Characterizing Bacterial Resistance and Microstructure-Related Properties of Carbon-Infiltrated Carbon Nanotube Surface Coatings with Applications in Medical Devices

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Characterizing Bacterial Resistance and Microstructure-Related Properties of Carbon-Infiltrated Carbon Nanotube Surface Coatings with Applications in Medical Devices

Stephanie Renee Morco

A dissertation submitted to the faculty of Brigham Young University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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ABSTRACT

Characterizing Bacterial Resistance and Microstructure-Related Properties of Carbon-Infiltrated Carbon Nanotube Surface Coatings with Applications in Medical Devices

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Doctor of Philosophy

Carbon-infiltrated carbon nanotube (CICNT) forests are carbon nanotube (CNT) forests infiltrated with pyrolytic carbon to increase durability by becoming a solid material. This material can be tuned to maintain the nanotube geometry of a CNT forest and can also be fabricated on a variety of materials and geometries. Additionally, the present work has indicated that CICNT forests may resist bacterial proliferation and biofilm formation. This phenomenon is not due to the CICNT chemistry; it is presumably due to the CICNT nanostructure morphology. Thus, both silicon and stainless steel substrates were used to investigate CICNT’s structural resistance to Methicillin-resistant Staphylococcus aureus (MRSA) biofilm. From in vitro experimental testing, CICNT on both these substrates resisted MRSA cell attachment and biofilm proliferation.

The discovery of this non-pharmaceutical biofilm resistance expands the potential applications of CICNT to include medical devices that are prone to infection and/or devices that contribute to infection. Two representative applications were investigated: external fixator pins and scalpel blades. CICNT-coated versions of these applications underwent additional MRSA biofilm resistance testing as well as mechanical testing. In particular, external fixator pins were identified as a high potential application of CICNT surface coating technology.

Previous work on both CNT and CICNT forests has largely been performed on planar structures. However, any potential medical device applications involve curved substrates. In particular, concave curvatures are challenging due to the potential for stress-related CICNT forest defects. Thus, the present work also included a study of the incidence rates and determining factors of these defects. SEM images of the cross-sections revealed different types of microscale forest defects while the top surface showed morphologies that are largely consistent with flat substrates. CICNT forest height and substrate curvature were identified as contributing factors to CICNT forest defect incidence rates.

Thus, the present work advances the understanding of bacterial resistance and microstructure-related properties of CICNT surface coatings, with applications in medical devices.

Keywords: carbon nanotube forest, CICNT, direct growth, bacteria resistance, MRSA biofilm, medical applications, concave substrate, CNT forest defects
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# NOMENCLATURE

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<tr>
<td>CNT</td>
<td>Carbon nanotube</td>
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<tr>
<td>CICNT</td>
<td>Carbon-infiltrated carbon nanotube</td>
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<tr>
<td>CVD</td>
<td>Chemical vapor deposition</td>
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<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
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<td>CICNT-Si</td>
<td>CICNT on a silicon wafer substrate</td>
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<tr>
<td>CICNT-SS</td>
<td>CICNT grown from a stainless steel substrate</td>
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<tr>
<td>MRSA</td>
<td>Methicillin-resistant <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>PJI</td>
<td>Periprosthetic joint infection</td>
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<tr>
<td>SS</td>
<td>Stainless steel</td>
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CHAPTER 1. INTRODUCTION

1.1 Motivation

Due to their surface morphology, CICNT coatings may provide a structural solution for bacterial infection, specifically for Methicillin-resistant *Staphylococcus aureus* (MRSA) biofilm infection. This is of particular interest because biofilm infections are a major concern in biomedical implant-related infections [1, 2] and are phenotypically tolerant to antibiotics [3-5], which makes them challenging to eradicate. An implant surface that resists initial biofilm attachment could dramatically reduce implant-associated infections, and a surface that accomplishes this based on its inherent structure (i.e., without the use of pharmaceuticals) could be the ideal solution. This work will provide the first *in vitro* experimentation to test if the CICNT structure resists MRSA biofilm attachment and growth.

Traditionally, CICNT material has been made with a silicon wafer as a substrate, but a flat surface is only suitable for some applications, and silicon itself is neither biocompatible nor biostable. Thus, the focus of the present work was on applications of CICNT as a standalone material or as a surface layer on more traditional implant material substrate (i.e., stainless steel, titanium, etc.). The CICNT material self-assembles, commonly expressed as “grows,” from a layer of catalyst particles added to the substrate. Catalyst particles can be layered onto nearly any surface geometry, so the number of useful applications can theoretically be expanded to products such as the inside of needles and the unique contours of medical implants. However, previous work has not addressed the structural effects of varying the substrate curvature for CICNT coatings, particularly for concave curvatures. Also, because antibacterial properties are associated with the top surface morphology, it is necessary to investigate whether the structure and surface morphology of a CICNT coating is independent of its substrate curvature.
Methods to grow CNTs using catalyst elements found in certain substrate materials, such as stainless steel, are being developed, which would have the potential to make a CICNT coating even more widely applicable. Stainless steel is tough, relatively easy to machine, and widely used in medical devices, so it would be an excellent substrate. The methods to grow CNT directly from stainless steel’s internal catalysts reduce the manufacturing time from five hours to two hours and the required equipment from four machines to one machine. Also, because the catalyst comes from an internal source, the coating would theoretically have exceptional adhesion to its substrate, which is critical for any coating but especially for medical implants. Using a manufacturing process to grow CICNT directly from stainless steel seems ideal; however, there is one major concern. The structure and surface morphology are different from the CICNT coatings made from external catalysts – it is not vertically aligned and is much less uniform. Since using these manufacturing methods would improve application versatility and coating adhesion, it is of interest to know if these coatings would exhibit MRSA biofilm resistance, similar to traditional external catalyst coatings.

1.2 Objective

The overall goal of the research presented in this dissertation was to advance the understanding and expand the application range of carbon-infiltrated carbon nanotube (CICNT) coatings as applied to biomedical implants. This goal was addressed through accomplishment of three distinct objectives:

1. Identify whether the surface morphology of CICNT coatings reduces MRSA biofilm attachment.

2. Determine whether CICNT coatings grown from the internal catalysts in stainless steel would be suitable and advantageous for medical devices.

3. Investigate how the structure and surface morphology of CICNT on concave substrates compares to that observed on planar substrates.
1.3 Organization

Following a review of the literature in chapter 2, the bulk of the research is found in chapters 3-5. Each of these research chapters comprises a full-length manuscript which has been or will be submitted for publication.

Chapter 3 includes experimental results showing that CICNT coated on silicon wafer substrates as well as CICNT coated directly onto stainless steel substrates resist MRSA cell attachment and biofilm proliferation. This phenomenon is not due to the CICNT chemistry; it is most likely due to the CICNT nanostructure. These results expand the possible applications of CICNT to include devices that are prone to infection and would benefit from a non-pharmaceutical solution, such as orthopedic implants. This manuscript was coauthored by Dustin Williams, Brian Jensen, and Anton Bowden and is currently under review at Journal of Orthopaedic Research.

Chapter 4 investigates scalpel blades and external fixator pins as potential applications for a CICNT coating. Various mechanical testing as well as MRSA biofilm resistance testing demonstrate that external fixator pins are a more viable application. This manuscript was coauthored by Jaclyn Larsen, Brian Jensen, and Anton Bowden, and it is published as a technical paper in the Society for the Advancement of Material and Process Engineering (SAMPE) 2020 conference.

Chapter 5 explores the geometric limitations of CICNT coatings on concave substrates. These are seen on the microscale as SEM images of the cross-sections reveal different types of defects in forests with tall CICNT and small substrate curvatures. The morphologies of the top surfaces are largely consistent with flat substrates, but internal forest defects likely affect certain mechanical properties of the forest. This manuscript was coauthored by Brian Jensen and Anton Bowden and is currently under review at Nanoscale.

Chapter 6 summarizes the dissertation and discusses suggested directions for future work in this area. Additional, unpublished research testing which may be helpful to others embarking on research in this area is included in Appendix A.
CHAPTER 2. BACKGROUND

2.1 Orthopaedic Infections

Even with advancements in sterile techniques and other preventative measures, bacterial infection continues to be a well-known risk in surgery, especially in orthopaedic implant surgeries. For example, the rate of deep infection – as opposed to the easier-to-treat surgical site infection – for total knee replacements is 1-3% [1-3], 0.97% for total hip replacements [4], 1.9% for internal fracture fixation plates [5], over 30% for open complex tibia fractures [5], and between 12-38% for external fixation pins depending on patient demographics and type of surgery [6]. By 2030, the number of people with periprosthetic joint infection (PJI) from knee and hip replacements alone could approach 100,000 infections in the US per year [7].

Because biofilms form on the surfaces of implants, a PJI or infection after fracture fixation cannot be treated with systemic antibiotics [8]. The biofilm must be physically removed, so the treatment options require at least one surgery: debridement and component retention (23 to 28% success rate), one-stage revision (81 to 89% success rate), or two-stage revision (91% success rate) [8, 9]. Naturally, these treatments are associated with increased morbidity and cost [10], so the impact of finding additional ways to reduce the infection rate would be tremendous for those who could escape this threat.

2.2 Approaches to Reduce Surgical Site Infection

One of the promising approaches to reduce orthopaedic implant infection rate is implant surface modification, but while there has been extensive research on the subject, the current market options are limited and may not be suitable as long-term solutions.

Antibiotic-loaded bone cement is a prophylactic treatment where an antibiotic, such as gentamicin or tobramycin, is mixed with the polymethylmethacrylate (PMMA) bone cement used
to secure many orthopaedic implants. The idea is that the antibiotic is released in the location most susceptible to infection, and it has been shown to reduce infection rates [11]. However, it has also been shown to be ineffective at reducing *Staphylococcus aureus* biofilm likely due to the antibiotic release kinetics offering a “window-of-effectiveness” that is too small [12, 13]. Antibiotics need to be effective for a duration long enough for the bone and other host cells to attach and proliferate on the implant surface before bacteria do. This “race to the surface” between host cells and bacteria is directly related to implant infection risk [14]. Although even if antibiotics can be released for a long period of time, an antibiotic-based solution may eventually lose its effectiveness. This is because *S. aureus* is attributed to approximately 50% of orthopaedic infections [15] and the prevalence of antibiotic-resistant strains of this bacteria, such as Methicillin-resistant *S. aureus* (MRSA), in orthopaedic infections is increasing [16].

Another clinically-available solution is having an implant coated with silver or a silver-impregnated coating like hydroxyapatite. It is biocidal against a broad spectrum of strains – both gram positive and negative – and the effect is long-lasting [17]. Silver-coated implants have been shown to reduce infection rates [18, 19]; however, long-term toxicity – particularly cytotoxicity to bone cells – is questionable [13]. Because silver ions are released, it is categorized as an active coating [17]. Another consideration is that although bacteria are less prone to develop resistance to silver than antibiotics [17], bacterial resistance to silver has been observed since the 1960s [20]. Widespread use would accelerate this resistance. Additional limitations include high cost and difficulty applying to many implants, so the application range is currently limited to oncology or other patients with high risk of infection [13].

The newest clinically-available solution is an antibiotic-loaded fast-resorbable hydrogel coating, and it has been shown to significantly reduce implant infection without any adverse side effects for closed-fracture osteosynthesis operations [21]. During the operation, a hydrogel is mixed with one or more antibiotics and then spread onto the implant, such as the stems of cementless hip prostheses and other areas that interface with bone [13]. The antibiotics are released after implantation within a designed 48 to 72 hour time frame [13, 21]. Although this hydrogel coating is a promising solution, it is similar to antibiotic-loaded bone cement in that it is limited by increasing antibiotic resistance [16].
Figure 2.1: Bactericidal and antifouling surfaces (Note: scale bars=200 nm unless otherwise noted). (a) Top view of the nanostructure of cicada wings [22]. (b) Gram-negative bacterium (Pseudomonas aeruginosa) sinking into the nanopillars of a cicada wing after it was punctured [23]. (c) Top and tilted views of black silicon [24]. (d) Top and tilted views of a dragonfly wing [24]. (e) Top view of a taro leaf [25]. (f) Top view of nanopatterned silk substrates [26]. (g) Top view of a lotus leaf [27]. (h) Tilted view of Sharklet AF [28].

2.3 Structural Bacterial Resistance

Some organisms have naturally-occurring micro- and nanostructures that protect them from bacteria and biofilm growth, which is a physical protection rather than a chemical protection. Antibacterial surfaces are classified as either bactericidal (kills bacteria) or antifouling (resists bacteria adsorption), and both occur from nanostructures in nature [22]. The nanopillars on cicada wings are bactericidal against gram-negative bacteria and have the following dimensions: 200 nm tall, 60 nm tip diameter, and 170 nm spacing [23, 29, 30] (Figure 2.1a and b). Dragonfly wings have nanopillars of similar size, but with much more variation in diameter and spatial distribution (Figure 2.1d). This makes them bactericidal against both gram-negative and gram-positive bacteria [24]. The nanostructure of lotus (Figure 2.1e) and taro (Figure 2.1g) leaves are antifouling [25] as well as shark skin [27, 28].

Researchers have found ways to create antibacterial surfaces using the micro- and nanostructure concepts found in nature. Black silicon is manufactured by reactive-ion etching a silicon wafer with SF$_6$ and O$_2$, which produces tall nanopillars [31] (Figure 2.1c). These
nanopillars have a bactericidal effect when they are produced with similar dimensions to dragonfly wings [24]. This material can be useful for certain applications; however, monocrystalline silicon is relatively expensive and very difficult to machine, so many geometries are not feasible. Additionally, the silicon itself has been demonstrated to be cytotoxic and is chemically unstable in biomedical environments [32, 33]. An example of a synthetic antifouling material is micro- and nanopatterned silk (Figure 2.1f), which is made by casting a silk solution onto micro- or nanopatterned Poly(dimethyl siloxane) (PDMS), and it has been shown to reduce Escherichia coli (E. coli) adhesion [26]. Sharklet AF (Figure 2.1h) is another synthetic antifouling material, and it is made by micro-patterning poly(dimethyl siloxane) elastomer (PDMSe). By changing the dimensions of the topography, the surface can be tailored to resist a specific organism, such as Staphylococcus aureus [28], which means that it may not resist a broad spectrum of bacterial morphologies [26, 34]. An additional concern with micropatterned antifouling surfaces is that they may not be clinically viable because of their reliance on a continual surface fluid flow to transport bacteria away from the surface. For most periprosthetic implant conditions, this requirement is not typical. To the author’s knowledge, the antifouling property of micropatterned antifouling surfaces under these conditions has not been demonstrated with in vivo testing.

2.4 CICNT Manufacture and Properties

The nanostructure of CICNT is similar to the antibacterial examples mentioned previously because the material is formed by growing an array of carbon nanotubes on a substrate material then infiltrating the array with amorphous carbon to a desired infiltration thickness [35-37]. It can be visualized as a composite material: CNT reinforced by amorphous carbon. This material has been called different names, such as carbon nanotube/carbon nanocomposite [38-40] and densified aligned carbon nanotube films [41], and it has been made into different forms, such as yarn [38] and CNT sheet [40]. Typically, the manufacturing process uses a silicon wafer substrate coated with 30-40 nm of aluminum oxide and 4-7 nm of iron that can be patterned with photolithography (Figure 2.2). The aluminum oxide layer is a diffusion barrier, and the iron is a catalyst for CNT growth. This wafer is then placed in a tube furnace and follows the time-temperature plot with gas flows shown in Figure 2.3 to grow a CNT forest and infiltrate it with amorphous carbon. The amount of time spent in the “growth” stage mostly determines how tall the forest is (i.e., thickness
Figure 2.2: A side-view of the CICNT manufacturing process with a photolithography step [37].

Figure 2.3: Typical time-temperature plot with gas flows to grow and infiltrate CICNT.
Figure 2.4: Schematic of a cross-section of four CNT and CNT with infiltration shows how the porosity of the material may be varied [42]. Also, because the CNTs are being completely coated with carbon, the chemistry of the CICNT surface is assumed to be that of the carbon infiltration.

of the CICNT coating), and the amount of time spent in the “infiltration” stage determines how much amorphous carbon coats the CNT (i.e., the diameter of the nanotubes). This is illustrated in Figure 2.4.

The carbon infiltration is responsible for the surface chemistry of CICNT, so understanding its chemistry is valuable. Because it is produced by chemical vapor deposition (CVD) with a hydrocarbon gas at a temperature between 1000K and 2500K, this material is classified as pyrolytic carbon [43]. Some use the term “amorphous pyrolytic carbon” [40]. Diamond-like carbon is also an amorphous carbon, so it can be easily confused with pyrolytic carbon; however, diamond-like carbon must be fabricated with plasma [43], such as plasma-enhanced CVD (PECVD) [44], in order to provide the high percentage of sp³ bonds required for this classification. The sp²/sp³ ratio (the ratio of carbon double bonds to single bonds) of the CICNT used throughout this research has not been measured. The material in this work is similar to a material that produced a ratio of 4 (80% sp² and 20% sp³) [40], but it cannot be assumed that this is the exact bond ratio of CICNT because the structure of pyrolytic carbon depends on fabrication parameters such as temperature, gas type and concentration, etc. [43, 45].

Although some of the chemistry specifics of CICNT are unknown, this coating has already demonstrated that it is promising for many applications, including medical implants. Because the carbon infiltration is pyrolytic carbon, the surface is non-reactive [37]. Many forms of pyrolytic
carbon are biocompatible and already used in implants like artificial heart valves [46] and finger joint replacements [47]. CICNT has even shown hemocompatibility [48], which is a stricter classification of biocompatibility. Additionally, a vacuum pyrolysis treatment can render CICNT superhydrophobic [49], which further increases the application range for this material.

Mechanically, CICNT as a stand-alone material has shown great strength. The ultimate strength has been measured at 903 ± 253 MPa in the direction parallel to the CNT forest and 140 ± 46 MPa in the transverse direction [35]. Although the strength in the transverse direction is not as strong as implant materials like stainless steel and titanium (310 and 830 MPa yield strength, respectively), it is stronger than bone cement (90 ± 10 MPa flexural strength [50]), and similar to the ultimate strength of cortical bone (135 ± 15.6 MPa in tension, 205 ± 17.3 MPa in compression [51]).

2.5 Growing CNT from Internal Catalysts

CICNT forests have also been produced using stainless steel (SS) as the substrate and CNT catalyst. This is especially valuable because it simplifies the manufacturing process and SS is already used in medical devices. The surface of the SS is pretreated before CNTs will grow. They grow either from the catalyst elements inside the substrate, such as iron, nickel, and chromium or from morphological changes at the nanoscale. Either an acid etch or an air heat treatment beforehand allows the SS to produce CNTs, but these are clearly different mechanisms. CNT growth after a 10-minute sulfuric acid etch on 304 SS suggests that the chromium oxide layer needs to be reduced or depleted for CNT to grow [53], but CNT growth after a 10-minute air heat treatment at 800 °C on 316L SS suggests that the smooth chromium-oxide layer needs surface restructuring before CNT will grow [54]. This surface restructuring takes the form of nano- to micro-sized oxide particles that form on the surface, and these are presumably where each individual CNT initiate growth. If the chromium-oxide layer already has an optimum surface structure though, CNT can grow directly from SS without pretreatment [52]. The idea of a physical surface affecting CNT growth is further supported by a study that grew better CNT forests after MgO underlayers were annealed to form nanoscale granular structures before the traditional silicon substrate growth [55].
Because implants are commonly made from 316L, this alloy is of special interest; however, it has been more difficult to produce vertically-aligned CNT with it. Vertical-alignment has been achieved on meshes [54] and inside tubing (Figure 2.6) [56], but not on sheets. Researchers have only produced random-oriented CNT on sheets of 316L (Figure 2.5) [57, 58]. Despite this, random-oriented CICNT on 316L has also been rendered superhydrophobic after a vacuum pyrolysis treatment [49], and it may resist biofilm attachment due to its nanostructure as well.

Figure 2.5: Random-oriented CNT on 316L SS sheet [57, 58].

Figure 2.6: Cross-sections of vertically-aligned CNT grown directly inside 316 stainless steel tubing with an inner diameter of 1.27 mm [56].
2.6 Nonplanar Surfaces

The manufacturing process of a CICNT coating allows for the substrate geometry to be varied, but little has been done to study the effects and limitations of substrate geometry changes on CNT forests. The geometry among medical implants and other devices is widely varied, but inferences to the behavior of CICNT coatings on complex geometries can be made by studying what happens on convex and concave substrates. Convex substrates have been studied previously by manufacturing CICNT on 3 mm diameter rods with forest heights ranging from 44 µm to 577 µm [59]. It was found that cracking was a significant issue, but it could be reduced by reducing the coating thickness (i.e., CNT height) because the circumferential stress increases as the forest height increases and before infiltration, the CNTs are held together by the weak Van Der Waals forces [60, 61]. With concave substrates, the circumferential stress would be negative – under compression – so cracking is not an issue; however, there are unanswered questions in the literature about what issues may arise from this compression. Vertically-aligned CNT forests have been grown successfully on fused silica concave optical lenses with 16 µm height and both 5.5 and 11.46 mm radius of curvatures [62]. Others have manufactured vertically aligned CNTs into flexible materials and later bent them into convex and concave substrates for flexible field emission devices [63, 64]. The parameters used in these studies are a CNT length of 130 µm with a 6 mm bending radius [64] and a CNT length of 5-15 µm with a 25 and 50 mm bending radius [63]. One group has achieved vertically-aligned CNT inside a stainless steel tube using the stainless steel as the substrate and CNT catalyst [56]. The inner diameter of the tube was 1.27 mm, and the CNT forest thickness ranged from 4 to 60 µm. A cross-sectional view of this tube shows the roughness and non-uniformity of the forest (Figure 2.6), but these defects have not been systematically studied.

2.7 Summary

Due to increasing bacterial resistance to antibiotics, current options for preventing orthopaedic infections may not be long-term solutions; however, there may be a structural solution rather than a chemical one. Some materials can resist biofilm with a nano- or micro-structured surface, and this mechanism theoretically offers a more long-term solution. A CICNT coating may be a good candidate for this because of its nanopillar structure. Additionally, its advantages include
the ability to be coated onto various materials and geometries as well as a biocompatible surface chemistry. If the CICNT coating can resist or delay biofilm formation on the surface of an implant, the body’s bone and other cells can win the “race to the surface” and prevent a deep infection.
CHAPTER 3. STRUCTURAL BIOFILM RESISTANCE OF CARBON-INFILTRATED CARBON NANOTUBE COATINGS

3.1 Abstract

Periprosthetic joint infection (PJI) is a devastating complication of orthopaedic implant surgeries, such as total knee and hip arthroplasties. Treatment requires additional surgeries because antibiotics have limited efficacy due to biofilm formation and resistant bacterial strains such as methicillin-resistant *Staphylococcus aureus* (MRSA). A non-pharmaceutical approach is needed, and examples of this are found in nature; dragonfly and cicada wings are antibacterial because of their nanopillar surface structure rather than their chemistry. Carbon-infiltrated carbon nanotube (CICNT) surfaces exhibit a similar nanopillar structure, and it is postulated that they might provide a structurally-derived resistance to bacterial proliferation and biofilm formation. The objective of this study was to test the biofilm resistance of CICNT coatings. Two types of CICNT were produced: a vertically-aligned CNT forest on a silicon substrate using a layer of iron as the catalyst (CICNT-Si) and a random-oriented CNT forest on a stainless steel (SS) substrate using the substrate as the catalyst (CICNT-SS). These were tested against SS and carbon controls. After 48 hours in a MRSA biofilm reactor, samples demonstrated that both types of CICNT coatings significantly (p<0.0001) reduced MRSA biofilm formation by 60-80%. Morphologically, biofilm presence on both types of CICNT were also significantly reduced. Clinical Significance: Results suggest that a CICNT surface modification could be suitable and advantageous for medical devices susceptible to MRSA cell attachment and biofilm proliferation, particularly orthopaedic implants.

3.2 Introduction

Advancements in sterile techniques and other preventative measures have made joint replacement surgeries highly successful [65, 66]; however, the infection rate of total knee and hip
arthroplasty (TKA and THA) continues to be approximately 1% [2, 4]. These periprosthetic joint infections (PJI) are particularly difficult to address because they are generally caused by biofilms [67, 68], which are phenotypically tolerant to antibiotics [69-71]. Treatment requires additional surgery: debridement, one-stage revision, or two-stage revision [8, 9]. Aside from being invasive and costly [72, 73], these surgeries also have a higher risk of infection [74]. Additionally, the overuse of antibiotics breeds antibiotic-resistant bacterial strains [17], and now, methicillin-resistant *Staphylococcus aureus* (MRSA) is present in over 25% of PJI [75]. As a result, there are calls for nonpharmaceutical solutions [76, 77].

There are surfaces found both in nature and the lab that offer a physical, rather than chemical, protection from biofilm based solely on their micro- or nanostructure. They are classified as either bactericidal (kills bacteria) or antifouling (resists bacterial adsorption) [22]. The nanopillar structures of cicada wings [23, 29, 30], dragonfly wings, and black silicon have a bactericidal effect [24], while the microstructures of lotus and taro leaves [25], patterned silk fibroin [26], nanorough titanium [78], shark skin [27], and Sharklet AF™ have an antifouling effect [28]. These bio-inspired surfaces are promising, but they have limitations. For example, black silicon is cytotoxic and its manufacturing processes have limited its application to surfaces that are generally planar [79], and Sharklet AF™ is limited because the dimensions of its microstructure are tailored to resist only one specific organism [28, 31].

We hypothesize that recent developments in carbon-infiltrated carbon nanotubes (CICNT) may provide a structural solution for preventing bacterial adherence/biofilm formation while offering biocompatibility and robust application across more complex geometries [48]. The material is fabricated in two steps: 1) a “nucleation and growth” step where a carbon nanotube (CNT) “forest” is nucleated on an underlying ceramic or metallic substrate and grown to a predetermined average CNT length ranging from 50-1000 microns, and 2) a subsequent “infiltration” step where bulk carbon is deposited on the CNT forest, essentially increasing the diameters of the CNTs and producing a nanopillar structure with a tailored infiltration diameter roughly corresponding to those in cicada and dragonfly wings [35-37, 42, 80-82]. The volume ratio is typically around 99% bulk carbon to 1% CNT. The CICNT material is structurally sound and can either remain bonded to the underlying substrate material as a surface modification (e.g. for
medical implant applications), or alternatively, can be detached from ceramic substrates for use as a free-standing material.

The nucleation and growth of the CNT forest can be accomplished using either an external or internal catalyst. Traditionally, a CNT forest is grown by applying a thin catalyst layer to a ceramic substrate such as a silicon wafer [83, 84], which we term an external catalyst, since it is applied externally to the substrate material. Recent work has shown that CNT forests can also be produced by using a pre-treatment process (acid etch or air heat treatment) on metallic substrates such as stainless steel (SS) [53, 54, 56], which we term an internal catalyst since it leverages structural and material features already present in the substrate. Internal catalyst processes are simpler, and anecdotally produce a stronger attachment between the substrate and the CICNT material. However, there are differences in the morphology of externally versus internally catalyzed CNT forests. Externally catalyzed CNT forests tend to nucleate and grow uniformly, producing vertically aligned CNT forests, while internally catalyzed CNT forests nucleate and grow in a randomly-oriented structure with a tortuous morphology [58, 85]. Exceptions to this have been vertically-aligned forests produced on meshes and inside tubing [54, 56], but not on planar substrates. However, the nanostructure at the surface of random-oriented CICNT on 316L SS (CICNT-SS) is similar to that of vertically-aligned CICNT on silicon substrates (CICNT-Si), so they may have similar biofilm resistance properties. This study determines whether both of these fabrication methods resist MRSA cell attachment and subsequent biofilm formation in vitro as compared to stainless steel and carbon controls.

3.3 Methods

*CICNT-Si fabrication:* A vertically-aligned CNT forest was grown and infiltrated by CVD on 1 cm square silicon wafer substrate layered with 30-40 nm Al₂O₃ (electron beam evaporated below 55 μTorr) then 7 nm Fe (thermally evaporated below 10 μTorr). The CVD process was performed by placing samples in a 1-in quartz tube furnace (Lindberg Blue M EW-33850) at atmospheric pressure and heated to 750 °C while flowing H₂ (311 sccm). At 750 °C, C₂H₄ (338 sccm) was added to grow the vertically-aligned CNT forest for 25 minutes. C₂H₄ was turned off while the furnace was heated to the 900 °C infiltration temperature then turned on for an 11 minute
infiltration. Then the process gases were stopped, and argon flowed (311 sccm) while the furnace cooled to below 200 °C. This process is illustrated with a time-temperature plot in Figure 3.1 and achieved CICNT diameters ranging from 100-140 nm.

CICNT-SS fabrication: A random-oriented CNT forest was grown and infiltrated on 1 cm square mirror-finish 316L stainless steel sheets (Stainless Supply 316L Stainless Steel Sheet #8 Mirror, 24G). First, a 2-minute air heat treatment at 800 °C with an air quench was performed. Next, the CNT were grown and infiltrated using a CVD process similar to the CICNT-Si fabrication and in the same tube furnace. Argon flowed throughout the process (311 sccm), and C\textsubscript{2}H\textsubscript{4} flowed at 800 °C for a 20 minute growth and again at 900 °C for a 3-7 minute infiltration. Infiltration time varied to achieve a comparable porosity to CICNT-Si. This process is also illustrated with a time-temperature plot in Figure 3.1 and achieved CICNT diameters ranging from 200-250 nm.

Stainless Steel and Carbon Control Preparation: In order to more closely replicate the roughness of an orthopaedic implant, SS bar stock of 4.75 mm thickness (McMaster-Carr #9083K35) was cut to 1 cm squares, cleaned by sonication in IPA, and rinsed with deionized water.
Pyrolytic carbon samples were created by over-infiltrating CICNT-Si samples to the point of complete saturation (i.e., no porosity), which was achieved by infiltrating for an additional 20 minutes. This provided the same chemical makeup as CICNT-Si with a different surface structure.

**MRSA Biofilm Growth:** A CDC biofilm reactor (Biosurface Technologies, Bozeman, MT, USA) was used to grow MRSA biofilms [86, 87]. In a sanitized biosafety cabinet, samples were fixed to reactor coupons randomly with either paraffin wax or silicone dispersion (NuSil MED-6607, Carpinteria, CA, USA), depending on adhesion capability, and the reactor was assembled. One mL of a 0.5 McFarland standard of freshly-cultured MRSA, approximately $7.5 \times 10^7$ bacterial cells [88], was inoculated into 500 mL of tryptic soy broth (TSB) (Fisher Scientific, Waltham, MA, USA) in the CDC biofilm reactor. The reactor used in this study is shown in Figure 3.2. The reactor was placed on a hot plate set at 34°C with rotation set at 130 rpm for 24 h. A 10% TSB flowed through the reactor at 6.94 mL/min for the next 24 hours, totaling 48 hours of growth.

![Figure 3.2: CDC biofilm reactor setup. Samples were positioned and fixed to coupons randomly, and MRSA biofilm growth was promoted for 48 hours.](image-url)
Figure 3.3: The 13 pre-determined locations for SEM imaging. Locations were navigated at 250x (white boxes) and imaged at 2,500x.

Sample Preparation for SEM Imaging: Samples were fixed, dehydrated, and sputter coated with gold in preparation for SEM imaging. Specifically, each reactor rod was aseptically removed, rinsed 3 times in 1x PBS (Fisher Scientific, Waltham, MA, USA), and samples separately submerged in modified Karnovsky’s fixative (2.5% glutaraldehyde and 4% formaldehyde in 0.2 M PBS, pH 7.4) for 2 h. Then they were dehydrated in 100% ethanol for 1 h, air dried for ~30 min, and sputter coated with ~3 nm gold (Hummer 6.2 gold sputter coater, Anatech LTD, Hayward, CA, USA).

SEM Imaging: Each sample was SEM imaged (Phillips XL30 S-FEG or ThermoScientific Verios G4 UC) in 13 pre-determined locations (Figure 3.3) at 2,500x magnification. This provided enough magnification to distinguish MRSA cells from other features and enough spread of locations to capture more of each materials’ response. Note, the edges were not imaged because stirring the nutrient broth causes fluid dynamic inconsistency which leads to different biofilm growth near the edges of a sample (known as the edge effect).

Image Processing: Automated cell counting functions in the software Cellprofiler proved inaccurate because images of CICNT samples are porous and irregular; therefore, a manual cell count was done instead. MRSA cells can be identified by their round shape, smooth texture, and size (roughly 0.8 µm diameter), but the cell counting functions of the software could not correctly distinguish between these cells and the features of CICNT. A 1600% difference from a manual
cell count was observed. Additionally, a manual count could include the minor contamination by a few rod- and fusiform-shaped cells separately. In order to do this, MRSA and contamination cells were marked in red and green, respectively, then automatically counted using a MATLAB program. It took approximately 20 minutes to manually mark the cells on each image (over 80 hours to mark over 250 images).

The manual cell count provided the most accurate results; however, the human aspect of this method adds some variability, so coefficients of variation were determined. This was done by marking one image of each material three times and computing the average and standard deviation for each image. The coefficients of variation were as follows: 1.9% for CICNT-Si, 13.9% for CICNT-SS, 2.5% for the SS control, and 3.4% for the carbon control. These percentages were multiplied by their respective means, and those values were added to the standard deviation error bars in Figure 3.6 in order to include this estimated standard deviation for measurement error.

3.4 Results

MRSA biofilm was allowed to grow on CICNT-Si, CICNT-SS, and control samples; then the samples were SEM imaged. These images showed MRSA cells adhered to each sample, and the early stages of biofilm formation were observed and quantified. Figure 3.4 shows SEM images of each material before and after the experiment (note: images with the highest cell counts from each material were selected). Examples of cell-marked images are shown in Figure 3.5. The images in Figure 3.4 and Figure 3.5 also show the MRSA biofilm morphology differences between the CICNT and control samples: less cell aggregation on the CICNT samples compared to the controls. After the cells in each image were counted, the value was divided by the image area to report in units of cells/mm², which provides a better visual of the biofilm spread.

The average cell counts for each material are plotted in Figure 3.6. A statistical analysis using one-way ANOVA confirmed that CICNT-Si and CICNT-SS showed significantly less MRSA cells than the SS control and carbon control (p<0.0001). Both CICNT materials reduced MRSA attachment and proliferation by 60-80% compared to both control materials.
Figure 3.4: SEM images of each material before the experiment (left) and the image location with highest MRSA cell count after the experiment (right). After 48 hours, MRSA cells are identified by their spherical shape, smooth edges, and roughly 0.8 µm diameter. Magnifications are 2500x and 10000x.

The breakdown of the average and maximum cell counts of the 13 image locations on each 1 cm square sample fixed to a coupon in the biofilm reactor are shown in Figure 3.7. The maximum cell counts show that the CICNT samples exhibited much lower MRSA cell density areas in contrast to the controls, which had a more robust biofilm formation [89, 90].

3.5 Discussion

Most solutions for reducing PJI and antibiotic resistance in orthopaedic implants involve applying antibiotics at the local level by adding antibiotics to implant coatings or bone cement [11, 18, 19, 21, 91]. This would reduce the dosages needed for systemic prophylaxis which would
Figure 3.5: MRSA cells marked in red for each material: CICNT-Si, CICNT-SS, carbon control and SS control. Image locations are the same as those in Figure 3.4. Scale bars are 5 µm.

Figure 3.6: Average number of cells per mm² for each material, includes both MRSA and contamination cells (grey error bars show standard deviation and red error bars include the estimated standard deviation for measurement error). * indicates p < 0.0001. Significance calculated by one-way ANOVA.
decrease antibiotic resistance [91], but these pharmaceutical or chemistry-based defenses are limited by the increase in antibiotic resistant bacteria and are not optimized against biofilms [16]. The work presented here offers a unique potential solution to reduce PJI rates and antibiotic resistance by deterring MRSA cellular attachment and biofilm growth based on the physical nanostructure of CICNT coatings.

MRSA biofilm formation was accelerated by starting with a high concentration of MRSA cells, so although this allowed cells to attach to all materials in this experiment, the differences between the materials are more consequential than the MRSA cell counts. The CDC biofilm reactor is robust and optimized for biofilm formation [92]. The strength of this method is that all samples reside in the same homogeneous broth, so each sample within the reactor has equal probability of forming biofilm [93]. This provides confidence that the cell count differences between materials seen in this experiment are meaningful.

Although CNTs are known for their bactericidal properties [94, 95], CICNT is a CNT composite with a volume fraction of over 99% carbon infiltration, so many of its properties come from the carbon infiltration [42]. The infiltration material is considered pyrolytic carbon [37], which is biocompatible and already used in implants like finger joint replacements [47], but it is
not antibacterial. This work shows that changing its nanostructure with a CNT forest reduces MRSA biofilm formation.

The significant difference between CICNT-Si and the carbon control clearly illustrates the effect of the CICNT nanostructure. Their chemistries are the same, and the fabrication is the same except the carbon control was infiltrated long enough to eliminate the porosity between the nanotubes. They are the same material with different nanostructures (Figure 3.4); the reduced formation of MRSA biofilm on CICNT-Si is most likely due to its nanostructure rather than its chemistry.

The effect that both CICNT-Si and CICNT-SS had on MRSA cells was likely antibiofouling. SEM imaging can identify a coating as bactericidal when cell degradation and lysis is observed [23, 96]. The current imaging techniques are not ideal for identifying this. However, since this was not seen on either type of CICNT surface (Figure 3.5), the biofilm reduction mechanism is likely antibiofouling. This is intriguing because other nanopillar structured surfaces, like the dragonfly and cicada wings mentioned previously, are bactericidal [23, 97, 98]. Both of these mechanisms have great potential to reduce infection by the unique physico-mechanical effect of nanopillars. Although an antibiofouling surface does not kill the bacteria like a bactericidal surface, an anti-fouling surface may be just as effective at reducing infection rates because biofilms that lead to PJI are prevented [71], and antibiotics administered systemically are optimized to eradicate planktonic cells that may be present.

This antifouling mechanism of CICNT coatings may be similar to nanopatterned silk fibroin where hydrophobic and size effects are combined [26]. Both of these effects achieve the condition where bacteria cells have less contact area for adhesion. Because the static contact angle of CICNT is approximately 90° [49], it is barely classified as a hydrophobic surface [26]. This suggests that most of the antifouling mechanism can be credited to the nanotubes’ size and distancing. This can happen when the feature size is smaller than the bacterial size [34], and this is the case for CICNT (100-225 nm diameter) and MRSA (~800 nm diameter).

A concern with an orthopaedic implant coating that resists biofilm attachment and proliferation is that it will also resist bone cell attachment and proliferation. If a prosthetic joint resisted osteoblast integration, the joint would loosen [99]. More comprehensive testing is underway, but initial in vitro experiments show that CICNT promote more human osteoblast
proliferation, vitality, and functionality than bare titanium [100]. The significant size differences between MRSA cells and osteoblasts, ~1 µm vs. ~10 µm, may account for the difference in cell behaviors on the nanostructured surface. If CICNT offers improved bone integration while limiting biofilm growth, it could reduce both septic and aseptic loosening in orthopaedic implants.

The biofilm resistance results for CICNT-SS are especially promising because it has potential for a broader application range and offers many advantages over CICNT-Si. SS is tough, relatively easy to machine, and widely used in medical implants. The methods to grow CNT directly from the internal catalysts of SS reduce the fabrication time from five hours to two hours and the equipment from four machines to one machine. Also, because the catalyst comes from an internal source, the coating would theoretically have exceptional adhesion to its substrate, which is critical for coatings. Additionally, despite its lack of uniformity and random-orientation, CICNT-SS resisted MRSA biofilm attachment and growth nearly as well as the highly uniform, vertically-aligned CICNT-Si.

There are limitations associated with this study. Although MRSA is highly relevant in PJI, there are certainly other bacteria of interest. Initial experiments with *Pseudomonas aeruginosa* (gram-negative rod) on CICNT-Si have also shown biofilm resistance, and future work on CICNT coatings will include additional strains. The second limitation is that the biofilm formation was evaluated at one point in time and with 2D images. Evaluating multiple time points, including a longer time point, and performing quantification by a 10-fold dilution series would provide greater insights into how MRSA biofilm react to CICNT and controls over time, which is an objective of future work. Although biofilms can develop within 48 hours under these conditions and the principle of preventing initial formation improves the chance of winning the “race to the surface” between bacteria and bone cells in external fixator pins [14, 86], a longer time point would provide greater confidence in the coating. Third, although CICNT resisted more bacteria than the stainless steel and carbon controls, it would be worthwhile to study how it compares to other biofilm resistant coatings, such as patterned silk fibroin, black silicon, cicada wings, etc. These materials are not readily available, but they would make an informative comparison in future work. Fourth, SEM images of biofilm provide a detailed picture of the biofilm morphologies on each material, but because it is unknown whether the cells are living or dead, the cell counts from SEM images represent the worst-case scenario where all cells are living. Finally, this was an *in vitro* study;
additional work will be required in vivo to validate benefits, but as mentioned previously, this is a robust method optimized for biofilm formation [92]. Despite these limitations, the promising results in this study justify future work including in vivo testing and mechanical testing for substrate adhesion, strength, wear, and cyclic loading.

3.6 Conclusion

In order to provide a solution to persistent PJI rates and antibiotic-resistant bacteria development, the ability of CICNT to resist MRSA biofilm formation was tested. This was done by subjecting samples to a biofilm reactor for 48 hours then counting MRSA cells from SEM images. Two types of CICNT were tested: a vertically-aligned forest using an externally-applied CNT catalyst on a silicon substrate (CICNT-Si) and a random-oriented forest using stainless steel as the substrate and CNT catalyst (CICNT-SS). They were compared to stainless steel and carbon controls. Both CICNT-Si and CICNT-SS resulted in substantially less MRSA cell attachment (60-80% reduction) and less mature MRSA biofilm formation than both controls. In fact, there was virtually a complete inhibition of mature biofilm formation on the CICNT samples. Because the carbon control had the same chemical makeup as CICNT, the statistically significant difference indicates that the biofilm resistance of CICNT is primarily due to a physico-mechanical effect from the nanostructure. This mechanism for resisting biofilm formation provides a unique way to potentially reduce PJI and prevent the development and spread of antibiotic-resistant bacteria.
CHAPTER 4. CARBON-INFILTRATED CARBON NANOTUBE COATING
APPLICATIONS: SCALPEL BLADES AND EXTERNAL FIXATORS

4.1 Abstract

A carbon-infiltrated carbon nanotube (CICNT) coating grown on stainless steel has a broad range of possible applications due to its non-pharmaceutical biofilm resistance [101]. This property may provide a solution to surgical site infection (SSI), so scalpel blades and external fixator pins were coated with CICNT and subjected to mechanical testing and MRSA biofilm testing. This scalpel blade coating is one of the first reports of CNT fabrication using 400-series SS as a substrate and a catalyst [102]. The CICNT coating did not prove to have superior anti-bacterial performance to a bare scalpel blade, but this work provides greater insight on CICNT properties. Hardness that was lost during the CICNT manufacturing process was recoverable, and the coating did not delaminate under the force of a diamond scribe. Although the CICNT on these scalpel blades did not exhibit MRSA biofilm resistance, new theories were offered on what factors are critical for the CICNT nanostructure to resist biofilm. The external fixator pin application proved to have significantly more promise than the scalpel blades. The bare stainless steel pin was completely covered in MRSA biofilm, while both the vertically-aligned and randomly-oriented CICNT-coated pins each allowed only a few cells to attach. Although there is more testing to be done, a CICNT coating may be an exceptional way to prevent infection while reducing antibiotic use.
4.2 Introduction

4.2.1 The Need for an Antibacterial Coating

Surgical site infection (SSI) continues to affect hundreds of thousands of people in the US each year [103]. With a 2-5% incidence rate and $3.5-$10 billion annual US cost, these infections are now the most common and costly type of hospital-acquired infection [103, 104]. For orthopaedic joint replacements, approximately half of SSIs are periprosthetic joint infections (PJIs) [105], which may be considered the most devastating complication of these surgeries [106]. The bacteria species that cause most SSI and PJI are part of the normal skin flora [107, 108], so although the evidence is limited, the scalpel may potentially cause some of these infections [109]. Therefore, coating a scalpel with an antibacterial coating may prove beneficial.

Along with addressing the scalpel as an infection source, external fixator pins have a remarkably high infection rate. The pin tract infection rate is 21% to 42% of cases [110]. Yet despite this risk inherent with the device’s transdermal pathway, they are still used because they play an important role in cases that need reduced soft tissue damage, avoidance of any infected areas, and adjustability [111]. Therefore, coating an external fixator pin with an antibacterial coating may also prove beneficial.

4.2.2 Challenges with Current Solutions

There are a variety of efforts aimed at addressing the challenge of SSI for external fixator pin infection. Coating pins with silver, iodine, or gentamicin-coated sleeves has shown evidence of reducing infection [112]. However, all of these options are based on their chemistry, so there is a concern of cytotoxic effects [113]. Additionally, antibiotic-based coatings are limited by drug-resistant bacteria strains, such as methicillin-resistant Staphylococcus aureus (MRSA), and biofilm formation that makes systemic antibiotics ineffective [113].

4.2.3 Benefits of a CICNT Coating

A carbon-infiltrated carbon nanotube (CICNT) coating may offer an improved solution. Recent in vitro testing on CICNT coatings provides evidence that this coating resists MRSA cell
attachment and biofilm formation due to its nanostructure rather than its chemistry [101]. Although additional testing is required, the results are promising, especially because it can be easily coated directly onto stainless steel (used in both scalpel blades and external fixation pins) and on different geometries. Other advantages of a CICNT coating include that it is low cost [86], heat stable, and would have a theoretically infinite shelf life. Because of this, potential applications are being investigated. The objective of this research is to test the feasibility and effectiveness of coating scalpel blades and/or external fixator pins with CICNT to resist bacterial infection.

4.3 Experimentation

4.3.1 Scalpel Blade Fabrication and Testing

CICNT was coated directly onto size 10 scalpel blades with a process optimized for this 400-series stainless steel (SS). The process sequence was a heat treatment, CNT growth, then carbon infiltration, all in a CVD tube furnace. The heat treatment was at 800 °C in argon (Ar, flowing at 250 sccm) for 30 minutes, the CNT growth was at 700 °C in hydrogen (H2, flowing at 230 sccm) and ethylene (C2H4, flowing at 250 sccm) for 15 minutes, and the carbon infiltration was at 850°C in Ar and C2H4 for 25 minutes. Controls were uncoated size 10 scalpel blades.

The CICNT coatings on scalpel blades then underwent various mechanical tests: cutting performance, hardness changes, and scratch durability. Cutting performance was tested by an experienced dissection technician cutting raw chicken with the skin intact using two coated and two uncoated blades. Four types of cuts were performed: plunge cut, 4-6 inch longitudinal cut, skin and tissue separation cut, and a cut that strikes the bone. This allowed for a subjective feedback on cutting feel and sharpness. Hardness change was tested on a Rockwell C scale in five locations (two on the body and three along the edge of the blade) on two of each type of treatment: (1) uncoated scalpels, (2) heat treated scalpels that underwent the time-temperatures protocol for growing CICNT, but without the carbon source, (3) CICNT-coated scalpels, (4) heat treated scalpels quenched in canola oil directly following the “infiltration” step, and (5) CICNT-coated scalpels quenched in canola oil directly following infiltration. The CICNT coating’s scratch durability was tested with a diamond scribe scratching a coated scalpel blade nine times: three
different pressures (light, medium, strong) in three locations. These were then SEM imaged for visual results.

Finally, the scalpel blade coating was tested for MRSA biofilm resistance. Three coated and uncoated scalpel blades were placed in a CDC biofilm reactor with MRSA-inoculated tryptic soy broth (TSB). The reactor was placed on a hot plate set to 34°C and 130 rpm for only 24 hours in order to capture the initial bacteria attachment that may bring bacteria into the body during surgery. To prepare for SEM imaging, samples were rinsed in phosphate-buffered saline (PBS), fixed with glutaraldehyde, dehydrated with ethanol, and coated with gold [86]. SEM images were taken at 5000x magnification in five locations: three along the sharpened edge and two on the body of the blade. This magnification was used to view the morphology and spread of MRSA cells.

4.3.2 External Fixator Pin Fabrication and Testing

External fixator pins were coated with CICNT using two CNT catalyst methods: layered and direct. The pins were 316L SS rods (McMaster 89325K91) with a 0.25-inch diameter and approximately 1-inch length. An uncoated SS rod was used as a control.

The layered method produced a CNT forest by using a traditional, externally-applied catalyst [114]. First, a diffusion barrier, an 80-nm thick layer of alumina, was deposited onto the pins by rotating the pins during an electron-beam deposition [114]. The pins were also rotated during the thermal evaporation of the CNT catalyst, a 4 nm thick layer of iron. A CNT forest was grown then infiltrated in a CVD tube furnace. The growth occurred at 750 °C with hydrogen (flowing at 230 sccm) and ethylene (flowing at 250 sccm) for 1 minute, and the infiltration occurred at 900°C under the same gases and flow rates for 22 minutes.

The direct method produced a CNT forest by modifying the SS substrate to be a catalyst – similar to the scalpel blade coating fabrication. This was performed in the CVD furnace as a continuous sequence: heat treatment, CNT growth, then CNT infiltration. The heat treatment occurred at 800 °C with air (flowing at approximately 200 sccm) for 5 minutes, the CNT growth occurred at 700 °C with hydrogen (flowing at 230 sccm) and ethylene (flowing at 250 sccm) for 15 minutes, and the CNT infiltration occurred at 850 °C with argon (flowing at 250 sccm) and ethylene (flowing at 250 sccm) for 25 minutes.
Three external fixator pins were tested for MRSA biofilm resistance – one of each type of pin. A pin that was coated by the layered method, one that was coated by the direct method, and one uncoated pin as a control were fixed to coupons using paraffin wax then placed in a CDC biofilm reactor under the same conditions as the scalpel blades for the first 24 hours. For the subsequent 24 hours, the nutrients in the broth were replenished by flowing fresh TSB diluted to 10% strength (~6.9 mL/min) in accordance with standard practice [87]. This additional 24 hours in a flow phase under a diluted broth promoted bacteria to shift into a biofilm state following their initial incubation or batch phase [86, 87]. After 48 hours in the reactor, the pins were prepared for SEM imaging using the same process as the scalpels above. SEM images were consistently taken at the apex of the pins at 1,000x and 10,000x to view the morphology and spread of MRSA cells.

4.4 Results and Discussion

4.4.1 Scalpel Blades

*CICNT Coating Morphology*

The CICNT coating on the scalpel blades was randomly-oriented and mostly uniform (Figure 4.1). There were no distinct patches of overgrown CICNT that are sometimes called bush-like growths [115]. Another observation was that the body of the blades had longer CICNT and more CICNT height variation than the edge of the blades. A randomly-oriented forest was expected because most methods for growing CNT using SS as the substrate and catalyst do not produce vertically-aligned forests [54]. Because the surface structure is still similar to vertically-aligned CICNT on the nanoscale, a randomly-oriented forest has previously shown evidence of biofilm resistance properties [101].

*Cutting Performance*

An experienced dissection technician performed the various cuts through raw chicken using the CICNT-coated scalpel blades to test their function. The coated and uncoated blades successfully dissected the chicken using a plunge cut, a 4-6 inch longitudinal cut, a skin and tissue
separation cut, and a cut that strikes the bone. Upon visual inspection, there were no black spots indicating detachment of CICNT in the chicken; however, the technician feedback indicated that the coated blades were less sharp than the uncoated blades. Since a blade’s sharpness over prolonged use is directly correlated to its hardness, this prompted a look into a hardness recovery process for the CICNT-coated blades. This is detailed in the next section.

**Scalpel Hardness**

The CICNT fabrication process may affect the scalpel’s hardness, and because a material’s hardness determines how sharp a blade stays, this property was investigated. The hardness of a blade with no treatment (“Uncoated”) was compared to four other treatments. Two treatments were treated and cooled in the furnace (“Heat Treat” and “CICNT”), and two treatments were quenched in oil after the 800 °C infiltration step in order to harden the blades (“Heat Treat & Quench” and “CICNT & Quench”). The oil quenched blades showed increased hardness (Figure 4.2). The blades that went through the CICNT process without carbon gas (“Heat Treat” and “Heat Treat & Quench”) each show decreased hardness compared to their CICNT counterparts (Figure 4.2).

The results indicated that the temperatures required to coat scalpels with CICNT significantly reduce the hardness of the blade, but this hardness can be almost completely
Figure 4.2: Scalpel hardness measurements for various treatments (left). Photo of the scalpel blades measured (right). Indentations show the measurement locations. Treatment pairs from top to bottom: uncoated, heat treated, CICNT-coated, heat treated and quenched, CICNT-coated and quenched.

recovered. The heat treatment without CICNT reduced the hardness from an average of 53 HRC to an average of 31 HRC. It is hypothesized that the ethylene during the growth and infiltration of CICNT carburized the blade and caused the hardness to increase to an average of 41 HRC. Approximately the same amount of hardness could be recovered by an oil quench directly following the “infiltration” step during the heat treatment (average of 43 HRC). The hardness was nearly recovered by combining the carburizing from the CICNT growth and infiltration with the oil quench directly following the infiltration step (average of 48 HRC). This shows that a CICNT-coated scalpel blade could be nearly as capable at maintaining a sharp edge.
Scratch Durability

CICNT-coated scalpel blades were able to withstand the hardness of a diamond scribe scratch with only a local disturbance rather than delamination. SEM images of the scratches are shown in Figure 4.3. As the force increased, both surfaces naturally exhibited a deeper and wider disturbance. Compared to bare scalpel, the disturbance was wider on the CICNT-coated blade than the uncoated blade. As measured in the previous section, the hardness of the CICNT-coated blades is reduced without an oil quench, so the wider disturbance may be due to the 12 HRC reduction in hardness.

MRSA Biofilm Resistance

Following 24 hours in the MRSA biofilm reactor, the CICNT-coated scalpsels allowed more biofilm attachment than the uncoated scalpsels (arrows in Figure 4.4); however, comparing these results to a CICNT coating that resists biofilm expands our understanding on critical dimensions needed for a nanostructure to resist MRSA biofilm. The main difference between the scalpel
coating and other CICNT coatings that have shown biofilm resistance (for example, Chapter 3) is that the CICNT heights on the scalpels were significantly more variable. This variation allowed the longer nanotubes to spread out throughout the surface, and this may affect the results in a variety of ways.

Because biofilm formation is affected by the shear stress caused by the fluid dynamics from the rotating paddle [92], the flow dynamics at the surface may be different on this CICNT-coating than on the CICNT-coated fixator pins (Section 4.2) or on the coatings reported in previous experiments [101]. The additional roughness caused by the extra-long CICNT may have altered the shear stress to a more optimal level for biofilm formation. The individual CICNT may have caused additional flow disturbances on the nanoscale as the broth flowed over all the nanotubes.

Because biofilm formation is also affected by the stiffness of its substrate [116], the unusual result could be caused by the lower stiffness in the longer nanotubes. There is an optimal stiffness for *S. aureus* biofilm formation [116], and MRSA most likely has the same response. Modeling the extra-long nanotubes as beams, their stiffness would be significantly reduced because the length of the beam, or nanotube, variable is to the fourth power. Therefore, although CICNT has a high modulus of elasticity [35], the stiffness of the scalpel blade coating may have changed enough to become optimal for MRSA biofilm formation.
4.4.2 External Fixator Pins

CICNT Coating Morphology

The CICNT fabrication method using a layered catalyst produced a vertically-aligned forest, and the method using a direct catalyst produced a randomly-oriented forest. SEM images of these are shown in the left columns of Figure 4.5. The CICNT forest from the layered method has relatively smooth and uniform surface, whereas the CICNT forest from the direct method shows bush-like growths.

MRSA Biofilm Resistance

Following 48 hours in a MRSA biofilm reactor, SEM images of the fixator pins showed significantly less biofilm formation for both types of CICNT coatings compared to the bare SS pin (Figure 4.5). MRSA cells were identified by their spherical shape, smooth edges, and roughly 0.8µm diameter, and then marked in Figure 4.6. The bare SS pin was entirely covered with MRSA cells (605 bacterial cells), while there are only a handful of cells on the pins with a CICNT coating (18 bacterial cells on the layered CICNT pin and 9 bacterial cells on the direct CICNT pin). The cell aggregation on the bare SS pin indicated mature biofilm formation on the surface, and the lack of cells attached to both types of CICNT coating indicated biofilm resistance.

4.4.3 Limitations and Future Work

As with any study of a new coating, there are limitations that provide more areas to research. More extensive testing must be done to further understand and verify the MRSA biofilm resistance seen from CICNT coatings. In general, these tests would include additional variables such as physical variations of CICNT, higher sample sizes, additional bacterial strains, and multiple experiment lengths. Specifically, since this study showed exceptional MRSA biofilm resistance on external fixator pins coated with CICNT, more testing must be done to ensure that the CICNT coatings promote skin-implant integration because infection is regularly the result of this lack of integration [117].

The disturbance of the CICNT coating caused by the diamond scribe during the scratch durability testing likely removed some CICNT from the substrate. This may be a problem in vivo
Figure 4.5: SEM images of each material before (left) and after (right) the biofilm experiment. After 48 hours, MRSA cells were identified by their spherical shape, smooth edges, and roughly 0.8µm diameter. Magnifications are 1,000x and 10,000x.

Figure 4.6: MRSA cells marked in red for each material: bare SS (605 bacterial cells), layered CICNT pin (18 bacterial cells), and direct CICNT pin (9 bacterial cells). Image locations are the same as those in Figure 4.5. Scale bars are 5 µm.

if the infiltration is removed from the CICNT to expose the CNT to the body. Although CICNT have shown biocompatibility, it is questionable if bare CNT is biocompatible because the evidence is inconclusive and there are multiple factors that affect its toxicity [118, 119]. Additional testing would need to be done.
4.5 Conclusions

Because CICNT-coating that has been shown to resist biofilm formation based on its nanostructure rather than its chemistry [101], a solution for SSI was proposed by coating scalpel blades and external fixator pins with CICNT and subjecting them to mechanical and MRSA biofilm tests. On the scalp blades, the CICNT coating did not exhibit superior performance to a bare scalpel blade, but this work provides greater insight on CICNT properties. The hardness that was lost during the CICNT manufacturing process was largely recoverable, and the coating did not delaminate under the force of a diamond scribe. Although the CICNT-coating on scalpel blades did not show MRSA biofilm resistance, the influence of CICNT length variation was indicated as a potential factor in biofilm resistance. The external fixator pin application proved to have significantly more promise than the scalpel blades. The bare SS pin was completely covered in MRSA biofilm, while both the vertically-aligned and randomly-oriented CICNT-coated pins each allowed only a few cells to attach. Although there is more testing to be done, we are optimistic that a CICNT coating will prove to be an exceptional way to prevent infection while reducing antibiotic use.
CHAPTER 5. CURVATURE-INDUCED DEFECTS ON CARBON-INFILTRATED CARBON NANOTUBE FORESTS

5.1 Abstract

A morphological study of the micro-scale defects induced by growing a carbon-infiltrated carbon nanotube (CICNT) forest on concave substrates was conducted. Two CICNT heights (roughly 60 µm and 400 µm) and 4 curvatures (1-4 mm ID) were studied in order to test the geometric limitations. Defects were categorized and quantified by scanning electron microscopy (SEM) of the tops and cross-sections. These deformities were categorized as increased roughness on the top surface, a corrugated (also called wavy or rippled) forest, a curved forest, an inside crevice where the forest separates, and increased forest density on the top surface. Roughness increased nearly 3-fold with the taller forest heights no matter the substrate curvature. Due to the geometric limitations of CICNT height and substrate curvature, all other microscale defects were significantly more present on samples with a small radius of curvature and a tall CICNT forest (p<0.05). These buckling and warping types of defects were attributed to the increase in circumferential compression as the forest grows as well as the Van der Waals interactions between the nanotubes. Because the fabrication process for CICNT involves growing a CNT forest and then infiltrating it with pyrolytic carbon, this work may be applicable to other CNT forests on concave substrates within these forest heights and substrate curvatures.

5.2 Introduction

The unique mechanical, electrical, and chemical properties of carbon nanotube (CNT) “forests” give this material an extensive range of practical applications [120]. A few of these include energy storage [121, 122], chromatography [123, 124], flexible electronics [125], and biomedical applications [44, 81, 126]. Although many potential applications require a CNT forest
coating on a rod or inside a tube, such as fluid flow applications [56], few studies on CNT forests have been conducted on curved substrates.

One of the concerns with growing CNT forests on non-planar substrates is that additional morphological defects will be induced because the order and alignment of the individual CNT within a forest ensemble influences the performance of the material on the macroscale. Microscale defects in CNT forests on flat substrates already affect their mechanical, electrical, and thermal properties [127]. For example, when forests exhibit more waviness – also termed as a rippled or corrugated forest – on the microscale, the stiffness of the forest as a whole is reduced by as much as three orders of magnitude [61]. This morphological defect may be a result of varying growth rates of adjacent CNT causing strain mismatch [128, 129], mechanical coupling from Van der Waals forces [127, 130], compressive stress [131, 132], or a combination of these [133, 134]. Understanding these defects and others leads to the understanding of forest growth and property changes.

A carbon-infiltrated carbon nanotube (CICNT) forest is a CNT forest that has been subsequently infiltrated with amorphous pyrolytic carbon [35]. The structure maintains its shape and can still be rendered superhydrophobic like CNT forests [49], but it is more durable as a cohesive material because CNTs cannot be brushed off the substrate. Under bending loads, CICNT have shown a strength of 102 MPa and a modulus of elasticity of 4.1 GPa in the direction perpendicular to the forest (i.e., pulling the nanotubes away from each other), and in the direction parallel to the nanotube axes, the achievable strength was 903 MPa with a modulus of elasticity of 11 GPa [35].

Because CNT, and subsequently CICNT, forests grow perpendicular to their substrate, a forest inside a tube or on any concave substrate experiences circumferential compression, while a forest on a rod or other convex substrate experiences circumferential tension. As these stresses increase, the forest will eventually deform and exhibit defects. Defects from circumferential tension were seen on 3 mm diameter rods of a CICNT forest as cracks parallel to the rod axis at the top of CICNT forests were observed and systematically studied [114]. For a forest height of roughly 150 µm, the length a forest section could be without cracking was 414 µm on average. This was attributed to the Van der Waals forces between the individual nanotubes and the increasing circumferential stress as they grow on a convex curvature.
Neither CNT nor CICNT forest defects caused by concave substrate curvature have been systematically studied. This is likely because most studies of CNT forests on concave substrates are comprised of relatively short CNT forest heights (< 16 µm) with relatively large curvatures (> 11 mm ID) [62, 123, 135, 136]. One exception is a study of CNT forests coated directly onto stainless steel tubing with an inner diameter of 1.27 mm and a CNT forest height ranging from 4 to 60 µm showed a relatively high surface roughness [56]. A cross-sectional view of this forest showed non-uniformity of CNT height, but this and other micro-scale morphological defects have not been systematically studied. The objective of this research was to characterize and evaluate the defects caused by the geometric limitations of coating CICNT on highly curved concave substrates.

5.3 Experimental

5.3.1 Design of Experiments Overview

Different combinations of radius of curvature and CNT height were tested in a 4 by 2 block design of experiments with a sample size of 6, or 48 samples in total. Four substrate sizes with radii of curvature ranging from 0.5 mm to 2 mm (i.e., 1 mm ID to 4 mm ID) in 0.5 mm increments were used. These roughly correspond to the internal diameters of gauge 17, 12, 9, and 7 needles and are also relevant to blood vessel sizes relevant to cardiovascular applications for CICNT materials. CNT height was controlled by the amount of time in the growth phase of the fabrication process [35, 36]. The growth times in this study were 1-minute and 10-minutes, which corresponded to heights of 63 ± 14 µm and 389 ± 61 µm, respectively. The length of the substrate and CICNT infiltration time were held constant.

5.3.2 Sample Fabrication

CICNT in quartz tubes were manufactured using a process that has been reported previously for flat, silicon substrates [37, 137]. The tubes were cut roughly 2 inches long and then in half lengthwise (Figure 5.2a) to expose the inside curvature to thin film deposition processes. This included a layer of Al₂O₃ as the diffusion barrier (30-40 nm thick, applied through electron
beam evaporation) followed by a layer of Fe as the CNT catalyst (4 nm thick, applied through thermal evaporation). These evaporation processes produce an even coating on flat substrates, but there are shielding effects that the concave substrates would be subject to. This caused the thicknesses on the sides, or “walls,” of the concave substrates to vary. Because of this, only the bottom 45°-60° (red lines in Figure 5.2) of the substrate was studied.

The fabrication process for CICNT growth and pyrolytic carbon infiltration is outlined in the time-temperature plot in Figure 5.1. In a 1-inch CVD tube furnace, the gas flow rates were as follows: hydrogen (H₂) at 235.5 sccm, ethylene (C₂H₄) at 249.3 sccm, and argon (Ar) at 220.6 sccm. The growth was performed at 750 °C for either 1 or 10 minutes, which correspond to the labels “short” and “tall,” respectively. The infiltration was performed at 900 °C for 16 minutes. The process for achieving “tall” growths was also used to coat CICNT on flat substrates with the purpose of comparing the CICNT density differences on the top surfaces of flat and concave substrates.

5.3.3 SEM Image Analysis

The samples were scored and snapped to examine the CICNT cross-sections under SEM (Figure 5.2c). Images at various magnifications ranging from 80x to 25,000x were used to identify and quantify any forest irregularities above the bottom central 45°-60° (red lines in Figure 5.2) as
Figure 5.2: (a) Photo of a 2 mm ID quartz tube cut in half lengthwise before CICNT growth and infiltration. (b) Photo after a tall CICNT growth and infiltration with an insert of tall CICNT on a flat substrate. (c) SEM image of a short CICNT growth on a 1 mm ID substrate. Scale bar is 500 µm. The CICNT forest grown along the area marked in red was used for analysis because the iron layer thickness was potentially variable along the sides due to shielding effects during the alumina and iron deposition processes.

discussed previously. These irregularities included any morphology that was not aligned and orderly even if the morphology is also seen on flat substrates. Because forest alignment affects the material properties of CNT forests [61], the irregularities were considered defects, and they were characterized and quantified for statistical analysis.

Surface roughness of the CICNT coating was calculated from SEM images using MATLAB image processing (Figure 5.3). An ideal surface would perfectly match the curvature of the underlying quartz tube, however localized non-uniformity in CNT growth rates produces a surface roughness that greatly exceeds that of the underlying quartz substrate. This surface roughness was obtained by comparing the deviation of the “actual” CICNT surface from an
idealized quadratic curve fit of the top edge (“ideal”). The subtraction of the “actual” and “ideal” images shows where they do not overlap (the black and white areas in Figure 5.3b), so these areas were divided by the length of the ideal curve to obtain a roughness value in units of µm. In order to better fit the circular curvature with a quadratic function, the roughness for the left and right halves of each image was calculated separately then averaged. Statistical significance was calculated by one-way ANOVA.

5.3.4 Density Calculation

Because the surface of a CICNT-coated device interacts with the environment, top-view SEM images of flat and concave substrates were analyzed for variation in CICNT density. Determining CICNT density is challenging due to the textural features of the CICNTs, thus a novel, automatic CICNT counting algorithm was developed based on image processing and geometric feature identification. Using this counting algorithm, the area density of CICNT (CICNT/µm²) on both flat and concave substrates was calculated by dividing the number of CICNT in an image by the image area. The algorithm approximated the tops of CICNT as circles, so its counts were verified by counting the circles in several images with a known quantity of circles (Figure 5.4) as well as manually counting CICNT in several locations on four different
Figure 5.4: Images with known numbers of circles used to validate the algorithm for calculating CICNT density. Note that many of the counts are lower due to parts of circles cropped from the image. The bottom-right is an example edge detection image that has been cropped to view the pixelation of the circle.

images (Figure 5.9). The locations were set as a grid of 9 squares with a 1 µm² area. The number of CICNT in each square was counted, and then the average, standard deviation, and range were computed to ensure the automatic computation results were within an acceptable range.

The first step in the algorithm was to find the circumference of the circles or CICNT by using a Canny edge-detection algorithm in MATLAB (approxcanny). This generated a black image with white pixels for the circle or CICNT edges. The circle verification is in Figure 5.4. Following the edge detection, the number of circles or CICNT in each image was calculated. The total circumference of all circles or CICNT in the image was first found by summing all the pixels that represent the edges in each image. The total circumference was then divided by 2.8 (modified pi) and the average circle or CICNT diameter in pixels to get the number of circles or CICNT in the image. Pi – the ratio of circumference to diameter – is slightly reduced because the pixelated
circles do not capture the full circumference of a circle (Figure 5.4) [138]. When this algorithm was tested on images with a known number of circles, there was a 1.3% error on average.

The density of a CICNT forest measured from top-view SEM images depends on the infiltration of that forest, so comparable images must have comparable CICNT diameters. Because of this, only four images were analyzed: two flat substrates, a concave substrate with a short CICNT forest, and a concave substrate with a tall CICNT forest (Figure 5.8).

5.4 Results

5.4.1 Roughness

Roughness values (µm) were calculated and plotted in Figure 5.5. The radius of curvature did not significantly affect the roughness, but the CICNT height did. Tall CICNT showed significantly more roughness than short CICNT (p<0.05). On average, the tall CICNT samples were over 3 times rougher than the short CICNT samples.

5.4.2 Microscale Defects

Four types of microscale defects were observed and recorded from the cross-section SEM images. These defects were designated as extra growth patches, corrugated forest, curved forest, and inside crevice. Examples of each defect are shown in Figure 5.6. If a defect or multiple defects were observed in the cross-section SEM images within the bottom 45°-60° zone, that sample was labeled with those defects. The defect presence was tallied and plotted with respect to the forest height and substrate curvature (Figure 5.7).

Extra growth patches were identified by several bundles of CICNTs growing taller than the rest of the forest. These can also be described as outgrowths of CICNT extending beyond the forest height. They were present in all CICNT height and curvature combinations, but they were seen in significantly lower quantities on the combination of wider curvature (3-4 mm ID) and shorter CICNT height. Specifically, short forests on 3-4 mm ID curvature had fewer samples with extra growth patches than tall forests on 1-2 mm ID curvature (p<0.0001), tall forests on 3-4 mm ID curvature (p=0.0002), and short forests on 1-2 mm ID curvature (p=0.006).
A corrugated forest defect was identified by microscale waves of CICNT with a wavelength of roughly 1-2 µm. These corrugations – or ripples – are akin to a high-order column buckling failure. The defect has also been seen with CNT forests on flat substrates under conditions where adjacent CNTs have different growth rates [128]. This defect was not present on any sample with short CICNT, and it was observed in significantly higher quantities on the samples with smaller curvature substrates (1-2 mm ID) and tall CICNT (roughly 400 µm). Specifically, tall forests on 1-2 mm ID curvature had more samples with a corrugated forest than short forests on 1-2 mm ID curvature (p=0.01) and short forests on 3-4 mm ID (p=0.01).

The curved forest defect was identified by CICNT that collectively arc away from the center of the forest. As the forest grew perpendicular to the substrate, the curvature caused the tips of the CICNT to push against each other as the available space decreased due to decreasing
Figure 5.6: SEM images of the types of defects seen. Top: a sample with extra growth patch, curved forest, and inside crevice defects. Scale bar is 500 µm. Bottom: a sample with a corrugated forest defect. Scale bar is 10 µm.

circumference. Rather than remain straight and perpendicular to the substrate, these forests bowed out and curved away from the center of the forest Figure 5.6. This defect was not present on any sample with short CICNT, and it was significantly more present on samples with smaller diameter substrates combined with a tall CICNT forest than on any other combination. Specifically, tall forests on 1-2 mm ID curvature had more samples with curved forests than short forests on 1-2 mm ID curvature (p<0.0001), tall forests on 3-4 mm ID curvature (p<0.0001), and short forests on 3-4 mm ID curvature (p<0.0001).
Figure 5.7: Percentage of samples where each defect was present (defect presence): extra growth, corrugated forest, curved forest, and inside crevice (* indicates p<0.05, and ○ indicates that no defect was observed for that combination).

The inside crevice defect is closely related to the curved forest defect, and in some ways, it is a more extreme version of the curved forest defect. An inside crevice was identified by a small gap or opening in the middle of the CICNT forest as if the tops of the forest were pushed together but the forest continued growing from the bottom causing the forest to bow and then separate under the pressure. The inside crevice had the same trends as the curved forest: no presence on any short CICNT samples and present significantly more on samples with smaller diameter substrates combined with tall CICNT than on any other combination. Specifically, tall forests on 1-2 mm ID curvature had more samples with curved forests than short forests on 1-2 mm ID curvature (p<0.0001), tall forests on 3-4 mm ID curvature (p<0.0001), and short forests on 3-4 mm ID curvature (p<0.0001).
5.4.3 Top Surface

The top surfaces of the concave substrates were compared with flat substrates of the same CICNT diameter. The surface morphology is essentially the same (Figure 5.8), but the density of the tall CICNT on a small concave substrate (10.35 CICNT/µm²) was higher than that on both the flat substrates (8.10 and 8.25 CICNT/µm²) and the short CICNT on a small concave substrate (8.01 CICNT/µm²).

These values computed by the density algorithm were validated by comparison with manual counting (Figure 5.9). The CICNTs within one square micron regions at nine equally-spaced locations in each image were manually counted, and the average, standard deviation, and range for each image was computed. All algorithm calculations fell within the range of the average ± standard deviation of each image, indicating consistency of the automatic calculation algorithm.

![Figure 5.8](image)

Figure 5.8: Each tile contains an SEM image of the top surface (top), image after edge detection algorithm (middle), and an overlay of the edge detection image on the SEM image (bottom). (a) Flat substrate with a 248 µm average CICNT diameter and a calculated density of 8.10 CICNT/µm² (b) Flat substrate with a 219 µm average CICNT diameter and a calculated density of 8.25 CICNT/µm² (c) Concave substrate with a 248 µm average CICNT diameter and a calculated density of 8.01 CICNT/µm² (d) Concave substrate with a 216 µm average CICNT diameter and a calculated density of 10.35 CICNT/µm².
Figure 5.9: Results from counting the CICNT by hand to verify the model. Values in the red boxes are the manual counts of CICNT in each corresponding 1 µm² area. The average, standard deviation, and range of the manual counts of CICNTs from the regions within each image were computed. All automatic computations fell within the range of the average ± standard deviation of each image. Scale bars at 2 µm.

### Discussion

As the CNT forests grew, the circumferential compression increased, and when geometric limitations of curvature and forest height were reached, certain types of defects occurred. These defects were characterized and quantified from SEM images of the top and cross-sectional views. The types of defects seen were increased roughness, extra growth patches, corrugated forest, curved forest, inside crevice, and increased CNT density on the top surface. Naturally, these were
seen significantly more on the combination of tall CICNT height (average roughly 400 µm) and small radii of curvature (1-2 mm ID).

Roughness was directly related to CICNT height; however, the variation observed in the present work may not impact future applications. Taller CICNTs displayed a significantly rougher surface than the shorter CICNTs. This may occur because all the CNTs in the forest normally grow at the same rate, but the longer they grow, the more variability can be introduced. There may be concern that the increased roughness on taller forests would impact potential applications, but the roughness averages varying between 1 µm and 3.5 µm in this study are relatively small and may not impact the desired outcomes. Certainly, this impact depends on the application. For a blood flow application, such as mechanical heart valves or coronary stents, roughness can be directly correlated with thrombus formation [139]; however, despite roughness changes between Ra=1.8 µm and 19.5 µm showing an effect on blood flow [140], surface roughness changes in this range have shown no effect on thrombosis [141]. This may also be the case for other flow applications.

Extra growth patches were seen significantly less on short CICNT with large curvature (3 and 4 mm ID) than any other combination, but this effect may not be an inherent feature of CICNT on concave substrates. These extra-growth patches may come from CNT growing on unstable corners and edges of the substrate and falling to the bottom of the tube. CNT are heavier when they are taller, so these would be more likely to fall. Additionally, because the 1 and 2 mm ID tubes were smaller and more difficult to cut down the center, their top surfaces were rougher and more imperfect. This could cause more unstable CNT to fall even for short growth heights.

The corrugated forest defect, also called rippled morphologies or vertical corrugations [128], occurred significantly more for long growth heights on the small IDs, but this defect is not unique to curved substrates. It has also been seen on flat substrates [128]. One possible explanation is that the CNTs buckled because of the extremely high aspect ratios of the CNTs (e.g. 2 nm diameter and 400 µm tall). However, compression tests done on other CNT forests show a different morphology than these [142-145], so this is not a likely cause. A more likely explanation is that if the corrugated forest area grew faster than its adjacent CNTs due to dispersity of CNT diameter, then the mismatch between the growth rates in that area and the entire forest will cause increased tortuosity due to mechanical coupling [60, 127, 128]. Simulations have shown that an increased tortuosity or waviness in a CNT forest reduces the effective modulus of elasticity (E) by up to
three orders of magnitude [61], and this is also illustrated in Eqn (1). In regard to potential coating applications, this defect may affect the strength under compression, but it should not interfere with the functionality because the function comes from the top surface structure.

\[ E = (\rho_N \times L \times \tau) \times K \]  

(1)

where \( \rho_N \) is the packing number density (CNT/area), \( L \) is the serpentine length of the CNT, \( \tau \) is the tortuosity (defined by \( L \) divided by forest height), and \( K \) is the effective spring constant [61].

The curved forest defect is a good indication that the CNT forest is too long for the radius of curvature. A possible explanation for this is that the CNTs cannot grow perpendicular to the substrate if other CNTs are blocking them. This may cause some CNTs to get smothered and stop growing as if they have had their iron capped [146], or they can expand outward creating a curve in the forest. The CNTs tend to grow parallel to each other because of van der Waals forces [147], but this is not always possible when the substrate is concave.

The results for the inside crevice defect were similar to those for the curved forest defect. This defect may also occur because as the tips of the forests run into each other, the middles are pushed out towards the sides as the CNTs continues to grow. Another explanation for this is that the inside crevice defect can also be present if there was a defect in the iron layer, such as a scratch or dust particle, that caused CNT not to grow there. This may have been the case for some of the samples in the present work, but post-hoc identification of such defects was not possible due to the fact that they are obscured by the surrounding CICNT.

Each of the defects described in the present work were more prevalent on tall CICNT forests with small substrate curvatures, so coating height, as a design factor for highly curved surfaces, is recommended to be as short as possible. Not only do shorter forest heights induce less defects, but they also reduce the interface stress between the CICNT material and the underlying substrate during use.

Many of the proposed applications of CICNT involve interaction with fluids, thus the top surface nanostructure is of special interest. Calculations of CICNT density have previously been quite challenging, thus in the present work we introduced a novel, image-analysis based methodology that is broadly applicable and quick to implement. Although this methodology is new
and still requires additional validation, it allows direct comparison of CICNT densities across samples.

There are several limitations of this work. First, CICNT were not coated inside a full tube, so only the bottom 45-60° of curvature was evaluated. The vapor deposition processes used to apply the CNT catalyst materials are unidirectional, which work well for planar substrates. The modifications required to use these processes for concave substrates are still under development and currently do not yield fully uniform catalyst material thicknesses. Future work may include improved processes for growing CICNT on the inside of full tubing. Thus, in the present work, we were constrained to analyze only a small fraction of the circumference. Nonetheless, the defects observed in this study are consistent with what may be induced by circumferential compression on a CNT forest. Another limitation is that the scope of this work was constrained to evaluation of the microscale defects found in CNT forests. More qualitative and quantitative work can be done to assess defects on the atomic and nanoscale [128]. Also, as is typical for CNT and CICNT-related work, growth heights in this study were indirectly controlled by holding CNT growth time constant, so there was variation in the final forest height. Despite these limitations, this study found significant differences between the number of defects in the groups of CNT height and substrate curvatures, and this data can be used to understand the microscale defects caused by substrate curvature.

5.6 Conclusion

Because growing a CNT forest on a concave substrate causes circumferential compression on the forest, the micro-scale morphological response to this stress was studied. CICNT forests were grown at different forest heights and substrate curvatures, and SEM images of the top and cross-sections were used to discover the geometric limitations. In general, the combination of a tall CICNT height (roughly 400 µm) with a small substrate curvature (1-2 mm ID) exhibited certain types of micro-scale defects: increased roughness, extra growths, corrugated forest, curved forest, and inside crevices. This combination was also associated with an increased CICNT density on the top surface. Because CICNT is a CNT forest infiltrated with pyrolytic carbon, this work likely applies to all CNT forests on concave substrates within these forest heights and substrate curvatures.
CHAPTER 6. SUMMARY AND FUTURE WORK

6.1 Conclusions

The present work has advanced the understanding and expanded the application range of CICNT coatings intended for biomedical implant-related applications. Key conclusions from the work are summarized in the sections below, along with recommendations for related future work.

6.2 MRSA Biofilm Resistance of CICNT-Si and CICNT-SS (Chapter 3)

6.2.1 Key Conclusions

Nanostructure can be used to render a material antibacterial regardless of its chemistry; however, the scope of nanostructure geometry that can resist biofilm attachment and proliferation has not been entirely investigated. The present work has demonstrated that CICNTs can resist MRSA biofilm regardless of the forest orientation and within a relatively large range of CICNT diameters (Figure 6.1). The work has also demonstrated that CICNT grown as both vertically-aligned forests from alumina and Fe deposited on silicon (CICNT-Si) and randomly-oriented forests directly from stainless steel (CICNT-SS) can resist MRSA biofilm. Besides their morphology, CICNT-Si and CICNT-SS also differ in their nanotube diameter: CICNT-Si were infiltrated between 100 and 140 nm diameter tubes while CICNT-SS were infiltrated between 160 and 225 nm diameter tubes.

Furthermore, the biofilm resistance of CICNT can be attributed to its physical nanostructure. The CICNT-Si and the carbon control had the same fabrication process other than the additional infiltration time to fill in the porosity for the carbon control surface. Because these were the same chemical makeup, the biofilm resistance of the CICNT-Si is considered to be due
to a physico-mechanical effect from the nanostructure. This non-pharmaceutical approach has the potential to reduce the need for antibiotics, and in turn, this would slow the evolution and spread of antibiotic-resistant bacteria, such as MRSA. Because parallel testing by others in our lab has shown CICNT to enhance bone cell attachment, medical devices – especially orthopedic implants with high PJI rates – would benefit greatly from this.

### 6.2.2 Future Work

*Additional Biofilm Testing*

Although biofilms can develop within 48 hours in a CDC biofilm reactor, additional time points would provide greater insight to the coating’s biofilm resistance. Evaluating multiple time points, including a longer time point (e.g., 1x per day for 7 days), could inform how long the initial formation prevention lasts. Because each reactor run is slightly different, both positive and negative controls need to be included in each run. Positive controls would be other biofilm resistant surface modifications, such as patterned silk fibroin, and negative controls would be the same used in this experiment or other materials that are commonly used in orthopedic implants, such as titanium/Ti-6Al-4V. The goal is to win the “race to the surface” between bacteria and bone cells, so the surface roughness of control materials must match the implant surfaces that interact with bone.
Evaluating more samples and time points requires a less time-intensive protocol. Future quantification can be done by performing a live/dead cell stain and using fluorescence microscopy and image analysis software to count biofilm area coverage [78], while SEM image analysis can be used to evaluate the morphology of the biofilms as well as verify the quantification. This quantification method would produce results quicker as well as remove the question of whether the MRSA cells are living or dead in SEM images.

Additionally, there are other biofilm-forming bacterial strains of interest. In Appendix A, an initial biofilm reactor test with *Pseudomonas aeruginosa* on CICNT-Si is reported, and the results were promising. Because it is a gram-negative rod-shape while MRSA is gram-positive sphere-shaped (coccus), these two strains can demonstrate the range of CICNT biofilm resistance – similar to a broad-spectrum antibiotic.

Future work must also assess the influence of nanostructure geometry on biofilm resistance. There are many feature sizes that can be controlled during the CICNT manufacturing process, such as diameter, density, height, etc., and these should be optimized for MRSA and *Pseudomonas* biofilm resistance. The current work shows that CICNT can resist biofilm formation on diameters between 100 and 250 nm, and there may be an optimal diameter within that range or just outside that range. These diameters are easily controlled by varying the time spent in the infiltration step and measured from SEM images. The density is controlled by changing the Fe thickness on CICNT-Si and by changing the air heat treatment time on CICNT-SS. Measuring this metric has not been established, but a method using SEM images in a MATLAB program is described in chapter 5. Methods for controlling and measuring the CICNT length variation have not been established, but since the increased length variation on CICNT-coated scalpel blades (chapter 4) was likely responsible for the lack of MRSA biofilm resistance, this should be studied and established in future work.

In order to further validate the claim that the CICNT biofilm resistance property is caused solely by the nanostructure, the chemistry must be changed and tested. One option is to test a CICNT forest that has been coated with a thin layer of gold, and another option is to test SiO$_2$ nanotubes. SiO$_2$ nanotubes are made by growing a CNT forest, infiltrating it with SiO$_2$ instead of pyrolytic carbon, and then burn out the CNT. This leaves the surface structure of CICNT without any of its chemistry.
Finally, although the 48 hours in a CDC biofilm reactor is a robust protocol optimized for evaluating biofilm formation, there are inherent limitations to all in vitro studies, and the next obvious step is to transition to an in vivo study in order to validate the benefits of CICNT surface coatings.

**Biocompatibility**

Arguably the biggest concern for biocompatibility of orthopedic implants is related to osseointegration. This is because the bone cells need to adhere to the implant surface before any bacteria can. As mentioned previously, initial testing by other researchers in our lab showed that osteoblast cells adhered well to CICNT, and future work should include more in vitro testing. For example, using a block design of experiments to optimize the range of CICNT diameters and density where the results are quantified by osteoblast cell count (live/dead cell staining) and cell functionality (Alizaren red staining and collagen type I staining). Ideally, these tests would only involve coating CICNT on substrate materials relevant to orthopedic implants, such as the direct coating on SS (CICNT-SS) and/or layering alumina and Fe to coat SS or titanium. Due to the low density of randomly-oriented forests (CICNT-SS), efforts may need to be focused on growing vertically-aligned forests directly on SS sheets, bars, or fixation pins.

While biocompatibility testing should include mechanical testing for substrate adhesion, strength, wear, and cyclic loading, there is also great deal of chemistry characterization and cytotoxicity that needs to be better understood. Amorphous carbon is known to take many forms (chapter 2), and properties vary widely within those classifications. In order to further investigate where CICNT fits in our knowledge of carbon, the following measurements are recommended:

- C NMR spectroscopy for the sp3/sp2 bond ratio [43]
- Polarized light microscopy to classify the microstructure as isotropic, lamellar, conical, etc. [43]
- Transmission electron microscopy for electron diffraction to verify the amorphous structure of the pyrolytic carbon [40, 148]
- Raman analysis for the Ig/Id ratio to measure the level of amorphous carbon in CICNT [40, 41, 148]
6.3 CICNT Applications (Chapter 4)

6.3.1 Key Conclusions

Because CICNT coatings have shown non-pharmaceutical biofilm resistance, knowledge of its mechanical and biofilm properties on specific applications is required. Methods for testing these properties on CICNT-coated scalpel blades and external fixator pins are described. To the author’s knowledge, the present work represents the first reporting of CICNT grown directly on a 400-series stainless steel (scalpel blades). This coating process reduces the hardness of the blade, as evidenced by the reduced cutting performance, but the hardness is recoverable through a heat treatment. The durability of the CICNT coating grown directly from stainless steel scalpel blades is excellent as it does not delaminate under the force of a diamond scribe. Although this CICNT-coating on scalpel blades does not exhibit MRSA biofilm resistance, both the vertically-aligned and randomly-oriented CICNT-coated fixator pins do, so the variables critical for a CICNT nanostructure to resist biofilm are better understood.

6.3.2 Future Work

Our results demonstrated that an external fixator pin application is a viable application of CICNT surface coating technology that could have a high impact. Recommended future work to validate this application includes the extensive biofilm and bone adhesion testing detailed in section 6.2.2 in order to further understand the exact nanostructure requirements for CICNT biofilm resistance. Additionally, we also need to know that CICNT will promote skin-implant integration because pin tract infection is regularly attributed to a lack of this integration [117, 149]. We anticipated that continuation of this work will lead to an in vivo animal model, such as rats or rabbits [150], to measure how well CICNT-coated external fixator pins resist infection and adhere to bone and skin tissue.

Mechanical testing of the CICNT coating on external fixator pins must also be completed. This would include the hardness and scratch tests described in the present work (chapter 4) as well as a conventional decohesion test performed on coatings to quantify the surface adhesion [151].
The disturbance of the CICNT coating caused by the diamond scribe during the scratch durability testing likely removed some CICNT from the substrate. Although contiguous CICNT surfaces have demonstrated biocompatibility, there has not been work to date validating the biological reaction to isolated CICNT fragments. Bare CNTs have a diameter comparable to the microtubules of eukaryotic cells and have exhibited evidence of cytotoxicity [118, 119]. In contrast, CICNTs have a diameter that is at least one order of magnitude higher and are not anticipated to exhibit the same response. However, further work is needed to validate this hypothesis.

Lastly, the bacterial resistance of CICNT on scalpel blades may also be tested using other methods. One option would be to grow biofilms on a material like collagen, slice the material with both CICNT-coated and uncoated scalpel blades, and then image the blades to see how much bacteria is on the surfaces. A more application-relevant option would add a step to test how much bacteria on the blades would transfer to the surrounding tissues.

6.4 CICNT on Concave Substrates (Chapter 5)

6.4.1 Key Conclusions

As CICNT forests are grown on non-planar substrates, circumferential stresses cause morphological defects. On concave substrates with small radii of curvature and tall CICNT forests, microscale defects include increased roughness, extra growths, corrugated forests, curved forests, and forests with an inside crevice. Additionally, CICNT density on the top surface increases slightly as the forest height increases and substrate curvature tightens, but this may or may not be considered a defect depending on the application. The strength and modulus of elasticity of forests with corrugated, curved, and inside crevice defects is theoretically reduced, but these defects do not occur when the forest is relatively short (63 ± 14 µm tall), even on the smallest curvatures (1 mm ID). The successful growths as well as the curvature-induced defects may apply to un-infiltrated CNT forests because the pyrolytic carbon infiltration in CICNT was added after the forest was grown.
6.4.2 Future Work

Because the processes used to grow CICNT were designed for planar substrates, modifications were required to use these processes for concave substrates. As a result, CICNT were not coated inside a full tube and only the bottom 45-60° of curvature could be evaluated. Future work may include developing processes for growing CICNT on the inside of full tubing to verify that the defects are consistent with what is observed in the present work and to prepare the coating for its potential applications. This may include a direct CICNT coating inside a stainless steel tube. Ideally, the CICNT would be grown as a vertically-aligned forest but studying a randomly-oriented forest inside a tube may also prove useful and interesting.

Increasing the circumferential stress on a CNT forest caused microscale defects, but it is unknown how much these defects affect the strength and other mechanical properties of a CICNT forest. Future work on CICNT with defects induced by concave substrates can involve measuring the stress-strain curves in tension and compression in multiple directions. These curves will also contribute stiffness measurements, and all these measurements can be compared to the values reported previously for CICNT [35].

There are many potential applications for CNT forests on concave substrates, so it would also be worthwhile for future work to add many more growth times to create a linear regression or other relationship model of CNT height, substrate curvature, and forest defects. This would inform future designers of the maximum CNT coating height for their application.

Lastly, the scope of this work was limited to the microscale defects found in CNT forests. More qualitative and quantitative work can be done to assess the nanoscale and atomic defects that may be induced by substrate curvature.

6.5 Overall Impact

With antibiotic resistance looming as one of the greatest threats to global health [152], alternative solutions are needed. CICNT may not be able to replace all forms of antibiotics, but can they replace the broad-spectrum antibiotics required for treating surgical site infections? That question cannot be answered yet, but the present work contributes to the knowledge necessary to allow CICNT to have that impact and more. Understanding the potential benefits of CICNT as
well as the various forms that it can take are foundational. Ultimately, the hope of this research is that it will be used as a stepping-stone to eventually eliminate the excessive suffering of those who may have otherwise contracted a periprosthetic joint infection, thrombosis, or any other ailment that CICNT may help avoid.
REFERENCES


APPENDIX A. ADDITIONAL OBSERVATIONS

Throughout the time spent running experiments for the research chapters of this work, additional observations were made without sufficient data for a publication. Because these are relevant to this work and to future researchers, they are reported in this chapter.

A.1 MRSA and Pseudomonas Biofilm Testing

By nature of experimental research, unforeseen complications are prone to emerge – especially in the beginning – resulting in inconclusive data. This was the case for the first set of MRSA and *Pseudomonas aeruginosa* biofilm reactor studies. These two bacterial strains can demonstrate the breadth of a treatment because MRSA is gram-positive and cocci-shaped (spherical) while *P. aeruginosa* is gram-negative and rod-shaped. After following the protocol for 48-hour biofilm growth outlined in chapter 3 using *P. aeruginosa* instead of MRSA, the samples were prepared for SEM imaging using a carbon coating instead of the gold coating that was later implemented. The carbon coating was thick and did not adhere well to many of the sample materials, so the results on these samples are unreliable (Figure A.1a and b). Figure A.1a shows the carbon coating peeling off and uncovering a biofilm, and Figure A.1b shows the carbon coating removing a biofilm as it peels off.

This carbon coating peeling was not prevalent on the SS control and CICNT-Si sample images, so the features on these images were generally identifiable and able to be analyzed. There were 3 specimens of SS control and 2 specimens of CICNT-Si. Using 25 SEM images from each specimen, the number of biofilm colonies larger than 50 µm were counted. The SS controls had 26, 15, and 25 colonies while the CICNT-Si had 6 and 12 colonies. These data are certainly limited by the small sample size, but it is also important to point out that the morphologies of the biofilms on these two materials was different. It was observed that colonies on the SS control samples were
Figure A.1: SEM images with arrows pointing to *Pseudomonas aeruginosa* biofilm. (a) Biofilm hidden under thick carbon coating on a carbon control sample. (b) Peeled carbon removing biofilm from a titanium control sample. (c) Biofilm on a SS control sample that protrudes from the surface. (d) Biofilm on a CICNT-Si sample that is roughly the same area as that on the SS control, but it is less bulbous. Scale bars are 50 µm.

generally more bulbous and taller than the biofilms on CICNT-Si samples even when their footprints are roughly the same size (Figure A.1c and d). Naturally, more extensive testing needs to be done, but these limited results are promising because there was less biofilm on CICNT-Si than the SS control which indicates some resistance to biofilm attachment and growth. These results with *P. aeruginosa* biofilm combined with the more extensive MRSA biofilm resistance results reported in chapter 3 suggest that CICNT may be a broad spectrum type of treatment.
A.2 Infiltrating CNT on Concave Substrates with CVD

Analyzing the defects in a CNT forest induced by a concave substrate (chapter 5) did not require an analysis of the infiltration as that was utilized for experimental convenience; however, a couple observations may be relevant to future work. These observations pertain to the variations seen while holding the infiltration time at a constant 16 minutes.

Although infiltration time is directly related to CICNT diameter size, there are other factors that need to be considered. The top surfaces of four concave substrate samples are shown in Figure A.2. Differences in CICNT diameters can be seen between the front and back of each sample (p=0.002) as well as between tall and short CICNT heights (p=0.01). There was no difference between narrow (1-2 mm ID) and wide (3-4 mm ID) curvatures. The fronts of each sample are more infiltrated that the backs, and the short CICNT heights are more infiltrated than the tall CICNT heights. This indicates either a reaction-limited or diffusion-limited process which can be resolved by changing how the CVD furnace system operates.

Figure A.2: SEM images of the top surface of CICNT grown on concave substrates showing the difference between the infiltration level of the front and back of the sample. Scale bars are 2 µm.
A.3 Growing CNT Directly on 316L Stainless Steel

Growing a CNT forest directly on 316L SS almost always results in a randomly-oriented CNT forest rather than a vertically-aligned CNT forest like those seen on substrates with an external catalyst layer. Under certain circumstances, vertical-alignment from a direct-growth method has been achieved on mesh and the inside of tubing [54, 56], but it has not been reported on sheets or other planar surfaces. This section describes our attempts to do this.

A.3.1 Heat Treatment Attempts

In a fabrication process similar to CICNT-SS, a vertically-aligned CNT forest was seen on a heat-treated 316L SS mesh [54]. More specifically, it was treated at 800 °C in air for 10 minutes which produced 100 nm oxide particles – also called nano-hills [52] – on the surface, and these particles are presumably where the CNT initiate their growth. We hypothesized that their size is critical for vertical alignment because the CNT forest must be dense enough to restrict the CNT to grow vertically.

Mirror-finish 316L SS sheet metal sheared to 1 cm by 1 cm chips were heat-treated in air at 800 °C for various time lengths with a room-temperature air quench. The time lengths attempted were 1.5, 2, 3, 5, 10, 20, and 30 minutes, and a handful of photographs and SEM images are shown in Figure A.3. The various color changes are indicative of the oxide layer thickening. The SEM images showed that, like the study on SS mesh, the oxide particles are non-uniform when the heat treatment is too short (1.5 minutes) and too large when the heat treatment is too long (10 minutes). The 2-minute heat treatment length produced uniform oxide particles that were the target size (roughly 100 nm).

Although the 2-minute heat treatment length produced the right size features, the CNT forests grown on these chips were still randomly-oriented rather than vertically-aligned. The forests were placed into the furnace at room temperature, heated to the CNT growth temperature flowing argon gas, grown flowing ethylene and a carrier gas for 25 minutes, and then cooled flowing argon gas. Two different carrier gases were used: hydrogen and argon. Two different CNT-growth temperatures were used: 750 °C and 800 °C. Argon carrier gas growing at 800 °C produced the most uniform (i.e., not bush-like) and dense CNT forests; however, they were not
vertically-aligned (Figure A.4). This may be due to the samples being heated to the CNT growth temperature from room temperature. The study on SS meshes was able to grow CNT directly following the air heat treatment, but this was not able to be safely done using our setup.

Figure A.3: Photos of SS chips after various air heat treatment times (top). SEM images of various SS chips (bottom). Scale bar is 1 µm.

Figure A.4: SEM images of CNT forests grown on 316L SS chips with various heat treatment times: 2, 5, 10, and 30 minutes.
A.3.2 Other Attempts

In addition to the air heat treatments with a room-temperature air quench described in the previous section, modifications to this heat treatment were attempted; however, none produced a vertically-aligned CNT forest. In the pursuit of making the oxide particles smaller, a water quench was used rather than room-temperature air. This was unsuccessful because the particles were the same size as those on the air-quenched chips. Another heat treatment modification was to heat the samples in argon rather than air, but this produced very large particles. The problem with using argon was that the tube could not be opened at 800 °C without letting air in, so although the chips were slid in and out of the heating zone for the actual heat treatment, they were in the furnace throughout the heating and cooling of the furnace. Ultimately, the particle size could not be reduced because they were at high temperatures for much longer than the air heat treatments.

Other experimentation done to produce smaller oxide particles included growing CNT on chips placed upside down in the furnace, cold-rolling chips, and using wires rather than chips. The idea of growing CNT on chips flipped upside down was to see if gravity played a role, and it did not produce vertical alignment. The idea of cold-rolling the chips before the air heat treatment was to add more dislocations, but the oxide particles were not uniform size and still too large. The idea of trying the process on a wire was to have a closer replication of the 316L SS mesh material, but the wires had the same problems as the cold-rolled chips.

Efforts to achieve the nano-hill structure were also made without heat treatments. One experiment was to roughen the surface with sandpaper and/or diamond paste in perpendicular directions. Even with Grade 1 diamond paste (14,000 grit equivalent), the feature size was still much larger than the ideal oxide particle size. Another experiment was to etch the SS with hydrochloric acid (HCl) because this method had previously been used in the lab to grow randomly-oriented CNT forests directly on SS. Chips were submerged in HCl for 5, 10, 15, 20, 25 minutes. There was some nanoscale pitting on the surface, but none of these etching times produced the desired morphology.
B.1 Taking SEM Measurements

%--------------------------------------------------------------------------
%Purpose:
%This code measures 2 features in an image with a scale bar in microns.
%Outputs are a=x-measurement, b=y-measurement, and c=hypotenuse of a and b.

%Written by: Stephanie Morco May 2018
%Updated 4/30/20

clear
clc
close all

%get the scale bar then the length from the image
filename = uigetfile('*.jpg');
image = imread(filename);
imshow(image);
[x,y]=ginput(6);

%scale bar conversion factor from pixels to um
scale = input('What is the scale bar length in um? ');
scale_length=(x(2)-x(1))/scale; %units of pixels/um

%object or distance #1 measurement
a1=x(4)-x(3);
b1=y(4)-y(3);
c1=sqrt(a1^2+b1^2);

%object or distance #2 measurement
a2=x(6)-x(5);
b2=y(6)-y(5);
c2=sqrt(a2^2+b2^2);

%change the units from pixels to microns
a1=a1/scale_length;
b1=b1/scale_length;
c1=c1/scale_length;
a2=a2/scale_length;
b2=b2/scale_length;
c2=c2/scale_length;
B.2 Red, Green, and Blue Cell Marks Counting

%--------------------------------------------------------------------------
% Purpose:
% This code quantifies and categorizes bacteria using a grayscale image
% with bacteria cells marked in red, green, or blue.
% Written by: Stephanie Morco May 2018
% Updated 6/22/18
clc
clear
close all

%---------------------------------------------------Read in Image------------------------
filename = uigetfile(".*");
image = imread(filename);
figure(1)
imshow(image)

%-------------------Threshold cells marked in color----------------------
imageR = squeeze(image(:,:,1));
imageG = squeeze(image(:,:,2));
imageB = squeeze(image(:,:,3));

%------------------Isolate each color mark---------------------------
red2bw=(imageR-imageG);
green2bw=(imageG-imageB);
blue2bw=(imageB-imageR);

%-------------------Clean up so marks aren't overlapping-------------------

%-------------------Red marks-----------------------------------
size_img=size(red2bw);
redMarks=zeros(size_img(1),size_img(2));
for i=1:size_img(1)
    for j=1:size_img(2)
        if red2bw(i,j)>=100
            redMarks(i,j)=255;
        else
            redMarks(i,j)=0;
        end
    end
end

%-------------------Green marks-----------------------------------
size_img=size(green2bw);
greenMarks=zeros(size_img(1),size_img(2));
for i=1:size_img(1)
    for j=1:size_img(2)
        if green2bw(i,j)>=100
            greenMarks(i,j)=255;
        else
            greenMarks(i,j)=0;
        end
    end
end
%-----------------------------------Blue marks-----------------------------------
size_img=size(blue2bw);
blueMarks=zeros(size_img(1),size_img(2));
for i=1:size_img(1)
    for j=1:size_img(2)
        if blue2bw(i,j)>=100
            blueMarks(i,j)=255;
        else
            blueMarks(i,j)=0;
        end
    end
end

%-----------------------------------Label and count marks-----------------------------------
[labels_r, numLabels_r] = bwlabel(redMarks);
[labels_g, numLabels_g] = bwlabel(greenMarks);
[labels_b, numLabels_b] = bwlabel(blueMarks);

disp(['Number of red cells: ' num2str(numLabels_r)]);
disp(['Number of green cells: ' num2str(numLabels_g)]);
disp(['Number of blue cells: ' num2str(numLabels_b)]);

B.3  CICNT Density

%---------------------------------------------

%Purpose:
%This code measures the density of CICNT SEM images by using edge detection models then counts the number of edges in all rows (total circumference), and then divides that by a modified pi for pixelated circles and diameter.

%Results for concave paper recorded in "Top Surface Comparison" PPT and "Finding pi for pixelated circles" XLS

%Instructions:
%Check the SEM image width size for pixel to um conversion.
%  9.8 um is the image width (SPEG at 25,000x)
%  6.97 um is the image width of 35,000x SPEG images
%  10.22 um is the image width of 25,000x verios images

%Written by: Stephanie Morco June 2020
%Updated 2/4/21

clear
cleanclose all

%input an image
filename = uigetfile('*.*');
I = imread(filename);
I = rgb2gray(I);

% Edge detection
BW_edges = edge(I,'approxcanny');
figure;
imshow(BW_edges)

% Horizontal lines will be taken from the image to count the edges
i=1;
j=1;
img_size = size(I);
img_height = img_size(1);
% Remove 7.5 percent because of the scale bar at the bottom of the image
img_height = img_height*(1-.075);

while i<=img_height
    edge_count(j)=sum(BW_edges(i,:));
j=j+1;
i=i+1;
end

% Total number of CICNT in the image is calculated (Note: the scale bar was
% removed by subtracting the bottom 7.5% of the image).
diameter = input('What is the CICNT diameter (pixels) ? '); %pixels/diameter
  %{small=54, medium=114, and large=299, 14spot=69,
  %105spot=90, 70spot=36, 36spot=45, 26spot=49, xsmall_vert=18,
  %CONCAVE_SHORT=32.10, FLAT=16.11, CONCAVE_TALL=27.93, FLAT2=14.33}
pi_pixl = 2.8; %the ratio of circumference to diameter is 2.8 for a
  %pixelated circle from our experiments and from data in
  %2017 Gharajeh paper on pixelated ellipses
CICNTcount = sum(edge_count)/pi_pixl/diameter

% Unit conversion from count to CICNT/um^2.
img_width = 9.8; %width of SEM image in um
pixl_size = img_width/img_size(2); %um/pixel
img_area = (i-1) * img_size(2) * pixl_size^2; % #rows*#columns*(um/pixel)^2
CICNT_density = CICNTcount/img_area %CICNT/um^2

B.4 Concave Substrate Roughness

%---------------------------------------------------------------------
% Purpose:
% This program is used to evaluate the roughness of the CI-CNT surface
% from an SEM image of the cross-section.
%
% Overview:
% A quadratic curve is fit to the top edge of the CI-CNT surface then
% it is subtracted from the actual top edge of the CI-CNT forest. The
% roughness is calculated by dividing the area outside the curve by
% the curve length.
%
% Inputs:
%  1. A grayscale SEM image of the cross-section called 'grayscale.jpg'
%  2. A black and white image of 'grascacle.jpg' where the area above
%     the top edge of the CI-CNT forest is white and the area below
%     is black called 'top_edge.jpg'. An
%     easy way to do this is to use
%     the "Quick Selection Tool" and paint the selection in Photoshop.
%
% Output:
% The variable "deviation" is the roughness in units of um^2/um.
% Variables:
% img_gray - grayscale image matrix
% size_img - size of the grayscale image matrix
% x_input - x-coordinates picked from the image for the scale bar
% y_input - y-coordinates picked from the image for the scale bar
% scale - SEM scale bar length
% top_edge - bw image matrix of the top edge
% e - image matrix where the top edge is the only thing turned "on"
% x_on - x coordinates for the image edge by which points are "on"
% y_on - y coordinates for the image edge by which points are "on"
% f - top curve fit
% f_eval - curve fit evaluated at
% i, j, & k - counter variables
% ideal - black and white image of the ideal top surface curvature
% d - difference image between the ideal and actual bw images
% area - number of pixels outside the ideal curve
% length_curve - length of the curve
% dx, dy, & l_segment - variables for calculating the curve length
% scale_length - conversion factor - units of pixels/um
% deviation - pixel area/pixel length in units of um^2/um

% Author: Stephanie Morco
% Created: 7/2015
% Updated: 7/2020
%---------------------------------------------------------------------
clear
clc
close all

%convert the SEM image to an array
img_gray=imread('grayscale.jpg');
size_img=size(img_gray);

%choose points from image
%order must be left edge of scale bar, right edge of scale bar
imshow(img_gray)
[x_input,y_input]=ginput(2);

%get the scale bar length
scale = input('What is the scale bar length (um)? ');

%define the top curve by edge detection
top_edge=imread('top_edge.jpg');
e=edge(top_edge,'Canny');

%create x and y coord. for the image edge by which points are "on"
k=1;
for i=1:size_img(2)
  for j=1:size_img(1)
    if e(j,i)>=1
      x_on(k,1)=i;
      y_on(k,1)=j;
      k=k+1;
    end
  end
end

% quadratic curve fit with these points (top)
f=polyfit(x_on,y_on,2);
f_eval=polyval(f,[1:size_img(2)]);

% show curve at the top of the forest
imshow(img_gray);
hold on
plot(f_eval)
hold off

% make an image for the ideal curvature (f)
% fill an array with zeros
for i=1:size_img(1)
    for j=1:size_img(2)
        ideal(i,j)=0;
    end
end

% fill in area above the ideal curvature with an arbitrary number: 100
for i=1:size_img(2)
    for j=1:f_eval(i)
        ideal(j,i)=100;
    end
end

% make sure the top_edge image is "black" (100) and white (255)
for i=1:size_img(2)
    for j=1:size_img(1)
        if top_edge(j,i)>100
            top_edge(j,i)=255;
        else
            top_edge(j,i)=100;
        end
    end
end

% get area of bumps by subtracting the top_edge from the ideal curvature
% 255-white
% 155-light gray
% 100-dark gray
% 0-black
for i=1:size_img(1)
    for j=1:size_img(2)
        d(i,j)=top_edge(i,j)-ideal(i,j);
    end
end

% show the subtraction
figure(2)
imshow(d)

% calculate the area that doesn't follow the curve (i.e., the white(255)
% and black(0) pixels
area=0;
for i=1:size_img(1)
    for j=1:size_img(2)
        if d(i,j)>255
            area=area+1;
        end
    end
end
elseif d(i,j) <= 0
    area = area + 1;
end
end
end

% calculate the length of the curve by square root of the sum of
% squares of each coordinate along the length of the curve
length_curve = 0;
k = size_img(2);
for i = 1:k-1
    dx = (i+1) - i;
    dy = f_eval(i+1) - f_eval(i);
    l_segment = sqrt(dx^2 + dy^2);
    length_curve = length_curve + l_segment;
end

% converting to microns
% calculate the scale bar length in pixels
scale_length = (x_input(2) - x_input(1)) / scale; % units of pixels/um
% change the units from pixels to microns
area = area / scale_length / scale_length; % units of um^2
length_curve = length_curve / scale_length; % units of um

deviation = area / length_curve % units of um^2/um