O₂, Fisher Scientific, Waltham, MA).
The bucket was placed inside the main stainless steel autoclave and the hydrothermal reaction was carried out for 1h at 80°C (ramp rate of 3°/min.). After the reaction was complete, the coupons were rinsed in DI water under sonication, dried in air and calcined in a split tube furnace (Lindberg Minimite™, Asheville, NC) at 700°C for 1h with a ramp rate of 1°/min.

3.2.2 Hydrothermal Processing of TiO$_2$ coated Ti Plates

The Ti plates from Henkel Corporation (Madison Heights, MI) had a thin film of TiO$_2$ deposited on them; they will be referred to as „Henkel plates” hereafter. The TiO$_2$ films on the Ti plates were made by a patented aqueous plasma electro-deposition (PED) process in which the Ti substrate is made the anode and titanium-bearing compounds in
solution are deposited and cured in situ by the plasma glow at the surface. In a typical PED process, a pulsed DC voltage (240 V) was applied for 10 ms on and 30 ms off at a current density of approximately 1500 A/m². This results in very adherent titania coatings on the cp Ti substrates. The as-received Henkel plates were cut into ½ x ½ in.² pieces and cleaned in 0.1 M HCl (Fisher Scientific, Waltham, MA, purity 37.5%) for 2 min. followed by rinsing in DI water and acetone, respectively, and air drying. Prior to autoclaving, the Henkel coupons were heated at 800°C for 4h in static air at a ramp rate of 10°/min. Retaining one for XRD and SEM analysis, the remaining coupon were chemically etched in 40% HF (Alfa-Aesar, MA, purity 40%) aq solution for 10s followed by rinsing in acetone and air drying. The heat treated Henkel specimens were divided into 4 batches.

One batch was subjected to autoclaving in hydrazine monohydrate (N₂H₄·H₂O) and another batch in 2.8 M tetramethyl ammonium hydroxide (TMAOH) both from Alfa-Aesar, MA, at 100°C for 2h at a ramp rate of 3°/ min. For autoclaving third batch, 5 M NaOH (Fisher Scientific, Waltham, MA) was used as the working reagent under the same conditions as above. In all three cases, after the reaction was complete, autoclaved coupons were washed and sonicated in acetone twice for 2 min.

3.3 Anodization of Ti Plates and Wires

A method of creating TiO₂ nanotubes by anodizing titanium foil (0.2 mm thick) in a mixture of 0.5 M H₃PO₄ and 0.14 M NaF solution at 20V under sonication has been reported by Mohapatra et al. In our work, we used a mixture of 0.5 M H₃PO₄ (Fisher
Scientific, MA) and 0.14 M HF (Alfa-Aesar, MA) instead. Also, cp Ti plate (2 mm thick) instead of foil was used and the voltage was maintained at 21 V. Sonication ensured that anodization took place evenly across the entire plate. Figure 3.5 shows the schematic and the actual setup used in this research.

Figure 3.5: Anodization setup: (top) schematic, (bottom) experimental design
In order to study the change in the quality of the oxide film formed on the surface, anodization was carried out for 1, 2 and 4h. Furthermore, one Pt wire on either side of the Ti plate was used to ensure that both sides got anodized. After each run, the anodized plate was thoroughly rinsed in DI water followed by chemical etching in a mixture of ethanol and 40% HF (5 ml each) for 5s and washing again with DI water. A Ti wire was used in place of the Ti plate in order to approximate the appropriate conditions required to produce the titania nanotubes. The Ti wire was also anodized for 1, 2 and 4h in 0.5 M H$_3$PO$_4$ + 0.14 M HF mixture at a voltage of 21V. Identical procedure was repeated with the actual implant specimen later.

All the autoclaved and anodized samples were characterized by structural, microstructural and quantitative analyses using the Philips XL30FEG SEM or Hitachi S-4800 High Resolution SEM, attached with the accessories capable of performing energy dispersive spectroscopy (EDS) as well.

### 3.4 Preparation of the *Escherichia coli* (*E.* coli) Culture

The *Escherichia coli* #23724 bacteria were obtained in freeze-dried form (American Type Culture Collection, Manassas, VA). The dry pellet was rehydrated in 2 ml of a super broth containing 32 g tryptone, 20 g yeast extract and 5 g NaCl (all from Fisher Scientific, Waltham, MA) per liter of aq solution made with ultra pure water with a conductivity of $5.5 \times 10^{-8}$ S-cm. The super broth solution was sterilized in an autoclave at 121°C for 10 min. at 18 psi, followed by cooling to room temperature (21°C) prior to storage in a refrigerator at 5°C.
The rehydrated *E. coli* was diluted with 100 ml of super broth and placed in a sterilized 250 ml flask. Sterilization was carried out by autoclaving the flask at 122°C for 20 min. at 18 psi followed by drying for additional 20 min. The suspension was incubated in a shaker at 37°C for 12 h. For the re-storage of the *E. coli*, the solution was spun down in a centrifuge until a pellet formed below a clear solution. The solution was decanted and pellet recovered to which 10 ml of 10% glycerol (99.8%, Fisher Scientific, Waltham, MA) solution of super broth was added to suspend the pellet again. 1 ml of suspension was aliquoted in 2 ml Eppendorf tubes and stored in a freezer at -80°C.

### 3.5 Bacterial Growth

For the bacterial growth, 100 ml of super broth was inoculated with a sterile 10 μl loop (Fisher Scientific, Waltham, MA), followed by agitation at 150 rpm for 12 h until the bacteria culture reached the stationary phase. To make the calibration curve, the cell concentration with a specific optical density value needed to be determined. A Perkin Elmer Victor 3 1420 Multilabel Counter was used to measure optical density at 600 nm (OD600) with a serial dilution of the *E. coli* in ultra pure water. The OD600 values were plotted against the dilution factor and a trendline fitting was attempted to obtain the predictive behavior of the data with a fair degree of confidence ($R^2 = .9934$). This is shown in figure 3.6.
Using this calibration, a serial dilution of 1:1 million E. coli dilutions was made. 100 μl of this solution was taken for use on three different super broth agar plates to incubate overnight at 37°C. Super broth with the additive agar consisted of 15 g of agar, 32 g tryptone, 20 g yeast extract, 5 g NaCl (all obtained from Fisher Scientific, Waltham, MA) per liter of ultrapure water. The super broth with agar was autoclaved and allowed to cool to 21°C and poured into 100 mm × 15 mm petri dishes to solidify and be stored at 5°C. The number of colonies on the plate was counted the following day to get the cell density (cells/ml) with time. Combining this information with the calibration and the trendline equation, the cell growth curve at a specific OD600 was plotted and is shown in figure 3.7. As can be seen, the cell growth follows the expected semi-log pattern and reaches saturation in about 6 h.

![Figure 3.6: Calibration curve for the E. coli cell concentration](image)
3.6 Staining of Bacteria

For staining purposes, an *E. coli* suspension containing $2.58 \times 10^8$ cells/ml was used. It was diluted with 100 μl of ultra pure water. Live/Dead BacLight Bacterial Viability Kits (Invitrogen, Carlsbad, CA) provide a novel two-color fluorescence assay of bacterial viability that is useful for diverse bacterial genera [35]. In this work, SYTO 9 and propidium iodide stains (1 μl of each) were used. The *E. coli* was left in complete darkness for 15 min. at 21°C to complete the staining. 40 μl of this solution was pipetted into 35 × 14 mm glass bottom microwell dish with No. 15 cover glass. To this, approximately 6 mg of the nanofibers were added. In order to view the fluorescence given off by these stains, the dish was positioned centrally in the multi-photon laser.
scanning confocal microscope (model TCS SP5, Leica Microsystems). Upon photo excitation, the *E. coli* stained with SYTO9 fluoresces green and that stained with propidium iodide fluoresces red. The excitation and emission of SYTO9 occurs at 480 and 500 nm, respectively. The excitation and emission of propidium iodide occurs at 490 and 635 nm, respectively.

### 3.7 Light Sources

Multiple light sources were used to photoactivate the titania nanostructures. The broad optical spectrum with the location and range of UV, visible and IR radiation is shown in figure 3.8.

![Figure 3.8: Wavelengths of various radiations in the light spectrum](image)

The energy of a single photon of light of a specific wavelength is given by the relationship:
\[ E = h \cdot \nu = h \cdot \frac{c}{\lambda} \]

Where the \( E \) is energy (J or eV; \( 1 \text{ eV} = 1.602176 \times 10^{-19} \text{ J} \)), \( h \) Plank’s constant \( (6.626 \times 10^{-34} \text{ Js}) \), \( \nu \) is the frequency \( (\text{s}^{-1}) \), \( c \) is the speed of light \( (3 \times 10^8 \text{ ms}^{-1}) \) and \( \lambda \) is wavelength \( (\text{m}) \). Various light sources used in this work and their relevant characteristics are listed in Table 3.1.

<table>
<thead>
<tr>
<th>Light Sources</th>
<th>( \lambda ) (nm)</th>
<th>Intensity (W/cm(^2))</th>
<th>Energy (J)</th>
<th>Energy (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV</td>
<td>365</td>
<td>( 350 \times 10^{-6} )</td>
<td>( 5.446 \times 10^{-19} )</td>
<td>3.40</td>
</tr>
<tr>
<td>MP IR Laser</td>
<td>820</td>
<td>( 2 \times 10^6 )</td>
<td>( 2.424 \times 10^{-19} )</td>
<td>1.51</td>
</tr>
<tr>
<td>Portable IR Laser</td>
<td>808</td>
<td>5</td>
<td>( 2.460 \times 10^{-19} )</td>
<td>1.54</td>
</tr>
<tr>
<td>IR Flashlight</td>
<td>830</td>
<td>unspecified</td>
<td>( 2.395 \times 10^{-19} )</td>
<td>1.50</td>
</tr>
</tbody>
</table>

As seen, the energy of the incident photons is inversely proportional to the wavelength. Consequently, the UV light source has higher energy per photon in comparison to the IR light sources. We will discuss the repercussion of this artifact later in the Discussion section.
### 3.8 Processing and Characterization of the Electrospun Fibers

After spinning was complete, small amounts of the as-spun composite fibers were used for viewing under scanning electron microscope (Philips XL30FEG SEM). This ensured the quality of fibers in terms of the absence of intertwining, twisting, branching, liquid globule entrapment, etc. The remaining fibers collected on the ceramic plates were fired at 700°C for 2h in static air as per the following heating rate-temperature-soak time profile: 25°C (room temperature) to 500°C at a rate of 0.5°C/min. with a hold at 500°C for 2h; 500°C to 700°C at a rate of 0.5°C/min. with a hold at 700°C for 2h, followed by cooling from 700°C to the room temperature at a rate of 0.5°C/min. The heating profile is shown in figure 3.9.

![Image showing the heating profile](image_url)

**Figure 3.9: Temperature-time profile used for firing the composite fibers**

The rather small heating and cooling rates were chosen to ensure the removal of organic components without destroying the nanofibrillar morphological features in the end product as well as to avoid the disintegration of the nanofibers into a powdery mass. The fired samples were thoroughly characterized by structural and microstructural analyses, using XRD (PANalytical X'Pert Pro MPD), SEM (Philips XL30FEG SEM), TEM
(Hitachi HD-2300 STEM) and EDS. As expected, the final product was TiO$_2$ in one case and TiO$_2$ doped with Fe$_3$O$_4$ in the other, the later being endowed with a brownish orange tinge.

3.9 Confocal Image Analysis: For Fibers

In order to photoactivate the titania nanofibers submerged in the *E. coli* suspension, the confocal multiphoton microscope system (Leica TCS SP5 MP, Leica Microsystems, Bannockburn, IL) was used. This instrument allows adapting the multiphoton system by optimally choosing from IR to picosecond or femtosecond laser and performing experiments with minimal phototoxicity. The reduced phototoxicity due to spatial confinement of excitation is ideal for living cells, which is crucial for this work in order to establish unequivocally that the observed bactericidal effect is due to the photocatalytic artifact of the titania nanofibers upon excitation and not due to photons alone.

In one experiment, the multi-photon beam ($\lambda = 820$ nm) generated by the in-built pulsed IR laser was turned on for 3 s and multiple real-time videos and still shots were recorded for 5 min. segments. The activity with respect to the bacterial colony was captured within the intervening period. The reason for using the MP beam ($\lambda = 820$ nm) is owing to its use in the photoactivation of green fluorescent proteins$^{[36]}$. After several trials it was found that only 3 s of excitation by the MP beam was adequate for causing effective bacterial death. In a simulated experiment, the *E. coli* broths containing titania nanofibers were also irradiated by a handheld portable IR flashlight ($\lambda = 830$ nm; [www.maxmax.com](http://www.maxmax.com), Carlstadt, NJ). In this case, the flashlight was turned on for 12 s;
longer exposure time (compared to the MP laser) is due to the low intensity of the IR flashlight. Since majority of bactericidal experiments using titania photocatalyst to date have employed a UV source \([16-18, 37-38]\), one set of data were created by using a handheld UV light source (\(\lambda = 365\) nm; Spectroline, model ENF-260C, Westbury, NY) for ready comparison of the results. In the case of UV exposure, excitation duration was varied between 3s and up to 20 min.

3.10 Confocal Image Analysis: For Ti Plates and Wires

In order to photoactivate (against the \(E. coli\) suspension) the Ti plates and wires onto which titania structure was created, an IR laser (\(\lambda = 808\) nm; 1W, \(www.freaklasers.com\)) was used. It should be pointed out that the IR laser is different from the IR flashlight which was used for activating the TiO\(_2\) fibers. The main reason for changing the source from IR flashlight (for fibers) to the IR laser (for films) is that the intensity of the beam from the flashlight was not strong enough to activate the TiO\(_2\) nanofibers or nanotubes formed on the latter. The IR laser used in this work is shown in figure 3.10.

![Hand-held IR laser source used in this work](image-url)
The focus beam size was adjusted to be about 5 mm diameter. This spatial confinement of the laser beam allowed the excitement of the titania plate alone while causing low or no direct phototoxity to the bacteria.

For the purpose of evaluating the bactericidal efficacy of titania films formed on the Ti plate and/or wire, the *E. coli* broth was placed inside a petri dish. The autoclaved and/or anodized Ti plate/wire was placed in the bacterial solution as shown below in figure 3.11.

![Experimental setup for the evaluation of bactericidal efficacy of titania films on Ti plates](image)

**Figure 3.11:** Experimental setup for the evaluation of bactericidal efficacy of titania films on Ti plates

The IR laser was placed ~1 inch above the Ti coupon and was turned on for 12 to 30 s. This allowed the titania film on the Ti plate to be photoactivated. After irradiation the plate was placed inside the petri dish containing the *E. coli* broth. The petri dish was then placed appropriately within the confocal microscope. The activity with respect to the bacterial colony was captured for the next several minutes (up to ~ 50 min.) The reason for using the IR laser (λ = 808 nm) is owing to its effectiveness in the photoactivation of green fluorescent proteins[^36]. After several iterations it was found that excitation by the
IR laser for 12 to 24 s was adequate for causing effective bacterial death. As will be discussed subsequently later in the thesis, majority of bactericidal experiments using titania to date, have employed UV source as the photoactivation agent. Therefore, one set of experiments was conducted by using a handheld UV light source ($\lambda = 365$ nm; Spectroline, model ENF-260C, Westbury, NY) also for ready comparison of the results; in the case of UV exposure, excitation duration was varied between 24 s and up to 20 min.
Chapter 4

Results and Discussion

4.1 Microstructural Features of Electrospun Fibers

4.1.1 As-spun Fibers

The morphological features of the as-spun cermer composite nanofibers from four different solutions showed consistent uniformity, open structure and non-woven features. The quality of the titania cermer composite nanofibers are shown in figure 4.1 (a through d). This also indicates that the formulations and spinning parameters was well-optimized such that no globules or clumps were formed.

Figure 4.1a: SEM images of the as-spun cermer nanofibers from solution B
Figure 4.1b: SEM images of the as-spun cermer nanofibers from solution C

Figure 4.1c: SEM images of the as-spun cermer nanofibers from solution D

Figure 4.1d: SEM images of the as-spun cermer nanofibers from solution E
4.1.2 Calcined Fibers

The intact nature of the nanofibers in layered non-woven format seen in the as-spun materials was retained in the calcined samples as well, by using the protocol of very slow ramp rate during the entire schedule of calcination. Even a slightly higher ramp rate was found to cause severe fiber rupturing, due to faster combustion of the polymer with a concomitant and sudden release of copious amount of gaseous products, rendering them into a powdery mass. The energy dispersive spectra collected on the fired compositions did not show any peak belonging to carbon, signifying that the heating profile selected in this work was able to eliminate the polymeric components quantitatively. SEM images of the fired titania nanofibers can be seen in figure 4.2 (a through d).

![SEM image of the calcined fibers B](image-url)

**Figure 4.2a: SEM image of the calcined fibers B**
Figure 4.2b: SEM image of the calcined fibers C

Figure 4.2c: SEM image of the calcined fibers D
4.2 Elemental Mapping/Quantification by Energy Dispersive Spectroscopy (EDS)

The elemental mapping in the as-spun and calcined fibers from solution D is shown in figure 4.3a and 4.3b, respectively; the atomic and weight percentage of the elements present in the as-spun and calcined fibers are shown in Table 4.1. It should be recalled that the fibers spun from solution D are iron doped. Accordingly, the elemental maps reproduced in figure 4.3a and 4.3b show (in addition to titanium and oxygen) the presence of iron in the as-spun and the calcined fibers both. In the as-spun fibers the source of oxygen evidently is the oxynitrate \([\text{O(NO}_3\text{)}_2]^4\) group from the titanium precursor, while that in the calcined samples comes from oxidation of fibers in air.

<table>
<thead>
<tr>
<th>Element</th>
<th>Concentration in as-spun fibers wt. %</th>
<th>Concentration in fired fibers wt. %</th>
<th>Concentration in as-spun fibers at. %</th>
<th>Concentration in fired fibers at. %</th>
</tr>
</thead>
<tbody>
<tr>
<td>O</td>
<td>71.19</td>
<td>33.67</td>
<td>88.45</td>
<td>61.26</td>
</tr>
<tr>
<td>Ti</td>
<td>21.87</td>
<td>48.21</td>
<td>9.08</td>
<td>29.3</td>
</tr>
<tr>
<td>Fe</td>
<td>6.94</td>
<td>18.11</td>
<td>2.47</td>
<td>9.44</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>
Figure 4.3a: Elemental mapping of the as-spun nanofibers D

Figure 4.3b: Elemental mapping of the fired nanofibers D
It should be pointed out that EDS provides only a semi-quantitative compositional analysis and the margin of error is about ± 3-5%. With that cognizance, the composition of the fired sample is in fair agreement with that calculated theoretically in 5 wt. % Fe doped-TiO₂.

4.3 Structural Characterization

X-ray diffraction patterns collected on the calcined powders of the pure and doped TiO₂ nanofibers are shown in figure 4.4; they are identical, conforming to a mixture of anatase + rutile phases (ICDD cards 78-2486 and 78-1508/09); the intensity of peaks belonging to anatase phase diminished significantly in the doped sample.

![XRD patterns of the pure TiO₂ and iron-doped TiO₂ after respective cermet composites were calcined at 700°C for 2h; A stands for anatase](image)

Figure 4.4: XRD patterns of the pure TiO₂ and iron-doped TiO₂ after respective cermet composites were calcined at 700°C for 2h; A stands for anatase
The irreversible transformation of the metastable phase of titania (anatase) into a thermodynamically more stable modification, viz., rutile, occurs upon high temperature calcination. The kinetics of this have been studied in great detail in the past. The transformation has been shown to be strongly influenced by particle size\cite{39}, cationic and anionic impurities and their nature\cite{40-51}, reaction environment and electric field \cite{52-54}, processing parameters\cite{55}, and other factors\cite{56}. More importantly, (i) small particles lead to the transformation at low temperatures and (ii) the conversion from anatase to rutile is also greatly assisted by the presence of impurities. In particular, cationic impurities of valence lower than 4+ accelerate the A to R transition by providing a charge compensation mechanism via oxygen ion vacancy creation that aids atomic transport. The extent of transformation was assessed by calculating the percentage of rutile phase in the two samples, using the Spurr-Myer equation\cite{57}:

$$F_R = \frac{1}{1 + 1.26 \times \left( \frac{I_A <101>}{I_R <110>} \right)}$$

Where $F_R$ is the mass fraction of rutile in the sample, and $I_A<101>$ and $I_R<110>$ are the integrated $<101>$ intensities of the anatase phase and $<110>$ intensities of the rutile phase, respectively. From the raw diffraction intensity data, values of 69.6% and 88.1% were computed for the rutile fraction in pure and Fe-doped titania nanofibers. Accordingly, presence of iron in the doped titania fibers led to the accelerated transition of anatase into rutile phase, though, due to rather low percentage, it was difficult to discern iron oxide in the XRD. The elemental mapping and EDS signature, however,
showed the presence of iron in the doped titania nanofibers, as shown in figure 4.3a-b, respectively.

**4.4 FESEM and TEM of Fe-doped Fired Titania Nanofibers**

The field emission scanning electron microscopy (FESEM) and transmission electron microscopy (TEM) images of the calcined iron-doped titania nanofibers (D) are shown in figure 4.5 and figure 4.6, respectively. It is evident that the heat treatment used in the present work has preserved the fibrous artifact in the processed material. Moreover, the titania fibers are porous and less than 100 nm across; they are comprised of interconnected nearly monosized grains (~20-25 nm) making the structure breathable and therefore quite amenable for the intended medical application.

![Figure 4.5: FESEM images of the Fe-doped titania nanofibers](image-url)
4.5 Microstructural Features of Titania Films on cp Ti Substrates

The titania films grown by various techniques described in the previous chapter on cp Ti coupons and wires as well as on Ti implants were also characterized by multiple techniques, such as, XRD, SEM, elemental mapping and energy dispersive spectroscopy (EDS). Identification of titania films grown on Ti substrates by hydrothermal processing and anodization, using XRD proved to be challenging, since the oxide coating in each of these cases was very thin and the X-rays penetrated past the TiO$_2$ surface, thereby
showing the diffraction pattern of titanium lying beneath. Hence, the XRD patterns are not shown. Collecting XRD patterns on wire samples was also challenging.

### 4.5.1 Hydrothermally Produced Titania Films

The SEM images of as-received Ti plate are shown in figure 4.7, while those of the plates hydrothermally processed in 30% aq H₂O₂ at 80°C/½ h are presented in figure 4.8.

![Figure 4.7: SEM images of the as-received Ti plate](image1)

![Figure 4.8: SEM images of cp Ti plate after autoclaving in 30% aq H₂O₂ at 80°C/½h](image2)

The elemental mapping with respect to titanium and oxygen is shown figure 4.9.
Figure 4.9: Elemental mapping of TiO$_2$ coating on cp Ti plates in 30% aq H$_2$O$_2$ at 80°C/½h; (O: red, Ti: green)

Keeping other parameters identical, if the time for autoclaving is increased, the morphology and the concentration of elements present in the film also change. This is clearly seen in micrographs shown in figure 4.10 and the elemental mapping in figure 4.11 for Ti coupons autoclaved at 80°C for 1 h.

Figure 4.10: SEM images of cp Ti plate after autoclaving in 30% aq H$_2$O$_2$ at 80°C/1h

Figure 4.11: Elemental mapping of TiO$_2$ coating on cp Ti plates in 30% aq H$_2$O$_2$ at 80°C/1h; (O: red, Ti: green)
This is corroborated by the atomic and weight percentage of the elements present in the two films, as shown in Table 4.2.

Table 4.2: Semi-quantitative compositional analyses of the films on autoclaved cp Ti

<table>
<thead>
<tr>
<th>Element</th>
<th>Autoclaved at 80°C/ ½ h</th>
<th>Autoclaved at 80°C/ 1 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wt. %</td>
<td>at. %</td>
</tr>
<tr>
<td>O</td>
<td>72.77</td>
<td>88.89</td>
</tr>
<tr>
<td>Ti</td>
<td>27.23</td>
<td>11.11</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Evidently, on hydrothermal processing for longer duration, greater oxidation of titanium is facilitated which leads to increase in oxygen content of the film. The composition of the film obtained after 1h of autoclaving at 80°C is closer to the stoichiometric titania; theoretically, a film of stoichiometric TiO$_2$ contains 60 wt. % (33 at. %) of Ti and 40 wt. % (67 at. %) of O.

However, the films formed in either case were found to be amorphous; to be photoactive, titania must be crystalline. Therefore, the Ti plates hydrothermally processed at 80°C for 1h were calcined at 700°C for 1h (ramp rate:1°/ min.) after cleaning with DI water under sonication. The SEM images and elemental mapping contours for Ti and O are shown in figure 4.12. Quantification is summarized in Table 4.3.

Table 4.3: Compositional comparison of the films on cp Ti and Henkel Ti coupons

<table>
<thead>
<tr>
<th>Element</th>
<th>Cp Ti autoclaved at 80°C/ ½ h and calcined at 700°C /1h</th>
<th>Henkel Ti calcined at 800°C /4h and autoclaved at 100°C/ 2h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wt. %</td>
<td>at. %</td>
</tr>
<tr>
<td>O</td>
<td>27.71</td>
<td>53.44</td>
</tr>
<tr>
<td>Ti</td>
<td>72.29</td>
<td>46.56</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>
4.5.2 Hydrothermal Processing of Henkel Ti Plates Results

As stated in chapter 3, the Ti coupons from Henkel Corporation (Madison Heights, MI) are coated with a thin film of TiO$_2$. Figure 4.13 shows the SEM images of the as-received Henkel samples. According to Henkel Corp., the titania film on the Ti surface is structurally brookite$^{[33]}$. These Ti plates were soaked at 800°C for 4h in static air at a ramp rate of 10°/min., whose microstructural features and elemental mapping are shown in figure 4.14; significant morphological changes are clearly seen. Quantitative elemental analysis by EDS is included in Table 4.3, which corresponds to TiO$_2$ composition within the permissible limits of errors.
Figure 4.13: Morphological features in the as-received Henkel plates

Figure 4.14: SEM and elemental mapping in the calcined TiO\textsubscript{2} film on Henkel Ti plate after calcination at 800°C/4h (O: red, Ti: green)

The calcined plates were chemically etched for 10 s in 40% HF aq solution, washed with acetone and air-dried. These were subjected to hydrothermal processing in different media as described in detail in chapter 3. The systematic microstructural evolution in these treatments is shown in figures 4.15 through 4.17. The compositional analysis predicted by EDS is presented in Table 4.4.
Figure 4.15: SEM and elemental mapping of Henkel plate autoclaved in hydrazine monohydrate at 100°C/2h (O: red, Ti: green)
Figure 4.16: SEM and elemental mapping of Henkel plate autoclaved in 5M NaOH at 100°C/ 2h (O: red, Ti: green)

Figure 4.17: SEM and elemental mapping of Henkel plate autoclaved in TMAOH at 100°C/ 2h (O: red, Ti: green)
Table 4.4: Compositional analyses of the films on Henkel Ti coupons after hydrothermal processing in different media at 100°C/ 2h

<table>
<thead>
<tr>
<th>Element</th>
<th>N2H4, H2O</th>
<th>5M NaOH</th>
<th>TMAOH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wt. %</td>
<td>at. %</td>
<td>wt. %</td>
</tr>
<tr>
<td>O</td>
<td>20.36</td>
<td>43.36</td>
<td>31.06</td>
</tr>
<tr>
<td>Ti</td>
<td>79.64</td>
<td>56.64</td>
<td>68.94</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

After the Hydrothermal processing on the different calcined Henkel plates was completed the microstructures indicated in figures 4.15 through 4.17 displayed that the Henkel plate autoclaved in 5M NaOH at 100°C/ 2h had formed nanostructures. The formation of nanofibrillar titania was produced with the Henkel plate that was autoclaved in 5M NaOH.

Hydrothermal processing has been employed by others also to create TiO2 coating on Ti plates. For example, Wang et al. [58] used hydrothermal treatment of Ti in 10M NaOH solution in the temperature range of 140-200°C for time periods varying between 2 and 6h. The autoclaved Ti substrates were soaked in 0.1M HCl solution for 12h followed by washing with DI water and annealing at 300-500°C for 2h. This helped to create TiO2 nanoarrays.

Zuruzi et al. [59] employed a process called “lift-off technique” to create titania nanoarrays on Ti surface, using aq H2O2 solution. The disadvantage of this procedure is the use of fluoroform, which is known to be a potent greenhouse gas in addition to being toxic.
In comparison, the procedure employed in the current work is quite mild where either hydrogen peroxide solution, TMOAOH or NaOH (5M) was used for short duration and at relatively lower temperatures.

4.6 Microstructural Features of Anodized Ti Substrates

Attempts were made to create nanostructured titania films on Ti substrates (wires and plates) via anodization as well. As stated in detail in chapter 3, these substrates were anodized in 0.5 M H$_3$PO$_4$ + 0.14 M HF mixture at a voltage of 21V for 1, 2 and 4 h. In the case of wire as well as plate, anodization led to the creation of titania nanotubes of almost uniform cross-section. This is shown in figure 4.18 for wire specimen, anodized for 1h.

Figure 4.18: SEM images of TiO$_2$ nanotubes created on cp Ti wire anodized for 1h
Elemental analyses on different locations of the wire anodized for 1h showed the presence of aluminum in one case in addition to titanium and oxygen (figure 4.19). This is due to the fact that commercially pure Ti contains small amount of aluminum; upon anodization, aluminum concomitantly migrates to the surface and also gets oxidized to Al₂O₃. This is corroborated by the data shown in Table 4.5.

**Figure 4.19: Location of EDS analysis (spot 1 and spot 2) on the anodized film**

**Table 4.5: Compositional analysis at spot 1 and 2 on the Ti wire anodized for 1h**

<table>
<thead>
<tr>
<th>Element</th>
<th>Spectrum 3 (spot 1)</th>
<th>Spectrum 4 (spot 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wt. %</td>
<td>at. %</td>
</tr>
<tr>
<td>O</td>
<td>37.58</td>
<td>62.28</td>
</tr>
<tr>
<td>Ti</td>
<td>55.05</td>
<td>30.48</td>
</tr>
<tr>
<td>Al</td>
<td>7.37</td>
<td>7.24</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Similar microstructural features were seen in the case of Ti plates (from Titanium Inc.) anodized for 1h. However, distribution of aluminum was far more widespread in this case compared to the wire; pure TiO₂ nanotubes were not generated. Availability of larger surface area in the case of plates facilitated the migration of aluminum to be more universal than localized. This could be seen by the presence of aluminum as small
intertwined rings on top of individual TiO$_2$ nanotubes, in the SEM images shown in figure 4.20.

Figure 4.20: SEM images of the Ti plate anodized for 1h

This results in exceedingly high counts for aluminum in the EDS. The spots where elemental analyses were carried out are shown in figure 4.21 and the data is summarized in Table 4.6.
Interestingly, the Ti plates anodized for 2 h under identical experimental conditions, showed the development of a consistently uniform nanotubular structure; the nanotubes are about 100-150 nm in diameter and ~ 300 nm in height. These results are presented in figures 4.22 and 4.23. Also, there was no presence of aluminum on the film, as evidenced by the EDS analysis (Table 4.7).
Figure 4.22: SEM images of the Ti plate anodized for 2h

Figure 4.23: SEM/EDS image of the Ti plate anodized for 2h
Table 4.7: Compositional analysis on the cp Ti plate anodized for 2h

<table>
<thead>
<tr>
<th>Element</th>
<th>Spectrum 3 (spot 1)</th>
<th>wt. %</th>
<th>at. %</th>
</tr>
</thead>
<tbody>
<tr>
<td>O</td>
<td>25.8</td>
<td>51.01</td>
<td></td>
</tr>
<tr>
<td>Ti</td>
<td>74.2</td>
<td>48.99</td>
<td></td>
</tr>
<tr>
<td>Al</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

It is likely that longer anodization leads to the dissolution of aluminum and/or aluminum oxide. This notion is strengthened by the results on anodization experiments carried out for even longer period (viz., 4h).

In the case of Ti plates anodized for 4 h, the characteristic nanotubular features were totally destroyed as can be seen readily from the micrographs shown in figure 4.24. In addition to variation in the morphological features, the titania entity also was destroyed as shown by the EDS analysis which confirms the presence of elemental Ti alone (figure 5.25 and Table 4.8); no aluminum was seen either.
Figure 4.24: SEM images of the Ti plate anodized for 4h

Figure 4.25: SEM/EDS image of the Ti plate anodized for 2h
Table 4.8: Compositional analysis on the cp Ti plate anodized for 4h

<table>
<thead>
<tr>
<th>Element</th>
<th>Spectrum 3 (spot 1)</th>
<th>wt. %</th>
<th>at. %</th>
</tr>
</thead>
<tbody>
<tr>
<td>O</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ti</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Al</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

This clearly shows that the optimum duration for Ti plate anodization is 2h and therefore, among all the plates, those anodized for 2h Ti plates were used in the bactericidal testing against *E. coli*.

Mor et al. [60] created TiO$_2$ nanotubes by anodization technique using Ti foil as the anode and Pt as cathode, in a range 3-20V in a mixture of 2.5 wt% HNO$_3$ and 1 wt% HF aq solution for 1-4h at temperatures ranging between 5 and 50°C; anodization at 20V and 50°C was reported to yield the best results. However, we could not reproduce these results in our laboratory on Ti plates.

Mohapatra et al. [34] reported creating highly ordered nanotubes of TiO$_2$ by anodization of Ti foils (0.2mm thick) in a mixture of 0.5M H$_3$PO$_4$ and 0.14 M NaF at room temperature and application of 20V. A slightly modified method adopted by us was successful in creating nanotubes on 2mm thick Ti plates.

**4.7 Evaluation of Bactericidal Efficacy**

A detailed systematic evaluation protocol was drawn and carried out in order to unequivocally establish that the bacterial necrosis was most effective in the presence of
free-standing titania nanofibers and the films on cp Ti plates and the implants, and, that both irradiation and photocatalyst were needed. For this purpose, confocal spectra were collected on the following systems with the *E. coli* suspensions illuminated with:

a. UV (\(\lambda = 365\) nm) radiation
b. multiphoton IR laser (\(\lambda = 820\) nm)
c. UV (\(\lambda = 365\) nm) radiation in the presence of pure titania nanofibers
d. multiphoton IR laser (\(\lambda = 820\) nm) in the presence of pure titania nanofibers
e. UV (\(\lambda = 365\) nm) radiation in the presence of Fe-doped titania nanofibers
f. multiphoton IR laser (\(\lambda = 820\) nm) in the presence of Fe-doped titania nanofibers
g. UV (\(\lambda = 365\) nm) radiation in the presence of Ti coupons decorated with hydrothermally produced titania films
h. UV (\(\lambda = 365\) nm) radiation in the presence of Ti coupons decorated with titania films produced via anodization
i. UV (\(\lambda = 365\) nm) radiation in the presence of Ti wires decorated with titania films produced via anodization
j. UV (\(\lambda = 365\) nm) radiation in the presence of TiAl6V4 implants decorated with hydrothermally produced titania films

### 4.7.1 Pure and Fe-Doped Titania Nanofibers

Figure 4.26 shows the aqueous suspension of titania nanofibers. Figures 4.27 through 4.30 show the time-dependent fate of bacterial (*E. coli*) colony in their water suspension, upon illumination for 3 s by UV radiation (\(\lambda = 365\) nm) and upon irradiation for 3 s by...
multiphoton (MP) IR laser ($\lambda = 820$ nm), respectively. The live cells are identified by green pixels which should turn red if dead. As can be seen, there is no indication of decrease in the microorganism population in any of these treatments, which demonstrates that with photo-irradiation (UV or MP IR laser) alone is not enough to cause bactericide.

Figure 4.26: Aqueous suspension of TiO$_2$ Fibers viewed by using DIC (Differential Interference Contrast)
Figure 4.27: Confocal images of the bacterial colonies at different times in DI water suspension without exposure to radiation
Figure 4.28: Confocal images of the bacterial colonies at different times upon exposure to UV radiation for 3 s
The bactericidal efficacy of the pure and Fe-doped titania nanofibers towards *E. coli* upon activation by UV ($\lambda = 365$ nm) and multiphoton IR laser ($\lambda = 820$ nm) is presented in figure 4.30, figure 4.31 and figure 4.32, respectively; again, the live cells are identified by green pixels and the dead ones by red pixels.
Figure 4.30: Confocal images of the bacterial colonies (conc. 2.15×10⁷ cells/ml) at different times upon exposure to UV radiation for 3 s in the presence of TiO₂ nanofibers B
Figure 4.31: Confocal images of the bacterial colonies (conc. $2.15 \times 10^7$ cells/ml) at different times upon exposure to UV radiation for 20 minutes in the presence of TiO$_2$ nanofibers B
The results shown in figure 4.33 suggest that if infrared (IR) beam is used in preference to the ultraviolet (UV) radiation, it is capable of causing bacterial necrosis even if their concentration is high.
Figure 4.33: Confocal images of the bacterial colonies (conc. $8.6 \times 10^7$ cells/ml) at different times upon exposure to MP radiation for 3 s in the presence of TiO$_2$ nanofibers B

The effectiveness of Fe-doped TiO$_2$ nanofibers (D) towards *E. coli* necrosis upon exposure to MP for 3 s and to an incident beam from handheld IR light for 12 s is shown in figure 4.34 and 4.35, respectively.
Figure 4.34: Confocal images of the bacterial colonies (conc. $2.15 \times 10^7$ cells/ml) at different times upon exposure to MP radiation for 3 s in the presence of Fe-doped TiO$_2$ nanofibers D

Figure 4.35: Confocal images of the bacterial colonies (conc. $2.15 \times 10^7$ cells/ml) in Fe-doped TiO$_2$ (D) suspension at different times after IR exposure with a handheld flashlight for 12 s

From the foregoing discussion, it is thus clear that a judicious combination of catalytic artifacts of titania and incident photons is quite effective in inhibiting bacterial colonization. While ultraviolet beam is effective in treating bacterial infection, it
however, necessitates longer exposure to be of quantitative value; in some cases the exposure could last from 20 to as long as 120 min. In contrast, present work clearly demonstrates that more quantitative bactericidal activities were observed with IR exposure for a far shorter duration (3-12 s).

This is perhaps the first investigation that makes use of IR light source to cause microorganism necrosis. Furthermore, a combination of titania in nanofibrillar format with infrared excitation in mitigating colonization is also reported for the first time. The results further demonstrated that the presence of iron oxide in small concentration as a second phase augments these activities. This opens up the possibility of using a non-woven mat of Fe-doped titania irradiated by a very short pulse of an infrared beam, as an infection mitigation device.

4.7.2 Ti Plates and Implants

Previously, we demonstrated that that the illumination of *E. coli* broth by the UV (\(\lambda = 365\) nm) and multiphoton (MP) IR laser (\(\lambda = 820\) nm) alone does not cause bacterial deactivation and necrosis. The multiphoton IR laser is integrated in the confocal fluorescence microscope and has an intensity of 2 W, while that of the portable IR laser flashlight (\(\lambda = 808\) nm) is 1 W. Thus, it appears that the use of a 1 W IR laser alone would not cause harm to the bacteria. However, with the help of the photocatalyst TiO\(_2\) film formed on the implant, the laser becomes an effective tool that could deactivate or kill the bacteria present on the implant. This would allow the patients to heal quicker and be less prone to an SSI.
It should be pointed out that unlike the free-standing fibers which are interconnected by particles whose average size is 30-40 nm, cp Ti and implant coupons used in this work were about 2 mm thick; even the Ti wire was 1.024 mm in thickness. Thus, it was imperative that to obtain appreciable bacterial mitigation, duration of photoactivation should be somewhat longer than that used in the case of nanofibers.

In the following are shown the confocal images of bacterial colonies subsequent to the exposure of the Ti plates that were processed hydrothermally and via anodization subjected to photoactivation for various durations by UV and IR beams. Green pixels indicate live cells and red pixels are the dead cells.

Figure 4.36: Confocal images of the bacterial colonies at different times in suspension containing cp Ti plate (autoclaved at 80°C/1h in 30% aq H₂O₂ and fired at 700°C/1h) and exposed to IR laser for 24 s
Figure 4.37: Confocal images of the bacterial colonies at different times in suspension containing Henkel Ti plate (autoclaved in 5M NaOH at 100°C/2h) and exposed to IR laser for 30 s

Figure 4.38: Confocal images of the bacterial colonies at different times in suspension containing Titanium Inc. Ti plate anodized for 1h and exposed to IR laser for 60 s
Figure 4.39: Confocal images of the bacterial colonies at different times in suspension containing Titanium Inc. Ti plate anodized for 2h and exposed to IR laser for 24 s

Figure 4.40: Confocal images of the bacterial colonies at different times in suspension containing Titanium Inc. Ti plate anodized for 3h and exposed to IR laser for 24 s
Figure 4.41: Confocal images of the bacterial colonies at different times in suspension containing Titanium Inc. Ti plate anodized for 2h, chemically etched in EtOH/HF mixture for 5 s (top) and 10 s (bottom) followed by exposure to IR laser for 24 s
The confocal results summarized above, led to the conclusion that among all titanium samples tested hitherto with the IR laser, two sets of results were most promising: (i) the Henkel Ti plates autoclaved in 5M NaOH, at 100°C for 2 h and, (ii) the plates from Titanium Inc. that were anodized for 2h and chemically etched for 5 s in EtOH/HF mixture. In order to make a realistic comparison of the data collected on IR-activated
specimen, parallel experiments were also carried out with UV light exposure on the two plates that worked well with IR beams. These results are shown in figures 4.43 through 4.45.

Figure 4.43: Confocal images of the bacterial colonies at different times in suspension containing Henkel Ti plate (autoclaved in 5M NaOH at 100°C/2h) and exposed to UV light for 20 min

Figure 4.44: Confocal images of the bacterial colonies at different times in suspension containing Ti plate from Titanium Inc. anodized for 2h, chemically etched for 5 s in EtOH/HF and exposed to UV light for 30 s
When titania is exposed to light, a multitude of free radicals are released to provide an oxidizing environment that is conducive for the destruction of a host of organic materials. The assessment of bactericidal effect of TiO$_2$ particle (in powder form) irradiated by UV light has been examined by several researchers to mitigate bacterial infections in clinical applications. Koseki et al. [37] irradiated a suspension of $S$. aureus (concentration: $1 \times 10^5$ cfu/ml) containing 19 µg/ml of TiO$_2$ particles with UV light (1.82 mW/cm$^2$) in one case and with fluorescent light (80 µW/cm$^2$) in the other, for 1h. It was found that the bacterial survival rate decreased steadily, reaching 9.4% and 10.9% after exposure to UV and fluorescent light, respectively.

Yu et al. [16] have reported the fabrication of Fe- doped TiO$_2$ films on stainless steel substrates through dip coating and their use as antibacterial agent for sterilization. In this
case, a suspension of *Bacillus pumilus* (concentration: $10^7$ cfu/ml) was placed onto the TiO$_2$-coated stainless steel plate and irradiated by a UV lamp (intensity: 630 µW/cm$^2$; $\lambda$ = 365 nm). The results showed that the active colonies of bacteria decreased by 50% after 2 h of exposure.

Recently, Oka et al. [17] studied the inhibition of bacterial colonization of methicillin-resistant *S. aureus* (MRSA; concentration: $1 \times 10^8$ cfu/ml) suspension on photocatalytic TiO$_2$ film prepared by direct oxidation of Ti substrate. Titania coating was created by etching the metallic plate with 5-10% HF solution followed by soaking in aqueous H$_2$O$_2$ for 48h. The MRSA suspension on the implant was exposed to a bulb emitting ultraviolet A (UVA) radiation for 1h and the number of bacterial colonies was counted. The bactericidal ability of the photocatalyst became apparent when only about 7% of bacteria survived after 1h of exposure. The number of colonizing bacteria on photocatalytic pins *in vivo* also decreased significantly.

The use of supermagnetic iron oxide nanoparticles (SPION) to prevent biofilm formation was demonstrated by Taylor and Webster [29]. They used maghemite ($\gamma$-Fe$_2$O$_3$) SPION against *S. epidermidis*. A BacLight Live/Dead Kit was used to stain the bacteria and *in situ* fluorescent images were taken to assess the formation of bacterial cluster and creation of biofilm. The results showed that SPION alone could be quite useful in the prevention of biofilm formation.
In the light of foregoing discussion, once again it can be readily concluded that the bactericidal activity of the titania coated plates was far more superior upon excitation by IR laser than by UV light. Moreover, the length of exposure time with the IR laser was also significantly less compared to that in the case of UV light. The evidence of bactericidal activity of titania coated Ti substrates is perhaps the first of its kind, where the effectiveness of infrared beam has been reported.

At this juncture it is worthwhile to point out the concern of using nanomaterials regarding their harmful side effects that can be caused to humans. It has recently been shown that TiO$_2$-based nanomaterial kills cancer cells leaving healthy cells unharmed $^{[61]}$. Another study pertaining to the assessment of TiO$_2$ nanoparticles towards their toxicity and cellular responses on intestinal cells showed that TiO$_2$ did not cause cell death $^{[62]}$. Use of nanophase ZnO and TiO$_2$ increased osteoblast activity while decreasing the $Staphylococcus epidermidis$ functions $^{[63]}$. These reports suggest that using TiO$_2$ nanoparticles for the mitigation of bacterial activities should be safe.
Chapter 5

Conclusions and Recommendations

Porous nanofibers of pure and iron-doped titania with interconnected grains were fabricated by the electrospinning technique, using a benign Ti$^{4+}$ precursor. The ceramic fibers were obtained from the ceramic-polymer composite by following a well-designed processing protocol and tested thoroughly for their structural integrity and microstructural features. Their efficacy for the inhibition of bacterial colonization of *E. coli* was evaluated by irradiating with multiphoton IR laser, UV and a handheld IR beam. The confocal microscopic results demonstrated that almost complete bactericidal activities were observed with IR exposure with the IR flashlight for a far shorter duration (3-12 s).

Several methods were developed for creating nanostructured coating of titania on cp Ti coupons and implants. The methods included hydrothermal processing under different experimental conditions using various media and anodization. In one, titanium coupons pre-coated with TiO$_2$ films were also used. These films action with IR laser demonstrated inhibition of the *E. coli*, thus opening the possibility of using Ti implants coated with TiO$_2$ nanocrystals or nanotubes as means of effective disinfectants for the prevention of major surgical site infections. As was seen with the nanofibers, the TiO$_2$ coating also showed great efficacy for the inhibition of bacterial colonization of *E. coli* when
evaluated by irradiating with the IR laser for 30 s and UV for 30 s and 20 min. The confocal microscopic results demonstrated that at least 40% of the bacteria were deceased with IR exposure. In both formats (nanofibers and coatings), the demonstration of the efficacy of IR light towards infection mitigation in terms of biocidal activity is the first of its kind.

Table 5.1, summarizes the results of the present work and compares them with those reported using UV radiation and other types of microorganisms. As was stated earlier, one can clearly see that the UV radiation has higher photon energy than the IR counterpart. However, calculations based on the intensity of the incident beam and the time of exposure in each case, shows that the number of photons incident per unit area during the entire exposure time is higher in the case of the present work using IR beam. This in turn, translates into higher efficiency of the IR light compared to UV by several orders of magnitude (photons/cm²), since there are more photons for the time duration in comparison to the UV; this explains why the IR method used in this work even for shortest period of exposure was much more effective than longer exposure by UV in the cases reported in the literature.

The survival rate in the present case was determined by using the imaging software called ImageJ (image processing and analysis in Java – National Institute of Health). This software allows one to count the bacteria in order to determine the amount of live cells compared to total number of cells.
Table 5.1 Summary of the results and comparison with the literature

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Light Source</th>
<th>λ (nm)</th>
<th>Exposure Time</th>
<th>Intensity (W/cm²)</th>
<th>Photon Energy (J)</th>
<th>Photon/area ‡</th>
<th>Survival Rate (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus aureus</td>
<td>UV</td>
<td>352</td>
<td>60 min</td>
<td>1.82×10⁻³</td>
<td>5.647×10⁻¹⁹</td>
<td>1.16×10⁻¹⁹</td>
<td>9.4</td>
<td>Koseki et al.#</td>
</tr>
<tr>
<td>Bacillus pumilus</td>
<td>UV</td>
<td>365</td>
<td>120 min</td>
<td>630×10⁻⁶</td>
<td>5.446×10⁻¹⁹</td>
<td>8.33×10⁻¹⁸</td>
<td>50</td>
<td>Yu et al.#</td>
</tr>
<tr>
<td>methicillin-resistant Staphylococcus aureus (MRSA)</td>
<td>UVA</td>
<td>365</td>
<td>60 min</td>
<td>1.1×10⁻³</td>
<td>5.446×10⁻¹⁹</td>
<td>7.27×10⁻¹⁸</td>
<td>7</td>
<td>Oka et al.#</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>IR/MP</td>
<td>820</td>
<td>3 s</td>
<td>2×10⁶</td>
<td>2.424×10⁻¹⁹</td>
<td>2.48×10²⁵</td>
<td>10</td>
<td>This work$</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>IR/laser</td>
<td>808</td>
<td>30 s</td>
<td>5</td>
<td>2.460×10⁻¹⁹</td>
<td>6.10×10²⁰</td>
<td>60</td>
<td>This work*</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>UV</td>
<td>365</td>
<td>20 min</td>
<td>350×10⁻⁶</td>
<td>5.446×10⁻¹⁹</td>
<td>7.71×10¹⁸</td>
<td>60</td>
<td>This work*</td>
</tr>
</tbody>
</table>
A recent research reported by Trouiller et al. \cite{64} on the use of titania nanoparticles on mice, paints a sobering picture. Their work showed that consumption of nanoscale titanium dioxide was damaging or destroying the animals' DNA and chromosomes, and could be linked to all the big killers of man, namely cancer, heart disease, neurological disease and aging. Consequently, more research needs to be done \textit{in vitro} in order to assess the usefulness of titania as an antimicrobial agent.

There are several avenues for improvement and development of even better titania nanofibers and nanofibrillar structures for the necrosis of bacteria. For example:

1. Develop a process of making silver-doped titania nanofibers since silver is a known antimicrobial agent \cite{65}.
2. Creating nanostructured films on Ti mesh using hydrothermal and anodization process. This will allow creation of flexible implant.
3. Evaluate the efficacy of titania towards \textit{Staphylococcus aureus}.
4. Use of nanostructures that work well at killing \textit{E. coli} and \textit{Staphylococcus aureus} in animal studies.
References


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