



SARA HAWI

FABRICATION AND FUNCTIONAL EVALUATION OF NATURE-
INSPIRED ANTI-BACTERIAL SURFACES

SCHOOL OF AEROSPACE, TRANSPORT AND MANUFACTURING

PhD

Academic Year: 2019 - 2023

Supervisor: Prof Saurav Goel

Associate Supervisor(s): Dr Claudiu Giusca and Dr Vinod Kumar

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ABSTRACT

The critical need to develop novel and efficient anti-bacterial strategies, particularly for biomedical implants, serves as a significant motivation for this research. The rise in antibiotic resistance continues to pose a threat to healthcare, making it increasingly important to explore alternative approaches to combat implant-associated surgical site infections (SSIs). These infections arise from bacterial attachment and biofilm formation on the surface of implants and medical devices, leading to costly treatments and high recurrence rates. The present research was aimed at investigating nature-inspired anti-bacterial surfaces through a rigorous fabrication and testing campaign.

The study investigated various nanofabrication techniques, such as femtosecond laser ablation, deep reactive ion etching, focused ion beam lithography and scanning probe lithography, for creating nature-inspired sub-micron features on stainless steel and silicon surfaces. The biological response of bacteria (*S. aureus*) and osteoblast-like cells (MG-63) was evaluated on these surfaces to test antibacterial as well as osseointegration response of the surfaces. *S. aureus* was chosen due to its high relevance to SSIs and its prevalence in infection and MG-63 cells served as a model for examining the osteoblast behaviour in laboratory studies.

The thesis established a novel scale-dependent relationship between surface topography and biological functionality, characterised by the dominating surface wavelength and fractal dimension. The anti-biofouling mechanism was influenced by surface topography, characterised through the fractal dimension, and it was consistently achieved and deemed more suitable for future applications due to the high anti-bacterial efficiency achieved compared to the mechano-bactericidal mechanism. High aspect ratio features (0.056-0.280 μm wavelength, 0.295-0.765 μm height, 0.045-0.046 μm diameter) did not induce mechano-bactericidal effects on *S. aureus* NCTC7791, indicating further research is needed. Moreover, the thesis demonstrated a predominant attachment of *S. aureus* and MG-63 cells on the crystalline silicon surfaces on the (111) orientation.

For feature sizes below 1 μm , the fractal dimension positively correlated with the anti-bacterial effect and MG-63 cell spreading. For sizes significantly larger than bacterial size ($> 2 \mu\text{m}$), no correlation was found with the anti-bacterial effect, but surface complexity positively correlated with MG-63 cell spreading. For feature sizes comparable to MG-63 cell size (10-40 μm), cell spreading was inhibited.

Femtosecond laser ablation emerged as a promising technique for commercial applications, while scanning probe lithography proved to be a cost-effective, flexible tool for prototyping and research-scale investigations.

In conclusion, the development and evaluation of nature-inspired anti-bacterial surfaces have revealed valuable insights into the scale-dependent relationship between surface topography and biological functionality. The findings from this research have the potential to improve the performance and safety of implantable medical devices by reducing the risk of implant-associated SSIs, ultimately benefiting patients and healthcare providers.

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LIST OF ABBREVIATIONS

ABR	Antibiotic Resistance
AFM	Atomic Force Microscope
Ag NPs	Silver Nanoparticles
bSi	Black Silicon
CCP	Capacitively Coupled Plasma
CFU	Colony Forming Units
CLSM	Confocal Laser Scanning Microscope
CPS	Capsular Polysaccharides
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic Acid
DRIE	Deep Reactive Ion Etching
EBL	Electron Beam Lithography
EDS	Energy Dispersive Spectroscopy
EPS	Extracellular Polymeric Substance
FEA	Finite Element Analysis
FE-SEM	Field Emission Scanning Electron Microscope
FIB	Focused Ion Beam
HSS	Hierarchically Structured Surface
hASC	Human Adipose Stem Cells
ICP	Inductively Coupled Plasma
LCP	Lock Compression Plate
LIPSS	Laser Induced Periodic Structured Surfaces

MEMS	Micro-Electromechanical Systems
NCTC	National Collection of Type Cultures
NEMS	Nanoelectromechanical System
OD	Optical Density
PBS	Phosphate Buffer Saline
PI	Propidium Iodide
PIII	Plasma Immersion Ion Implantation
RF	Radio Frequency
RIE	Reactive Ion Etching
ROS	Reactive Oxidative Stress
SEM	Scanning Electron Microscope
SPL	Scanning Probe Lithography
TSB	Tryptic Soya Broth
Triton-X-100	4-(1,1,3,3-Tetramethylbutyl)phenyl-polyethylene glycol
UH	Ultra-High
UV	Ultraviolet
WCA	Water Contact Angle
XDLVO	Extended Derjaguin, Landau, Verwey, Overbeek
XPS	X-Ray Photoelectron Spectroscopy
XRD	X-Ray Diffraction

1 Introduction

1.1 Motivation for Research

The outstanding ability of natural surfaces to perform complex functions with high efficiency has attracted the attention of scientists worldwide. Nature inspired surfaces, which mimic the unique structures and properties of their natural counterparts, have the potential to enable various fields, from healthcare and biotechnology to material science and engineering to come together and drive multifarious benefits through the adoption of Engineering Biology as a new theme being promoted by the Research Councils of the UK. Nature has been perfecting its designs and functionalities over millions of years through the process of evolution. Examples such as the self-cleaning and anti-biofouling properties of lotus leaves, the adhesive capabilities of gecko feet, and the remarkable anti-biofouling abilities of shark skin, among others, have inspired scientists to develop innovative materials and surfaces that replicate these properties, with some solutions being developed into nature-inspired products that have penetrated the niche market [1]. Examples of these nature-inspired products are shown in Figure 1-1.

The study of natural surfaces and consequent investigation of nature-inspired surfaces not only allows us to harness the inherent advantages of these complex biological systems but also fosters a deeper understanding of the underlying principles governing their performance.

One of the most significant motivations for this research is the critical need to develop novel and efficient anti-bacterial strategies, especially in the context of metallic biomedical implants. This need has become even more pressing in recent years as the rise in antibiotic resistance continues to pose a threat to healthcare [2,3]. Nature inspired surfaces, such as those mimicking the hierarchical topography of the lotus leaf (microfeatures with superimposed nanofeatures) and the nanostructured topography of the butterfly wings

(hundreds of nanometre ridges linked together with smaller transverse nanoripples) have demonstrated self-cleaning capabilities.



Figure 1-1 Commercially available nature-inspired products. The green circles represent functional mimetics, the yellow circles represent feature mimetics and the cyan represents world remarkable architecture inspired by nature. Reprinted under CC BY 4.0 License from [1].

The self-cleaning capabilities of such surfaces, which rely on the ease of foulant removal, may also contribute to an anti-bacterial functionality through a similar anti-adhesion or anti-biofouling mechanism. Additionally, dragonfly wings' nanotopography (high aspect ratio nanofeatures) has shown promising anti-bacterial properties, more specifically mechano-bactericidal properties. These topographical surface modifications inspired by nature hold immense promise as

next generation anti-bacterial surface modification strategies, since they do not promote the development of anti-bacterial resistance in any known pathways to date [4,5]. By studying these surfaces and their interactions with bacteria, the development of innovative anti-bacterial solutions can be accelerated, potentially leading to more effective and sustainable implant technologies.

Applying nature-inspired anti-bacterial surfaces strategies to biomaterial surfaces (e.g., biomedical implants, instruments, surfaces and equipment) comes with manufacturing difficulties, especially when considering the length scales of fabrication required being micro/nanoscales and the difficulty of machining materials of interest (e.g., metals) at such scales. The fabrication challenges are exacerbated by difficulties associated with characterising the topography of these complex surfaces and further testing their biological functionality (interaction of surface with bacteria and human cells). These challenges arise due to the diverse protocols and methodologies employed by researchers, making direct comparison between studies a complex task. Consequently, there is a pressing need for research that addresses these challenges and advances the understanding of the effective fabrication, characterisation and testing of nature-inspired surfaces for anti-bacterial implant applications.

1.2 Thesis Aim and Objectives

The primary aim of this research work was to design, fabricate and test nature-inspired anti-bacterial surfaces using a range of nano processing techniques, and to characterise their topographic and biological performances to draw inferences about nature inspired surfaces for engineering and societal applications in products such as medical implants, medical devices, high traffic areas including airports, train stations, hospitals to eradicate pathogens in a sustainable manner.

The specific objectives of the research were:

- 1) To survey the landscape of various ultra-precision nanomanufacturing technologies capable of producing nature-inspired anti-bacterial surfaces with robust and reliable accuracy, repeatability and performance.

- 2) To understand the biological response of bacteria and bone-like cells to nature-inspired surfaces, offering an understanding of the cellular interactions with the topographies of anti-bacterial surfaces.
- 3) To investigate the relationship between physical dimension and biological (bacteria and bone-cell related) functionality of the fabricated surfaces, providing insights into how scale-dependant surface topography influences the performance.
- 4) To evaluate the scalability potential of the developed approaches for uptake of the research results for societal applications.

1.3 Thesis Structure and Overview

This thesis is organised into eight chapters one of which is this introduction chapter itself.

Chapter 1 sets the context for the research by presenting the motivation, the aims and objectives of the thesis. It also provides an overview of the structure of the thesis, highlighting the logical flow of the chapters.

Chapter 2 serves as the foundation for the thesis by reviewing the relevant literature on bacterial attachment to surfaces, and anti-bacterial modification approaches for surfaces with a focus on surface topography modifications on the micro and nanoscale, inspired from natural anti-bacterial surfaces. To understand the wider anti-bacterial surface modification landscape, a brief review was presented concerning chemical anti-bacterial surface modifications. The chapter then presents various techniques used to evaluate the anti-bacterial functionality of surfaces. It then highlights manufacturing methods for engineering nano and micro-scale surface features and techniques for surface topography/texture measurements which ultimately aid in the investigations of the relationship between the geometric characteristics of surfaces and their biological functionality. The knowledge gaps identified in this chapter inform the research questions addressed in subsequent chapters.

Chapter 3 outlines the research methodology. Specifically, it drafts the materials and methods used, the fabrication techniques employed, the surface characterisation techniques, the biological evaluation techniques and the

statistical analysis carried out keeping in mind the extant literature. The methodology was designed to address key research questions and objectives identified earlier.

Chapter 4 begins the exploration of nature-inspired sub-micron and hierarchical nano/micro scale features. This investigation draws from the literature review about the structured anti-bacterial natural surfaces and emphasises ultrafast laser ablation as a fabrication technique. The chapter presents results and discussions on how the fabricated features affect bacterial coverage and osteoblast attachment, shedding light on the approach's effectiveness. Moreover, it tackles the challenge of correlating surface parameters with the biological functionality of structured surfaces and examines the relevance of surface parameters across varying scales.

Chapter 5 extends the investigation of nature-inspired surface modification techniques by focusing on the fabrication and characterisation of nanoscale silicon structures using deep reactive ion etching. The chapter assesses the effects of these structures on bacterial viability, attachment, and osteoblast cell behavior, further contributing to the understanding of anti-bacterial surface modifications inspired from nature.

Chapter 6 delves into alternative nanofabrication techniques, specifically scanning probe lithography and focused ion beam lithography, which were tailored for focused research applications. These techniques were employed to create nature-inspired nano-scale features on stainless steel, offering a diverse range of surface modification approaches. The analysis and discussion presented in this chapter shed light on the impact of these techniques on bacterial morphology and adhesion, thereby expanding the breadth of understanding and potential applications of surface modification strategies. This comprehensive investigation demonstrates the significance of these alternative techniques in the context of bacterial adhesion.

Chapter 7 briefly examines the use of silver ion implantation as a chemical anti-bacterial surface modification technique, an alternative to anti-bacterial

topographical surface modification. The purpose of this concise chapter is to provide a comparative perspective, as the main focus of the thesis lies in topography modification. The results and discussion of this chapter demonstrate the potential of this modification strategy on a commercially relevant scale, enabling a comparative analysis between the different modification approaches in the general discussion and conclusions chapter of this thesis. This chapter acknowledges the use of a commercial technique (as silver implanted implants are popular in market) to help identify the potential of physical anti-bacterial mechanisms over the existing chemical anti-bacterial mechanisms.

Chapter 8 is the final chapter of the thesis. It synthesises the findings from various fabrication techniques and surface modifications investigated throughout the thesis. It provides an overall discussion, conclusions drawn from the research, and recommendations for future work, demonstrating the significance of the research in the context of anti-bacterial surface modifications.

2 Literature Review

2.1 Introduction

Nature has long acted as a rich source of inspiration for humanity, thanks to its countless well-organised, engineered, and skilfully created processes, algorithms, materials and designs. The study of biomimicry which involves taking inspiration from nature's designs has been around since the 1950s and was popularised in the 1990s by scientist Janine Benyus who is considered a pioneer in the field [6]. As highlighted in previous work [1], nature inspired designs are split into surface design and structural design. Surface design mainly comprises of surface-bound modifications which lead to surface-bound effects such as change in wettability or light absorbance, while structural design comprises of a bulk-type modification which affect bulk material properties such as material toughness [1].

There are two approaches to nature-inspired design: the problem-driven approach and the solution-driven approach. These approaches vary in their initial stages, where inspiration and creativity significantly influence the design, but eventually lead to the same result. Once natural models are identified, the process becomes methodical. The problem driven approach is outlined below as based on eight main steps, developed by biomimicry design spiral [7], expanded by Fayemi et al.[8] for biomimetics and by Katiyar et al. [1] for nature-inspired design:

1. Problem analysis: Evaluate the situation when no problem has been identified or describe the previously identified issue.
2. Abstract technical problem: Recognise the context and limitations to establish the required function.
3. Transpose to nature: transform the required function into a question directed at nature and explore how nature accomplishes that objective. Proper question formulation is crucial since the outcomes are highly sensitive to it.

4. Identify potential natural models: Conduct a literature review to identify natural models, including biological ones. The knowledge gained at this stage on both technological and natural levels may require revisiting the first three steps, creating an iterative loop.
5. Selecting a natural model of interest: Select a natural model from the identified models.
6. Abstract natural strategy: Comprehend the functioning of the chosen natural models and separate them from the natural entity. Combining multiple natural strategies is essential for solving the initial problem through a transferable functional model, as direct transition from nature to technology is often impractical.
7. Transpose to technology: Express the natural solution in technical terms, with technological knowledge being crucial for implementation in the technical domain.
8. Implementation and testing: converting natural strategies to technology, followed by implementation and testing, will lead to a successful completion of the cycle and the introduction of a nature-inspired design. If the results are unsatisfactory, the process is repeated in either phase 1 (steps 1 to 3) or phase 2 (steps 4 to 8).

Nature-inspired design has proven to be a very successful approach for solving existing problems such as the inability of human body to regulate its temperature in extreme environments [9], creating a reusable, dry adhesive with a strong yet easily reversible bonding mechanism [10], ultrabroadband suppression of mid-infrared reflection losses [11], etc.

Antibiotics are crucial in the treatment of bacterial infections. However, the misuse of these drugs has empowered bacteria to develop tolerance and resistance by, most prevalently but not exclusively, secreting a shielding biofilm to prevent therapeutic access. Antibiotic resistance (ABR), a subset of antimicrobial resistance [12] is a threat to human health and human quality of life globally. As the development of novel antibiotics and antimicrobials has been diminishing, new approaches to combat ABR have become a necessity [13].

Moreover, treatment without prevention is insufficient, especially with regards to biomaterial-associated infections, as this can dictate the fate of implant surgeries by tipping the scale in favor of bacteria in the “race for the surface” against eukaryotic cells [14]. Clinical statistics suggests that the percentage of post-operative infections of orthopedic implants ranges between 0.7 % and 4.2 % [15–17] and can be as high as 30% for complex trauma cases [18,19] as the surfaces of these implants are at high risk of bacterial contamination. The ABR of pathogenic bacteria such as *Staphylococci* and *Streptococci*, among others, renders existing antibiotic treatments for post-operative infections futile, with resistance starting to emerge for last resort antibiotics such as vancomycin [20] (widely used in orthopedics), polymyxin B and colistin [21]. Protective biofilms can form within hours of implantation and therefore, agile preventative methods are required, which are being currently explored [22,23]. Such methods have been directed towards preventing bacterial contamination and biofilm formation by modifying the surfaces using various techniques which are termed as “anti-bacterial”. Contrary to the common practice in non-biology (especially engineering) related fields, the term anti-bacterial is not exchangeable with anti-microbial, but a subset of it. Herein, a classification is offered (Figure 2-1) to highlight anti-microbial surfaces by their specific functions. Based on this classification, anti-microbial surfaces can be categorised as follows:

- Self-cleaning surfaces that have the ability to remove microbes (i.e., bacteria, viruses, fungi) or foulants after adhesion,
- Anti-biofouling or anti-adhesion surfaces that repel microbes and prevent them from adhering to the surface and
- Biocidal surfaces that can kill or suppress the growth of microbes.

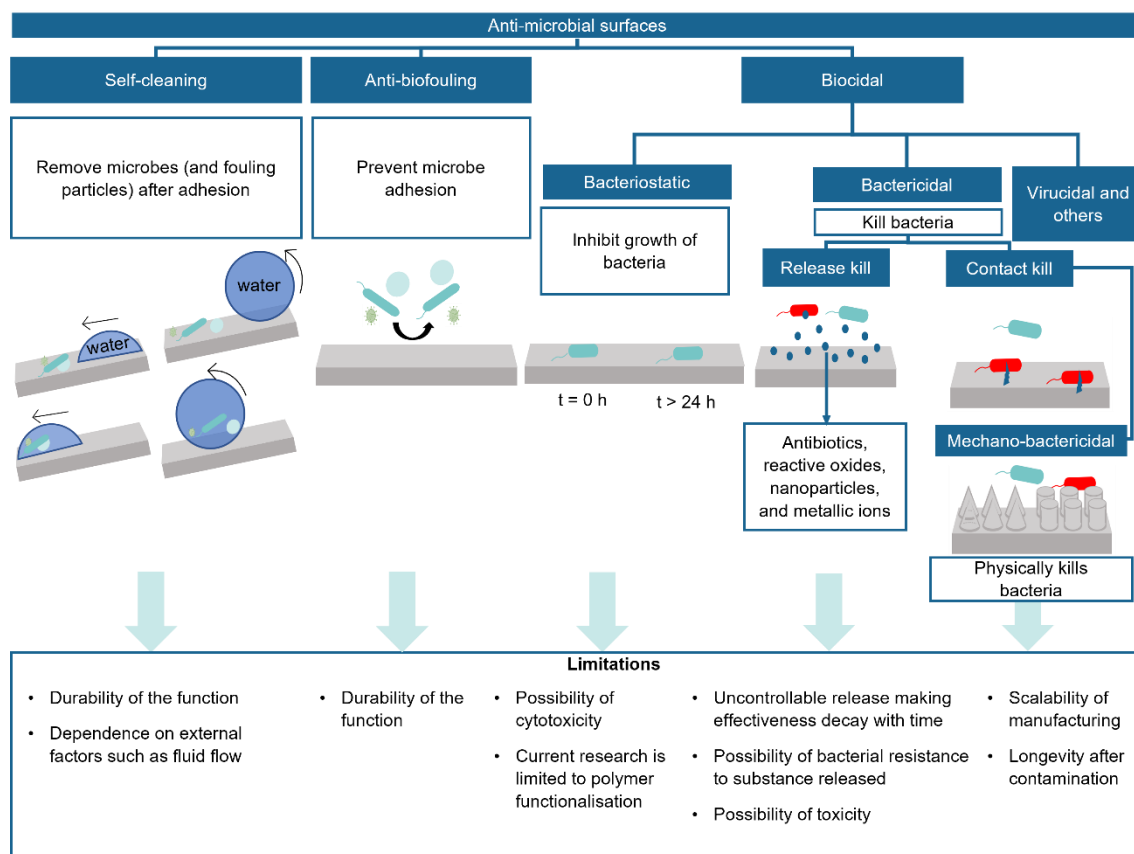


Figure 2-1 The landscape of anti-microbial surfaces

Biocidal surfaces can be further divided as (i) bacteriostatic, which can prevent the proliferation of bacteria, (ii) bactericidal which can kill the bacteria either through release or contact kill mechanisms and (iii) antiviral or virucidal surfaces that are lethal to viruses. Focusing on bactericidal surfaces, release kill and contact kill surfaces have slight differences between them. Release kill surfaces are those that cause ozone exposure triggered by metallic ions [24,25] (ionic silver, ionic copper, graphene nanosheets, carbon nanotubes etc.) or that can release antimicrobials from the surface into the surrounding fluid to kill the bacteria. Contact kill surfaces are those which can kill the bacteria merely by their physical presence either by virtue of the surface chemistry (coating, immobilised antimicrobial agents etc.) or their surface topography (physical geometry, nanostructure, nanotexture, etc.), often referred to as mechano-bactericidal.

Several limitations arise for the different techniques and are presented in Figure 2-1. These limitations drive active research topics. For instance, research into

longevity of the surface function [26] and into controlled release in case of chemical modifications [27] are being conducted. Ultimately, as the research into these aspects evolves, the persisting limitations will guide the application of each type of surface.

Of the several factors that effect bacteria-surface interaction, the anti-bacterial strategies rely on two main factors that have a big effect in dictating the interaction. Those are the surface chemistry and the surface topography. The modification of the surface topography has a big advantage over the modification of chemistry as it is considered a passive method which, according to current literature, doesn't induce bacterial resistance development, either in attachment prevention strategies or killing strategies.

The surface topography modification solutions have been inspired from the plethora of anti-bacterial textured surfaces found in nature.

These strategies hold great promise to achieve efficient anti-bacterial surfaces for a variety of applications. However, in order to understand how these strategies can be leveraged and optimised, it is first essential to get an insight into how a bacterium senses, attaches and grows on the surface.

2.2 Bacteria Recognition and Response to Surface Contact

2.2.1 Bacteria and their anatomy

When discussing anti-bacterial surface and bacteria-surface interactions, understanding of the bacterium's anatomy (Figure 2-2) becomes increasingly important.

The main structural elements constituting a bacterium are the cytoplasm, the peptidoglycan and the membranes (cytoplasmic and outer). The cytoplasm is a matrix that is composed mostly of water (80 %) and contains enzymes, nutrients, wastes, gases, ions and cellular components such as ribosomes, plasmid (if any), bacterial DNA, etc. It has been reported to behave as a glass-forming fluid in some studies [28], but generally its material properties remain uncharacterised.

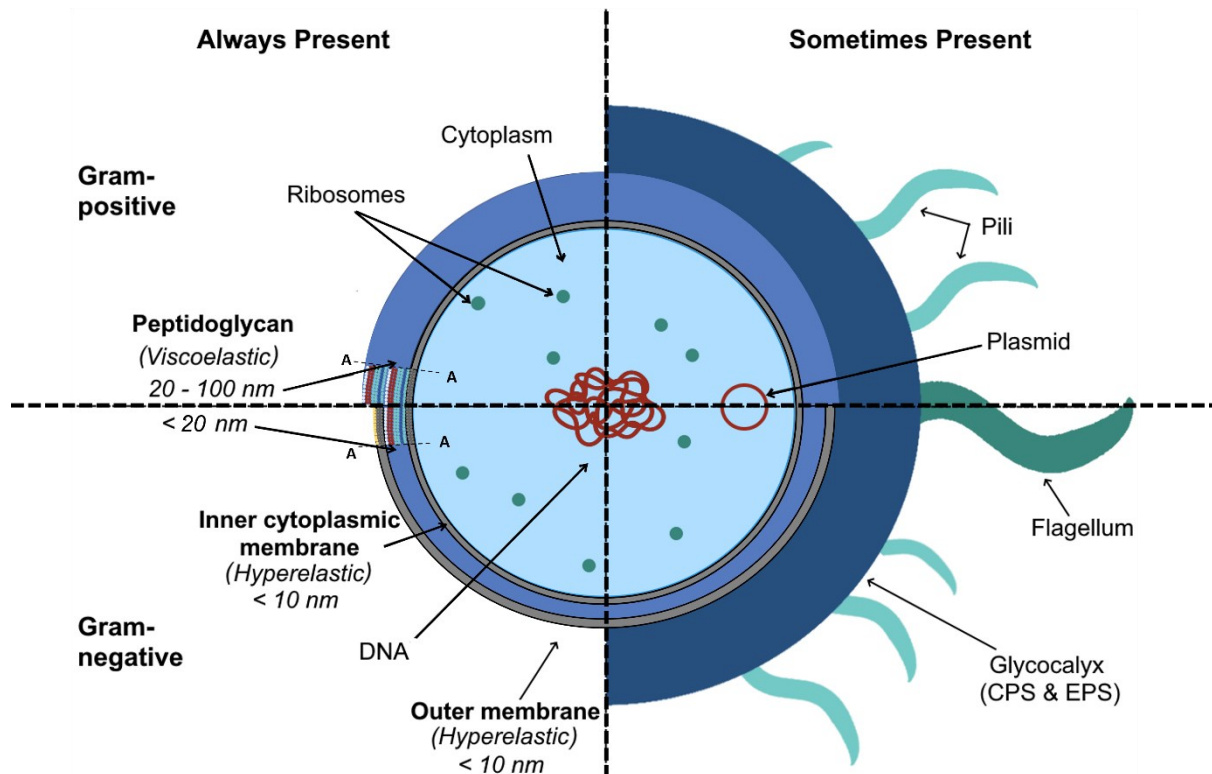


Figure 2-2 The anatomy of a bacterium. Section A-A depicts the basic structure of the peptidoglycan, the cytoplasmic membrane and the outer membrane. The grey circles represent the lipid bilayer, the turquoise (light blue circles) represent the alternating units of N-acetylglucosamine and N-acetylmuramic acid, with the N-acetylmuramic acid residues cross-linked to peptides. The yellow circles represent the lipopolysaccharides. CPS and EPS stand for capsular polysaccharides and extracellular polymeric matrix respectively.

The peptidoglycan is a very robust protective layer that is made up of repeating disaccharides of N-acetylmuramic acid and N-acetylglucosamine that are linked by $\beta(1-4)$ glycosidic linkages [29]. A recent report [30] claims that the latter exhibits viscoelastic behavior when subjected to indentation load under an atomic force microscope (AFM).

The cytoplasmic membrane, or inner membrane of a bacterium, is a phospholipid bilayer whereas the outer membrane (in case of Gram-negative bacteria) is principally made up of lipopolysaccharide. Measuring the mechanical properties of the individual components of the bacterial cell wall continues to be a great

challenge due to the cellular complexity. However, cellular membranes consisting of lipid bilayers and lipopolysaccharides have been reported to behave elastically under large deformations [31], making them hyperelastic materials.

2.2.1.1 *Staphylococcus aureus*

Staphylococcus aureus (*S. aureus*) is of particular interest in this work as it is an opportunistic pathogen associated with the majority of orthopaedic SSIs, community and hospital acquired infections [32,33].

S. aureus is a spherical or round-shaped Gram-positive bacterium that typically measures between 0.5 and 1.5 μm in diameter [34]. However, the exact size of individual cells can vary depending on growth conditions, age of the culture and other factors. The peptidoglycan layer of *S. aureus* has been reported to range between 10 to 30 nm [35]. *S. aureus* are negatively charged and hydrophobic in nature due to the presence of highly negatively charged and hydrophobic teichoic and lipoteichoic acid sites which are some of the main constituents the bacterium's cell walls [36,37].

Some studies have characterised the mechanical properties of different *S. aureus* strains in different conditions (e.g. air dried, fixed with 0.01 % poly-L-lysine, immersed in brain heart infusion) through atomic force microscopy [38–43]. The modulus of elasticity was generally calculated according to the hertzian contact mechanics model. The different values of the moduli are presented in Figure 2-3. However, other sources also report extremely high modulus values (i.e. 88 - 95 MPa) [44].

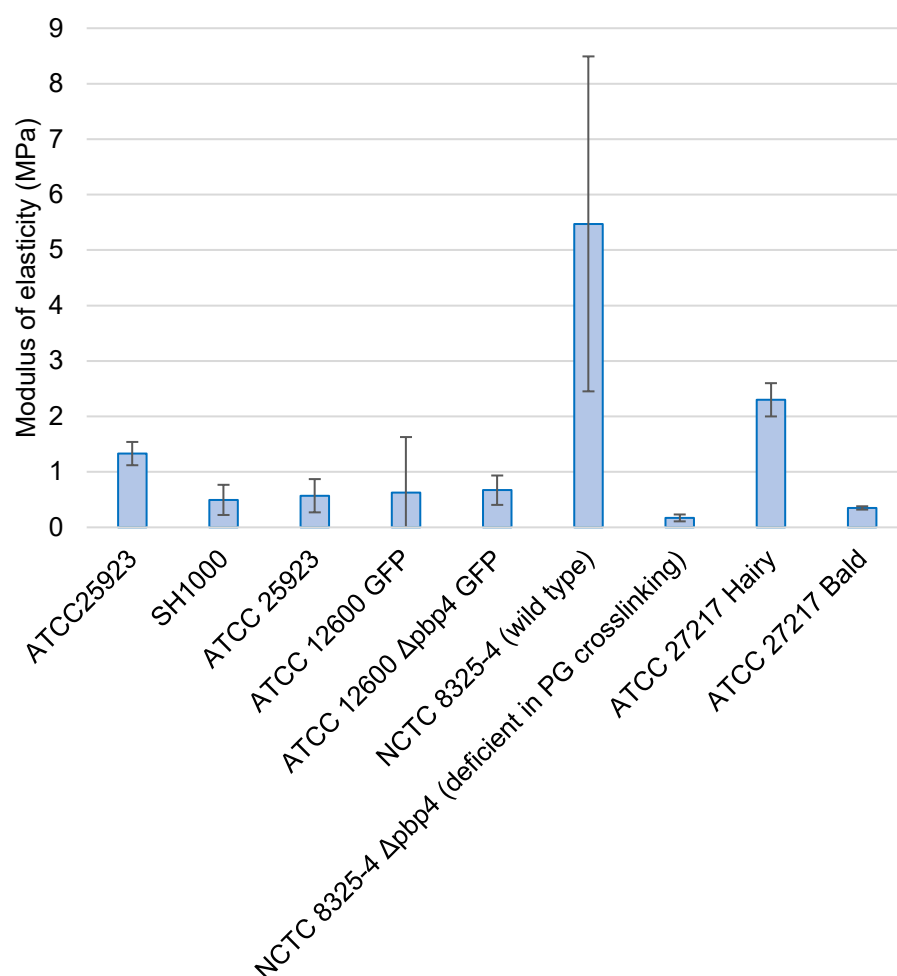


Figure 2-3 Modulus of elasticity of different *S. aureus* strains

2.2.2 Bacterial attachment on biomaterial surfaces

In a solid-liquid interface, bacteria tend to adhere and form biofilms in two main phases:

2.2.2.1 Phase 1: Reversible and instantaneous

2.2.2.1.1 Reaching the surface

To initiate attachment to a surface, some bacteria in a planktonic state (free-floating) exhibit directional motility, driven by the flagella (filamentous protein extension of the cell) in response to environmental cues, such as chemical signals, light, temperature, magnetic fields and oxygen [45]. Other bacteria are passive and get transported by external forces such as the flow in the bulk liquid,

Brownian motion and gravitational forces [46]. Adhesion is an interplay between characteristics of the environment or fluid, the bacteria and the surface they adhere upon as presented in Figure 2-4.

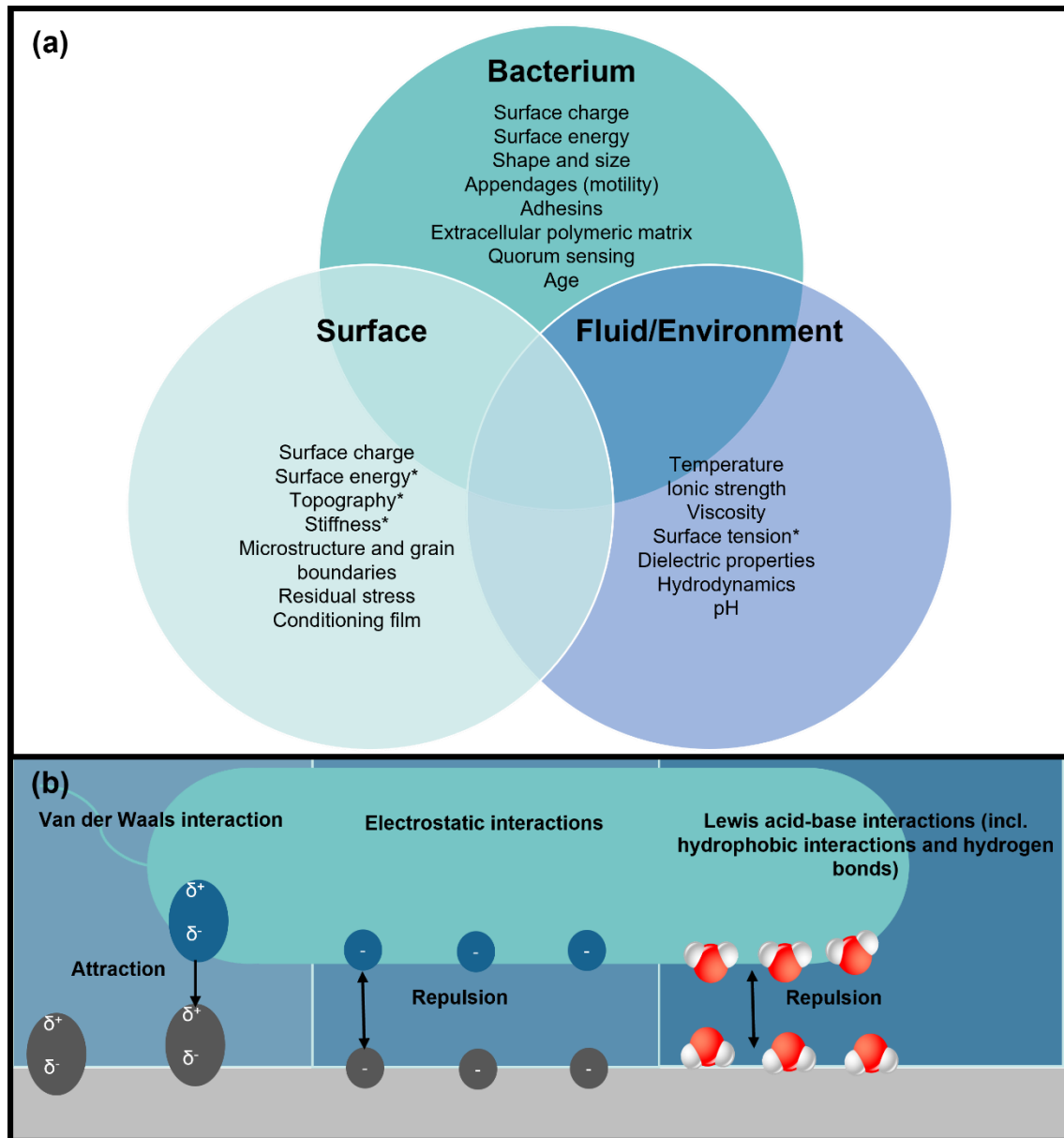


Figure 2-4 (a) The interplay of factors affecting bacteria/surface interaction between the bacteria, surface and the surrounding environment [3,4,5] and (b) physicochemical forces that affect the attachment adapted from [50].

The bacteria that reach the near surface environment experience amplified Brownian motion which results in them having circular trajectories [51]. This slows

down the velocity of the bacteria and gives them more time in proximity of the surface. Thus, more opportunity to initiate direct contact with the surface.

2.2.2.1.2 Initial contact, positioning and attachment

Bacterial flagella, fimbriae, curli and pili aid the adhesion to the surface be it directly or indirectly. The flagella, for instance, provides a physical contact means and functions as an adhesin [6,7]. However, due to the complexity and versatility of the bacteria and surfaces to adhere on, the possession of many surface appendages does not always equate to an efficient adhesion.

In addition to these appendages, other physicochemical forces and interactions also play a role in the initial contact, positioning and attachment of bacteria to surfaces. These forces include van der Waals forces, electrostatic interactions, and Lewis acid-base interactions, which collectively contribute to the overall adhesion process (Figure 2-4 (b)). The Extended Derjaguin-Landau-Verwey-Overbeek (XDLVO) theory integrates these forces and provides a framework for understanding bacterial adhesion to smooth surfaces [54].

Van der Waals forces are weak, attractive forces that occur between molecules and are influenced by their proximity to each other. Electrostatic interactions, on the other hand, are based on the attraction or repulsion between charged particles on the bacterial cell surface and the target surface. Lewis acid-base interactions involve the donation and acceptance of electron pairs between the bacterial cell and the surface, contributing to the overall adhesion process.

In this reversible phase, the initial contact and positioning is polar as presented in Figure 2-5.

2.2.2.2 Phase 2: Irreversible and time-dependent

To generate a permanent attachment and transition from the reversible phase to the irreversible phase, the bacterium repositions from the initial polar attachment to a longitudinal positioning and begins the synthesis of protein or polysaccharide adhesin molecules. The repositioning occurs to stop the bacterium from spinning around its axis and detaching from the surface and to maximise the contact

between the surface and the bacterium. Repositioning does not always occur as some bacteria possess very strong localised polar adhesins that can secure them an irreversible polar attachment [45], or in the case of spherical bacteria such as *S. aureus*. As the undeformed bacterium adhere to the surface, adhesion forces arising from the substrate/bacterium interaction gradually deform it until counteracted by opposing forces arising from the rigid bacterial cell wall and increased intracellular pressure.

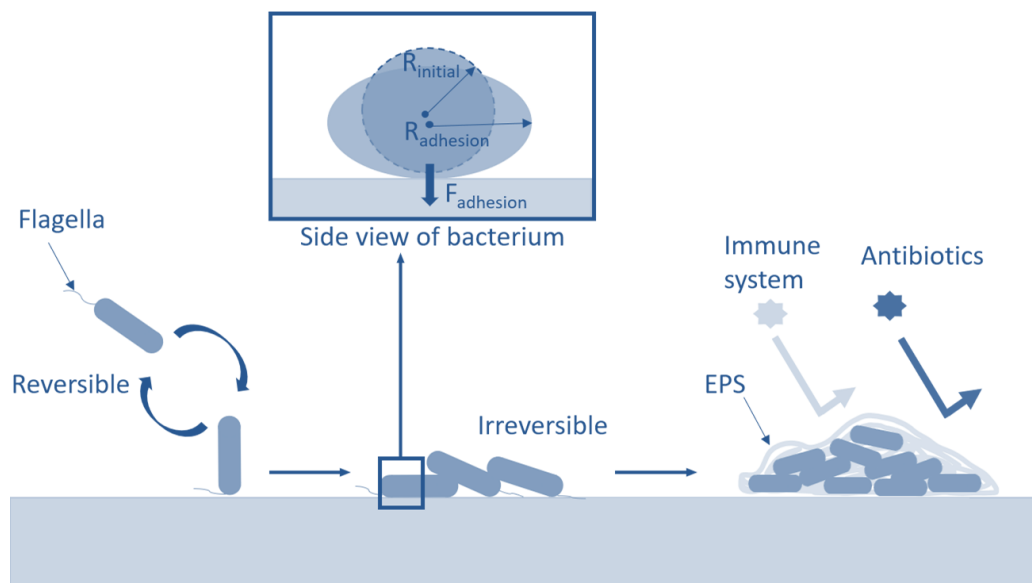


Figure 2-5 Reversible and irreversible attachment of bacteria to a surface

In this phase, the bacteria commit to a biofilm mode of growth. They start to colonise and produce extracellular polymeric substances (EPS) to form a mature biofilm (Figure 2-5).

By definition, a biofilm is a bacterial community in which its cells are attached to a substrate (solid surface), an interface or to each other [55]. They are embedded in a matrix of EPS, exhibit altered behaviour with regards to growth, gene expression and protein production [55]. Upon reaching a certain cell density, a mechanism is triggered to initiate the degradation of the extracellular polymeric matrix to enable the release of embedded cells within the biofilm. This is known as the dispersal stage which leads to the spreading of the infection and re-initiation of biofilm formation in a different site, ensuring the bacterial survival and

proliferation. The drive for the bacterial species to maintain survival and proliferation, in accordance with Darwin's theory of evolution, is the driving force behind biofilm formation of bacteria.

Because biofilms serve as an impermeable protective shield for bacteria, they are one of the major reasons behind antibiotic tolerance. Thus, the window of action of any anti-bacterial surface would have to be during the initial attachment, prior to biofilm formation.

2.3 Anti-bacterial Modification Approaches of Surfaces

As highlighted in sections 1.1 and 2.1, the growing threat of antibiotic-resistant bacteria has emphasised the importance of developing innovative anti-bacterial surface modifications. A particular area of interest in this domain is nature-inspired surfaces, which seek to emulate the inherent anti-bacterial properties found in natural systems. This section delves into various approaches to creating nature-inspired surfaces.

Section 2.3.1 focuses on nature-inspired surfaces that employ topographical features to influence bacterial adhesion and growth. These designs are derived from the study of biological systems, which have evolved effective mechanisms to combat bacterial colonisation. This section will explore the principles and examples of topographical modifications, drawing inspiration from nature through two subsections.

Subsection 2.3.1.1 discusses anti-biofouling surfaces, which are designed to prevent bacterial adhesion by utilising specific surface properties. This approach minimises the opportunity for bacteria to establish themselves on the surface and, thus, reduces the likelihood of biofilm formation and subsequent infection.

Subsection 2.3.1.2 investigates mechano-bactericidal surfaces, which rely on physical mechanisms to kill bacteria upon contact. By replicating natural structures and geometries, these surfaces have the potential to be highly effective in reducing bacterial colonisation without promoting resistance.

Section 2.3.2 shifts the focus to chemical modifications, which alter the surface chemistry of materials to imbue them with anti-bacterial properties. This section presents an alternative approach to nature-inspired surface designs, emphasising the importance of understanding the competitive landscape in developing effective anti-bacterial surface modifications.

Lastly, Section 2.3.3 examines the various techniques used to evaluate anti-bacterial surfaces and bacteria-surface interactions. This includes a discussion of the current methodologies and the challenges faced in determining the efficacy of different surface modifications.

2.3.1 Nature-inspired Topographical Surface Modifications

Following the nature-inspired design approach presented in 2.1, two potential natural surface strategies or mechanisms have been identified: the anti-biofouling strategy and the mechano-bactericidal strategy. The next step in the design strategy is to understand their workings in order to be able to transpose them to technology, implement and test them. Herein the two strategies are discussed in sections 2.3.1.1 and 2.3.1.2.

2.3.1.1 Self-cleaning and Anti-biofouling Surfaces

Before exploring the history and theory behind self-cleaning and anti-biofouling surfaces, it is crucial to distinguish between the terms. Self-cleaning refers to a surface's ability to remove debris (including biological organisms) upon adhesion, which often requires an external hydrodynamic factor, particularly in submerged surfaces [56]. For instance, magnetic assisted cilia exhibit self-cleaning capabilities when actuated [57]. For passive self-cleaning surfaces, mainly inspired by natural surfaces, self-cleaning is heavily linked to the wetting of the surface [58]. For example, on superhydrophobic surfaces water droplets roll off, carrying debris from the surface and cleaning it. On superhydrophilic surfaces, water is able to spread out very well on the surface to get in between the debris or adhered particles and the surface, displacing them from said surface and rendering it clean. Anti-biofouling, on the other hand, describes the ability of a

surface to resist or prevent fouling by biological organisms, decreasing their adhesion levels. Some researchers have coined the terms active and passive anti-biofouling where they described passive anti-biofouling similarly to the anti-biofouling notion described previously, which delays or prevents the fouling process [59]. Whereas active anti-biofouling overlaps with self-cleaning surfaces as it is defined by the ability to remove bioaccumulation on demand [59].

It is crucial to recognise that a self-cleaning surface does not inherently possess anti-biofouling properties, especially that anti-biofouling is a scale specific function due to the varying sizes of the biological foulants whereas self-cleaning is more or less specific to water/fluid contact. Although self-cleaning and anti-biofouling properties are often interlinked through surface wettability, influenced by surface chemistry and topography, these properties might not always coexist. For the purpose of this thesis, anti-biofouling will refer specifically to the prevention of fouling, while self-cleaning will denote the ease of foulant removal after adhesion.

Research into self-cleaning surfaces started several decades ago, with some of the earliest examples dating back to the 1960s when investigations of marine organisms (e.g., oysters) that are able to attach themselves to underwater surfaces without being fouled by other organisms began. However, the texturing aspect of the anti-biofouling approach began even earlier, around the 1970s, when research into the topography of the lotus leaf was initiated by Botanist Wilhem Barthlott after he noticed that water droplets on the lotus leaf form nearly perfect spheres and roll off of its surface, as reported by Neinhuis [60]. At this stage, the lotus leaf was considered a self-cleaning surface due to its observed wetting behaviour.

Wetting behavior, as characterised by the equilibrium contact angle of a liquid droplet on a surface (and described later in this section), is intrinsically linked to the surface free energy of the material; a lower contact angle indicates higher surface wettability and, consequently, a higher surface free energy, which can

directly influence the antibacterial behavior of the surface as will be evidenced in the coming paragraphs.

The link between wetting (contact angle) and surface energy can be mathematically expressed according to several models, most prominently the Owens-Wendt-Rabel & Kaelble model [61] model as presented below:

$$\frac{\gamma_l(\cos \theta_Y + 1)}{2\sqrt{\gamma_l^d}} = \sqrt{\frac{\gamma_s^p \gamma_l^p}{\gamma_l^d}} + \sqrt{\gamma_s^d} \quad (2-1)$$

Where γ_l is the total surface energy of the liquid, γ_l^d and γ_l^p are the dispersive and polar component of the liquid respectively, and γ_s^d and γ_s^p are the dispersive and polar components of the surface.

The understanding of the unique wetting behaviour of the lotus leaf, among other natural surfaces, was enabled by surface wetting models as it is crucial to understand surface fouling. The degree of wetting for an ideal flat surface was explained by Thomas Young in 1805. This model also known as Young's model defines the equilibrium contact angle (angle between the surface-solid contact line and tangential line to the liquid droplet) between a liquid and a solid to be a balance of surface tensions at the three-phase contact line: solid, liquid and gas as is shown in Figure 2-6 (a).

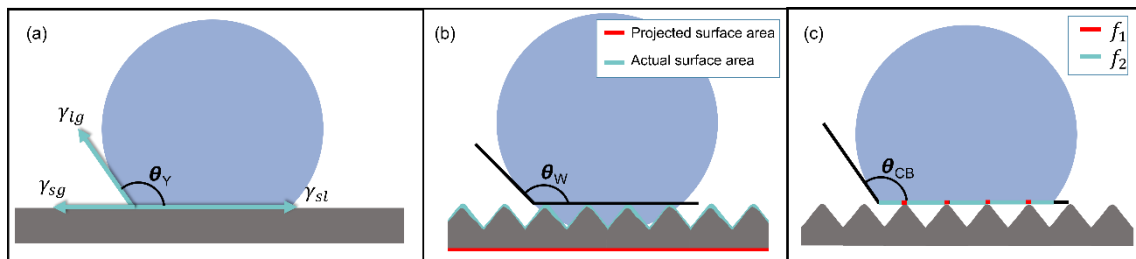


Figure 2-6 The wetting Models: (a) The Young's model considering an ideally flat surface: γ_{sg} is the solid-gas interface, γ_{sl} is the solid-liquid interface and γ_{lg} is the liquid-gas interface, (b) The Wenzel model considering full wetting of surface roughness features, and (c) The Cassie-Baxter model considering air pocket

entrapment within the surface roughness features. f_1 is the solid fraction of the surface in contact with the liquid and f_2 is the gas fraction.

Young's equation for wetting can be described as [62]:

$$\theta_Y = \cos^{-1} \left(\frac{\gamma_{sg} - \gamma_{sl}}{\gamma_{lg}} \right) \quad (2-2)$$

Where γ_{sg} is the solid-gas interface, γ_{sl} is the solid-liquid interface and γ_{lg} is the liquid-gas interface.

In 1936, the Wenzel model was introduced to account for the fact that no surface is perfectly flat and all surfaces have a roughness that should be accounted for. The Wenzel model considers that the full scale roughness of a surface is fully wetted by the liquid and suggests that the roughness of a surface amplifies its inherent wettability. It suggests that due to the surface roughness, there are two contact angles involved: the young's contact angle and the apparent or measured contact angle (as is shown in Figure 2-6 (b)). Wenzel's equation relating Young's contact angle to the measured contact angle is [63]:

$$\cos \theta_w = r \cos \theta_Y \quad (2-3)$$

Where r is defined as the roughness ratio between the actual and projected solid surface area. For a smooth surface, the roughness ratio is 1 and bigger than 1 for a rough surface.

Later on, the Cassie-Baxter model was developed (Figure 2-6 (c)). It considers that the roughness features are not fully wetted by the liquid but that there are air pockets present within it. This leads to a composite wetting state that can exhibit extreme wetting properties.

The Cassie-Baxter equation is expressed by [64]:

$$\cos \theta_{CB} = f_1 \cos \theta_Y - f_2 \quad (2-4)$$

Where f_1 and f_2 are the ratios of the contact areas between the drop and solid area, and the drop and the air respectively.

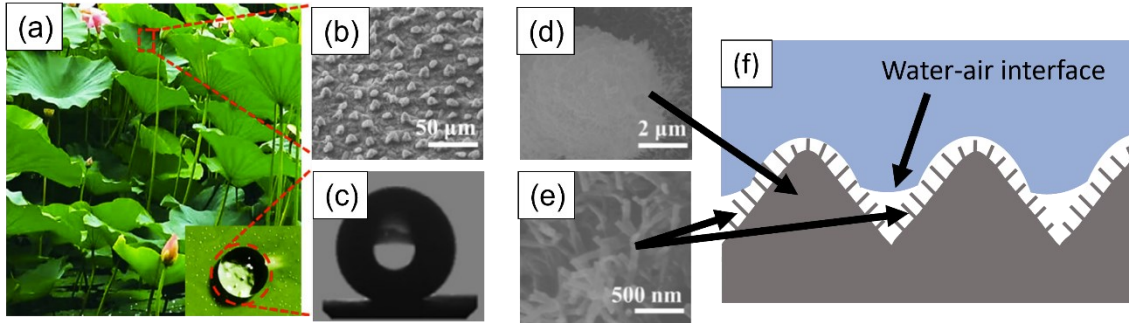


Figure 2-7 (a) The superhydrophobic lotus leaves and the spherical water droplet in contact with the leaf surface. (b) SEM image of the lotus leaf tilted at an angle of 45°. (c) The high contact angle (154°) of the water droplet on the lotus leaf surface. (d, e) Higher magnification SEM images of a single microfeature denoted as papilla and the superimposed nanofeatures or nanotubes. Reprinted from [65] Copyright (2020), with permission from Elsevier. (f) Schematic of the hierarchical structure of the lotus leaf.

In regards to the lotus leaf, further investigation into its surface topography revealed multiscale hierarchical features described as large microfeatures in the range of 5-10 μm with superimposed nanofeatures of 0.1-0.2 μm in diameter [65]. These investigations also revealed the anti-biofouling ability of the lotus leaf surface which manifested by reduced fouling or adhesion of *Escherichia coli* [65].

The underlying self-cleaning mechanism was determined to be a consequence of the wetting behaviour of the multi-scale topography of the surface combined with its chemical composition (wax) which induces droplet roll-off [66]. The anti-biofouling mechanism was determined to be an effect of the multiscale surface topography inducing air entrapment at the liquid/solid interface (following the Cassie-Baxter model), minimising the contact area and potentially reducing biofouling.

Organically, the study of structured anti-biofouling surfaces branched out from the study of self-cleaning structured surfaces as the functionalities were found to be interlinked in many instances. Naturally structured surfaces that have

presented self-cleaning abilities are presented in Figure 2-8, some which have proven anti-biofouling abilities too.

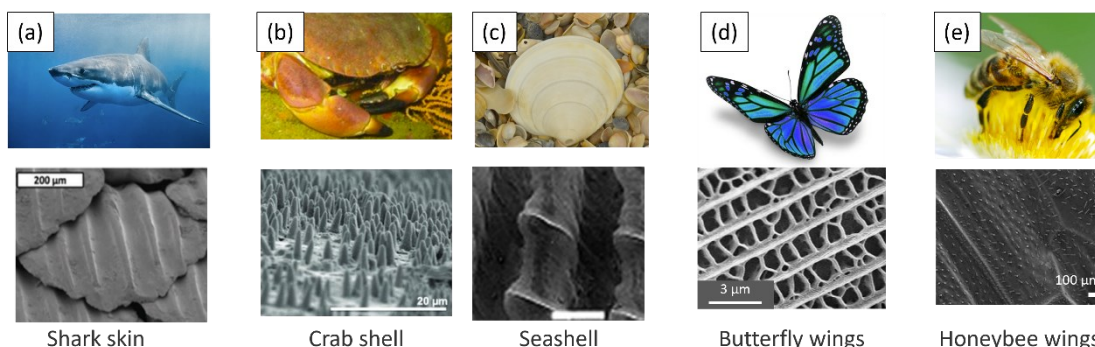


Figure 2-8 Natural structured surfaces including (a) Shark skin, (b) crab shell, (c) seashell, (d) butterfly wings and (e) Honeybee wings that inspired synthetic self-cleaning and anti-biofouling surfaces. Reprinted with permission from [67–70].

The anti-biofouling abilities of these natural surfaces is scale dependent which influences the intended application of synthetic surfaces inspired from nature. For instance, shark skin features are in the scale of hundreds of microns which make them suitable against the fouling of large marine organisms. However, the relatively large scale of features, compared to bacteria, were found to actually promote early stage bacterial attachment yet prevent bacteria from developing biofilms [71,72]. Hence the interest in replicating the shark skin surface topography for both maritime and biomedical settings, with sharklet being at the forefront of the commercial development [73]. There are instances where naturally structured superhydrophobic surfaces, such as rose petals, exhibit high fluid adhesion rather than self-cleaning properties, yet still possess anti-biofouling capabilities [74,75]. This reinforces the idea that self-cleaning surfaces and anti-biofouling surfaces are not mutually exclusive.

The observation of wetting behaviour of these natural surfaces, their topography and the anti-biofouling effect begs the question of the correlation between these parameters. Specifically, it is worth investigating whether the anti-biofouling effect is primarily influenced by surface wettability or surface topography. Herein, several attempts have been done to replicate the surface-topography-driven anti-

bacterial (anti-biofouling specifically) functionality of natural surfaces. In this regard, a collection of water contact angles or WCAs (wettability) and anti-bacterial efficiencies of nature-inspired textured surface is presented in Figure 2-9.

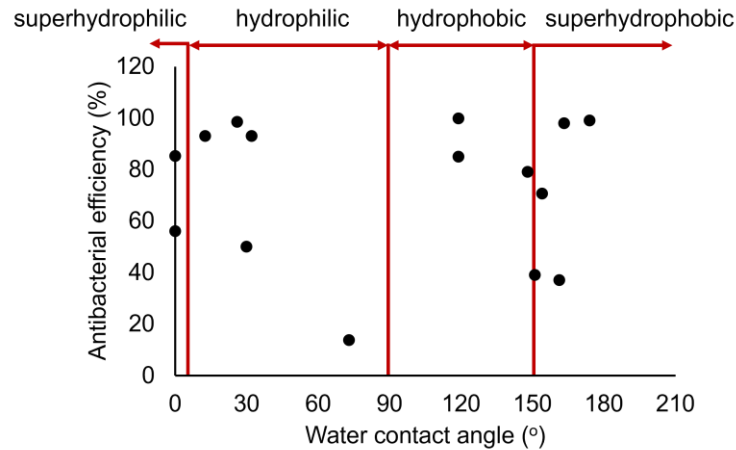


Figure 2-9 The relationship between the water contact angle and the anti-bacterial efficiency of textured surfaces. Data was extracted from [65,76–83]. Most data points correspond to the anti-bacterial efficiency against *S. aureus* bacteria, yet points (26, 98.5) and (119, 99.8) correspond to the efficiency against *E. coli*.

As can be seen from the data collected from the literature [65,76–83], textured surfaces with both low and high WCA have exhibited anti-bacterial functionality. Concurrently, both low and high WCA surfaces have shown low anti-bacterial efficiency too (< 50%). This indicates that surface wettability, even if topography-induced, might not be the most influential factor in dictating anti-bacterial efficiency of a surface when a surface topography is present.

For instance, while studying the anti-biofouling effect of hierarchical surfaces fabricated through nanoimprint lithography, Francone et al. [84] concluded that *E. coli* was more susceptible to the surface topography while *S. aureus* was more susceptible to the wettability of the investigated surfaces. Additionally, Epperlein et al. [85] found that two surfaces with the same wettability but different topographies had differing bacterial attachment.

Yet to date, very few studies have directly looked at the influence of the surface topography versus the influence of surface wettability in this regard, especially considering the complexity of the subject and the subjectivity of the results on the bacterium used as a model, as will be further discussed in section 2.3.1.2.1.1. Not only that, but to date no direct correlation was evident between specific topographical parameters and the anti-biofouling effect as will be evidenced in section 2.5.3.

2.3.1.2 Mechano-bactericidal Surfaces

Mechano-bactericidal surfaces have gained significant interest in the past decade (Figure 2-10) due to their premise of transferability to different biomaterials. The bactericidal mechanism(s) behind their action remains unclear due to complexities that accompany bacteria-surface experimental investigations and a lack of uniformity in the reported data.

Conflicting correlations have been made between bactericidal efficiency and: (i) surface wettability, (ii) surface roughness and (iii) nanostructures, among others. It is however categorically established that the mechanism is independent of surface chemistry [86] and relies on the mere topography of the surface leading to bacterial killing via a physical rather than a chemical mechanism, hence the prefix “mechano”. Moreover, it has been suggested that the biocidal action can be expanded to affect other microbes i.e., viruses [87,88] and yeast [89] cells.

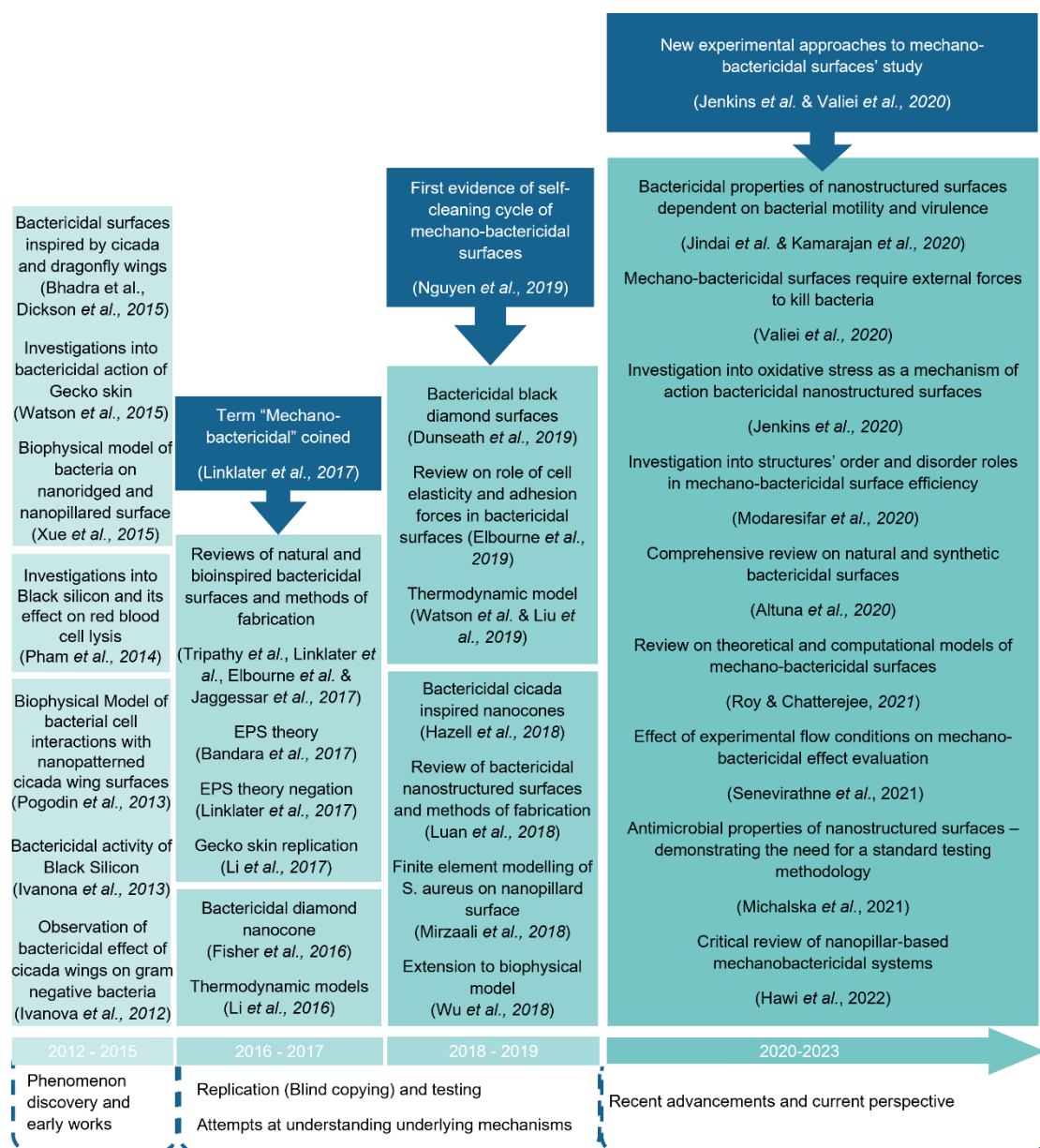


Figure 2-10 Timeline showing progressive learning about the bactericidal surfaces based on the topography of cicada and dragonfly wings. [88,90,99–108,91,109–118,92,119–123,93–98]

2.3.1.2.1 Mechano-bactericidal Mechanisms

In the quest to elucidate the mechanism(s) underlying mechano-bactericidal activity, two research approaches have been adopted which are based either on experimental data or simulations. In the next sections, the mechanisms revealed

by these experimental approaches, the mechanisms revealed by simulation approaches and the factors playing into the effect of mechano-bactericidal structures are presented. The limitation of experimentation and simulations will both be discussed in their prospective sections.

(a) Mechanisms Revealed by the Experimental Approaches

Through experimental approaches, nanostructured surfaces gained their reputation as mechano-bactericidal surfaces, independent from chemical effects [86] as the functionality (bacteria killing ability) was shown to persist across different materials.

Early research relied on observations from techniques such as scanning electron microscopy (SEM) imaging to form the hypothesis that nanostructures puncture bacterial cell walls leading to the release of cytoplasmic fluid (Figure 2-11 (a)). This was due to compromised cells taking the shape of the nanopillars, revealed by the SEM images which led to the conclusion that the nanostructures puncture the bacteria. Techniques of evaluation then progressed into methods that can reveal the nanostructure/bacteria interaction through cross-sectional imaging e.g., Focused Ion Beam (FIB). Recently, slice-by-slice FIB-SEM data reconstruction was used to better observe the interaction of nanopillars with single cells [124] leading to an improvement from the previous postulations. Several hypotheses then emerged, one of which is specific to Gram-negative motile bacteria [101]. This hypothesis suggests that the biocidal activity is mediated by an extracellular polymeric substance (EPS). This mechanism was later refuted as it disregards the fact that the bactericidal effect occurred within a few minutes of contact whereas EPS secretion is thought to take significantly longer (hours to days) [102,119].

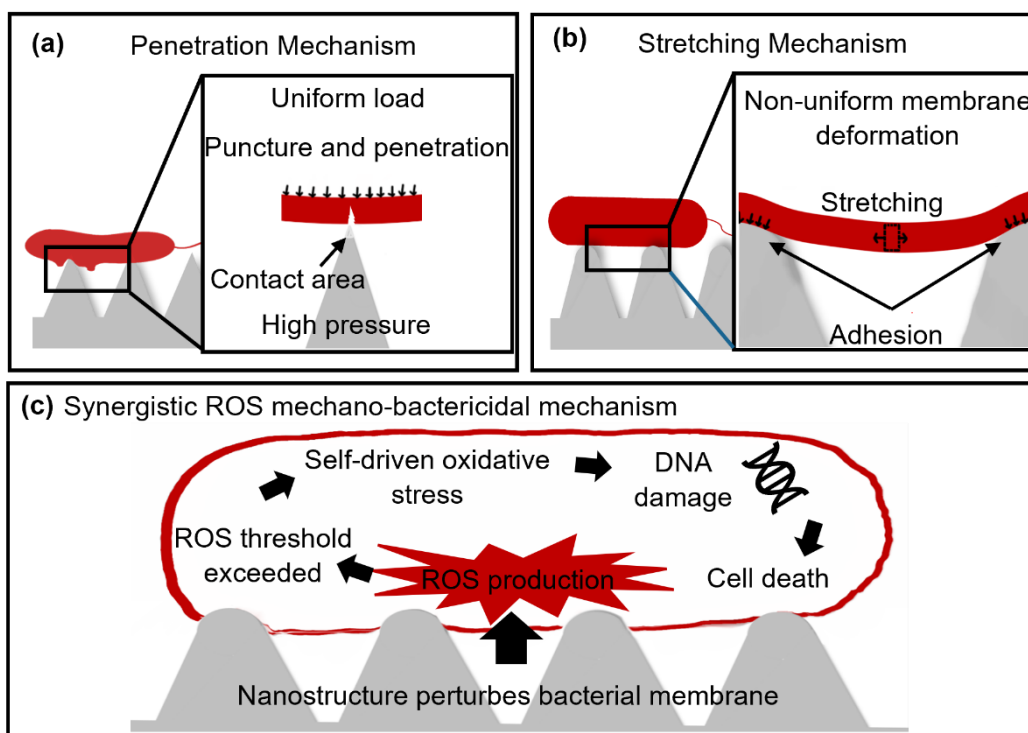


Figure 2-11 (a) The penetration mechanism based on sharp nanostructures, (b) The stretching mechanism proposed and (c) the synergistic ROS mediated mechano-bactericidal mechanism.

Amidst the development of these hypotheses, it was experimentally established that the effusion of intracellular fluid does occur for *Escherichia coli* on nanostructured surfaces using fluorescent proteins [125]. This important finding confirmed that rupturing of the bacterial membrane is involved in the bactericidal action presented by the nanostructures.

Meanwhile, Ivanova et al. [91] suggested that the bactericidal activity of nanostructures is actually driven by the mechanical and structural responses of the bacterial cell as it adheres to the nanostructures. Particularly, these responses create inelastic stress imposed by the surface nano-topography on the peptidoglycan cell wall and inner membrane of the bacterial cells. Herein, the surface ineffectiveness against Gram-positive bacteria was attributed to the thickness of the peptidoglycan layer in the cell wall as it is four to five times thicker than that of Gram-negative bacteria [126]. This was named as the “stretching”

mechanism (Figure 2-11 (b)) and thought to compete the “penetration” mechanism.

Exhaustively, all these hypotheses were inferred from indirect evidence from the same experimental approaches (mainly confocal laser scanning microscope, SEM and FIB-SEM) with interpretations based on *a priori* knowledge, which introduced a certain bias towards the previously conceived stretching and penetration mechanisms. It is imperative to look at the problem from an alternative perspective proposed here.

A few recent studies have shed light on the shortcomings of the experimental approaches employed in understanding the underlying mechanisms of the mechano-bactericidal effect. One such study by Jenkins et al. [117] provided a baseline for a reactive oxygen species (ROS) mediated mechanism as bacteria exposed to the nanostructures (be it Gram-positive or Gram-negative) were found to be oxidatively stressed. This occurs when bacteria are subjected to a lethal stressor which triggers production of ROS as a self-destruction mechanism [127]. Bacteria can detoxify low levels of reactive oxygen species (except hydroxyl radical) through several protective enzymes (e.g. superoxide dismutase, catalases) [128]. However, if the ROS levels surge above a certain threshold, the death process becomes self-driven and irreversible even if the initial stressor is removed [127]. This synergistic ROS-mediated mechano-bactericidal mechanism, illustrated in Figure 2-11 (c), sheds a light on the importance of expanding the experimental approach to look beyond the “penetration” or “stretching” mechanisms. In other words, the mechanism, although shown to be surface chemistry independent, does involve chemistry at a bacterial level contrary to the currently presented pure mechanistic models.

Nonetheless, understanding the proposed mechanisms is imperative to evaluate their role in the bactericidal pathway of the nanostructures. “Penetration” as a term has repeatedly been used to explain that the failure of the bacterial membrane in the area that is directly in contact with the nanostructures. However, the term penetration in its essence indicates that stress concentrations leading to

fracture occurs at the failure site which in turn requires a very sharp rigid structure. For “penetration” or puncture to mechanically occur, the degree of freedom of the penetrated object should be restricted which is mostly not the case in this scenario of bacteria-nanostructure interaction. Thus, if failure occurs at the apex of the nanopillar, it is due to the local stretching or pressure on the membrane in that region. The question remains as to where the critical action site is within the mechano-bactericidal mechanism and what is the driving force for that deformation. As direct observation and quantification of such aspects is extremely difficult with the techniques and instruments available, simulation studies have been deployed to find the right answers.

(b) Mechanisms Revealed by Simulations

The simulation models developed so far can be categorised into analytical models and computational (numerical) models. In analytical models, equations based on surface energy considerations, thermodynamic equilibrium and energy minimisation are formulated and solved by introducing geometric constraints relating to both the bacteria and the nanostructured surface. The deformation of the adsorbing bacteria, in these models, is considered as a necessity to reach thermodynamic equilibrium. In other words, adsorbing bacteria continue to envelop the nanostructures until equilibrium is reached. These models differ in the way they define the change in free energy (based on local stretching degree and stretching modulus [92,107]; membrane tension and strain tensors [129]; work required to bend the bacterial wall around the nanopillar shaft [111]; and variation of the adhesion contact angle as the cell migrates into nanopillars [112]).

In computational models, the bacteria-nanostructure interaction is modelled with finite element method based on a major assumption that the bacteria have no fluid surroundings. The simulation models developed so far have thus assumed bacteria-surface interaction in vacuum – an aspect which needs to be evaluated properly. Also, failure of a bacteria is based on the maximum strain in the

membrane which is assumed to occur when the local strain value exceeds the permissible strain threshold.

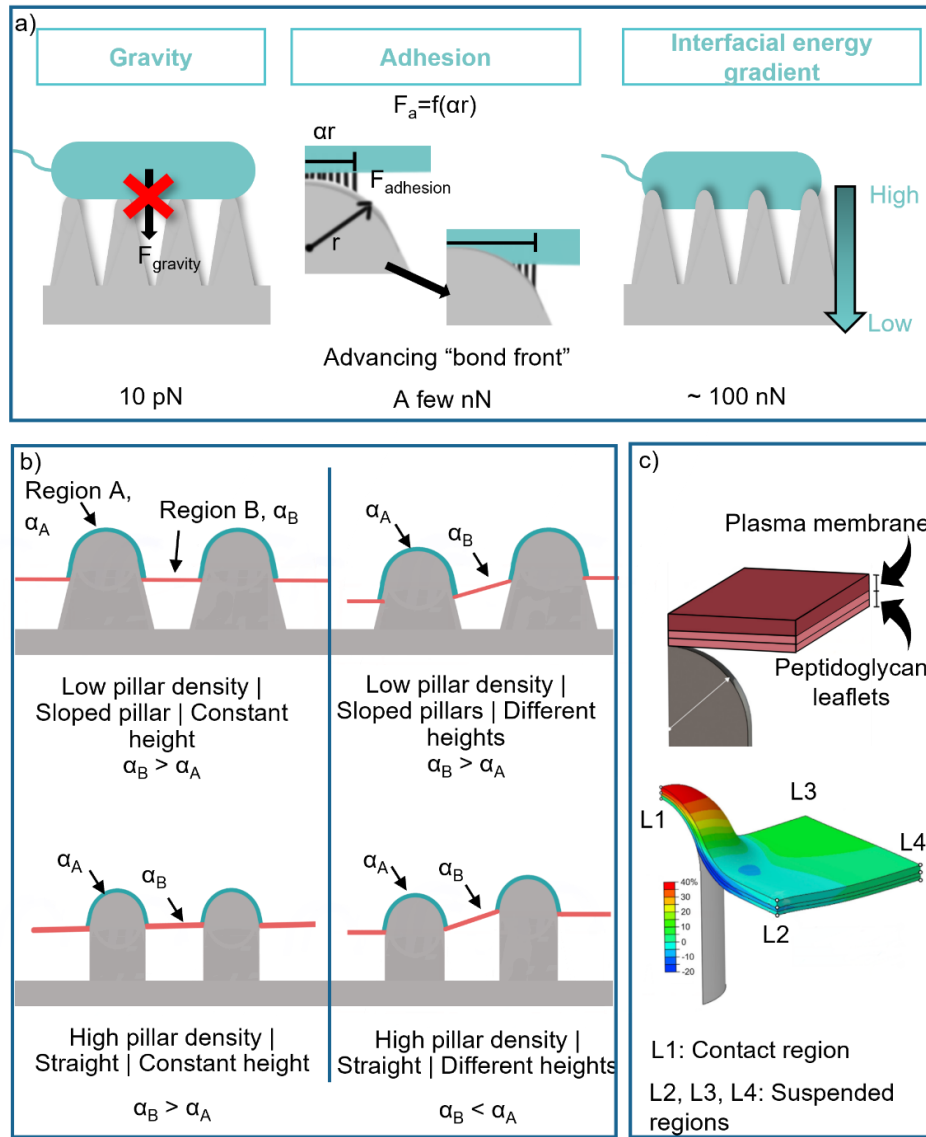


Figure 2-12 (a) Forces proposed to drive bacteria towards the nanopillars, b) Energy-based analytical model of bacterial cell adhering and stretching on nanostructured surfaces in four configurations depending on the pillar density, the slope and the height. α_A and α_B are the stretching degrees in regions A and B respectively. The larger stretching degree indicates the critical action site of that said configuration,[92,107] c) a contour plot of longitudinal uniaxial strain for an adhered bacterial envelope. The contact region is probed by L1, the suspended regions are probed by L2, L3 and L4. At each location, in-plane uniaxial strains are

probed at three points through the thickness, representing each of the three layers. For the plasma membrane, the maximal through-thickness value is found at the top plane. For the outer leaflet, the maximal through thickness value is found in the suspended region. Adapted from [130] with permission from Elsevier. Copyright 2021.

The value of this strain threshold reported in the literature shows high percentage variability > 50%, which indicates that the currently reported finite element analysis (FEA) studies are incoherent. For example, Velic et al. [130] based the rupture strain range on experimentally reported values of the rupture strains of different organisms [131,132], bacterial threads [133] and values obtained from coarse grain modelling of mimetic lipid systems [134] (0.18-0.65 as longitudinal strain for the peptidoglycan layer and 0.05-0.35 as areal strain for the outer membrane). Mirzaali et al. [108] considered the rupture strain value of 0.5 through the work of Thwaites and Mendelson [135] which was based on the elongation of *B. subtilis* bacterial thread rather than the elongation of a single bacterium. To add to this dilemma, other studies [5] have reported vivid values (0.08 for *S. aureus*, 0.12 for *E. coli* and 0.05 for *P. aeruginosa*). Clearly, this important information which is a prerequisite for meaningful FEA studies needs reinvestigation. The inconsistency reported so far is largely due to the fact that the constitutive modelling of a bacteria is ill-defined, and the methods employed to evaluate the mechanical properties of the bacteria were not comprehensive enough. A full set of data can be obtained by fully developing a set of AFM nanoindentation experiments, especially accounting for the anisotropy presented by a bacterium. Clearly one such anomaly which can be seen as an example in the reported literature is the assumption of a gravitational force [94] acting on the bacteria which is an incorrect assumption since a bacterium is not of its own but is a freely floating particle in a fluid. Therefore, any future modelling studies needs to consider these aspects as well as other aspects, such as the boundary conditions, loading type, strain type etc. very carefully.

The Driving Forces

To develop any simulation whether analytical or computational, assumptions must be made as to what are the forces driving/governing the phenomenon that is being modelled. Gravity, as shown in Figure 2-12(a), has been incorrectly considered as the main driving force in some studies. Hence, the question arises as to whether the magnitude of a bacterium's gravity is enough to cause its rupture. Xue et al. [94] studied the stretching of bacteria due to gravity on two different geometries: nanopillars and nanoridges. In their model, the stretching degree of the bacterial membrane exceeded the calculated critical stretch value. The rupture of bacterial membrane was inferred to be more likely on nanopillars than nanoridges. Additionally, an FEA study conducted by Velic et al. [136] found that the force of gravity on an *S. aureus* bacterial cell, coupled with the force from the water column above the bacterium (tens of piconewtons) strains it enough, as it contacts a nanostructured surface, to exceed the assumed strain threshold of 0.5. In both studies, the stiffness of the bacterial membrane was assumed to be in the range of a few pascals to a few kilopascals.

This is inconsistent with AFM studies which have shown a wide range of stiffness (i.e., 0.2 to 95 MPa) [137], as evidenced for *S. aureus* in section 2.2.1.1. Additionally, AFM studies show that bacterial cells including *S. aureus* can withstand forces of several nanonewtons before their rupture. Based on this, gravity can be ruled out as the main driver for cell rupture.

Liu et al. [112] suggested that cell adsorption is driven by the differential energy gradient (i.e. between Gibbs surface free energy and deformation surface energy) along the height of the nanopillars (i.e. high at the tip of the nanopillar and low at the base). The force induced by this gradient (100 nN) drives the bacterial cell downwards towards the nanopillars, instigating the elastic deformation of the bacterial cell wall. If pressure or stress exceeds the yield strength of the wall, the latter undergoes creep until rupture. This model can be used to justify the bactericidal effect of a nanostructure on bacteria yet implies a non-cytotoxic effect of such surfaces on mammalian cells. Since the force stems from a differential energy gradient of the nanopillars, the smaller volume of the

cell in contact, the larger the effect of the pressure induced on it by a single pillar. It is known that bacterial cells have sizes in the range of few hundreds of nanometers to a few micrometers, whereas mammalian cells are usually in the range of tens to hundreds of micrometers. Thus, according to this model mammalian cells undergo reduced amounts of pressure which do not cause damage to their membranes.

Another driving force suggested to cause the rupture of bacteria is the surface energy released as the cell wall binds to the surface [111]. If the total surface binding energy is larger than the stretching and bending energy, the bacterial membrane drops below the equilibrium drop height, the tensile stress exceeds the tensile strength of the bacterial membrane, then the membrane tears. In the case of slow binding, influenced by active interactions and responses of the bacteria towards the surface such as hydrophobic interactions, the bacterial death occurs gradually. In the case of fast binding, induced by physical-chemical forces (i.e., van der Waals forces and hydrogen bonding), the rupture occurs instantaneously as the bacterial cell wall binds to the top of the nanopillar with a surplus of energy.

Adhesion-driven deformation of the bacterial envelope as it adsorbs onto a nanopillar was also considered in the computational work of Velic et al. [130]. In this model, the adhesion was considered as an evolving “bond front” and applied as a downward pressure load on an area that increased in size incrementally.

When considering the motility of some bacterial strains or bacteria in flow conditions, the frictional behavior in contact with such surfaces becomes of high importance. Studies report frictional instabilities experienced in contact with nanostructured surfaces where shear tracing showed irregular and sharp peaks across such structured surfaces compared to smooth surfaces [138]. These sharp spikes can present lateral force contributing to the disruptive action of nanoscale features on the bacterial cell wall influencing the bacterial stress response that could manifest in turgor pressure fluctuation and ROS production. The frictional behavior of nanostructured surfaces is highly influenced by the surface specific

parameters such as surface roughness parameters [139], nanofeature geometries and their densities [140]. In this light, studying nanostructured surfaces exhibiting different frictional behaviors and correlating it with their bactericidal performance, could give new insight into the optimisation of the mechano-bactericidal surfaces.

Critical Action Sites

The competing “penetration” and “stretching” mechanisms can be considered analogous to the competing critical action sites of a bacterial membrane in analytical and computational modelling. In most energy-based models, the critical action site is in the area suspended between the two pillars [92,97,98,107,141]. That is because in those models, the bacterial membrane deforms non-uniformly. The stretching of the suspended bacterium occurs to accommodate to the active adsorption onto the nanopillar. In an extension to work by Pogodin et al. [92], Wu et al. [107] found that by varying the heights of adjacent nanopillars, a higher stretching degree is induced on the membrane region adsorbed onto the nanopillar than on that suspended region. This model is illustrated in **Figure 2-12** (b). In most computational models, however, the entire membrane undergoes the same force (body force) and the strain across the entire membrane is uniform. The presence of the nanopillars restricts that deformation and induces tension in the membrane at the pillar apex. Interestingly in the computational model that considered adhesion-driven rupture, the critical action sites did not coincide between the layers of the cell wall model of a Gram-negative bacterium (**Figure 2-12** (c)). Uniquely, the cell wall was modelled as a plasma membrane and peptidoglycan made up of two leaflets. The critical action site for the whole cell envelope was found at the pillar apex as both the inner leaflet and plasma membrane fail at that location with a strain much greater than that transpiring anywhere in the outer leaflet. The critical action site of the outer leaflet of the peptidoglycan, which is in direct contact with the pillar, remains in the suspended region. That is due to the strongly modelled bond between this leaflet’s adsorbed region and the pillar, that forces the reallocation of deformation to the suspended region. Unlike most models, this study did not over-simplify all the complexities

of a bacterial membrane and its components nor assumed it to be a planar elastic layer (plane strain). It instead brought attention to (i) the importance of a more detailed multi-layered cell wall model, (ii) the need for three-dimensional analysis of this non-developable problem and (iii) the significance of force application as a “bond front” mimicking adhesion.

In layered bacterial membrane models, as in reality, the stresses that can be withstood by different layers (different materials) vary. In addition, some materials can resist compression more than tension or vice versa. Knowing these material details can help shape the design of the nanostructure to engineer the location of the critical action site appropriately. This can be done by understanding and adjusting the geometrical considerations of nanostructures. It implies the need for developing material constitutive models of bacterial membranes in order to model complex membrane structures reliably.

Nevertheless, considering the complexities of the bacteria-nanostructure interaction, factors beyond geometrical considerations can be influential as discussed next.

2.3.1.2.1.1 Factors Influencing the Bactericidal Activity of Nanostructured Surfaces

The mechano-bactericidal effect is a complex interplay of bacteria and substrate-dependent factors (see Figure 2-13), which may be classified into four main categories: geometric, biological, electric and interfacial physical factors.

The structural dimensions including the nanofeature radius, shape, interspacing/pitch/nanofeature area density and height/aspect-ratio are the main constituents of the geometric factor.

Bacteria-nanofeature Contact Area: Radius and Shape

The tip size of the nanofeature is of great importance because it is the first point of contact between the bacteria and the surface. Studies suggest that a smaller tip radius induces higher pressure on the bacterial membrane and enhances the bactericidal effect of a nanostructured surface [91,94,108,142]. Some other

studies based on the analytical models, as previously discussed, alluded to the fact that a larger radius provides a wider contact area. This pushes the suspended region of the membrane to try and accommodate for the perimeter change by stretching and ultimately rupturing [129,130].

It is evident that there is a limited range of radii in which nanopillars exhibit enhanced bactericidal effects. According to simulations, that range falls between (50 to 80) nm [98], where the radii is big enough to anchor the bacterial membrane, yet small enough to push bacterial membrane deformation to rupture.

The shape of the nanofeatures relates directly to the available contact area (see Figure 2-13 (a) and 2-14 (A)). Mo et al. [143] tested micro and nano arrays of cones and pillars and found that nano-cones, as opposed to pillars, possess enhanced bactericidal activity due to the tip sharpness. The slope presented by the nano-cone features also plays a role in enhanced bactericidal activity as it increases the contact area along the vertical region of the feature [107].

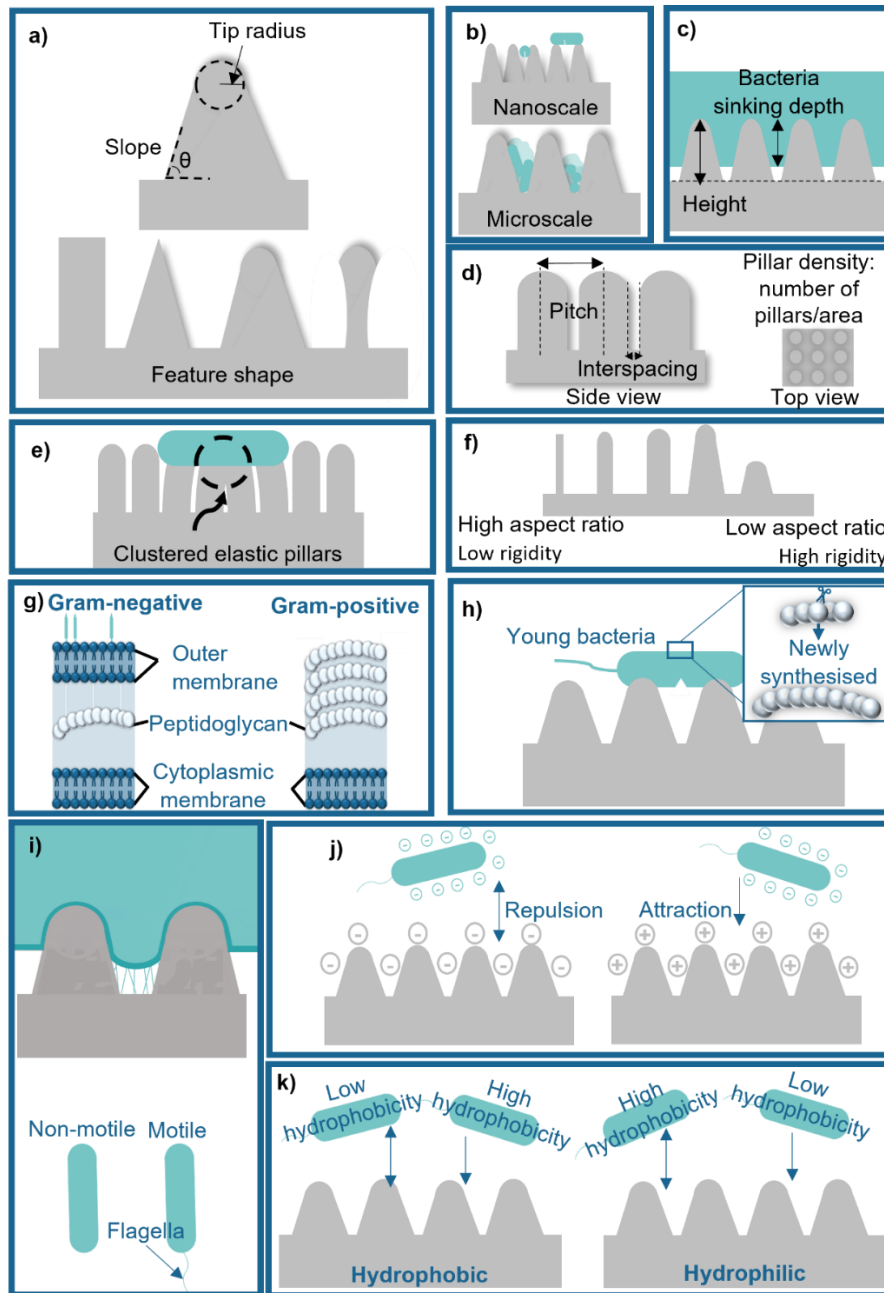


Figure 2-13 Factors affecting the efficacy of a mechano-bactericidal surface for a specific bacterial strain. (a) Contact area controlled by feature radius and shape, (b) length scale of the bacteria/structures, (c) height of the structures, (d) the nanofeature interspacing and array density, (e) elasticity of the pillars/structures, (f) aspect ratio-controlled rigidity, (g) Gram stain of the bacteria, (h) age of the bacteria, (i) Surface appendages of the bacteria, (j) electrical charge of the surface and bacteria, (k) the bacteria/surface interfacial-physical factors.

Nanofeature Interspacing and Array Density

For a nanostructured surface to exert stress on a bacterium, the interspacing of the structures needs to be smaller than the diameter of the bacterium, be it cocci or rod-shaped. The bacterium can otherwise align to the spaces between the nanopillars, proliferate and develop a biofilm (see Figure 2-13 (b) and 2-14 (Be)) [144]. The height of nanopillars should also accommodate the maximum stretching of the bacterial cell wall and be larger than the sinking depth of the bacterium as shown in Figure 2-13 (c), otherwise the bacterium will only experience elastic deformation and then rest on the bottom surface with no additional mechanical deformation to cause lysis.

Simultaneously, conflicting reports on the role of interspacing in bactericidal efficiency can also be seen. For instance, Mirzaali et al. [108] suggested that higher interspacing increases the stretching degree of the bacteria which improves the bactericidal efficiency of the surface but warns that an increased interspacing could lead to cytotoxicity rendering it unsuitable for *in-vivo* applications.

Their FEA study predicted a combination of width and interspacing ($W = 50$ nm, $IS = 300$ nm) to be vital in inducing bactericidal effect against *S. aureus* while avoiding cytotoxic reactions. Conversely, Modaresifar et al. [116] investigated the effect of interspacing of silicon nanostructures on the viability of *S. aureus* and found that a lower pitch of interspacing led to an improved bactericidal activity with the highest bactericidal efficiency achieved for a spacing of 100 nm.

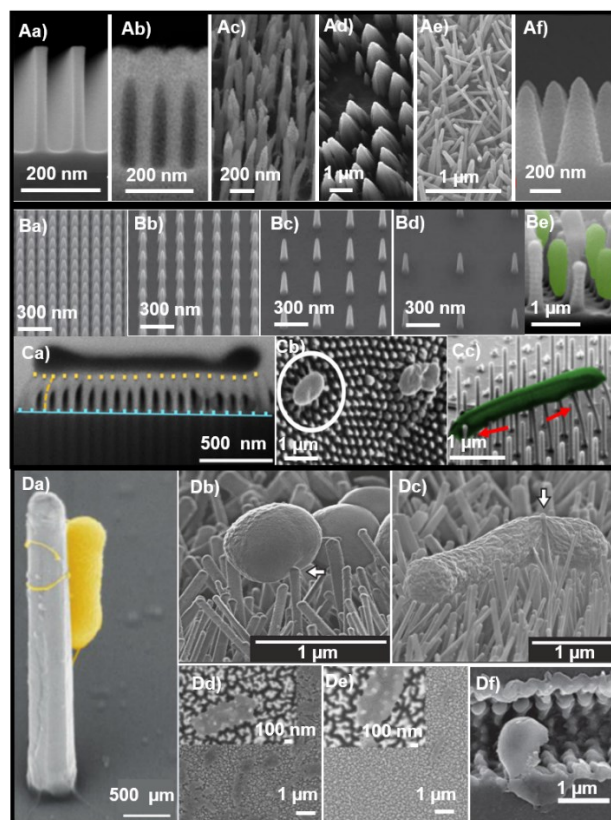


Figure 2-14 A: Ranging Ranging nanostructure shapes and tip radii; Aa) blunt tip nanopillars [129], Ab) cotton-swab shaped nanopillars [129], Ac) sharp nanopillars[91], Ad) sharp conical nanofeatures, reprinted from [99] by permission of AIP Publishing. Copyright 2016, Ae) nanowires [117], Af) cicada-like conical nanofeatures, reprinted from [145] by permission of ACS Publications. Copyright 2015. B: Ranging interspacing and feature densities [116]; Be) *P. aeruginosa* aligned between nanopillar interspacing, reprinted from [144] by permission of ACS Publications. Copyright 2010. C: Elastic and high aspect ratio structures; Ca) enhanced bactericidal effect, reprinted from [146] by permission of PNAS. The yellow dots represent the bacteria-structure interface, blue line represent the alignment of the substrate. Cb, Cc) Impeded bactericidal effect. Cb: Reprinted from [143] by permission of Elsevier. Copyright 2020. Cc: Reprinted from [147] by permission of ACS publications. Copyright 2016. D: Biological and interfacial physical factors affecting bactericidal efficiency; Da) bacterium (i.e. *S. oneidensis*) flagella wrapped around micro-sized pillar to maximize the contact area, reprinted from [148] by permission of ACS Publications. Copyright 2013. Db) Gram positive *S. aureus* hardly perturbed on nanowire-like structure [117], Dc) Gram-negative *E.*

coli severely deformed on nanowire like structure [117], Dd, De) superhydrophobic structures induce higher hydrophobic-*P. aeruginosa* attachment and death than superhydrophilic structured surface, reprinted from [102] by permission of ACS publications. Copyright 2017. Df) Positively charged nanostructured surface induces rupture of gram-positive *S. aureus* [149].

Velic et al. [130] agreed that a reduced interspacing increases the bactericidal effect of a nanostructured surface. The authors argued that the simulation studies suggesting the larger interspacing increases the deformation of the envelope had assumed that a “constant” load was being applied. This assumption results in load distribution over a given number of pillars, thus directly inducing more deformation with further spaced pillars and less contact points. On the other hand, in the case of bacterial interaction with nanostructures, the interaction forces are not distributed, but developed at each individual nanopillar.

This signifies that more contact points will induce more deformation and a smaller interspacing is bactericidal enhancing.

Some studies suggested evaluating the area density of nanofeatures (see Figure 2-13 (d)), instead of their interspacing or pitch, as an important parameter in the adhesion pattern and therefore the stretching degree of the attached bacterial cells [107]. Kelleher et al. [150] observed a linear correlation between bactericidal activity and the number of nanostructures with which a bacterial cell comes in contact with. In their findings however, the surface that exhibited the highest bactericidal efficiency (and the highest pillar density) was the surface with the highest feature aspect ratio (i.e., ratio of height over width) of around 1.54 with height of 241 nm, pitch 165 nm and diameter 156 nm. In this case, the height and pillar density parameters cannot be decoupled and the distinction of which parameter had a more significant effect on the bactericidal activity was not possible.

Aspect Ratio and Rigidity

Few studies have investigated the effect of the aspect ratio and the rigidity of the nanopillars on the bactericidal efficiency of mechano-bactericidal nanostructures.

When dealing with elastic pillars, interpillar adhesion and clustering could partially compensate for the force exhibited on the bacterial membrane [146,151]. This can hinder the bactericidal ability of a surface which is shown in Figure 2-13 (e) and 2-14 (Cb), however the flexibility of high aspect ratio structures has been shown to enhance elastic energy storage in nanofeatures. They release this energy by bending when in contact with bacteria, which improves the bactericidal activity of the nanostructured surface as shown in 2-14 (Ca, Cc) [152]. Thus, a threshold exists by which the deformation of the elastic pillar remains favorable to the bactericidal action. The deformation of these nanofeatures is dependent on surface material properties, nanofeature dimensions and the adhesion force of the bacterium to the substratum surface and can be expressed as (in the case of nanopillars) [153]:

$$\Delta x = F_{adhesion} / k \quad (2-5)$$

Where k is the pillar stiffness defined as [153] :

$$k = \pi E_{material} \frac{3r^4}{4l^3} \quad (2-6)$$

where r is the radius of the nanopillar and l is its length.

As opposed to the elastic modulus of a bulk material, stiffness or eigenvalue of a structure, a nanostructure pillar in this case is influenced significantly by its aspect ratio (Figure 2-13 (f)). The high aspect ratio nanostructures on the wings of *Palapsalta eyrei* (cicada species) possess an enhanced bactericidal activity and short kill time compared to wings from other cicada species that contain lower aspect ratios, namely *Psaltoda claripennis* and *Aleeta curvicosta* [154]. The height of the nanofeatures varies from 100 nm to 2 μ m, and this largely contributes to the aspect ratio while the diameter is controlled in a limited margin within the fabricated nanopillars. Ivanova et al. [146] reported that a height of 360 nm yielded the most efficient results against both *P. aeruginosa* and *S. aureus* with little to no clustering of the pillars.

Generally, the work done in replication and fabrication of mechano-bactericidal structures has failed to report the repeatability in the feature dimensions and geometries which limits direct comparison of results produced by different research groups [25]. However, of the few studies that have successfully reported characterised morphology of the bactericidal surface, the dimensions of effective surfaces were in the range of 100 nm to 1 μ m height, (10 to 300) nm diameter and less than 500 nm spacing [155].

Biological Factors

As the mechano-bactericidal effect is surface and bacteria dependent, it is imperative to pay attention to the biological factors that might influence the bactericidal action observed. These factors, unlike geometric ones, are not static and most of the time cannot be controlled.

For instance, the composition of the bacterial cell wall, as classified by the Gram stain, plays a role in governing its susceptibility to the mechano-bactericidal surfaces. Figure 2-13 (g) contrasts the differences between a Gram-positive and Gram-negative bacterial membrane structure. Gram-positive bacteria possess an inner membrane and a thick peptidoglycan layer (20-100 nm), whereas Gram-negative bacteria possess outer and inner membranes and an intermediate peptidoglycan layer (only a few nanometers thick) [156]. Gram-negative bacteria are thus more susceptible to the killing effect of the nanostructures than Gram-positive bacteria (see 2-14 (Db, Dc)). Pogodin et al. [92] found that exposing Gram-positive bacteria (*B. subtilis*, *Planococcus maritimus* and *S. aureus*) to microwave radiation reduces their rigidity and increases their vulnerability towards the mechano-bactericidal effect of the nanostructured cicada wing surface. In the case of sharp-edged surfaces like black silicon (bSi) with very small tip radii, the sharp edge is able to disrupt both Gram-positive and Gram-negative bacteria leading to their death [91].

In addition to the Gram stain, the possession of surface appendages has also been identified as an influencing factor (see Figure 2-13 (i)). In a recent study by Ishak et al. [157], bacterial surface proteins have been observed to play a role in

facilitating the deformation of the cell wall as represented in Figure 2-13 (i) (top). Additionally, the motility and possession of motile appendages (e.g., fully active flagellum) has been associated with a higher probability of bacteria being affected by the bactericidal surface [158]. Jindai et al. [119] suggested that since the flagellum makes the first direct contact with the structured surface, it gets entangled with the surface nanofeatures leaving the bacteria some space to move. The bacteria then suffer abrasions after hitting the surface repeatedly causing effusion of intercellular fluid and death. An SEM micrograph presented in 2-14 (Da) shows how the flagellum of a bacterium wraps around a micro-sized pillar.

Aside from the Gram stain and motility, a bacterium's membrane will pass in phases of fluctuating rigidity at a young age (< 6 h [159]) as shown in Figure 2-13 (h). This fluctuation occurs when existing peptidoglycan sacculi breaks to incorporate new material and synthesis of a new layer of peptidoglycan is under progress [159]. Thus, younger bacteria are more susceptible to the bactericidal action of nanostructured surfaces than matured bacteria.

A few studies have been conducted to test the effect of mechano-bactericidal nanostructures on the adherence and differentiation of eukaryotic human or human-like cells. A common denominator between all these studies is unusual elongated morphology of the cells adhered on these surfaces along with the lack of cellular spreading compared to control smooth surfaces [160]. However, Le Clainche et al. [161] reported that hASCs were able to proliferate and maintain their ability for trilineage differentiation on such nanostructured surfaces. Additionally, Bhadra et al. [78] showed that human fibroblasts are able to proliferate and provide a high area coverage on such surfaces. Modaresifar et al. [162] found that the metabolic response of preosteoblast cells was reduced after 14 days on the most efficient bactericidal (40 % dead *S. aureus*) surface tested compared to control flat surfaces. Additional standardised studies to study the cytotoxicity of mechano-bactericidal surfaces are needed in order to be able to compare the performance of these surfaces across the board and their applicability to implant surfaces.

Electric and Interfacial-physical

From the mechanisms formerly discussed, the consensus is that mechano-bactericidal activity is a contact phenomenon. Mechano-bactericidal activity can only be invoked by contact, adsorption and adhesion between the bacteria and the surface. The adhesion of the bacteria with the surface is therefore a noteworthy factor in making the surface bactericidal. Adhesion is heavily affected by the electrostatic charge of the bacteria and the substrate and their respective wettability, among other factors (i.e. Lifshitz–Van der Waals forces and Brownian movement forces) as explained by the extended DLVO (XDLVO) theory [54].

Bacteria are generally negatively charged. If the surface is also negatively charged, repulsion will be dominant (Fig. 8 (j)) and the surface might be called anti-biofouling. For mechano-bactericidal surfaces relying on adhesion to kill bacteria [155], positive charges can be beneficial as they advocate for the attraction of the bacteria to the surface. Chen et al. [149] combined surface structuring by femtosecond laser and positively charging the surface by Layer-by-Layer polyelectrolyte coating in order to enhance the bactericidal effect of the borosilicate glass surface against *S. aureus* and *E. coli* as is presented in 2-14 (Df).

Generally, the more hydrophobic the bacteria, the higher is its affinity to a hydrophobic surface and greater is the adhesion force between the bacteria and surface as shown in Figure 2-13 (k) and 2-14 (Dd, De). Bacteria of different strains can have differing wettability (hydrophobic or hydrophilic) however, hydrophobicity/hydrophilicity of bacteria can alter depending on the environmental changes and bacterial stage of growth [163]. Both hydrophobic and hydrophilic surfaces can exhibit bactericidal actions against a range of bacterial strains. This leads to the inconclusive role of wettability in the mechanism of mechano-bactericidal action [102,164].

It must be noted here that the evaluation of different factors impacting the bactericidal effect of nanostructures was performed either through simulations or experiments. During simulation investigations, the models were based on

stretching/penetration mechanisms, which discount the biologically dynamic effects of bacterial cells. In the experiments, the techniques used to evaluate the bactericidal activity of a surface were limited to detection of live/dead bacteria. This limits the understanding of the effect of changing geometrical and electric and interfacial physical factors to how those changes are physically affecting the rupture/death of bacteria. It does not explore the effect of varying those factors on any possible biological mechanism that is leading to bacterial death. In section 2.3.3, various experimental techniques that have been used to evaluate bactericidal surfaces and the interaction between bacteria and nanostructured surfaces are further discussed.

In the following section, a concise summary of chemical surface modifications is provided to broaden the comprehension of the anti-bacterial surface modification landscape.

2.3.2 Chemical Surface Modifications

The use of anti-bacterial agents, particularly in nano-sizes, such as silver (Ag), copper (Cu) and Zinc (Zn) exhibit potent bactericidal properties through two primary mechanisms: (i) the release of ions which bind with proteins and enzymes found on the bacteria's cellular surface, destabilise the cellular membrane and disrupt the metabolic pathway and (ii) the production of reactive oxygen species (ROS) at the cell membrane which leads to irreversible DNA damage [8,9]. As silver exhibits both anti-bacterial and osteogenic properties [166], the impregnation of implant materials with silver has gained a lot of traction not only in research but also in industrial scale applications [167].

The impregnation of silver onto implant materials generally comes in the form of a coating with few micrometres of thickness. For instance, Accentus medical Ltd. has introduced Agluna® [168] as a surface treatment technology that utilises a modified anodization process to create silver containing micro-pits on implant surfaces which is already being implemented in a clinical setting [169]. Other techniques such as electroplating silver on medical implants are being applied clinically [170]. However, these solutions are still not widely implemented on

implant surfaces requiring osseointegration [170]. Due to the adverse effect of coating delamination, research into other techniques of introduction of anti-bacterial material into implant materials have been underway. Ion implantation has been a front runner in this regard.

Ion implantation is a method in which ions are accelerated in vacuum, passed through a magnet that separates them according to a desired species and charge, then passed through a series of electrostatic and magnetic lens elements and targeted towards a substrate. As these energetic ions penetrate the substrate, they lose their energies due elastic (low energy ions) and inelastic collisions (high energy ions). The depth of penetration is related to the energy of the ions which is usually between several keV to few MeV [171], and the higher the energy the deeper the penetration. But, generally, the modification is confined to the near surface environment and the bulk properties of the material are maintained [172]. The parameters that define ion implantation are the ion species, beam energy (ion energy), fluence (number of ions that bombard a surface) and beam current or flux. This method serves as a means of immobilisation of Ag particles.

There are several ion implantation techniques [173–178], as presented in Figure 2-15. Beam-based techniques use a focused or mass-filtered ion beam to implant ions which have several limitations including line-of-sight restriction, non-uniform implantation on freeform surfaces, low throughput (except for ion shower implantation) and possible sample damage.

Among the various ion implantation methods presented, plasma Immersion Ion Implantation (PIII) stands out as a versatile and promising technique for modifying the surfaces of materials with complex shapes, free from the line-of-sight restrictions, non-uniform implantation and sample damage seen in conventional ion implantation approaches. Typically, a PIII system consists of a vacuum chamber containing a workpiece stage, a plasma source, and a high-voltage pulse modulator [121]. During PIII processing, the samples are submerged in a high-density plasma and subjected to a high negative pulsed potential relative to

the vacuum chamber wall. This potential repels electrons from the samples while attracting the positive ions in the plasma, forming a plasma sheath around the samples [178].

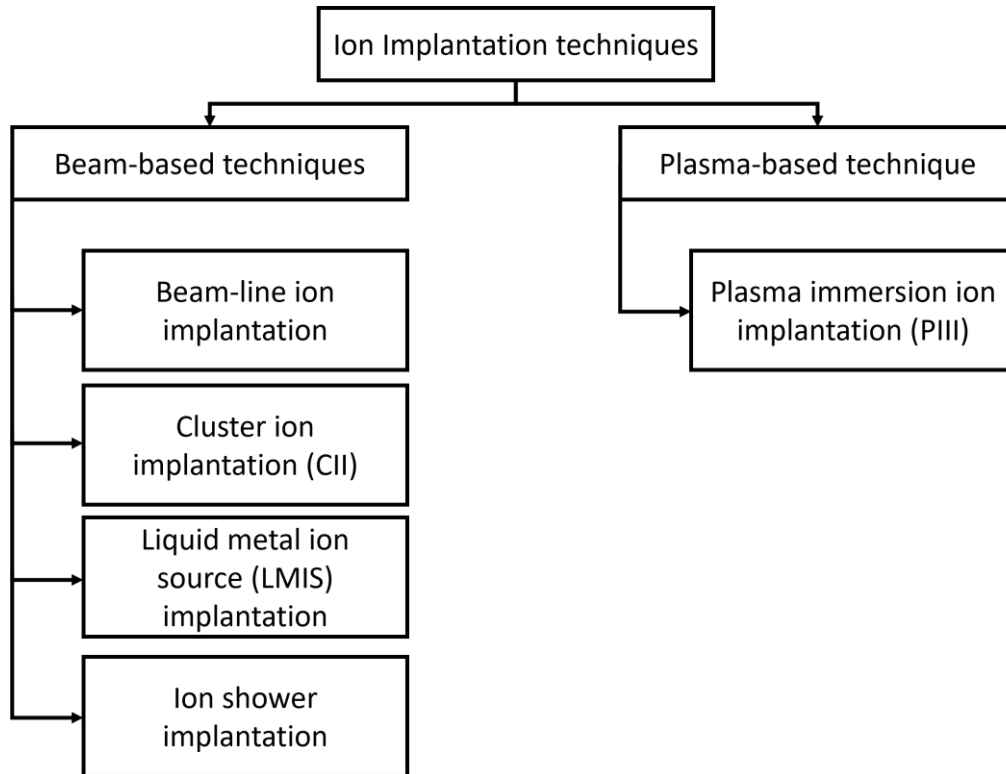


Figure 2-15 Ion implantation techniques

Silver nanoparticles (Ag NPs) are gaining more attention in this regard due to their increased reactivity compared to bulk metallic silver owing that mainly to their larger active surface area [179]. Under environmental and physiological conditions, Ag NPs oxidise and releases Ag^+ ions, which are theorised to be the hub of the bactericidal action of silver by disrupting bacteria membrane and reducing their viability [179,180]. This happens through strong interactions between the ionic Ag^+ and thiol groups of cystine residues of proteins present in bacteria cell walls [181]. Some studies suggest that the bacterial walls get pitted and exasperated with Ag^+ particles leading to their death [182]. According to the kinetic model developed by Zhang et al. [180] based on the hard sphere theory using the Arrhenius equation, the rate of Ag^+ released is directly related to the dose of Ag implanted. Mei *et. al.* [183] investigated the release of Ag ions of

samples implanted with different doses of silver incorporated in TiO₂ nanotubes using low-voltage Ag PIII. They concluded that silver release from low concentration samples was higher than that of high concentration samples in early stages, but the high concentration samples show a more sustainable release in long term. Consequently, the samples with higher dosage of Ag implanted were more bactericidal [184]. This has been corroborated by several studies in which the implantation dose was varied using metal vapor vacuum arc implantation methods [185–188]. A collection of silver ion implanted surfaces' anti-bacterial efficiencies in function of their silver ion implanted dosages is presented in Figure 2-16.

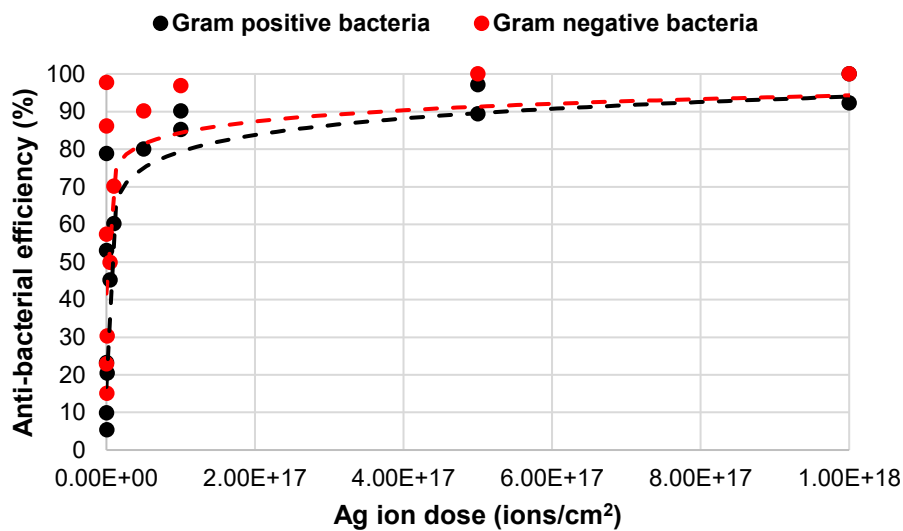


Figure 2-16 Anti-bacterial efficiency of silver implanted materials, in function of Ag implanted dose, for both Gram positive and Gram negative bacteria. Data for the plot were extracted from [185,189,190].

In addition, to having good anti-bacterial activity, Ag ion implanted substrates exhibit good osseointegration tested in-vitro through osteoblast-like cells (MG63), human epithelial cells and fibroblast [183], good cytocompatibility tested through human bone marrow stromal cells (hBMSCs) and in-vivo with observation of direct bone formation around femur implant in rats [184].

Although anti-microbial materials for biomaterial surface applications have only recently been on the uprise, some anti-microbial materials have been employed a long while ago in applications such as disinfecting solutions, creams and wound dressing [191,192]. The most prominent example of that would be silver, it is especially prevalent in the treatment of burns and chronic wounds infections [191]. However, bacterial resistance to silver has been recently detected in *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Staphylococcus aureus* Clinical isolates [193]. Additionally, a recent scoping review has identified bacterial resistance to heavy metals including copper and zinc and a correlation to antibiotic resistance [194]. In addition to the potential resistance bacteria can build, anti-bacterial metals have the potential of being cytotoxic in high concentrations [195].

Due to the increasing threat of anti-bacterial resistance to chemical modifications, it is becoming timelier to explore topography-based anti-bacterial strategies or surface modifications.

2.3.3 Techniques Used to Evaluate Anti-bacterial Surfaces and Bacteria-Surface Interactions

As bacteria-surface interactions, specifically regarding nature-inspired textured surfaces, remain largely unknown in the light of evolving experimental procedures, a range of techniques have been used to study anti-bacterial surfaces mostly at discrete time points.

To study this interaction, anti-bacterial surfaces are first incubated with a volume of bacterial suspension (*i.e.*, a nutrient-rich broth containing bacteria). This can be done using one of the three main set-ups presented in Figure 2-17 (a); the overlay or drop-test method where a droplet with large volume is deposited onto the nanostructured surface [196], the spray method where the bacterial suspension is sprayed over the surface [197] and the immersion method where the nanostructured sample is fully immersed in the bacterial suspension [81]. The overlay and spray methods introduce surface tension at the perimeter of the

droplets which might induce an external force that is able to drive the bacteria towards the nanostructures, hence stimulating a high mechano-bactericidal reaction (Figure 2-18 (d)). Since the overlay method consists of one large drop instead of smaller dispersed droplets as in the case of spraying, it is more suitable to use when evaluating mechano-bactericidal surfaces. The high bactericidal effect-inducing surface tension at the droplet perimeter can be eliminated from consideration when analysing the results. These two methods cannot be used to test the functionality of the surfaces for long periods of time as they are prone to evaporation. However, they are suitable for studying initial bacteria-surface interaction. The immersion method, on the other hand, provides the possibility of long duration functional analysis, continuous durability analysis (> 24 h), studying potential self-cleaning cycles of mechano-bactericidal surfaces [115] and eliminating surface tension-inducing bubbles by using dynamic incubation [198].

To assess and compare the anti-bacterial performance of various nanopatterns, it is critical to consistently test the *non-specific* bacterial response using the same type strains and growth conditions. However, due to vast diversity of bacteria, which is reflected in differences of the bacterial cell wall composition and thickness [199,200], wettability and *specific* host interactions of clinical isolates, different strains that belong to the same species, may respond differently to the same nanopattern [201,202].

One important aspect to study, which is largely absent in literature, is the protein preconditioning of anti-bacterial nanostructured surfaces, especially when considering end applications such as biomedical implants. As these implants are inserted into the body, the first thing that comes into contact with them is blood and this results in instantaneous formation of a protein film. This protein film might mask or reduce the effect of the mechano-bactericidal surface as it can fill the spacings between the nanostructures rendering them ineffective. That is why, it is of utmost importance to study serum preconditioning of the surfaces prior to incubation with bacteria. Understanding how that protein film forms on anti-bacterial structured surfaces can help the structure optimisation process to be more efficiently anti-bacterial.

After incubation, the first objective to seek when studying a mechanically designed bactericidal surface is to evaluate the effectiveness of this surface towards a specific bacterial strain. For that, it would be beneficial to know the number of bacteria attached to the surface (dead and alive). Additionally, it is imperative to ensure that the surface is bactericidal by detecting the count of dead bacteria over a timespan and, to quantitatively determine its bactericidal efficiency expressed as:

$$\eta_{bactericidal}(\%) = \left((A - B) / A \right) \times 100 \quad (2-7)$$

where A is the number of dead bacteria on the test sample surface and B is the number of dead bacteria on the reference sample surface.

Previously, bactericidal efficiency has been evaluated using the colony counting or colony forming unit method (CFU). It is a method used to quantify the number of colonies that can grow in a nutrient medium (agar) from a swab of bacteria-exposed test surface or after rinsing the bacterial test surface and replating the suspension medium. This method can indirectly quantify the number of live and attached bacteria on the test surface.

However, the CFU method does not guarantee complete removal of all bacteria from the surface and often times, the technique for removal (e.g., sonication, enzyme) may cause cell death. Additionally, CFU underestimates the true killing efficiency of the surface as it fails to account for the number of dead bacteria on the surface and the dead bacteria that could have been released out onto the suspension after death [123,203]. This method evaluates the ability of a bacteria to attach and proliferate on the surface rather than the killing ability of a surface. That is why this technique might be better suited to evaluate the anti-biofouling functionality rather than the bactericidal efficiency of a test surface, unless accompanied by other forms of viability quantification such as Live/dead staining. Although theoretically, there are distinct definitions of bacteriostatic versus bactericidal surfaces, in practice they are ill-defined. For instance, when discussing bacterial killing agents or antibiotics, a general consensus is that in an

incubation duration of (18 to 24) hours, if the agent is able to kill (90 to 99) % bacteria, it is called bacteriostatic and if it is able to kill >99.9 % bacteria, it is considered bactericidal [204]. This is to account for the *in vivo* factors in infection with time. However, when discussing bacteria killing surfaces, the quantitative value is not specified for the designation between bactericidal and bacteriostatic. This constitutes an empirical need to identify the clinically relevant threshold above which the rate of bacterial death is quicker than the multiplication of bacteria in the deep prosthetic surgical site infection. Understanding this threshold will allow for the consistent labelling of anti-bacterial nanostructured surfaces in the biomedical field.

The durability of the anti-bacterial function is under question and experimental tests can be intriguingly used to understand the fate of bacteria after they become non-viable on the surface in the case of mechano-bactericidal surfaces. Intuitively, one would think that the non-viable bacteria would remain in the pits of the mechano-bactericidal structured surface and constitute a means of adhesion and nutrition for newly adhering bacteria. Another possible fate of the non-viable bacteria however would be the detachment and return to bulk fluid. In the latter case, considering the number of non-viable bacteria present in the bulk liquid would be important to quantify the bactericidal efficiency of the test surface. It is also vital to quantify the time of the killing of a bacterium to judge the degree of surface's lethality against high bacterial loads.

The experimental techniques used to address these points are relatively straightforward. For instance, the number of bacteria adhering to the surface and/or present in the bulk liquid can be evaluated by different types of assays in conjunction with labelling methods such as fluorescent staining, dye staining and several others that would allow the differentiation between live and dead bacterial cells as presented in Figure 2-17 (e). Most commonly, Confocal Laser Scanning Microscopy and BacLight viability testing kits (Syto9™ fluorescent green & Propidium iodide fluorescent red) are used for live/dead assays as shown in Figure 2-17 (a). Through this method, Nguyen et al. [115] tracked *P. aeruginosa*

bacterial cells on silicon mechano-bactericidal surfaces, observing their detachment as presented in Figure 2-18 (b).

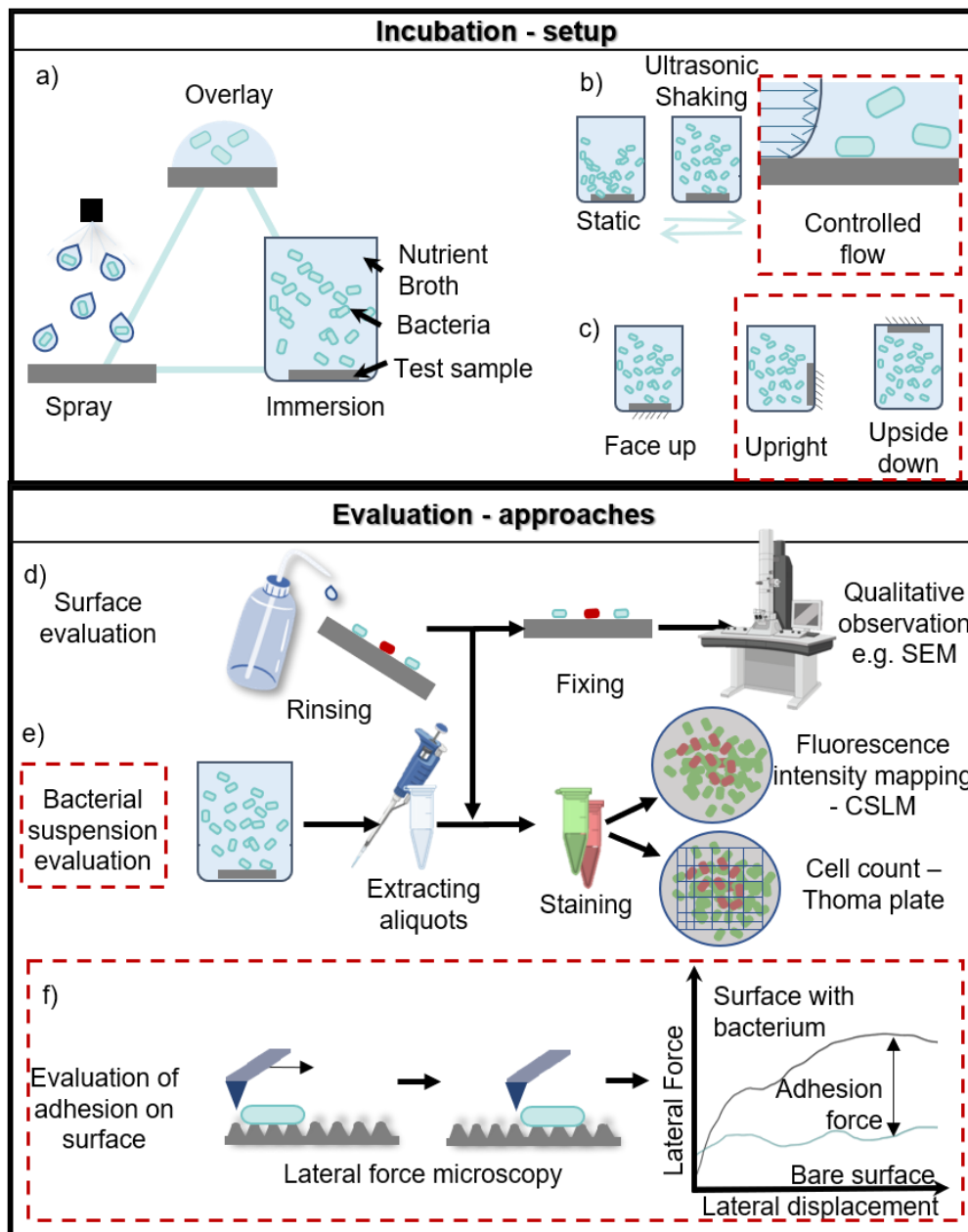


Figure 2-17 The incubation set-up including a) the method and volume of bacterial suspension contact with the sample surface: the overlay, spray and immersion methods , b) the flow conditions during incubation: static and dynamic comprising ultrasonic shaking and controlled flow, c) the sample orientation: face up, upright and upside down, d) Surface evaluation by rinsing, e) the bacterial suspension live/dead cell count methods, f) the evaluation of bacterial adhesion on

nanostructured surfaces using lateral force microscopy. The dotted red rectangle denotes the areas that are least explored or unexplored as of yet.

This test method is based on the assessment of the membrane permeability of bacteria. Syto9™ is a dye that can enter live and dead cells alike, while Propidium iodide or PI can only stain dead cells. There have been a few problems associated with the use of these dyes in determining cell viability, for instance in dye bleaching due to overexposure, low intensity of PI especially at high dead bacterial loads and orange-yellow coloring of some bacteria which is attributed to the slow permeability of PI after SYTO9 staining. That is why care must be taken while performing measurements with such dyes, by for example quickly and efficiently taking the measurements and closing the microscope shutter in between captures to delay the bleaching effect. Additionally, AFM was used to quantify this detachment cycle using quantitative imaging mode (QI™) to avoid applying any significant lateral forces to the cells (Figure 2-18 (c)) [115,205].

Another important objective should be to evaluate how bacteria die on the mechano-bactericidal surfaces. As it has been shown by Jenkins et al. [117] and Ishak et al. [157], the number of bacteria dying from penetration does not necessarily equate to the total number of dead bacteria. That is why, to understand what mechanisms are at play in the bactericidal action of the test surface, it is important to quantify the number of bacteria that are dead specifically from penetration or deformation and compare it to the total number of dead bacteria. Bactericidal mechanisms, other than penetration and stretching, that surfaces can be exhibiting should be investigated to see if they are occurring, such as oxidative stress or others. Additionally, the driving force of the mechano-bactericidal activity needs to be investigated. For instance, samples can be incubated in different orientations to evaluate the gravity effect on the bactericidal activity and if it is indeed the driving force for it. The adhesion force between the bacteria and the surface can also be investigated to see whether it is enough to invoke the rupture of the bacterial membrane.

Furthermore, bio-AFM can be used to quantify the adhesion force between the bacteria and the surface by lateral force microscopy as presented schematically in Fig. 4 (f), and to quantify the elastic properties of the bacterial cell by AFM force spectroscopy through indentation and retraction [38,206,207].

These points are harder to address as techniques that could be used to do so require expensive, special, and sophisticated equipment and multidisciplinary training. Furthermore, investigating the detailed workings of mechano-bactericidal surfaces, specifically, ideally requires real-time monitoring and in-depth microbiological investigations. This research area is very fertile, and many basic discoveries are yet to occur. For example, investigation into whether cell death was mediated by ROS on mechano-bactericidal surfaces was only done recently through protein extraction, proteomic analysis and H₂O₂ labelling assays [117]. Cross-sectional observation of surface and bacteria contact performed through SEM-FIB or transmission electron microscope can aid in analysing if bacterial membranes are being deformed and penetrated by the nanostructures present on mechano-bactericidal surfaces and to what extent (Figure 2-17 (d)).

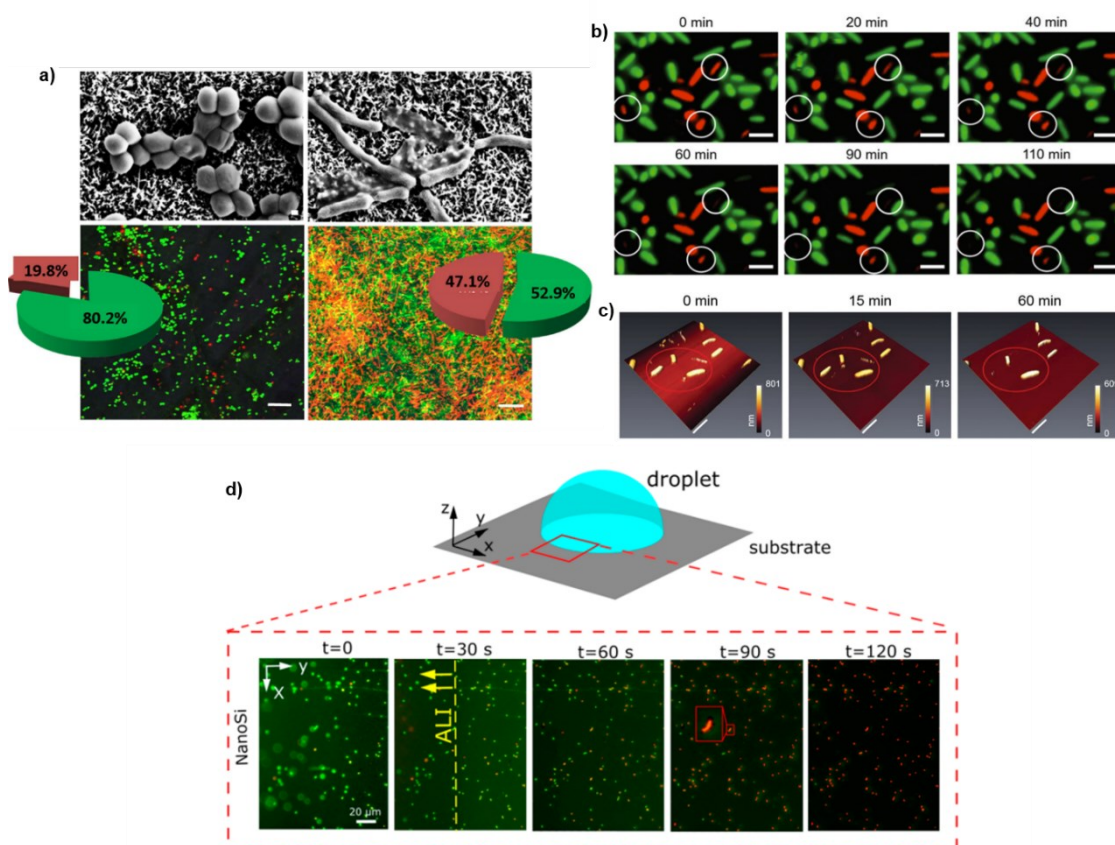


Figure 2-18 (a) CLSM images of viable and non-viable *S. aureus* and *P. aeruginosa* on nanostructured surfaces [96] (b) CLSM and (c) AFM tracking of non-viable bacteria on mechano-bactericidal nano-arrayed surface. Reprinted from [115] by permission of Royal Society of Chemistry. Copyright 2019. (d) Overlay/drop-test on Nano-Silicon surface: Viability of bacteria as a function of time as the bacterial droplet is subject to evaporation (ALI is the air liquid interface). Live cells are green and non-viable cells are red. Reprinted from [198] by permission of ACS Publications. Copyright 2020.

The effects of the flow on attachment and death of bacteria have not been extensively explored as most experimental studies follow static incubation protocols (see Figure 2-17 (b)). On one hand, the efficiency under static protocols can be underrated compared to the efficiency in flow conditions. That is because in static conditions, a build-up of bacteria may occur. Yet on the other hand, the efficiency under static protocols can also be overrated as stated by Valiei et al. [118]. They argued that in static conditions, air bubbles present between the

surface and the bacteria suspension could expand during microscopic evaluation and induce an external force that drives the killing effect of nanostructures. Whereas in dynamic conditions, these bubbles are simply eliminated by ultrasonic shaking. In fact, static conditions do not mimic reality. In biomedical applications there is fluid flow surrounding biomedical implants. Therefore, the determination of anti-bacterial efficiency should be done under realistic flow conditions (measured shear flow rate). It is however important to keep in mind that under dynamic conditions, the adhesion of bacteria decreases, and detachment increases compared to static conditions which might affect the results. It is also possible to postulate that under flow conditions, the bacteria experience advective flow which causes their collision with nanopillars with a significant impact force that might increase the killing efficiency in mechano-bactericidal surfaces [208]. On the other hand, a recent study by Kamarajan et al. [209] has reported an increased adhesion and survival rate for *P. aeruginosa* PAO1 on nanostructured surfaces under flow conditions. They attributed this behavior to the increased secretion of EPS adopted by the bacteria which masked the nanopillar tips. This goes to show that understanding the response of bacteria to anti-bacterial nanostructured surfaces in flow conditions is vital in determining the surfaces' true efficiency. A more detailed discussion of experimentation under flow conditions can be found in a review by Senevirathne et al. [123].

Additionally, substrate orientation (Figure 2-17 (c)) is important to help determine the driving force of the mechano-bactericidal mechanism.

A summary of the techniques used to evaluate and investigate mechano-bactericidal surfaces to date highlighting their advantages, limitations and the errors that could possibly occur by their usage can be found in the appendix 9A.1.

2.4 Engineering Nano and Micro Scale Surface Features

The advancement of micro and nano engineering techniques for surface structuring has enabled several technological advancements in various fields. For

instance, nano-engineering techniques have led the development of nanoparticles that can be used to deliver drugs to specific cells in the body, some which are being investigated for use in cancer treatments among other conditions [210,211]. Additionally, lab-on-a-chip devices and microfluidics are micro-engineered devices that have the potential to revolutionise medical diagnostics [212], environmental monitoring [213] and food safety testing[214] applications, to name a few. Furthermore, micro and nano engineering fabrication techniques have propelled the creation of functional textured surfaces including plasmonic biosensors [215], anti-bacterial surfaces [88,216], optical surfaces [145] and bioinspired dry adhesives such as the gecko tape [217] among numerous others.

To fabricate micro/nanostructured surfaces inspired from nature as described in section 2.3.1, a range of manufacturing methods have been investigated such as nano-imprinting, photolithography, micro milling, multi-tip diamond turning, hydrothermal treatments, micro-injection moulding, reactive ion etching, electron-beam lithography and atomic force microscopy [104,218]. There are however limitations for techniques such as these, which range from constraints on which materials can be processed, limitations in fabricating complex hierarchical structures on multiple scales (micro and nanoscale), inability to extend the process to modify complex curved surfaces (freeform surfaces) and low throughput as is presented in Figure 2-19.

In this work, structured surfaces were fabricated through a few selected advanced techniques such as ultrafast laser micromachining, deep reactive ion etching and nanolithography prototyping techniques. The following sections focus on a detailed review of each of these techniques and the advances which allowed them to be used to produce nano and micro scale structured surfaces.

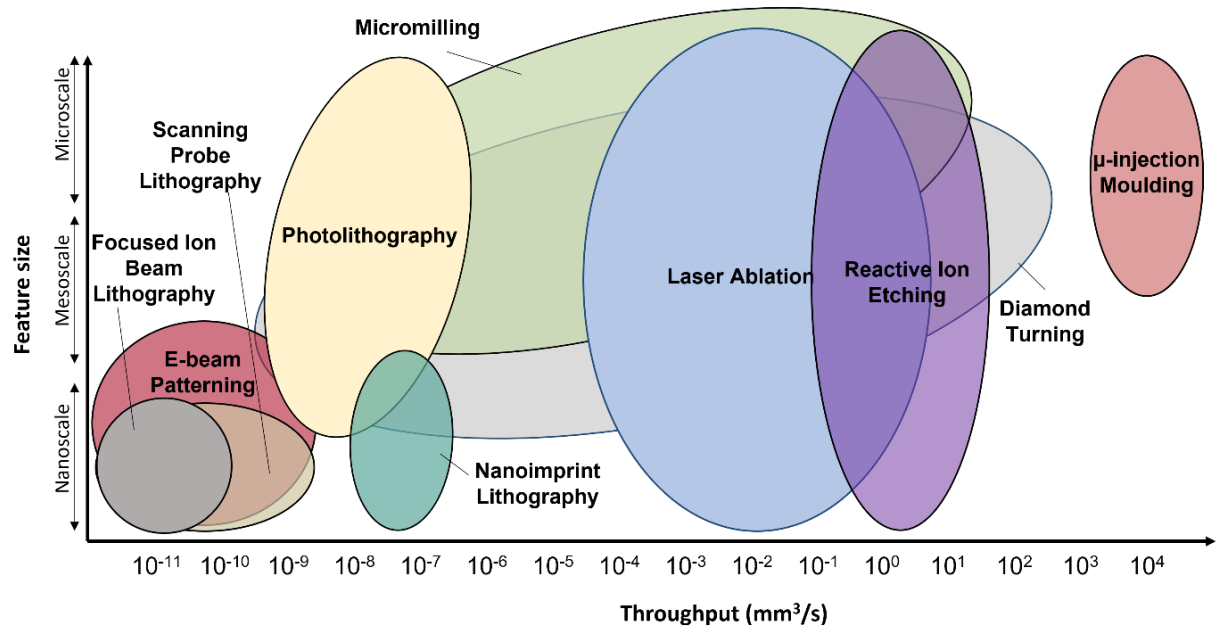


Figure 2-19 Ashby style chart of feature size versus throughput of various micro/nano fabrication methods. This chart is based on general literature, however highly specialised equipment set-ups could push the magnitudes of feature size and throughput further. Additionally, limitations on studies where the effect of laser wavelength can be directly compared has limited the subsection of laser ablation into femto-, pico- and nanosecond laser ablation [26,219,228–237,220,238,221–227].

2.4.1.1 Ultrafast (femtosecond) Laser Ablation

Laser ablation is a subtractive machining process that uses high power laser beams to ablate and remove material from a solid surface. Ultrafast laser ablation uses ultrashort laser pulse (typically on the femtosecond and picosecond timescale) to rapidly deliver energy to a target substrate leading to material removal with very little heat input into the rest of the bulk material. Irradiating materials using multiple laser pulses allows for a range of processing methods such as drilling, cutting, thin film removal and micro/nanostructuring.

The process of ablation is dependent on both the laser parameters and the substrate material. The laser parameters that impact the ablation process include the laser power, the pulse duration, the beam polarisation and the central wavelength of the laser source. Compared to longer pulse lasers, femtosecond

laser ablation has been shown to result in higher quality ablation with minimal evidence of heat affected zones, no recast molten material and improved surface finish when processing metals [239–241].

Femtosecond laser ablation can be used to process a wide range of materials such as metals [241], semiconductors [242], ceramics [243], polymers [244] and even biological materials [245]. Highly reflective materials may not be suitable for processing as the laser energy may possibly be reflected, causing damage to the laser system. Additionally, materials with low absorption coefficients (α) possess higher ablation threshold or threshold fluence F_{th} (Figure 2-20), meaning that they require more energy to be ablated the ablation which could increase the risk of thermal damage [246].

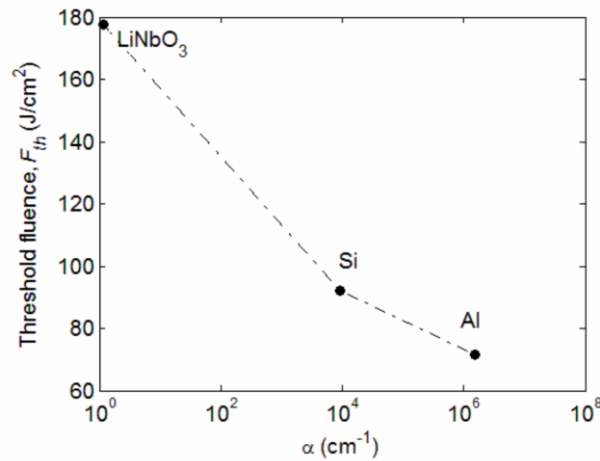


Figure 2-20 The relationship between the absorption coefficient and the threshold fluence [246].

Generally, there are three main ablation processes applied: The punching process, scribing process and milling process. For fabricating directly ablated structures, the milling process is used. The milling process involves machining of several parallel lines with a defined spacing called the hatch distance or hatch pitch d_h . The overlap of consecutive pulse along the milling line O_{line} is a function of the beam radius ω , repetition rate f_{rep} and the scan speed v_{scan} [247]:

$$O_{line} = \frac{v_{scan}}{f_{rep} 2\omega} \quad (2-8)$$

The overlap of the pulses in the direction perpendicular to the scan direction can be determined by:

$$O_{cross} = 1 - \frac{d_h}{2\omega} \quad (2-9)$$

Several layers N_L or machining passes can be conducted in order to create deeper structures. Typically, to perform the milling process the laser head needs to have a range of motion in two lateral directions. This is commonly achieved by employing a galvanometric scan head [248]. The galvanometer allows the laser beam to move rapidly and accurately, allowing for precise control of the beam's position and intensity.

Ultra-short pulse lasers have been employed to mainly produce two types of structures. The first one being directly ablated structured surfaces where the feature dimensions are in the same range as the laser wavelength and the second one being laser-induced periodic surface structures (LIPSS) where the surface feature sizes are much smaller than that of the laser wavelength and thus spot size. While a comprehensive theory behind LIPSS formation is yet to be established, some mechanisms are shown to influence the formation such as plasmon coupling with the incident light, surface self-organisation and the coupling of the incident electromagnetic wave with the surface roughness [249]. LIPSS shapes can be modified to a certain extent by changing the beam polarisation [250] and the media in which the sample is being processed [251]. However, the formation of LIPSS remains a self-reorganisation of the surface topography, limiting direct control as in the case of direct ablation. Additionally, the size and periodicity of the LIPSS features is dependant of the laser wavelength. For that reason, most LIPSS features are limited to around 200-300 nm in size. However, with the use of femtosecond laser pulses in deep UV, features below 200 nm have been achieved on stainless steel [252]. Limitations

in the feature size are also being overcome by the rise of direct laser interference patterning (DLIP) technique which exploits the interference between a number of laser beams to directly ablate features smaller than the single beam size [76]. In recent years, both LIPSS surfaces and hierarchical structured surface have been fabricated by femtosecond laser ablation to functionalise surfaces whether for controlled surface wettability in anti-icing and energy harvesting applications [253], anti-biofouling applications [81,197,254] and even bactericidal applications [149], among many others. Recently, freeform hierarchical structuring of neural interfacing electrodes was performed with the use of a combination of tip-tilt stage on a XYZ-translation stage [255], demonstrating the promise of femtosecond laser structuring in processing complex geometries.

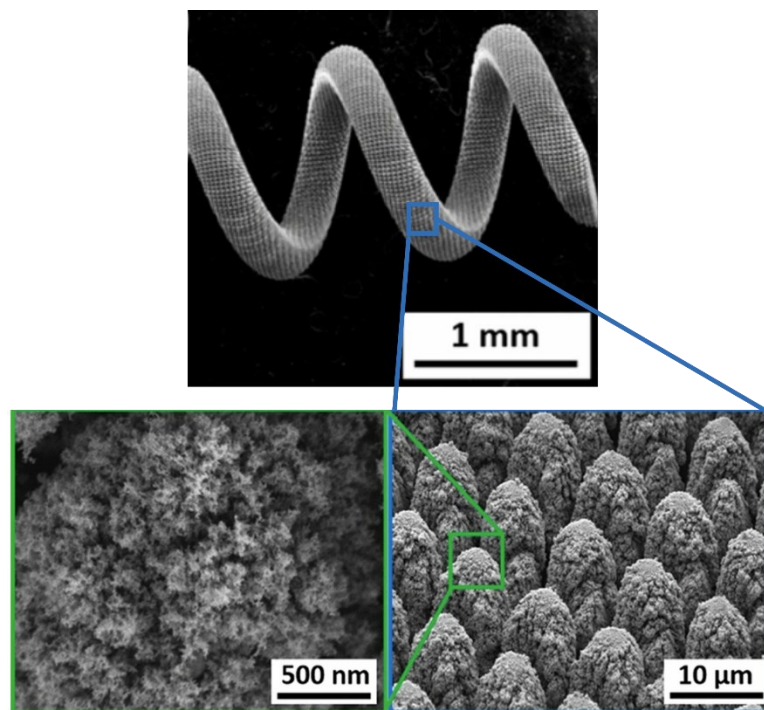


Figure 2-21 Femtosecond laser ablated hierarchical structures on 3D complex geometry of biomedical electrodes [255].

2.4.1.2 Deep Reactive Ion Etching

Reactive ion etching (RIE) is a dry etching technique that involves bombarding the substrate surface with reactive ions (plasma) to selectively remove material. Typically, the plasma used contains oxygen and other reactive gases that react

with the substrate material surface and chemically etch it. RIE is commonly used in micro and nanofabrication processes to etch precise pattern, often with the help of a masking layer determining the pattern dimensions. Deep reactive ion etching (DRIE) is a subset of RIE generally used for fabrication of high aspect ratio features through a cyclic etching/passivation process [256]. RIE is heavily used in micromechanics, microelectronics and semiconductor fabrication as well as the in the fabrication of integrated circuits. High aspect ratio nanostructured silicon surfaces, also known as black silicon (bSi), fabricated using RIE or DRIE, gained a lot of interest for their use in photovoltaics as they are very good at absorbing light due to their light trapping topography [257]. Since the emergence of mechano-bactericidal surfaces, black silicon has been named as the most efficient synthetic mechano-bactericidal surface with bacteria killing efficiencies reaching above 90 % for both Gram positive and Gram negative bacteria [102]. In the following section, the black silicon formation mechanism, as understood to date, is presented.

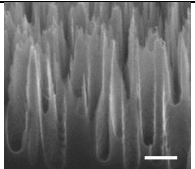
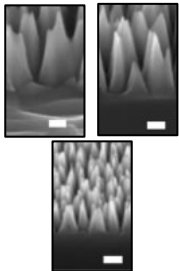
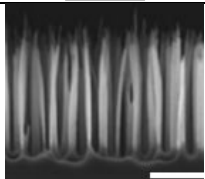
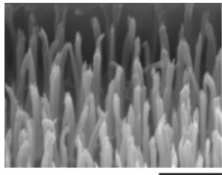
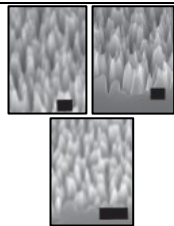
2.4.1.2.1 Black Silicon Formation

RIE etching systems can be categorised according to their power source as either inductively couple plasma known as ICP or capacitively coupled plasma known as CCP. The substrates are positioned on an electrode that is powered by radio frequency (RF) and coupled capacitively. The electrode obtains a negative charge due to the generation of a direct current self-bias voltage resulting from the impact of electrons on the electrode following plasma ignition. The gases are then introduced into the reactor where the ICP RF power energises the electrons and, through collisions with atoms and molecules, causes ionisation and dissociation. The formation of silicon is done through a maskless etching technique at low pressure. Fluorine-based plasma is widely used although the use of chlorine and bromide have also been successfully explored [258–260]. Fluorine-based plasma, particularly SF₆/O₂ plasma has been used to fabricate black silicon topographies that can be considered as the research standard for mechano-bactericidal surfaces [91,142,261].

The origin of black silicon formation, in this case, is a constant competition between fluorine radicals that etch the silicon and oxygen radicals that passivate it. It is currently hypothesised that, once they are introduced into the chamber, SF₆ and O₂ gases are dissociated into SF_x⁻, F⁻ and O²⁻ radicals.

As a result, volatile SiF₄ is formed while non-volatile SiO_xF_y is formed and acts as a passivating layer on the etch profile. The varying SF₆ flow allows later etching of this silicon oxyfluoride layer using SF_x⁺ ions through the formation of volatile SiO_xF_y gases [258]. Native oxide, dust and silicon oxide particles adsorbing on the silicon surface during etching serve as micro-masks for the fluorine radicals to etch around. At a certain SF₆/O₂ ratio balance between etching and passivation is achieved which results in the formation of high aspect ratio features. The height of the resulting features is dependent on the etch time [258,259]. The morphology of the features can be varied by changing parameters such as the flow rate, system temperature, substrate bias and RF power. Table 2-1 depicts different processing parameters used to fabricate black silicon in the literature. The different morphologies are imbedded in the table as SEM micrographs.

Table 2-1 Parameters used to fabricate black silicon by DRIE. The scales bars are 1 μm , 100 nm, 2 μm , 2 μm , 200 nm and 200 nm, respectively.

ICP power (W)	Temperature ($^{\circ}\text{C}$)	Pressure (mTorr)	Etch duration (min)	SF_6/O_2 ratio (sccm/sccm)	Reference	SEM micrograph
750	-40, -32	15	0.5 - 10	60:60	[257]	
150	-	-	15, 30, 45	35:45	[102]	
100	20	35	10 - 60	65:54	[262]	
100	20	35	25	65:44	[263]	
150	-	7	15, 30, 45	35:45	[142]	
100	20	35	5	65:44	[264]	

2.4.1.3 Focused Ion Beam Lithography – FIB

Focused ion beam (FIB) lithography uses a focused beam of ions to scan the surface of a specimen to sputter material. This process is generally performed in a vacuum chamber fitted with both electron and ion optical columns known as a FIB-SEM system (Figure 2-22). To perform milling with the FIB in a FIB-SEM system, the sample has to be rotated (typically 52° – 55°) for the surface of the sample to be coincident with the ion beam.

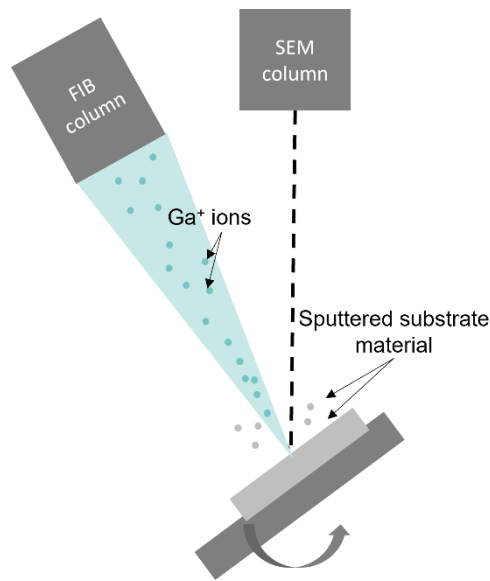


Figure 2-22 A schematic of the FIB-SEM system

In the milling process, several parameters are important for the performance of the FIB system. They can be categorised into three main parts: the scan method/strategy, the beam parameters and the processing parameters. The beam parameters are the ion species, the beam energy, the accelerating voltage and the ion current (spot size and current density). The processing parameters are specific to the incident angle, dwell time and beam pitch or overlap. FIB-SEM software is equipped with simplistic milling designs where the dimensions can be customised such as circle, donut, square, rectangle, polygon, etc. In new FIB-SEM systems, computer aided design or CAD can now be utilised for patterning very complex structures [265].

As mentioned above, one of the important parameters in defining the quality of the mill is the ion source. Ion sources can vary; however, the gallium liquid metal ion source is the most prevalent. Gallium ion FIB systems are generally considered very advantageous in terms of defined milling as the operating current ranges from 1 pA to 10 nA, with a minimum spot size of tens of nanometres. The beam can be finely focused further to achieve a spot size smaller than 10 nm by adjusting the current passing through the lens [266]. Compared to electron beam lithography (EBL), FIB lithography provides a higher resolution even when considering the same spot size. This is due to the lack of backscattering effects, diminished forward scattering and lateral diffusion of secondary electrons which are common in EBL [267]. Additionally, ions, being heavier than electrons, can penetrate the sample with greater energy, resulting in quicker exposure of the resists and faster processing nanostructures, which is especially useful in photonics [268]. A downside of Gallium ion based systems is the possible redeposition and ion implantation into the substrate which could lead to amorphization, phase changes, damage and surface roughening. To overcome these limitations, plasma-based FIB microscopes utilising noble gases of heavy ion species such as Xe can be used. These systems are suitably employed for milling large volumes quickly [269]. However due to their relatively large beam diameter compared to Ga systems, they are not very well suited to mill defined features of tens of nanometres in scale [270].

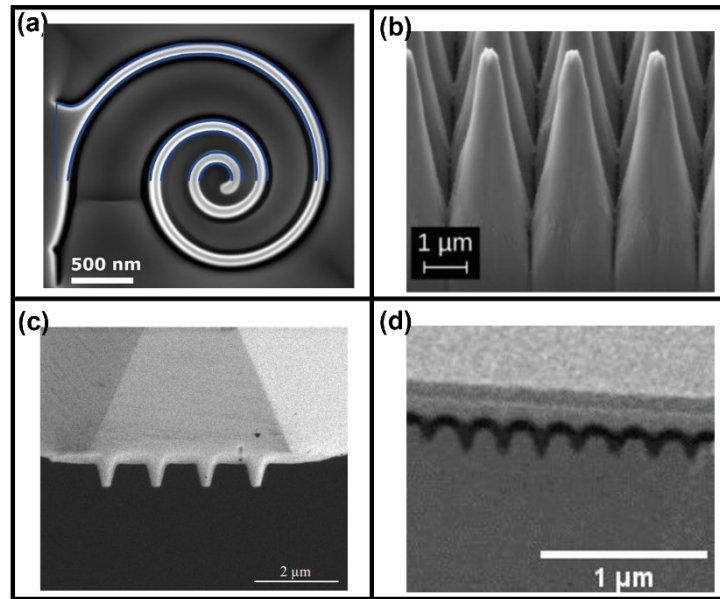


Figure 2-23 (a) CAD/CAM FIB milled complex features on silicon. (b) High aspect ratio periodic patterns on GaSe as ultrabroadband anti-reflective surface [11]. (c) Nanoscale multi-tip single crystal diamond tool [271]. (d) Cross-section of FIB milled stainless steel for cardiovascular stent applications [272].

Examples of features fabricated with FIB lithography are shown in Figure 2-23. FIB patterning has been explored for several applications such as MEMS/NEMS, magnetic head trimming, mechanical tools, biological tools and functional 2D and 3D structures [273] on a variety of materials including silicon, silicon nitride, glass, among others. Yet, only one study reported patterning key orthopaedic implant materials such as stainless steel substrates. The published study attempted the fabrication of a $400 \times 400 \mu\text{m}^2$ area nano-pit features on stainless steel 316L with 180 nm diameter and 240 nm pitch with 30 keV and 0.92 nA (Figure 2-23 (d)) and found that endothelial cells had reduced adhesion on FIB patterned areas compared to flat surfaces [272,274].

2.4.1.4 Scanning Probe Lithography (SPL)

Nanofabrication with scanning probe lithography (SPL) is conducted with the atomic force microscope set-up. The atomic force microscope is generally used for measuring nano scale topography of a surface. It works by detecting displacements on a cantilever with a probe tip as it is run across (contact mode)

or tapped (tapping mode) across a surface. During the scan, a laser is reflected off the back of the cantilever onto a diode that detects the change in position. This is then converted into a map of heights which can be visualised as a 3D representation of the surface topography. An AFM has a small range of scanning typically $< 100 \times 100 \mu\text{m}^2$. A recent review by Fan et al. [275] highlighted different fabrication mechanisms enabled by SPL. Of particular interest is the mechanical scanning probe lithography approach or M-SPL with its subset called static ploughing lithography. Through this fabrication technique, the atomic force microscope is used to remove material selectively from the substrate by using an applied normal force on the probe to scratch or mill a pattern on the surface. In this case the tip acts as a cutting tool, moving in a translational motion and capable of inflicting plastic deformation on the substrate with enough normal force. This technique has only been employed in early nanoscratching experimental investigations so far.

Several studies have employed static ploughing lithography to fabricate nanostructures with precise control over depth and width, on a variety of materials including polymers, soft metal and semiconductors. Examples include the fabrication of nanostructures on GaAs, achieving sub-20 nm-width lines with nearly 1 nm depth on GaAs surface [276] and creating nanoscale hole arrays with a 55 nm periodicity on GaAs/AlGaAs heterostructure substrate [277]. Patterning aluminium films was conducted by Filho et al. [278]. In that study it was shown that the feature depth correlates positively with the applied normal force, negatively with the tip scanning velocity, and correlates positively with the duration of the force applied. The research in this area is in its early stages and the parameter space to explore is enormous, considering the machining needs of different materials.

2.5 Surface Topography/Texture measurements

Surface topography, or texture, significantly influences the functionality of surfaces. In this section, the focus is on the methods and parameters used to characterise surface topography, specifically pertaining to anti-bacterial surfaces.

Subsection 2.5.1 outlines contact-based and non-contact techniques for surface measurement. Contact-based methods, such as stylus profilometry (2.5.1.1.1) and atomic force microscopy (AFM) (2.5.1.1.2), are compared to non-contact techniques, including interferometry (2.5.1.2.1) and confocal microscopy (2.5.1.2.2). Advantages and limitations of each technique are discussed in 2.5.1.3, followed by an examination of filtering (2.5.1.4) for data analysis.

Subsection 2.5.2 focuses on surface parameters for characterising topography, emphasising the fractal dimension (2.5.2.1), which quantifies surface complexity and could be employed in anti-bacterial surface performance studies.

Finally, subsection 2.5.3 investigates the role of surface topography in anti-bacterial surfaces, examining the prevention of bacterial adhesion and biofilm formation. This section integrates previous research to highlight the importance of topography measurements in advancing anti-bacterial surface development.

2.5.1 Techniques for Surface Topography/Texture Measurements

2.5.1.1 Contact-Based Techniques

Contact-based techniques for surface topography measurement involve the physical interaction between a probe or tip and the sample surface. In these methods, the probe moves along the surface, detecting variations in height, which are subsequently converted into a topographical profile. Although contact-based techniques can provide high-resolution data, they carry the potential risk of sample damage or alteration due to the direct contact between the probe and the surface. Nevertheless, these techniques remain valuable tools in the field of surface characterisation and have been extensively employed in various applications. In the following sections two contact-based techniques surface measurement are presented: stylus profilometry and atomic force microscopy.

2.5.1.1.1 Stylus Profilometry

Stylus profilometers are one of the frequently used equipment that measure surface texture [279]. They are equipped with a probe that detects the surface

and physically moves along a straight line on the surface to acquire height data using a displacement transducer [279] as is depicted in Figure 2-24 (a).

2.5.1.1.2 Atomic Force Microscopy (AFM)

It is typical for the AFM to be used in tapping mode for measuring surface topography. In this mode, the cantilever tip is oscillated at or near its resonant frequency while scanning across the sample surface [280]. As the AFM tip approaches the sample surface, the amplitude of the oscillation decreases and there is intermittent close contact between the tip and the surface. The change in amplitude is then detected by a position-sensitive photodetector and used to create the height map [281]. This method is sometimes considered a non-contact method due to the very limited contact that occurs between the tip and the surface and the non-destructive nature of the technique [282].

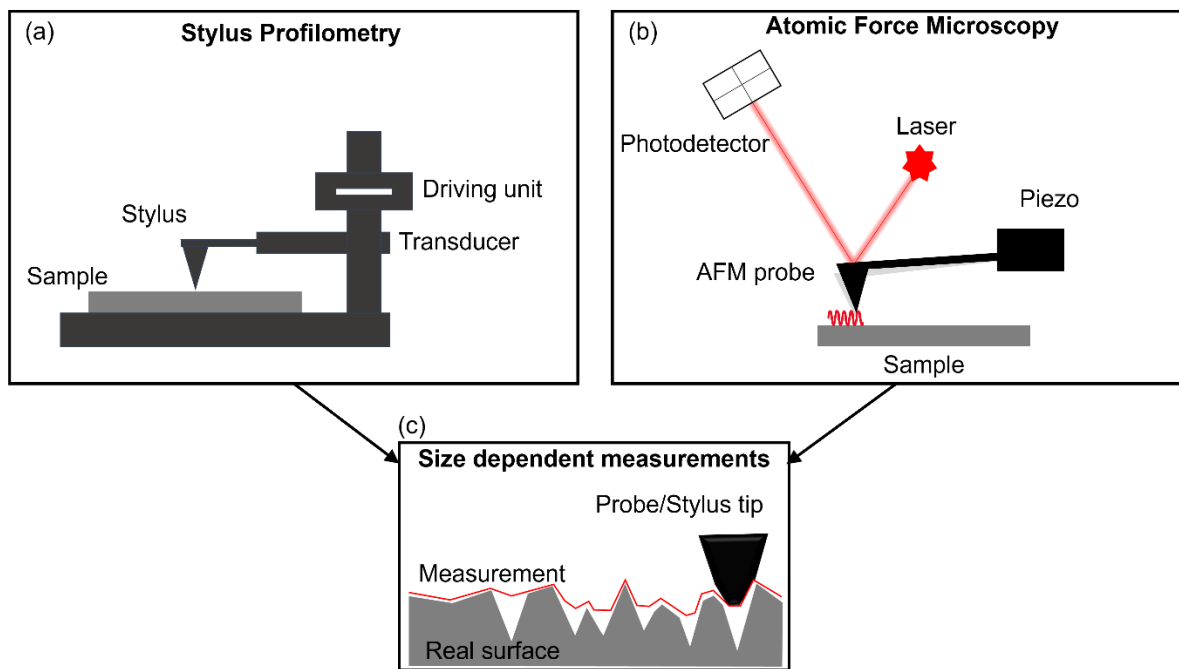


Figure 2-24 Schematic of contact surface measurement techniques including (a) stylus profilometry, (b) Atomic force microscopy (c) The dependence of the measurements on the probe or tip size in contact surface measurement techniques.

The imaging resolution in both contact techniques discussed (Stylus profilometry and AFM) is limited by the geometrical properties of the tip including its shape and size [283]. For instance, if the tip is too big the measured surface deviates quite a bit from the actual surface as is illustrated in Figure 2-24 (c). To avoid this, proper tip selection must take place or alternatively, non-contact methods can be used.

2.5.1.2 Non-Contact Techniques

Non-contact techniques generally use light instead of the stylus used in contact techniques. The instruments vary depending on the principle of measurements used and include white light interference and the confocal principle.

2.5.1.2.1 Interferometry

Interferometry is a technique that utilises the interference patterns of light to measure surface topography of a sample [279]. The working principle of interferometry is depicted in Figure 2-25. First, a light beam is split into two parts using a beam splitter. One part is directed toward the sample surface while the other part is directed toward a reference surface. The light waves that are reflected from the two surfaces recombine and form an interference pattern which is recorded by a detector (typically a CCD) and analysed using software to extract the topographical information [284]. A very common type of interferometry used to measure surfaces is white light interferometry (WLI). In WLI, a broad-bandwidth light source is used, and the interference pattern is created by overlapping of light waves reflected from different depths within the sample [285].

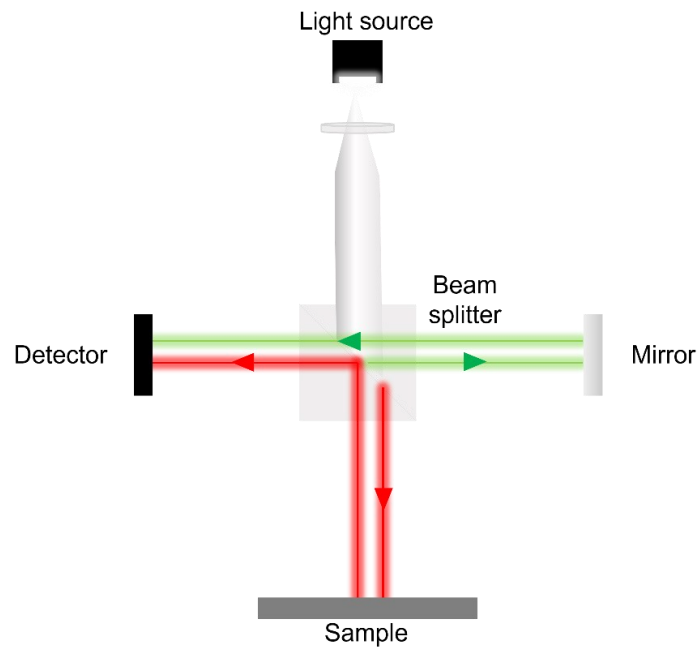


Figure 2-25 Interferometer working principle.

2.5.1.2.2 Confocal microscopy

In confocal microscopy, a laser beam is usually used to scan the surface to be measured and is then reflected off that surface and captured by a detector. By focussing the laser beam at different depths, a series of images is obtained at different focal planes [286]. The laser intensity is recorded for each image at different focal planes. The captured images are then processed to generate a surface height map using software algorithms that analyse the intensity and the location of the laser beam at each focal plane to identify the surface of the sample [286]. The resulting height map shows the height of the surface at each point on the samples with brighter areas representing higher points and darker areas representing lower points [287].

2.5.1.3 Comparison of Techniques

Table 2-2 depicts the advantages and limitations of the topography measurements techniques presented. Typically, aerial topographical information

gives a greater understanding of the surface topography which is more beneficial for surfaces that do not have a high degree of anisotropy and particularly in the investigation of bacteria-surface interactions. Resolutions of a few nanometres to tens of nanometres are ideal considering the size of most bacteria concerned in biomaterial associated infections (0.5 to 3 μm) including *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* [34,288,289]. Therefore, AFM measurements appear to be essential when studying bacteria-surface interactions.

Table 2-2 Comparison of different surface topography measurement techniques. Information on lateral and vertical resolutions were extracted from [290].

	Technique	Advantages	Limitations
Contact	Stylus Profilometry	<ul style="list-style-type: none"> • Relatively fast and cheap • High vertical resolution (0.25 nm) 	<ul style="list-style-type: none"> • Destructive on soft samples • Limited lateral resolution (50 nm) • Highly probe-dependent (best suited for features ranging from microns to tens of microns) • Limited to profile measurements
	Atomic Force Microscopy	<ul style="list-style-type: none"> • High vertical resolution (0.1 nm) • Good lateral resolution: can be as low as 2 nm, but typically is around 10 nm. • Aerial measurements capability • Less destructive than stylus profilometry due to controlled load on the tip 	<ul style="list-style-type: none"> • Highly probe-dependent lateral resolution • Relatively slow
Non-Contact	Interferometry	<ul style="list-style-type: none"> • High vertical resolution (0.3 nm) • Aerial measurements capability • Non-destructive 	<ul style="list-style-type: none"> • Needs robust sample preparation. • Sample reflectivity • Depth of field • Limited lateral resolution (500 nm)
	Confocal Microscopy	<ul style="list-style-type: none"> • Good vertical resolution (1-20 nm) • Non-destructive 	<ul style="list-style-type: none"> • Needs robust sample preparation. • Depth of field • Limited lateral resolution (500 nm), dependent on the numerical aperture and lateral sampling. • Sample reflectivity • Sample transparency

2.5.1.4 Filtering

In order to analyse the surface texture in relation to a surface function (wear resistance, fluid retention, water repellence, etc.), it is often important to decouple different wavelength depending on what is relevant for the investigated application. Decoupling is a term meaning selecting the analysis range or rejecting irrelevant information and it is done through filtering. Filters select the profile/surface features according to the scale on the x-axis when considering 2D or profile measurements and x-y axis when considering 3D or aerial measurements. This is considered filtering in terms of surface wavelengths or spatial frequencies. A filter that rejects short wavelengths while retaining longer ones is called low pass filter as it preserved the low frequencies or “lets them pass”. In metrology, a low pass filter is termed s-filter and is always applied to “denoise” the surface measurement resulting in what is called the “primary surface” [291]. A filter that rejects high wavelengths is termed high pass or an L-filter. A filter that has a low pass and high pass component is known as band pass filter resulting in an S-L filtered surface [291]. In most applications, it is more appropriate to use a band pass filter to reveal features of interest [292]. For S-filters, Gaussian or Spline filters are recommended to be used. For L-filters, the Gaussian filter is standardly utilised however, in some cases the robust gaussian filter is more appropriate as it preserves edges and features in the surface by assigning lower weights to outliers in the data [293,294].

2.5.2 Surface Parameters for Characterising Surface Topography/Texture

After the topographical measurements are filtered adequately, corresponding surface parameters can be calculated. Generally, three types of surface parameters exist, the first one is the type relating to the amplitude of the texture, the second relates to the spatial frequency or wavelength of the texture (spacing), while the third one is a combination of the two and thus termed hybrid. Additionally, functional parameters have been introduced to try and link some functional behaviours with surface topography. A graphical representation of

useful surface topography parameters is presented in Figure 2-26 while in Table 2-3 the description of these parameters is outlined. The surface topography parameters presented are divided into amplitude, spacing, hybrid and functional parameters.

The functional parameters presented in Table 2-3 are generally relating more closely to amplitude parameters however they do take into account feature spacing to a degree.

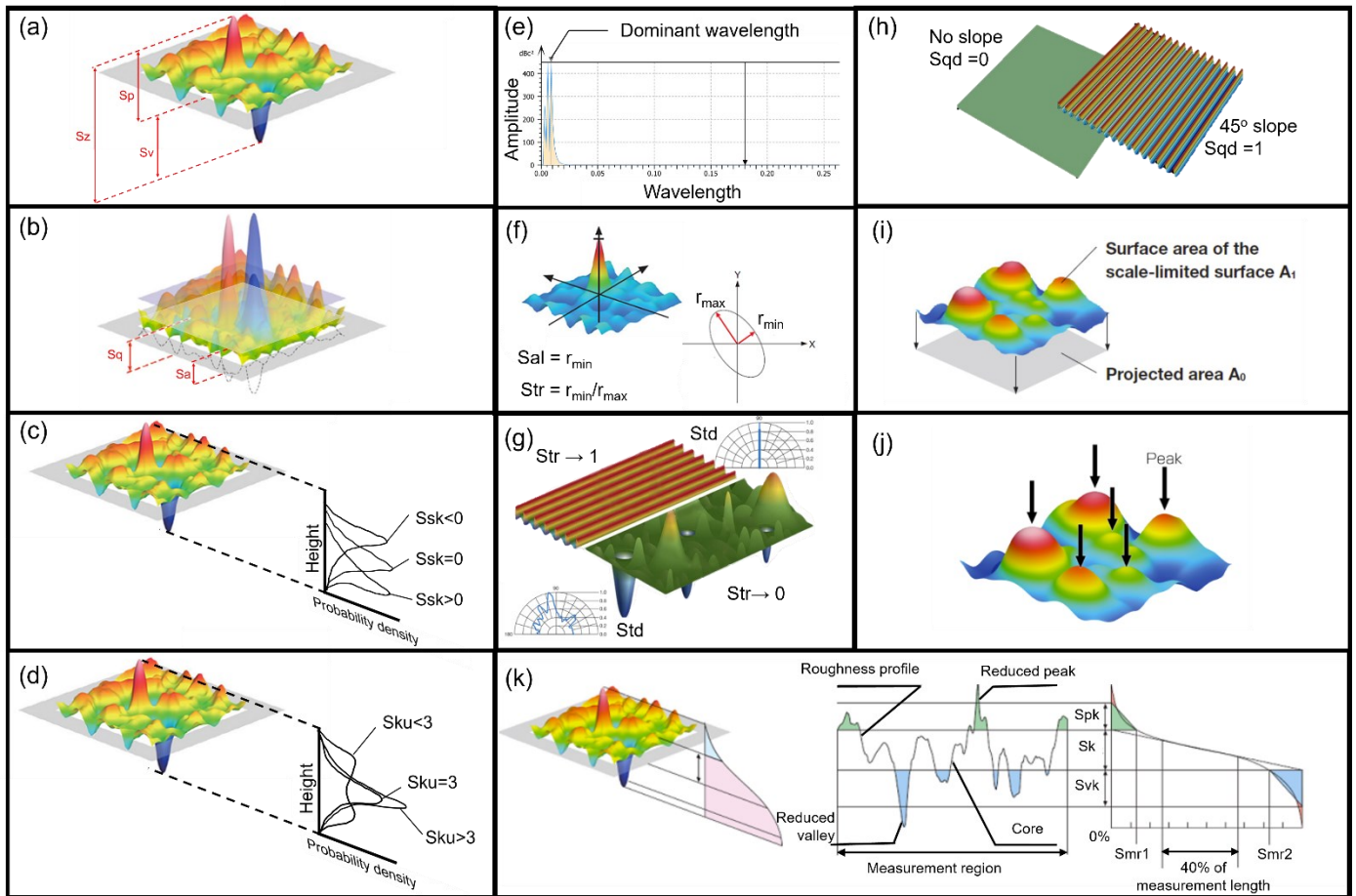


Figure 2-26 Graphical representation of amplitude, spacing, hybrid and functional areal surface roughness parameters. Adapted from [295].

Table 2-3 Commonly used amplitude, spacing, hybrid and functional areal surface roughness parameters.

Type of parameter	Parameter	Description	Possible or common use	Figure
Amplitude Parameters	Maximum peak height R_p , S_p	The height of the highest point of the profile/area from the mean line/plane	Indicates poor material or poor processing: sharp spike or burr, cracks, scratches	Figure 2-26 (a)
	Maximum valley depth R_v , S_v	The deepest point of the surface below the mean line within the sampling area.	Surface wear and defect analysis	
	Maximum height R_z , S_z	The difference between the maximum peak height and maximum valley depth within the sampling area.	Quality control and wear analysis	
	Arithmetic mean deviation R_a , S_a	The average absolute deviation of the surface from the mean line.	General indicator of surface roughness	Figure 2-26 (b)
	Root mean square deviation R_q , S_q	The root mean square of the height deviations from the mean line.	General indicator of surface roughness, particularly popular in optics.	
	Skewness R_{sk} , S_{sk}	The symmetry of the height distribution relative to the mean line.	Indicator of the presence of dominant peaks or valleys on the surfaces	Figure 2-26 (c)
	Kurtosis, R_{ku} , S_{ku}	The sharpness of the height distribution relative to a Gaussian distribution.	Indicator of distinct peak or valley characteristics	Figure 2-26 (d)

Spatial Parameters	Power spectral density function, PSD	The distribution of surface height variations as a function of spatial frequency.	Popularly used in optics and tribology	Figure 2-26 (e)
	Autocorrelation length, S_{al}	The average distance over which the surface heights are correlated.	Indicator of the spatial structure of the surface texture	Figure 2-26 (f)
	Texture aspect Ratio S_{tr}	The ratio of the dominant horizontal texture feature size to the dominant vertical texture feature size.	Used in the characterisation of anisotropic surfaces	Figure 2-26 (f, g)
	Texture direction S_{td}	Indicates the dominant orientation of the surface texture features.	Friction and wear analysis	Figure 2-26 (g)
Hybrid Parameters	Root mean square of surface slope S_{dq}	The average square of the local slopes across the surface.	Adhesion and wetting studies	Figure 2-26 (h)
	Developed interfacial area ratio S_{dr}	The ratio of the developed surface area to the projected area. Important in applications involving surface contact, such as adhesion and lubrication.	Adhesion and lubrication studies	Figure 2-26 (i)
	Peak density S_{pd}	The number of peaks per unit area.	Tribology and optical studies	Figure 2-26 (j)
Functional Parameters	Reduced peak height S_{pk}	The mean height of peaks above the core surface.	Tribology and lubrication studies	Figure 2-26 (k)
	Core height S_k	The difference between the minimum height and the maximum height of the core surface.		
	Reduced valley height S_{vk}	A measure of the valley depth below the core roughness.		

	Peak material portion Smr1	The ratio of the area of the peaks to the total surface area	Wear or erosion studies	
	Valley material portion Smr2	The ratio of the area of the valleys to the total surface area	Lubrication studies	

The exclusive use of amplitude or spacing parameters, although very common in the literature, is an oversimplification of the surface topography as these parameters result in an averaged amplitude-specific or wavelength-specific value over the whole measured area which can often obscure underlying patterns or surface features. This hinders the correlation of surface parameters with functional features. For instance, two profile measurements could possess the same R_a yet they will have significantly different functional behaviour [296]. For example, if sharp spikes were present on the surface instead of blunt dimples, the wear resistance will be significantly impacted. Hence the importance of characterising surfaces with a combination of surface parameters encompassing amplitude and spacing parameters: hybrid parameters.

While surface roughness parameters give an insight into the geometric features of a surface at different scales, the fractal dimension provides a quantitative measure of the surface's self-similarity and roughness over multiple length scales, making it a useful tool for characterising complex and irregular surfaces.

2.5.2.1 The Fractal Dimension

“Clouds are not spheres, mountains are not cones, coastlines are not circles, and bark is not smooth, nor does lightning travel in a straight line.” – Benoit Mandelbrot [297].

Fractal geometry is distinct from the simple figures of classical, or Euclidean, geometry; the square, the circle, the sphere, etc. It is used to study complex structures and shapes that have fractional dimension. Fractal geometry was first introduced by the mathematician Felix Hausdorff in 1918 and then developed, to what is known today, by the mathematician Benoit Mandelbrot [298]. The term fractals refer to self-similar objects that have the same details across different scales such as the Mandelbrot fractal. The application of Mandelbrot's work on engineering surfaces was developed by Professor Christopher Brown of Worcester Polytechnic Institute where scale-dependent analysis was introduced to infer functional correlations [293] particularly in adhesion and wear [299]. The fractal dimension, measuring the complexity of a surface is generally used in this

context. The use of the fractal dimension as a functional surface indicator was thereafter applied in a few areas related to engineering surfaces. For instance, it has been used in fracture mechanics as an estimator of the theoretical strength of materials [300], and a predictor for fracture energy of fractured surfaces [301]. Additionally, it has been applied to study the topography and film growth mechanisms of thin films [302] and to the study of fluid flow behaviour in microtubes [303]. The fractal dimension was also found to be useful in the study of archaeological surfaces [304].

The fractal dimension can be determined by various techniques like walking-divider method, box-counting method, prism-counting method, Epsilon-Blanket method, perimeter-area relationship, power spectrum method, etc [305]. Among the several techniques to estimate the fractal dimension, box-counting or its 3D equivalent, the cube-counting method and power spectrum methods are more popularly used techniques [302]. The accuracy and invariance of the result depend on the selection of the most suitable method for a particular problem. Particularly, the cube-counting method [302] was found to be more robust in calculating the fractal dimension for surfaces generated through Weierstrass-Mandelbrot (W-M) function as compared to other techniques such as power spectral density, structure function and autocorrelation function while remaining computationally adequate [306].

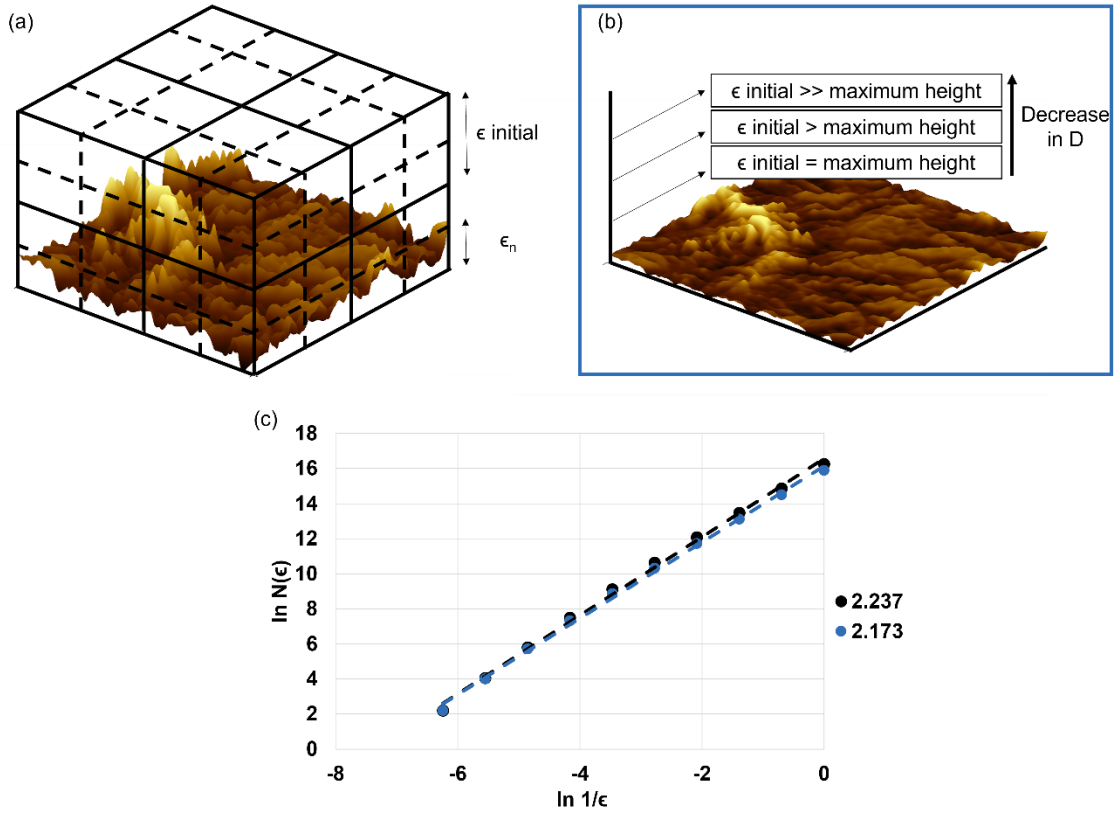


Figure 2-27 The cube counting method for determining the fractal dimension of a surface. (a) Example of a sectioned surface with different box dimensions. (b) The effect of initial box dimension on the fractal dimension computed. (c) The log-log plot and extracted fractal dimension for the surfaces presented in (a) and (b) according to the initial dimension being the maximum surface height.

The cube counting method [307], works by superimposing the image to be analysed with boxes (squares/cubes) as is presented in Figure 2-27 and then counting the number of boxes needed to cover the fractal image completely. Repeating the process with boxes of varying dimension (ϵ) and counting the number of boxes ($N(\epsilon)$) to cover the entire volume of the image.

A log-log graph is then plotted (Figure 2-27) with $\ln \frac{1}{\epsilon}$ along the x-axis and $\ln N(\epsilon)$ along the y-axis:

$$N(\epsilon) \propto \epsilon^{-D} \quad (2-10)$$

Where D is the fractal dimension. Taking the logarithm, equation (2-3) becomes:

$$\ln N(\epsilon) = D \ln \frac{1}{\epsilon} + c \quad (2-11)$$

The fractal dimension can then be calculated from the slope of the $\ln N(\epsilon)$ vs. $\ln \frac{1}{\epsilon}$ graph.

The value of the fractal dimension is sensitive to the initial box dimension (ϵ). Therefore, the selection of $\epsilon_{\text{initial}}$ is very important. The preferred value for the initial box dimension is the maximum height of the surface and in most modern software is applied accordingly. This makes the analysis of the fractal dimension specific to each surface analysed which permits the comparison of the fractal dimension between surfaces independent of the z-scale of each [308].

2.5.3 Surface Topography/Texture in Anti-bacterial Surfaces

When investigating the interactions between bacterial cells and a material's surface, the primary concern is micron and submicron wavelengths of surface texture evaluations. A large number of research has already been conducted into the effect of surface topography on surface-bacteria interactions. 28 studies published in the period from 2005-2022 who looked at the effect of surface topography (in the submicron scale) on bacteria-surface interactions were evaluated [36,76,310–319,77,320–325,78,81,107,162,197,264,309]. It was found that most studies evaluated the surface roughness in terms of R_a and R_q , while few of the recent studies expanded the evaluation to include 3D topographical parameters S_a (20.8 %), S_q (16.6 %), S_{ku} (12.5 %), S_{sk} (12.5 %), S_{pd} (20.8 %). However, the use of hybrid parameters such as S_{dr} and S_{dq} was still lacking. Furthermore, the fractal dimension has not been used in this regard. Among the studies evaluated, 48.3 % of the studies have found positive correlation between the amplitude surface roughness parameters evaluated and the anti-bacterial effect of the surface evaluated, 41.4 % found a negative correlation and 10.3 % did not find any correlation. This reinforces the need to expand the surface topographical characterisation to encompass parameters that are more descriptive of the surface architecture. Indeed, for engineering

applications Stout et al. [326] proposed a set of 14 roughness parameters to comprehensively describe a surface. The set is referred to as the Birmingham 14 within the literature. Later on, Crawford et al. [327] condensed the Birmingham 14 parameters into a set of minimum parameters to use for standard surface characterisation in microbiological applications. These parameters are S_q , S_{dr} and S_{pd} and have been recommended considering that any minimum set of parameters should include descriptors of both the amplitude and spatial properties.

Most of the studies that evaluate or correlate the surface roughness to the anti-bacterial effect of the corresponding surfaces are specifically investigating the anti-adhesion or anti-biofouling effect rather than mechano-bactericidal effect. For studies evaluating mechano-bactericidal surfaces, the roughness parameters were rarely evaluated, and when evaluated there was no attempt at correlating them with the surface functionality, except for the density of peaks in some instances [142]. As discussed in section 02.3.1.2.1.1, the critical surface parameters investigated in relation to the mechano-bactericidal phenomenon are related to the specific geometry of the nanopillars: Tip diameter, height and the spacing between the pillars which could be related to the density of pillars or of peaks.

In conclusion, this section has demonstrated the significance of surface topography and texture in the context of anti-bacterial surfaces. By reviewing various techniques and parameters, it underscores the necessity for a comprehensive approach to surface characterisation. Through the evaluation of more diverse parameters and a deeper understanding of the relationships between surface properties and anti-bacterial performance, researchers can contribute to the development of increasingly effective anti-bacterial surfaces.

2.6 Summary

The literature review highlighted the potential of nature-inspired surface structuring for use in anti-bacterial applications using two distinct strategies: the anti-biofouling and mechano-bactericidal strategies. Despite the valuable insights

gained from existing literature studies on both these strategies, there are still several gaps in the understanding of their anti-bacterial functionality that is hindering the swift advancement of the field on both a research scale and a commercial scale. Additionally, the effectiveness of both anti-bacterial strategies has not been compared to probe a direction for future research.

From a manufacturing point of view, the literature has not fully explored the precise manufacturing processes that result in the observed anti-bacterial effects. While various fabrication techniques have been investigated, the literature lacks a comprehensive understanding of how different manufacturing methods may impact the anti-bacterial performance of the resulting surfaces. Additionally, it has not thoroughly evaluated the most suitable manufacturing methods for producing nature-inspired anti-bacterial surfaces at both the research and commercial scales. More research is needed to identify the advantages and limitations of different techniques and determine their feasibility for large-scale production.

Moreover, it remains unclear whether the anti-bacterial effects observed on nature-inspired surface topographies are primarily chemical or physical in nature especially when considering anti-biofouling surfaces. There is a need for more in-depth studies to elucidate the specific mechanisms underlying these effects and determine how they might be influenced by surface topography and wettability.

Finally, although several surface parameters have been identified as important for describing nature-inspired structured surfaces, a clear understanding of the critical parameters and their relevance across different scales is still lacking. The literature does not provide a comprehensive framework for correlating specific topographical parameters with biological functionality.

Addressing these gaps will help advance the understanding of nature-inspired anti-bacterial surfaces and inform the development of more effective anti-biofouling strategies for various applications.

2.6.1 Research Questions

In light of the existing literature, the following research questions arise:

- 1) What is the manufacturing fingerprint that causes the observed anti-bacterial effects?
- 2) Are the anti-bacterial effects observed on nature inspired surface topographies chemical or physical in nature?
- 3) What are the critical surface topography parameters necessary for adequately describing nature-inspired structured surfaces and correlating them with their biological functionality and at which scales do these parameters become most relevant?
- 4) Which manufacturing method is more suitable to produce nature inspired anti-bacterial surfaces at the research scale and at the commercial scale?

3 Research Methodology

3.1 Materials and Methods

3.1.1 Biological materials

Staphylococcus aureus NCTC 7791 provided by the Cardiff University Dental hospital's microbiology department, purchased from Culture Collections, UK Health Security Agency, UK was employed for all the bacterial tests reported here.

The choice of *Staphylococcus aureus*, specifically the NCTC 7791 strain, as the model bacterium for this research is grounded in multiple compelling factors. Foremost among these is the clinical significance of *S. aureus* in healthcare settings. This bacterium is implicated in over 50% of post-operative infections associated with orthopaedic implants in hospitals [328,329].

Additionally, the non-motile and non-sporulating characteristics of *S. aureus* make it a suitable candidate for controlled, lab-based in-vitro testing. While *S. aureus* comprises a diverse range of 110 characterised strains [330], the NCTC 7791 strain was specifically selected for this study. This choice was made to facilitate straightforward comparison with benchmark studies in the existing literature, thereby enhancing the relevance and impact of our research findings.

Osteoblast-like MG-63 cells were used as a model cell line to study the biocompatibility of the fabricated surfaces due to their relevance to bone tissue engineering. These cells are derived from human osteosarcoma affected bone cells, which is a type of bone cancer [331]. They have similar characteristics to human bone-forming cells called osteoblasts [332], which play a crucial role in bone regeneration and remodelling. Despite the primary focus of this work on exploring the anti-bacterial efficacy of hierarchically structured and nanostructured surfaces, it is crucial to gain insight into the possible response of human cells to such surfaces. This understanding serves as a foundation for

future applications and underscores the significance of assessing cytocompatibility alongside anti-bacterial properties.

3.1.2 Substrate Materials

In this work, stainless steel substrates and silicon substrates were both used to fabricate textured anti-bacterial surfaces. Stainless steel is widely used in biomedical applications due to its excellent properties, corrosion resistance and biocompatibility. Additionally, orthopaedic implants manufactured using stainless steel are usually employed to provide directionality and fixation to the fractured bone, like in the case of forearm plates. These types of implants are therefore not required to accommodate for bone growth, unlike implants made with titanium such as hip joint implants. Hence, stainless steel was a good candidate material for focusing investigating nanostructured anti-bacterial surfaces without concerns regarding osseointegration.

Silicon, on the other hand, was chosen as a test material for investigating the anti-bacterial mechanism mediated by high aspect ratio nanopillars (mechanobactericidal mechanism). To date, nanostructured silicon surfaces, also known as black silicon, have been shown to be the most efficient synthetic mechanobactericidal surface studied, with reported killing efficiencies of up to 97% for Gram-positive bacteria [102].

In chapter 4, rolled stainless steel plates were supplied by LASEA Ltd. (Belgium) and cut into 0.5 x 0.5 x 0.3 cm³ squares. The chemical composition of the stainless-steel samples is presented in Table 3-1.

Table 3-1 Chemical composition of the as-received stainless steel samples measured through EDS

Element	Fe	Cr	Mn	C	O	Si
Weight percentage (%)	67.58 ± 1.61	13.44 ± 0.59	9.11 ± 2.52	7.59 ± 2.01	1.66 ± 0.55	0.61 ± 0.50

In chapter 5, 100 mm p-type silicon wafers of orientation (100) and (111) were used as substrates for nanopillar fabrication. The chemical composition of the silicon samples is presented in Table 3-2.

Table 3-2 Chemical composition of the silicon wafers measured through EDS

Element, in weight percentage (%)	Si	C	O	F
Si (100)	84.46 ±	14.85 ±	0.61 ±	0.083 ±
	0.16	0.43	0.41	0.091
Si (111)	86.66 ±	12.66 ±	0.67 ±	N/A
	2.45	2.54	0.49	

In chapter 6, a stainless steel 316L sheet was purchased from Smiths metal centres Ltd., UK and cut into 10 x 10 x 2.5 mm samples. The subsequent samples were ground and polished to reach a roughness (R_a) below 5 nm.



Figure 3-1 Polished stainless steel samples used for SPL and FIB prototyping.

In chapter 7, Striker orthopaedic (Michigan, US) implant samples including lock compression plates (LCP) composed of stainless steel, spiral blades composed from titanium aluminium niobium and the femoral component of a knee replacement implant composed of cobalt chromium (pictured in Figure 3-2) acquired from Milton Keynes University (MKU) Hospital were subsequently processed with silver ion implantation.

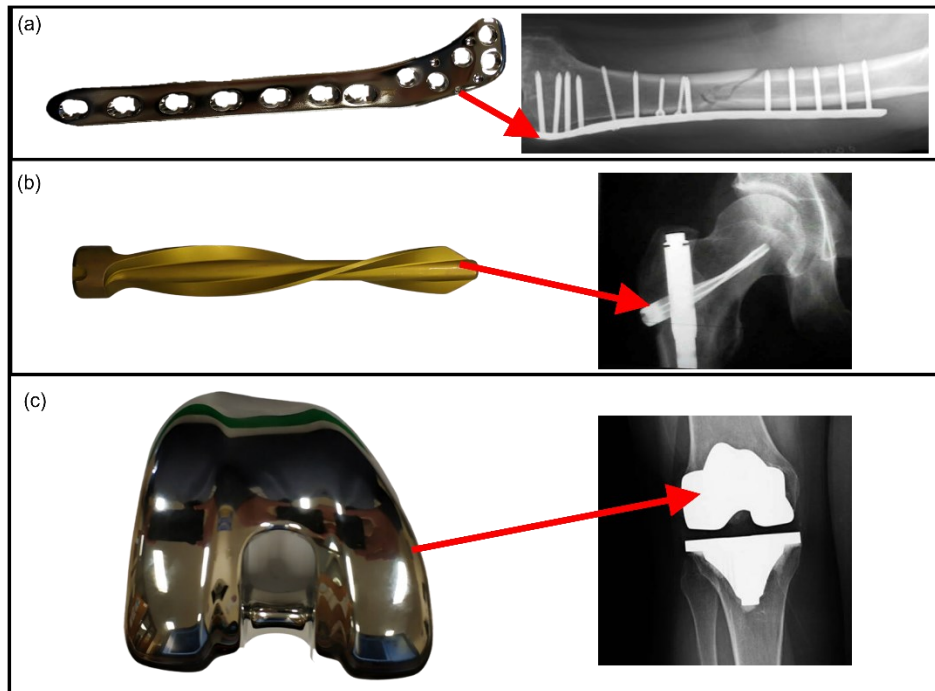


Figure 3-2 (a) Lock Compression Plate (stainless steel) and its application [333], (b) spiral blade (Titanium Aluminium Niobium) and its application [334] and (c) knee replacement femoral component (Cobalt chromium) and its application [335].

3.1.3 Nanofabrication Selection

Following the literature review and microscopic examination of the length scales of natural anti-bacterial surfaces, inverse design of anti-bacterial surfaces was conducted.

Nanofabrication techniques were carefully evaluated based on several factors, including their alignment with broader healthcare objectives. Specifically, the chosen techniques propose an innovative, physical means to minimise or eradicate bacterial growth. This is in line with the healthcare priorities of the UK Research Council and has the potential to reduce the need for antibiotics, thereby contributing to combating antibiotic resistance [336–338].

The economic feasibility of the chosen fabrication processes was also considered; they were adapted from existing manufacturing methods. While initial costs for these advanced surface treatments may be high, long-term clinical and economic benefits suggest that such an investment could serve as

both an immediate patient safety measure and a long-term economic strategy for healthcare systems. Furthermore, the risk of implant-associated infection extends beyond the immediate post-operative period, adding another layer of importance to the advanced surface treatments as a long-term safeguard.

Ultrafast laser ablation was selected for its high throughput capability and the ability to fabricate features in a wide size range and on a variety of materials, but it has less precise geometric control and limitations in achieving high aspect ratio features such as nanopillars. Additionally, deep reactive ion etching (DRIE) was chosen as the research standard for fabricating mechano-bactericidal surfaces. Although this technique can achieve high throughput, the nanopillar formation mechanism lacks precise geometric control and is limited to certain materials. Finally, scanning probe lithography (SPL) and focused ion beam (FIB) lithography were chosen for their capability to exert precise geometric control on nanofeatures, but both have a low throughput. These techniques were utilised as benchmarking methods to compare the capabilities of contact and non-contact prototyping nanofabrication techniques in the creation of anti-bacterial nanostructures.

In collaboration with Eurecat, the Technological Centre of Catalunya, silver ion implantation was applied to orthopaedic implants obtained from MKU hospital in the aim of comparing chemical modification techniques with topography modification techniques.

A comprehensive economic analysis and larger scale trials are recommended for future studies to fully evaluate the economic and clinical efficacy of these techniques.

Figure 3-3 presents the methodology of the thesis in form of a flowchart.

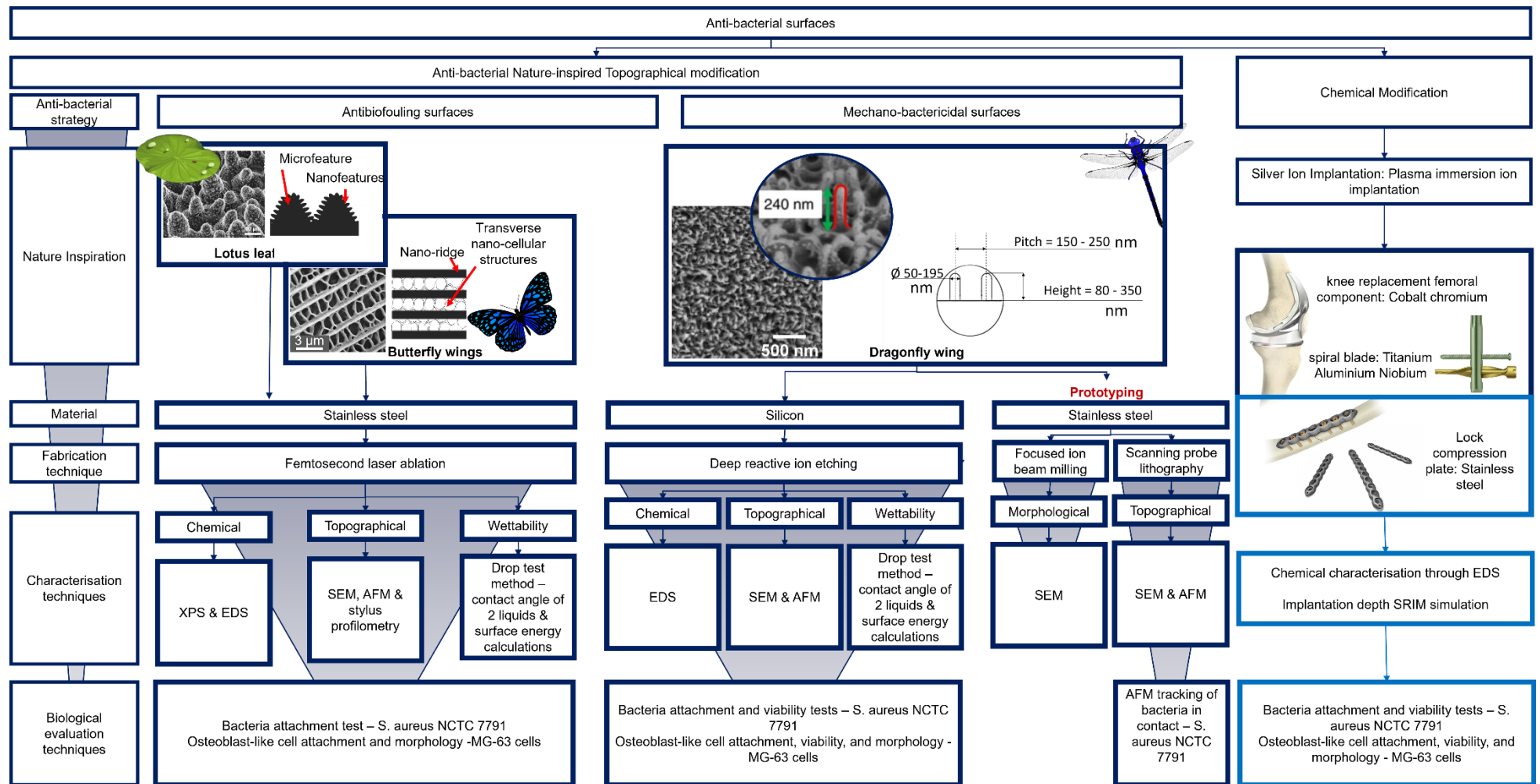


Figure 3-3 Flow chart of the methodology of the thesis

3.2 Fabrication Techniques

3.2.1 Femtosecond Laser Ablation

Laser irradiation was performed on the stainless steel samples supplied by LASEA Ltd. with a LASEA LS4 machine equipped with an amplitude systems satsuma laser source (wavelength = 1030 nm, pulse duration = 400 fs). The laser output was firstly directed through a polariser and half-wave plate for fine adjustment of the laser pulse energy, prior to a beam expander to achieve an appropriate beam size (Figure 3-4). A galvanometric scanning head (LS scan) and a 100 mm focal length f-theta lens were then employed to focus the laser beam on the specimen surface.

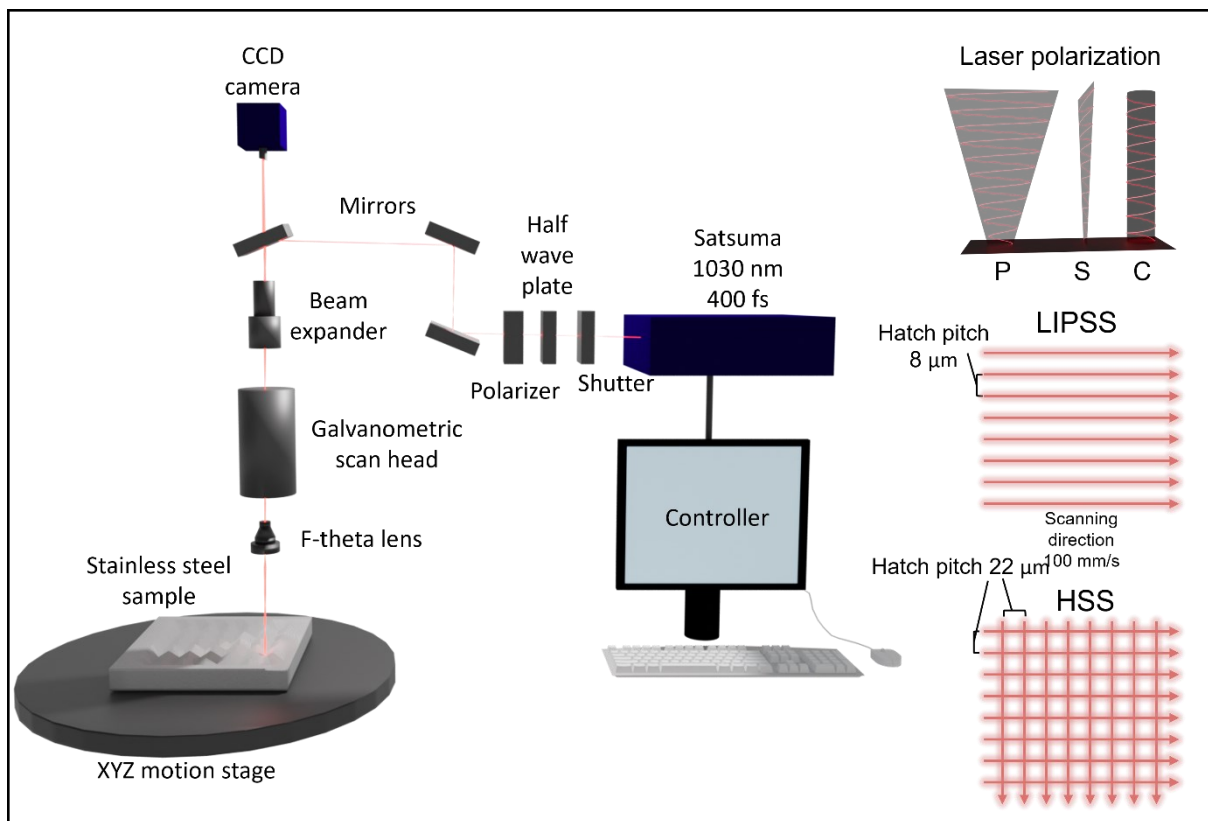


Figure 3-4 Laser setup and processing strategy

Two types of laser treatments were applied to generate nanostructured surfaces and hierarchical micro-nano structured surfaces herein referred to as laser

induced periodic structured surface (LIPSS) and hierarchically structured surfaces (HSS), respectively.

Table 3-3 details the parameters used to generate the distinct types of surfaces.

Table 3-3 Laser parameters used for fabrication of structured surfaces on stainless steel.

Parameters	LIPSS	HSS
Wavelength (nm), pulse duration (fs)	1030, 400	
Average power (W)	0.1	0.7
Repetition rate (kHz)	125	
Scan speed (mm/s)	100	
Hatch pitch (μm)	8	22
Passes	1	10 or 40
Polarisation	P, S, or C	P
Fluence (J/cm ²)	0.385	2.695

Herein, the LIPSS surfaces will be referred to according to the beam polarisation used to fabricate them; LIPSS_P, LIPSS_S and LIPSS_C. Additionally, the hierarchically structured surfaces will be referred to according to the number of laser passes applied; HSS_10 and HSS_40.

The fluence was varied between LIPSS and HSS processing but was maintained above the ablation threshold of stainless steel (0.1 J/cm²) [76]. It was calculated according to the following equation:

$$F = \frac{2E_p}{\pi\omega_0^2} \quad (3-1)$$

Where E_p is the energy per pulse (0.8 μJ for LIPSS and 5.6 μJ for HSS) and ω_0 is the focal spot size in focus (23 μm).

3.2.2 Deep Reactive Ion Etching

Fluorine based reactive ion etching was performed using a Surface Technology System (STS) Multiplex Advanced Silicon Etcher, shown in Figure 3-5, to etch the silicon wafers of orientation (100) and (111).

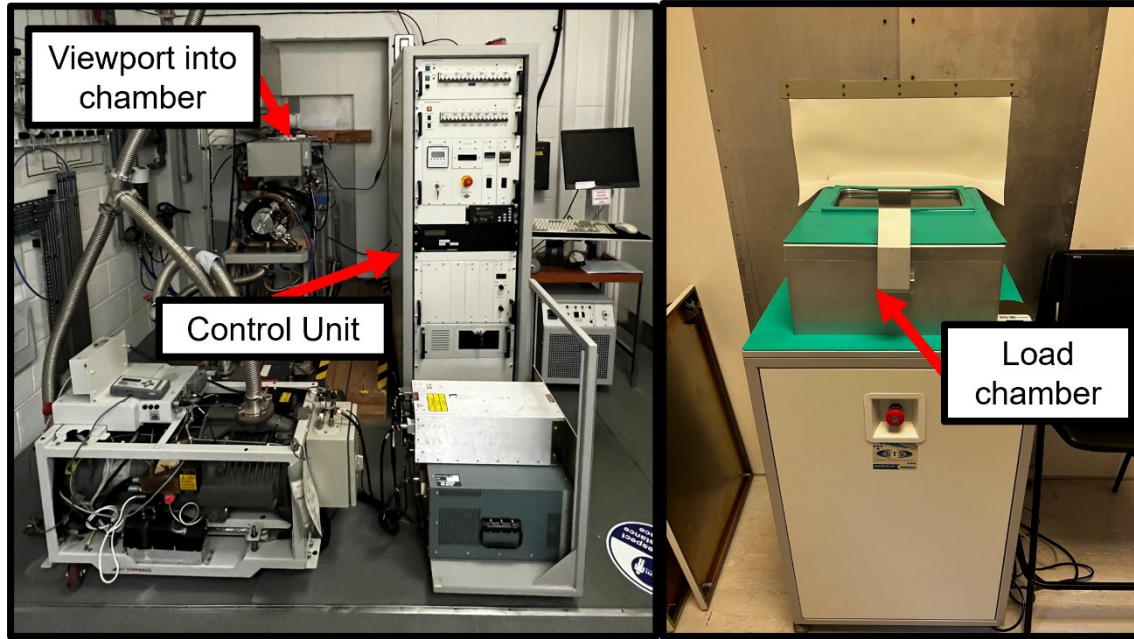


Figure 3-5 Surface Technology System (STS) Multiplex Advanced Silicon Etcher used to fabricate black silicon

O₂ gas flow was maintained at 90 sccm. The etching was done in three rounds by varying SF₆ gas flow from (60 to 40) sccm with 10 sccm increments. The RF power for ICP and RF generators were 600 W and 5 W respectively. The chamber pressure was kept at 94 mTorr. The lid temperature was maintained at 20 °C. Total etching time was 8 min. The unetched control and black silicon surfaces were cut into 1 x 1 cm² samples by diamond scribing.

3.2.3 Focused Ion Beam Milling

The Helios NanoLab 650 FIB SEM system (FEI, USA) was used for patterning a stainless steel sample. An array of 25 x 33 x 33 pillars was milled through an automated Helios scripting language. A datum target was set, and drift correction was done by automated periodic image recognition of said datum target. The

depth of mill is set for the mill rate for silicon. The beam parameters and pattern definitions (depth, inner and outer diameters) were tuned experimentally and are presented along with the milling strategy in Figure 3-6.

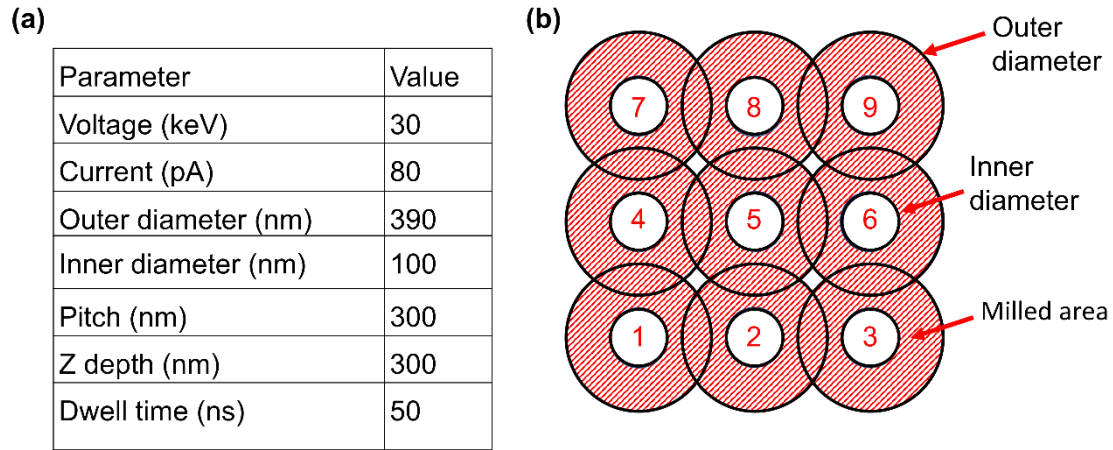


Figure 3-6 (a) FIB milling parameters, (b) FIB milling strategy

3.2.4 Scanning Probe Lithography

A stainless steel 316L sheet was acquired from Smiths metal centres Ltd. (UK) and cut into $10 \times 10 \times 3 \text{ mm}^3$ pieces for patterning. The samples were then ground and polished to achieve a roughness (R_a) of around 5 nm.

The experimental set-up includes a Dimension Icon atomic force microscope (Bruker, USA) and a high accuracy stage. The stage control system was used to set a velocity of $60 \mu\text{m/s}$ with a normal applied load of $50 \mu\text{N}$. The AFM system was fitted with a boron-doped diamond tip (NC-LC type probe, Bruker, USA – 20 nm nominal end radius, a nominal spring constant of 100 N/m, nominal frequency of 450 kHz and nominal length of $125 \mu\text{m}$). The tip was selected after experimenting with a lower stiffness coefficient probe (DT-NCLR, Nanosensors, Switzerland).

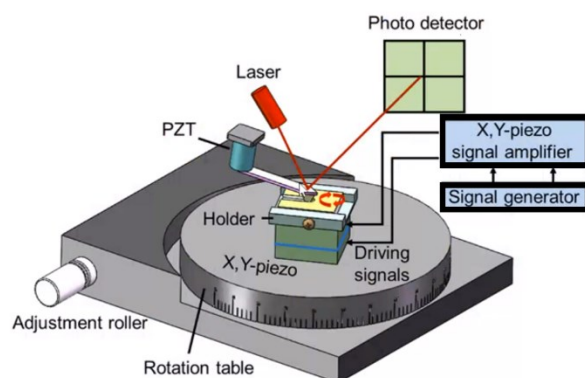


Figure 3-7 The set-up used for the SPL process

The milling was done in a hatched fashion to create a nanopillar pattern on a $50\text{ }\mu\text{m} \times 50\text{ }\mu\text{m}$ area by nano-milling in one direction then rotating the sample stage by 90° .

3.2.5 Silver Ion Implantation

Silver implantation processes of orthopaedic implants parts were carried out in a pulsed filtered cathodic vacuum arc system (PFCVA-450 from Plasma Technology Limited, Hong Kong). In order to reduce the incorporation of detrimental microparticles in the samples, this system is provided with two pulsed filtered cathodic arc sources, where the generated arc plasmas are guided into the vacuum chamber by an electromagnetic field applied to the curved ducts. The samples were positioned in the centre of the chamber (240 mm away from the exit of the filtered duct), and they were negatively biased to a high pulsed voltage. More details of this equipment can be found elsewhere [339]. A schematic and picture of the system are presented in Figure 3-8.

Two high-purity silver cathodes (99.95 %) were co-evaporated on a rotating sample. The cathodes were ignited at 50 V, using a pulse duration of $450\text{ }\mu\text{s}$ at a frequency of 7 Hz, generating a mean arc current of about 0.17 A. An argon flow of 2 sccm was introduced through each arc source. The implantation process was carried out at working pressure of 0.03 Pa, for 60 min and at $175\text{ }^\circ\text{C}$ substrate temperature. The samples were biased to a pulsed potential of -20 kV (pulse

frequency 7 Hz, pulse width 600 μ s). Under these conditions, the bias current was around 34 mA, which corresponds to an ion dose of 4.0×10^{17} ions/cm², approximately.

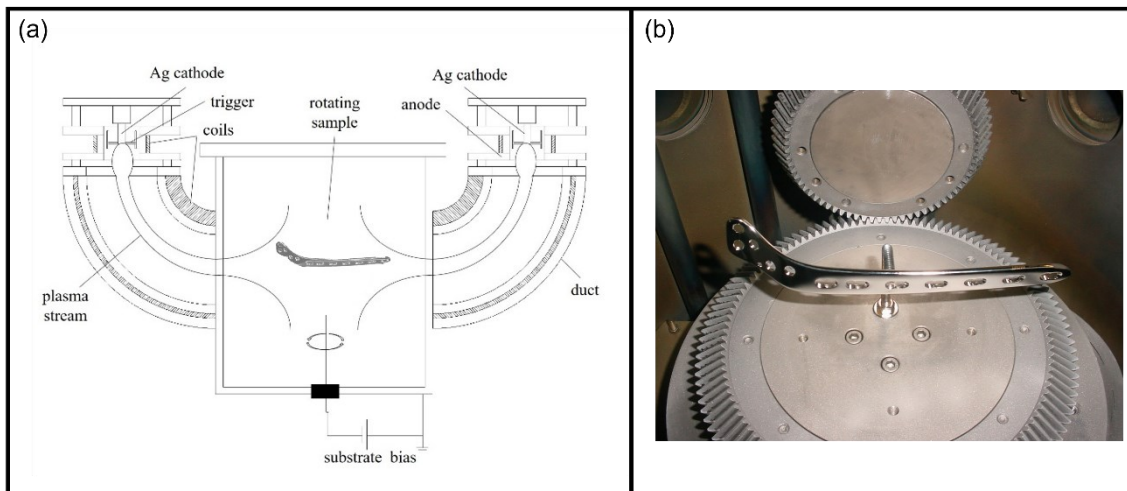


Figure 3-8 (a) Schematic of the pulsed filtered cathodic vacuum arc system. (b) Placement of the locking compression plate (LCP) prosthesis on the rotating sample holder.

All fabricated samples were cleaned by sonication with acetone for 15 min, sonication with isopropanol for 10 min and then immersed in 70% ethanol (for around 2 min) in a 24 well plate. The samples were then moved to a fresh well plate and were allowed to air dry prior to characterisation and biological evaluation.

3.3 Surface Characterisation techniques

3.3.1 Scanning Electron Microscope

All samples were analysed with a Tescan S8215G field emission (Tescan, Czechia) scanning electron microscope (FE-SEM) to observe the morphology of the fabricated structures.



Figure 3-9 The S8215G field emission SEM utilised in this work.

The stainless steel samples were imaged at 10 keV and 63 pA. The silicon nanostructured samples were sputter coated with 10 nm of gold to provide a conductive surface and then imaged in UH-resolution mode at 20 keV and 1 nA. Tilted images were also taken for black silicon nanostructured samples and later analysed with imageJ (version 1.53e, National Institutes of Health, Bethesda, MD, USA) to obtain the tilted length of the pillars. The actual length was calculated according to the following equation:

$$h_{actual} = \frac{h_{tilt}}{\sin \phi} \quad (3-2)$$

where ϕ is the tilt angle.

In some cases, the SEM was also used to observe the morphology of bacteria on the surfaces after attachment.

3.3.1.1 Surface preparation for SEM bacteria imaging

Following bacterial tests for FIB structured surfaces, the adhered bacteria were fixed in 10 % formalin for 10 min while the SPL milled surfaces were fixed in a 4 % Glutaraldehyde solution overnight in order to fix the adhered bacteria while retaining their morphologies through protein crosslinking. After that, they were rinsed and dried again for 1 hour then placed in different concentrations of

ethanol for 10 min successively (30 %, 50 %, 70 %, 90 % and 100 %) and dried in a class 2 flow hood [197]. Following submersion in ethanol, the samples were sputter coated with a 10 nm gold layer to ensure conductivity for SEM imaging.

3.3.2 Atomic Force Microscope

3.3.2.1 Laser Structured Surfaces

A (Veeco/DI) Bruker Dimension 3100 with a Nanoscope V controller (Bruker, USA) atomic force microscope (AFM) was used for assessing the roughness scale parameters of all the laser structured surfaces, presented in Figure 3-10.

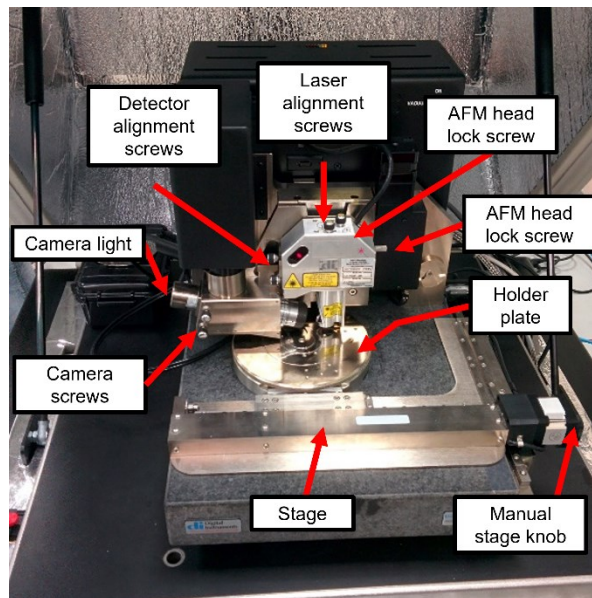


Figure 3-10 The AFM used for topographical measurements.

The AFM measurements were conducted in tapping mode. The AFM was fitted with a nanoSensors AFM Probe PPP-NCHR-10 tip with a nominal radius of <10 nm and nominal length of $125\text{ }\mu\text{m}$. The AFM scans were performed for an area of $(5 \times 5)\text{ }\mu\text{m}^2$ in tapping mode. Particularly for the hierarchically structured surfaces, the AFM tip was centred with the top of the microfeature and an area of $(5 \times 5)\text{ }\mu\text{m}^2$ was subsequently scanned as shown in the diagram in Figure 3-11.

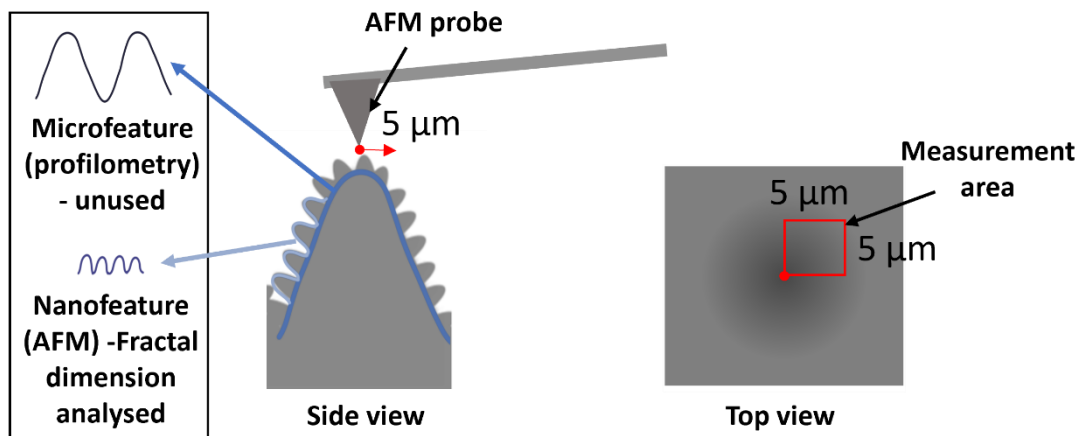


Figure 3-11 Diagram of the AFM measurements conducted for the hierarchically structured surfaces. The red dot indicates the point at which the AFM tip was centred. The red square is the measurement area.

The scan resolution was chosen to be 512 x 512 as that would result in a 9.76 nm sampling size which is suitable for the tip size used. Three measurements were taken for each sample. Post processing was performed using the TalyMap Gold software (7.4, Taylor Hobson Inc., UK) [340].

The AFM scans were first levelled line-by-line according to the direction of scanning. Then an S-filter was applied to denoise the measurement with a cut-off of 0.06 μm [291]. The robust Gaussian filter was then used to assess the surface parameters in varying scales (0.125:0.125:1 μm and 1:1:5 μm).

3.3.2.2 SPL Patterned Surface

A sharp silicon tip (VTESPA-300, Bruker, USA) with a 5 nm nominal tip radius and a tip height ranging from 9-12 μm was fitted on a Veeco Dimension 3100 AFM (Bruker, USA).

The AFM scans were performed for an area of (10 x 10) μm^2 in tapping mode. The scan resolution was 256 x 256 which results in around 39 nm sampling size. Polynomial background levelling was applied. Post processing was performed

using the Gwyddion software (version 2.6, Czech Metrology Institute, Czechia)[341].

3.3.2.3 DRIE Surfaces & SPL Patterned Surface

For all types of surfaces, areal topography parameters such as average areal surface roughness (S_a), root-mean-square roughness (S_q), developed interfacial area ratio (S_{dr}), density of peaks (S_{pd}), texture aspect ratio (S_{tr}) and the fractal dimension (D) were calculated using the TalyMap Gold software (7.4, Taylor Hobson Inc., UK) [291]. For the DRIE samples, the root-mean-square gradient S_{dq} was also a parameter of interest. The reader is requested to refer to section 2.5.2 for a detailed explanation of the significance of each parameter.

Unless stated otherwise, three measurements were taken for each sample and the results are reported as average value $\pm$ the standard deviation.

3.3.3 Profilometry

Profilometry was used to measure the profiles of the hierarchical laser structured stainless steel surfaces since the microfeatures present on these surfaces were too large to measure with the AFM. A Form Talysurf 120L surface profilometer, shown in Figure 3-12, (2.5 μm radius and 60° cone angle diamond tip probe) was used to measure 200 μm profiles from both HSS_10 and HSS_40 surfaces. Three measurements were taken for each sample and the microfeature heights (Peak-to-peak) were then determined.

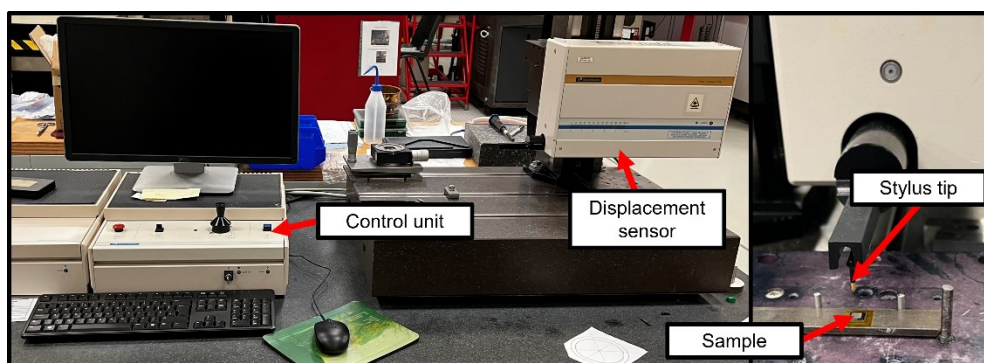


Figure 3-12 Form Talysurf 120L stylus profilometer set up

3.3.4 Energy Dispersive Spectroscopy (EDS)

Energy dispersive spectroscopy analysis was performed on all substrates by means of an Oxford Instruments Ultim® X-Max 100mm EDS (Oxford Instruments, Abingdon, UK) fitted on a Tescan S8215G (Tescan, Czechia) SEM to study the chemical composition of the samples.

3.3.5 X-ray Photoelectron Spectroscopy (XPS)

X-ray photoelectron spectroscopy (XPS) analysis was performed, on laser structured surfaces, using a UHV-XPS which is a FlexMod system with a monochromated 400 W Al K anode (SPECS XR-50M) (SPECS surface nanoanalysis GmbH, Germany) as shown in Figure 3-13. The Analyser is a Phoibos 150 with 1D delay line detectors.

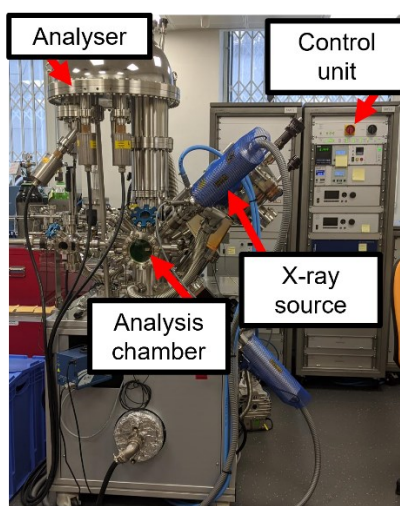


Figure 3-13 The XPS system used in this work

Measurements were carried out at 10^{-9} mbar. The hydrocarbon component of the C 1 s peak (BE 284.8 eV) was used as a reference for charge correction. Survey spectra were collected in 2 scans with 50 eV pass energy and recorded at 0.5 eV/step and 0.1 s dwell time, while the region spectra were collected in 9 scans with 30 eV pass energy taken at 0.1 eV/ step and 0.1 s dwell time.

The Tougaard algorithm was used to measure the background core level spectra and chemically distinct species in the high-resolution regions of the spectra were

resolved using synthetic Gaussian–Lorentzian components and asymmetric line function where applicable (Cr metal and Fe metal) after the background was removed (using CasaXPS software, v. 2.3.25). The relative atomic concentration of elements determined using XPS was quantified on the basis of the peak area in the selected high-resolution region, with the appropriate sensitivity factors for the instrument being used. High-resolution scans were performed across each of chromium 2p (Cr2p) and iron 2p (Fe2p) peaks.

3.3.6 Surface Energy – Sessile Drop method

Deionised water and diiodomethane static contact angles were measured via the sessile drop method with an OCA 25 goniometer shown in Figure 3-14 (DataPhysics Instruments GmbH, Germany) and analysed with SCA20 software (DataPhysics Instruments GmbH, Germany). A droplet volume of 2 μL was employed for tests regarding laser structured surfaces, while 1 μL was used for DRIE surfaces. At least three measurements were taken for each sample after (8-10) s of contact with the sample surface. The results are reported as average value $\pm$ the standard deviation.

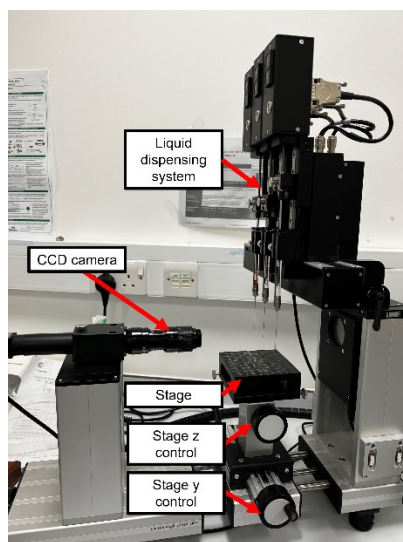


Figure 3-14 The goniometer set up employed

The roughness corrected contact angle (Young's contact angle) was calculated according to the following equations [63,342]:

$$\cos \theta_m = r \cos \theta_Y \quad (3-3)$$

$$r = 1 + \frac{S_{dr}}{100} \quad (3-4)$$

Where θ_m is the measured contact angle, θ_Y is young's contact angle, r is the roughness factor and S_{dr} is the developed interfacial area ratio.

The surface energy (SFE) was then calculated based on the Owens-Wendt-Rabel & Kaelble model [61] as presented in equation (2-1).

The parameters used to compute the surface energy are shown in Table 3-4 [343,344].

Table 3-4: Surface energy components for water and diiodomethane

Liquid	γ_l (mJ/m ²)	γ_l^p (mJ/m ²)	γ_l^d (mJ/m ²)
Distilled water	72.8	46.8	26
Diiodomethane	50	2.6	47.4

It must be noted that the size of the area evaluated through the droplet method is significantly higher than the area evaluated to calculate S_{dr} values. Additionally, since the AFM scans from which S_{dr} values were extracted are $5 \times 5 \mu\text{m}^2$, unincompassing the full microfeatures for the hierarchical laser structured surfaces (HSS surfaces), S_{dr} cannot be used to calculate the corrected contact angle for these surfaces. However, for LIPSS surfaces and DRIE silicon surfaces, given that their dominant wavelength is encompassed within the measurement, S_{dr} values can be used. Therefore, S_{dr} was evaluated at different scales to assess the suitability of its usage to compute the corrected contact angle for these surfaces.

3.3.7 X-ray Diffraction (XRD)

X-ray diffraction (Siemens D5005, Germany) analysis was performed under ambient conditions to confirm the orientation of the silicon wafers and any changes in orientation/crystallinity due to etching. The samples were scanned over the $2\theta = 10-90^\circ$ at a scanning rate of 0.5° per minute using Cu-K α radiation, ($\lambda = 0.15406$ nm). To confirm the expected XRD spectrum 2θ value, Bragg's law was applied:

$$n\lambda = 2d \sin \theta \quad (3-5)$$

Silicon with orientations (100) and (111) was simulated in AtomsK and visualised in OVITO. The lattice parameter was then extracted and used to calculate the $2\theta_{100}$ and $2\theta_{111}$.

3.4 Biological Evaluation techniques

3.4.1 Bacterial Growth, Retention and Viability

To ensure a consistent and pure bacterial culture for each experiment, *S. aureus* NCTC 7791 (Culture Collections, UK Health Security Agency, UK) bacterial cultures were prepared from a single colony on tryptone soya agar (TSA) and grown overnight in tryptone soya broth (TSB) at 37°C , 5 % CO_2 .

10 mL of the overnight bacterial culture was transferred to a universal container and centrifuged at 3000 rpm for 5 min. This step was conducted to isolate the bacterial cells from the culture medium. The bacterial pellet was then resuspended with phosphate buffered saline (PBS) and centrifuged again at 3000 rpm for 5 min. The pellet was resuspended in PBS to an optical density (OD_{600 nm}) of 0.09 (corresponding to approximately 1×10^7 CFU/mL). This standardised the bacterial concentration for each experiment.

The overlay method as described in section 2.3.3 was conducted as follows: a 15 μL droplet of the inoculum was added to each sample surface in a 24 -well plate and incubated for 1 hour (laser structured surfaces) and 3 hours (DRIE,

SPL, FIB and Ag-implanted surfaces) at 37 °C, 5 % CO₂. This allowed for controlled interaction between the bacterial cells and the various surface designs.

After incubation, the samples were then transferred to a fresh 24-well plate and washed in 1 mL of 0.85 % NaCl. Live/dead staining was performed according to the manufacturer's instructions (LIVE/DEAD BacLight Bacterial Viability Kit, Thermofisher Scientific, UK). This step enabled the differentiation between live and dead bacterial cells on the surface.

The stained samples were imaged using an AX-70 fluorescent microscope (Olympus, Japan) at magnifications of x10, x 20 and x40. The percentage area coverage or bacterial coverage (BC) was quantified using the ImageJ software as outlined in a previous publication [15] using the x10 magnification images to ensure the largest sample surface area was analysed.

3.4.2 AFM Bacterial Cell Tracking

S. aureus NCTC7791 bacteria were grown overnight in tryptone soya broth (TSB) at 37 °C, 5 % CO₂. An aliquot of 1 mL was centrifuged to a pellet (11999 rpm for 3 min), washed and resuspended in PBS with an OD₆₀₀ of 1.5.

The SPL structured stainless steel sample was mounted in a liquid cell of a JPK nanowizard® 4XP bioscience AFM (JPK instruments, Bruker, USA). A droplet of distilled water was added to the sample surface and the sample was imaged in QI mode at 5 nN.

For bacteria-surface observation, a 20 µL droplet of bacterial suspension was added to the sample. The surface was then continuously scanned. Once a bacterium was located, a timer was started and continuous scans were conducted to monitor the response of the bacteria to the surface pattern. Similar scans were conducted on a flat area of the stainless steel surface for comparative purposes.

3.4.3 Osteoblast Cell Culture, Attachment and Morphology

In order to assess the biocompatibility/cytotoxicity of the manufactured surfaces, human MG-63 osteoblast-like cells (Sigma Aldrich, UK) were used. Cell attachment was studied to determine favourable surface features for encouraging bone formation, and cell morphology was studied to determine whether surface features may induce cytoskeletal changes and therefore influence osteogenic potential. Cells (passages 29–32) were maintained in T75 cell culture flasks in culture medium comprising of alpha minimum essential medium (α -MEM) supplemented with 10 % (v/v) heat-inactivated foetal bovine serum (FBS) and 100 units/mL penicillin G sodium, 0.1 μ g/mL streptomycin sulphate and 0.25 μ g/mL amphotericin (Thermofisher Scientific, UK). The cells were incubated at 37 °C, 5 % CO₂ with the medium changed every 2–3 days. Cells were cultured until they reached approximately (80–90) % confluency before being used for subsequent experiments.

The cells were detached using trypsin-EDTA, counted and seeded directly on the control surfaces, the laser, DRIE and Ag-implanted surfaces in a 24-well plate at a density of 100 cells/ μ L (approximately 1500 cells per sample). Samples were incubated for 1 h at 37 °C, 5 % CO₂ to allow attachment. The wells were then flooded with 500 μ L of complete culture media and incubated for a further 24 hours.

The next test procedure (CCK-8 assay) was only applied to DRIE surfaces. The CCK-8 assay is a colorimetric method used to assess cell viability and proliferation, which works by measuring the metabolic activity of living cells through the reduction of a water-soluble tetrazolium salt, producing a coloured formazan product that can be quantified using a spectrophotometer [345]. Following the 24 hours incubation with attached MG-63 cells, the media was removed and the samples were washed three times with PBS. They were then transferred to a fresh 24-well plate and 200 μ L of fresh media were added to each well along with 20 μ L of CCK-8 reagent. The samples were then incubated for

3 hours at 37 °C, 5 % CO₂. After incubation, 100 µL of media were transferred to a 96 well plate and the absorbance at 450 nm was measured using a FLUOstar Omega plate reader.

Subsequently, the laser structured samples, DRIE samples and Ag-implanted samples were washed three times with PBS to remove non-adherent cells and incubated in 500 µL of 10 % formalin overnight at 4 °C to fix the attached cells. Following incubation, the formalin solution was removed and the samples were washed three times with tris-buffered saline (TBS). The cells were then permeabilised with filtered 1 % Triton X-100 (Sigma Aldrich, UK) for 30 min at room temperature. After permeabilisation, the samples were washed three times with TBS. Incubation of the samples for 1 hour at room temperature with a solution of 1 % bovine serum albumin (Vector Laboratories, UK) in TBS was performed to block non-specific binding sites. Following incubation, the actin filaments of the cells were stained with freshly prepared fluorescein isothiocyanate (FITC) labelled phalloidin (diluted 1:50 in TBS, Sigma Aldrich, UK) and incubated in the dark for 40 mins at room temperature. The samples were then washed three times with TBS and allowed to air dry for 5 min. Subsequently, 10 µL of 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories, UK) was added to each sample surface to stain the nuclei of the cells. Images of the cell actin filament and nuclei were taken at magnifications of x10, x20, x40 using an AX70 Olympus fluorescent microscope (Olympus, Japan). FITC and DAPI channels were superimposed using ImageJ. Cell attachment and morphology analysis was performed on the fluorescent images to semi-quantify the area of the cells (CA), the circularity of the cells (CC) and the cell number (CN), where applicable using imageJ image processing software.

3.5 Statistical Analysis

Contact angle measurements, AFM measurements and cell culture experiments were performed three times for all surfaces. Bacterial attachment experiments were performed twice, and three images (x10) were taken at random and analysed for each sample. The Shapiro wilk test was conducted on all results to

validate the normal distribution for all experiments. All data were subject to a one-way analysis of variance (ANOVA) to test for statistical significance. For data including two groups only (some of the DRIE data), p values were calculated by two-tailed paired Student's t test. In all cases, a p-value of < 0.05 was considered significant whereas in the case of a p-value of > 0.05 , the results were reported as observational. In the case that the ANOVA-obtained p-value was found to be less than 0.05, Tukey's multiple comparisons test was performed to obtain pairwise comparisons between data means. All data were expressed as the mean accompanied by the standard deviation.

For laser structured surface, to determine the correlation between bacterial attachment, surface energy and topographical roughness parameters, as well as the correlation between surface energy, topographical roughness parameters and MG-63 cell-related parameters correlation matrices were constructed using the spearman correlation factor. The MG-63 cell-related parameters included the number of attached cells (CN), cell area (CA) and cell circularity (CC).

For comparison of all the fabricated textured surfaces, the spearman correlation was used. However, since the measurements techniques vary between the surfaces, the results were considered observational.

4 Fabrication of Nature-Inspired Sub-micron and Hierarchical Nano/Micro Scale Features Using Laser Ablation

4.1 Introduction

The work presented in this chapter describes the use of femtosecond laser ablation to fabricate nature-inspired structures on stainless steel on two length scales: nanoscale and hierarchical micro-nanoscale. The structures fabricated were inspired from the butterfly wings which are comparable to the laser induced periodic structured surfaces (LIPSS) and the lotus leaf structures (hierarchical structured surfaces, HSS) presented earlier in section 2.4.3. The fabricated surfaces were extensively characterised to investigate the influence of surface topography and surface energy on the resulting biological functionality (i.e. ability to inhibit bacterial attachment, whilst maintaining healthy osteoblast attachment). The two important questions this chapter attempts to answer are:

- (i) which surface physical parameters are most indicative of the biological functionality of nano/micro-structured surfaces and at what scales?
- (ii) to what extent do the physical changes to structured surfaces influence the biological (bacterial and osteoblast cell) responses compared to surface energy changes?

4.2 Results

4.2.1 Characterisation of Micro/Nanopatterned Surfaces

4.2.1.1 Laser Induced Periodic Structured Surfaces - LIPSS

The SEM micrographs presented in Figure 4-1 (a) provide evidence that surfaces subjected to low fluence laser irradiation undergo reorganisation, resulting in the formation of a distinct surface morphology known as "laser-induced periodic structured surfaces" (LIPSS).

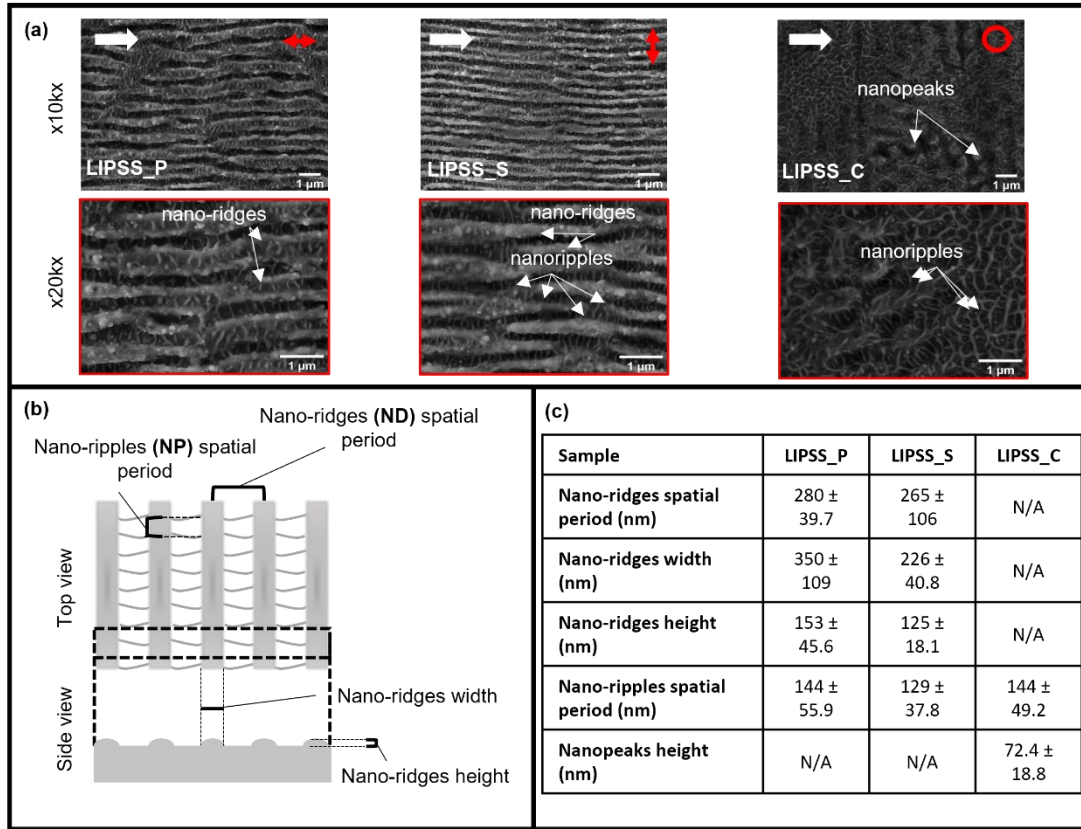


Figure 4-1 (a) SEM micrographs of the three types of LIPSS surfaces. The red arrows indicate the laser polarisation, the white arrows indicate the scanning direction. (b) Diagram depicting the typical linearly polarised LIPSS morphology (c) geometrical measurements of nanoripples and nano-ridges of LIPSS surfaces measured from SEM image processing. The heights were measured from line scans extracted from AFM measurements.

As stated previously, the LIPSS surfaces are referred to according to the beam polarisation used to fabricate them; LIPSS_P, LIPSS_S and LIPSS_C. Additionally, the hierarchically structured surfaces are referred to according to the number of passes applied: HSS_10 and HSS_40. A flat is an as-received steel sample which was used as a reference sample.

LIPSS_P and LIPSS_S surfaces were both exposed to a linearly polarised laser beam whereas LIPSS_C was exposed to a circularly polarised laser beam. The direction of the incident electrical laser field is indicated with red arrows and a circle and the scanning direction is indicated with the white arrow. LIPSS_P and

LIPSS_S present a similar pattern of parallel nano-ridges linked together by short nano-ripples as depicted in Figure 4-1 (b). Nanospheres can also be observed on LIPSS_P and LIPSS_S. The structure of LIPSS_C is a nano-ripple dominated structure with a few visible circular nano-peaks. In some instances, nanoripples can be seen clustered together longitudinally (top right of LIPSS_C 10kx SEM micrograph in Figure 4-1 (a)).

The dimensions of nanoripples present on the LIPSS surfaces were estimated from SEM image analysis which are shown in Figure 4-1 (c). The nano-ridges spatial period was found to be lower for LIPSS_S than the LIPSS_P. The width and height of nano-ridge for LIPSS_P were found to be higher than that of LIPSS_S. The spatial period of the nano-ripples on surfaces LIPSS_P and LIPSS_C were of similar dimension however, LIPSS_S possesses slightly lower spatial period between its nanoripples. The nano-ripples' heights (obtained from AFM scans) were low for LIPSS_C, higher for LIPSS_S and highest for LIPSS_P.

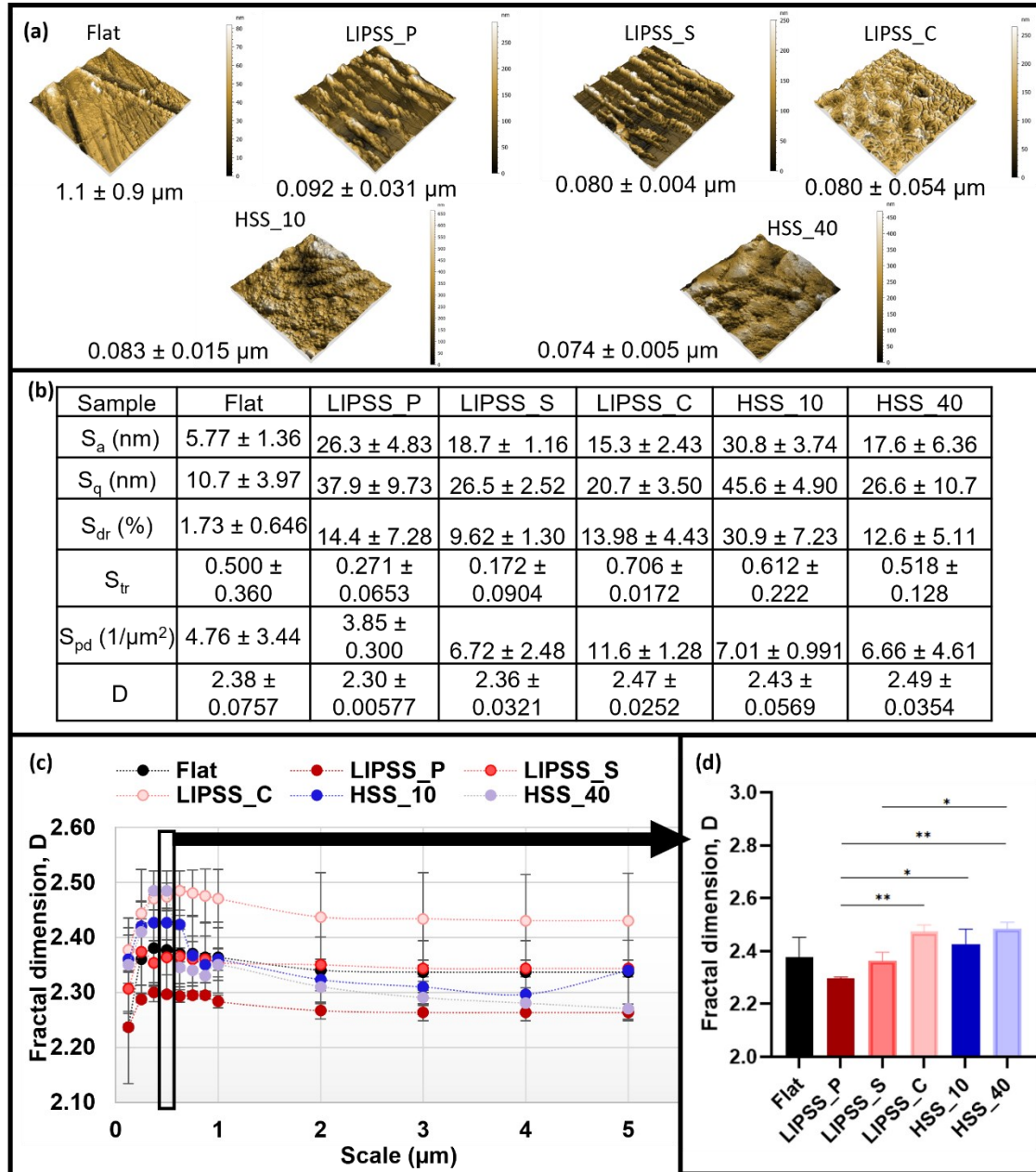


Figure 4-2 (a) AFM height maps of all tested surfaces indicating the corresponding surface's wavelength based on the power spectrum density calculations. (b) Surface topographical parameters of the surfaces at the 0.5 μm scale (c) The fractal dimension of the samples at varying scales. (d) The fractal dimension of the sample at the 0.5 μm scale. The p-value for pairwise comparison between samples and the flat sample are represented by * ($p < 0.05$) and ** ($p < 0.005$).

The AFM height maps of the different surfaces are shown in Figure 4-2 (a), along with the dominant surface wavelength obtained through the power spectrum

density method. Evidently, the structured surfaces showed a significantly lower dominant wavelength than the flat surface. It is crucial to consider this aspect for understanding the relevance of feature wavelength (scale) in relation to bacteria-surface and MG-63-surface interactions that will be discussed later on.

The roughness parameters, on varying scales as described in chapter 3, were extracted from filtered AFM height maps. In particular, the roughness parameters at the 0.5 μm scale are shown in Figure 4-2 (b), respectively.

On all scales, including 0.5 μm , LIPSS_P possesses higher S_a than LIPSS_S which corroborates with the nano-ridges height measurement. The developed interfacial area ratio increases from a flat surface to a structured surface. The highest S_{dr} was in the case of LIPSS_P for all scales. The peak density parameter was lowest for LIPSS_P, followed by the flat surface, then LIPSS_S and highest for LIPSS_C. The fractal dimension, describing the surface complexity, was highest for LIPSS_C as shown in Figure 4-2 (c, d).

4.2.1.2 Hierarchical structured surfaces – HSS

Surfaces that have been exposed to high fluence laser revealed a hierarchical structure that consists of regularly spaced micron-sized bumps superimposed with nanoridges, nanopeaks and nanoripples as presented in the SEM micrographs in Figure 4-3 (a). A few deep micro-holes can be seen on the surface exposed to 40 laser passes (HSS_40).

The HSS surfaces surface topography presents two features, which can be viewed in short and long range wavelengths – the short is one amenable to AFM measurements whilst the long one is amenable to profilometry measurements as shown in Figure 4-3 (b). The long wavelength component was composed of microfeatures while the short wavelength component comprised of nanofeatures sitting on top of the microfeatures. Together they are referred to as hierarchical structures.

Profilometry line scans depicting the primary profile of the hierarchical surfaces are presented in Figure 4-3 (c). The height of the microfeatures of HSS_10 was

lower than that of HSS_40, as expected, due to the increased number of laser passes.

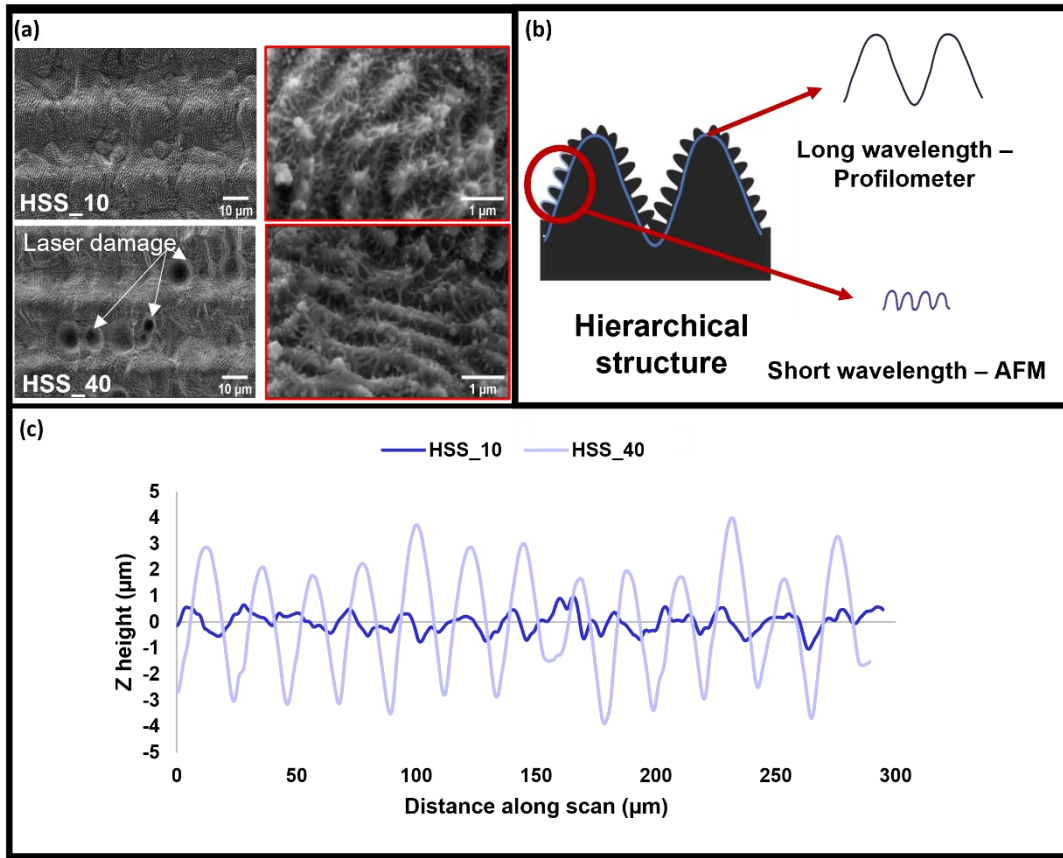


Figure 4-3 (a) SEM micrographs of HSS surfaces. (b) Diagram depicting the dual scale topography of HSS surfaces. (c) Profilometry line scan of HSS surfaces.

Figure 4-2 (b) presents the results of the roughness measurements, the fractal dimension and the surface energy (SFE) of HSS surfaces. The S_a for HSS_10 was comparable to LIPSS_C whereas that for HSS_40 was lower. S_{dr} is higher for HSS_10 than HSS_40. However, S_{pd} was higher for HSS_40 than all other surfaces including HSS_10. The fractal dimension for HSS_10 was lower than HSS_40 owing to the complexity of the surface subjected to 40 laser passes.

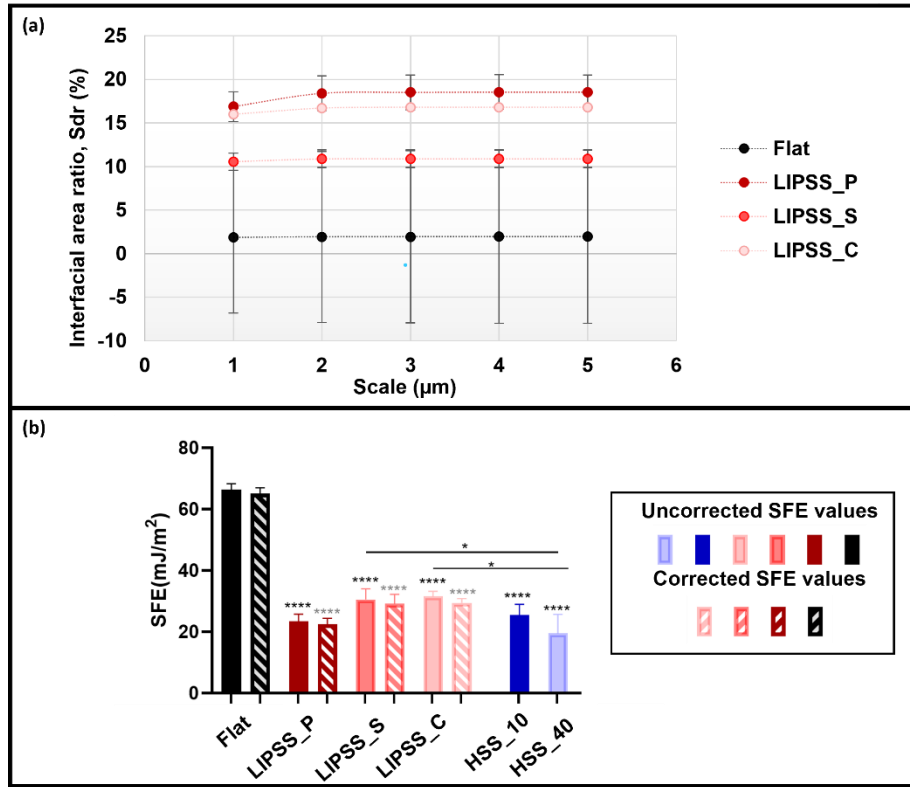


Figure 4-4 (a) Surface interfacial area ratio S_{dr} values for Flat and LIPSS surfaces over varying scales. (b) Surface energy (SFE) value calculated from the apparent contact angle for all surface along with roughness corrected surface energy values for LIPSS surfaces. The p-value for pairwise comparison between samples and the corresponding flat sample SFE value are represented by * ($p < 0.05$) and ** ($p < 0.0001$).**

S_{dr} values were plotted against all evaluated scales for LIPSS surfaces (Figure 4-4 (a)). Since S_{dr} values for all samples level off above the 3 μm scale, the levelled values were used to calculate roughness-corrected contact angles and corrected SFEs. According to this correction factor, the corrected surface energy for LIPSS surfaces is presented in Figure 4-4 (b). Since the HSS surfaces contain large microfeatures that are not accounted for within the AFM measurements, it was not possible to correct the SFE values for these surfaces. For comparison, uncorrected surface energy values are also shown for all surfaces.

All the structured surfaces exhibited significantly lower SFEs compared to the flat surface. Within LIPSS surfaces, LIPSS_P exhibited the lowest SFE of 23.4 mJ/m². Within HSS surfaces, the surface energy was lowest for the HSS_40 surface, while it was comparable to LIPSS_P for HSS_10.

EDS analysis was performed on all tested samples and is presented in Figure 4-5 (a). The measurements of different elements did not show significant changes between the samples except for the case of iron and carbon where there was a significant decrease between the flat sample and LIPSS_C for iron, and LIPSS_C and HSS_10 for both iron and carbon.

To better understand the chemical nature of the surface modification, X-ray photoelectron spectroscopy (XPS) measurements were conducted. The survey XPS spectra of the representative laser structured surfaces (LIPSS_P and HSS_10) and the flat sample are presented in Figure 4-5 (b) and the composition of the surface layer of the corresponding samples are given in Figure 4-5 (c). It was observed that the carbon content in all sample types was significant due to surface contamination. However, the laser structured samples showed approximately 30 % more carbon content than the non-structured samples.

This increased carbon content on the laser structured samples is likely due to the larger surface area of the sample and the presence of more surface sites for carbon contamination. When a surface is exposed to air, carbon-based compounds in the air can react with the surface to form a carbon layer. As the laser-textured samples had a larger surface area, they may have had more exposure to carbon-based compounds in the air, resulting in a greater amount of carbon contamination.

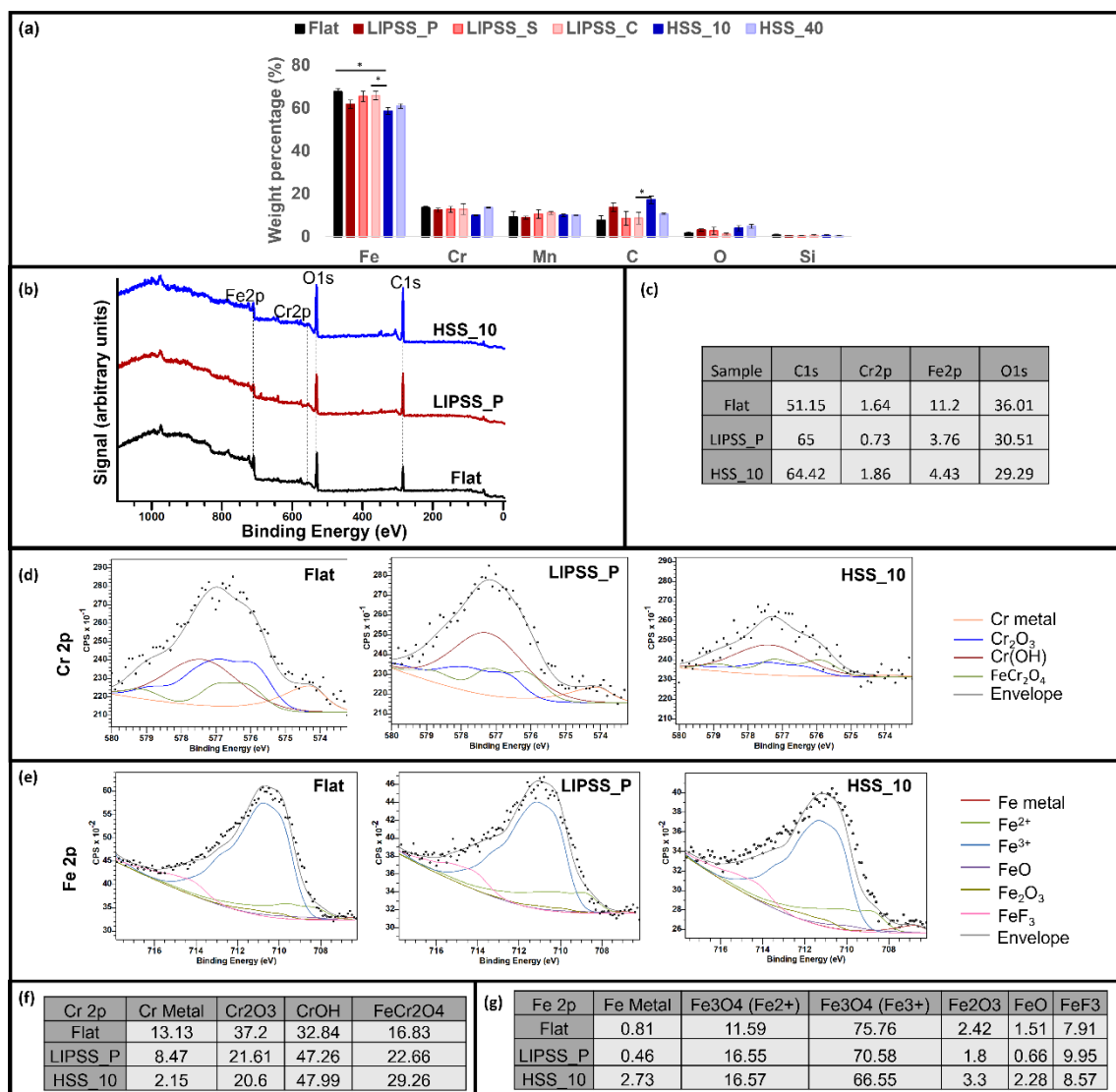


Figure 4-5 (a) Chemical composition of tested samples obtained by EDS. (b) XPS survey spectra of representative samples; flat, LIPSS_P and HSS_10. (c) High resolution Cr 2p spectra. (d) High resolution Fe 2p spectra. (e) Surface chemical composition of the samples. (f) Fractions of chromium compound and (g) Iron compound components for the representative tested surfaces.

This difference in carbon content is also evident in the survey spectra, where a significantly larger carbon peak can be observed for the laser-structured sample. The high-resolution spectra of the chromium and iron peaks confirmed the presence of an oxide layer on all three types of samples. Figure 4-5 (d) shows the high resolution spectra of Cr 2p peaks and their deconvolutions. The spectra

have been fitted with four major components; Cr metal, Cr_2O_3 , $\text{Cr}(\text{OH})_3$ and FeCr_2O_4 according to data found in the literature [346]. The high resolution Fe 2p spectra are shown in Figure 4-5(e). The peak has been fitted with six components belonging to Fe metal, FeO, Fe^{2+} and Fe^{3+} of Fe_3O_4 , Fe_2O_3 and FeF_3 , according to data found in the literature regarding peak positions, peak line shapes and widths of individual components [347]. The deconvolution allowed the identification of the different oxidation states of iron and chromium present on the sample's surface and the quantification of their relative amounts as presented Figure 4-5 (f, g). The results reveal that a very small amount of Cr metal is present on the surfaces of the laser structured surfaces, while Fe metal is barely present on all surfaces and that the surfaces are composed of mostly Cr_2O_3 , $\text{Cr}(\text{OH})_3$, FeCr_2O_4 , and Fe^{3+} with small amounts of Fe^{2+} , Fe_2O_3 , FeO and FeF_3 present. Further explanation of these results is made in the section 4.3.

4.2.2 Bacterial Coverage

Fluorescent imaging results following the live/dead assay on the tested surfaces are presented in Figure 4-6 (a).

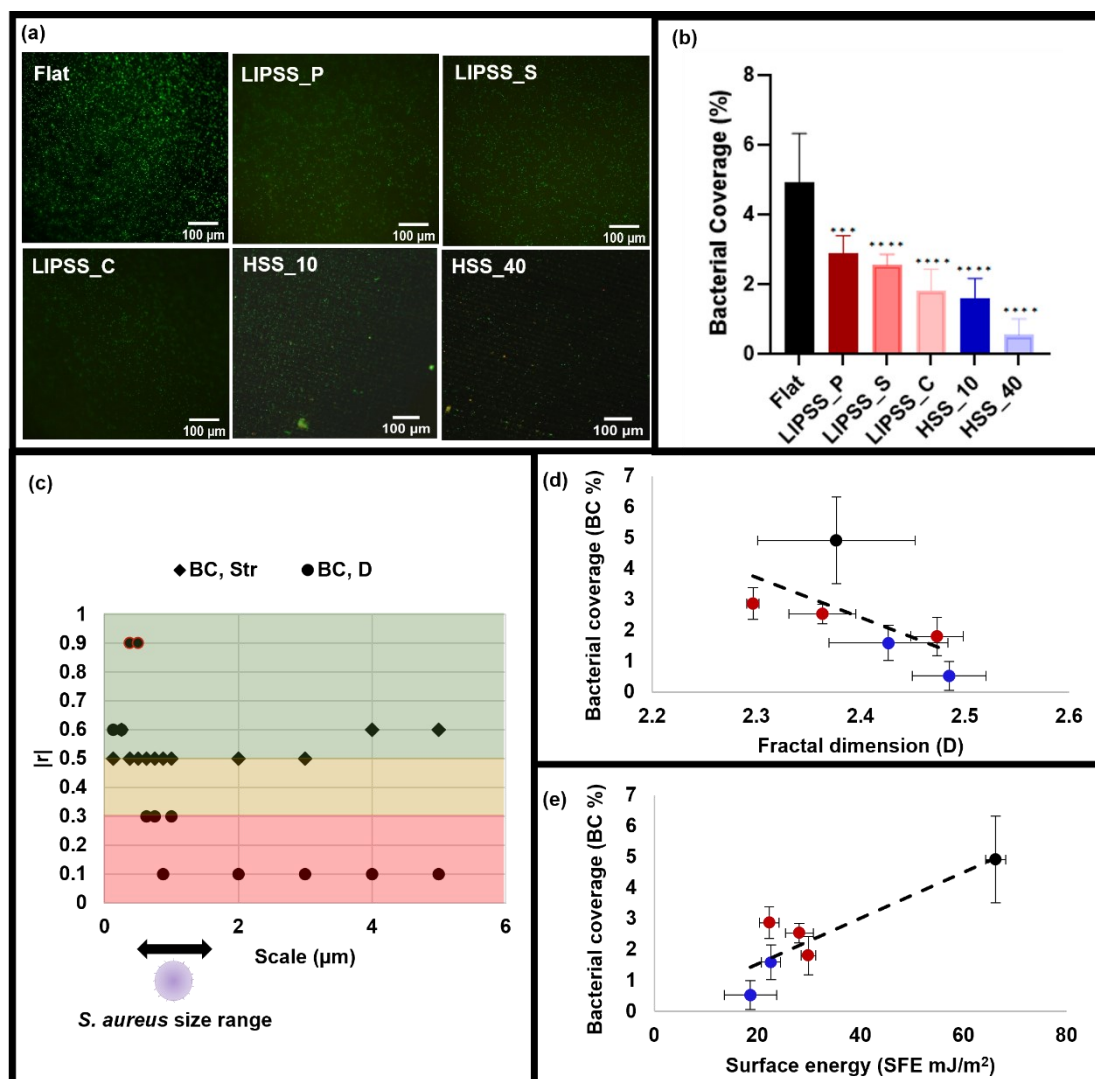


Figure 4-6 (a) Fluorescent images of *S. aureus* bacteria attached to the tested surfaces. **(b)** The bacterial coverage on the tested surfaces. The p-value for pairwise comparison between samples and the flat sample are represented by *** ($p < 0.0005$) and **** ($p < 0.0001$). **(c)** The absolute value of correlation factors between the bacterial coverage, the texture aspect ratio and the fractal dimension across scales. Red region: no correlation. Yellow region: weak correlation. Green region: strong correlation. **(d)** The variation of bacterial coverage with the fractal dimension at 0.5 μm analysis scale. **(e)** The variation of bacterial coverage with the surface energy.

The coverage or attachment of bacteria as seen in Figure 4-6 (a, b) decreased for all the structured surfaces compared to the flat control surface. The result

clearly showed that the highest number of attached bacteria was on the flat unstructured surface with a bacteria coverage percentage of around 4.91 %, followed by LIPSS_P (2.87 %), LIPSS_S (2.53 %) with the lowest attachment for LIPSS type surfaces being on the LIPSS_C surface with 1.80 % bacterial coverage. The HSS surfaces exhibited even lower bacterial coverage with 1.59 % for HSS_10 and 0.53 % for HSS_40.

High negative correlation was mainly observed on the lower scales (0.125 – 0.5 μm) for both S_{tr} and D with the bacterial coverage as shown in Figure 4-6 (c). The correlation of BC and D was only statistically significant ($p < 0.05$) at the 0.375 μm and 0.5 μm scales. Figure 4-6 (d, e) depict the variation of bacteria coverage with the fractal dimension at the 0.5 μm analysis scale and the surface energy, respectively.

4.2.3 Osteoblast Attachment and Cell Morphology

Following seeding and 24 hours incubation of MG-63 cells on the structured surfaces, the attachment was observed using fluorescent microscopy which is presented in Figure 4-7 (a). The cell area (CA), cell number (CN) and cell circularity (CC) were evaluated for all surfaces. The cell area analysis showed a significant difference between most surfaces presented in Figure 4-7(b). The average cell area on the flat surface was highest while surface HSS_40 exhibited the lowest significant reduction in cell area. The number of cells attached, although not significantly different among the samples, was highest on the flat surface and lowest on HSS_40. The number of cells attached on HSS_10 was around double the number of cells attached on HSS_40. The cell circularity analysis did not show evident significance either however, the circularity of MG-63 cells on HSS_40 was the highest.

A plot depicting the absolute values of the correlation parameters between surface topographical parameters and the MG-63 cell area is presented in Figure 4-7 (c). High positive correlation between CA, S_{pd} was found at very low scales ($< 0.25 \mu\text{m}$) and at high scales ($> 4 \mu\text{m}$).

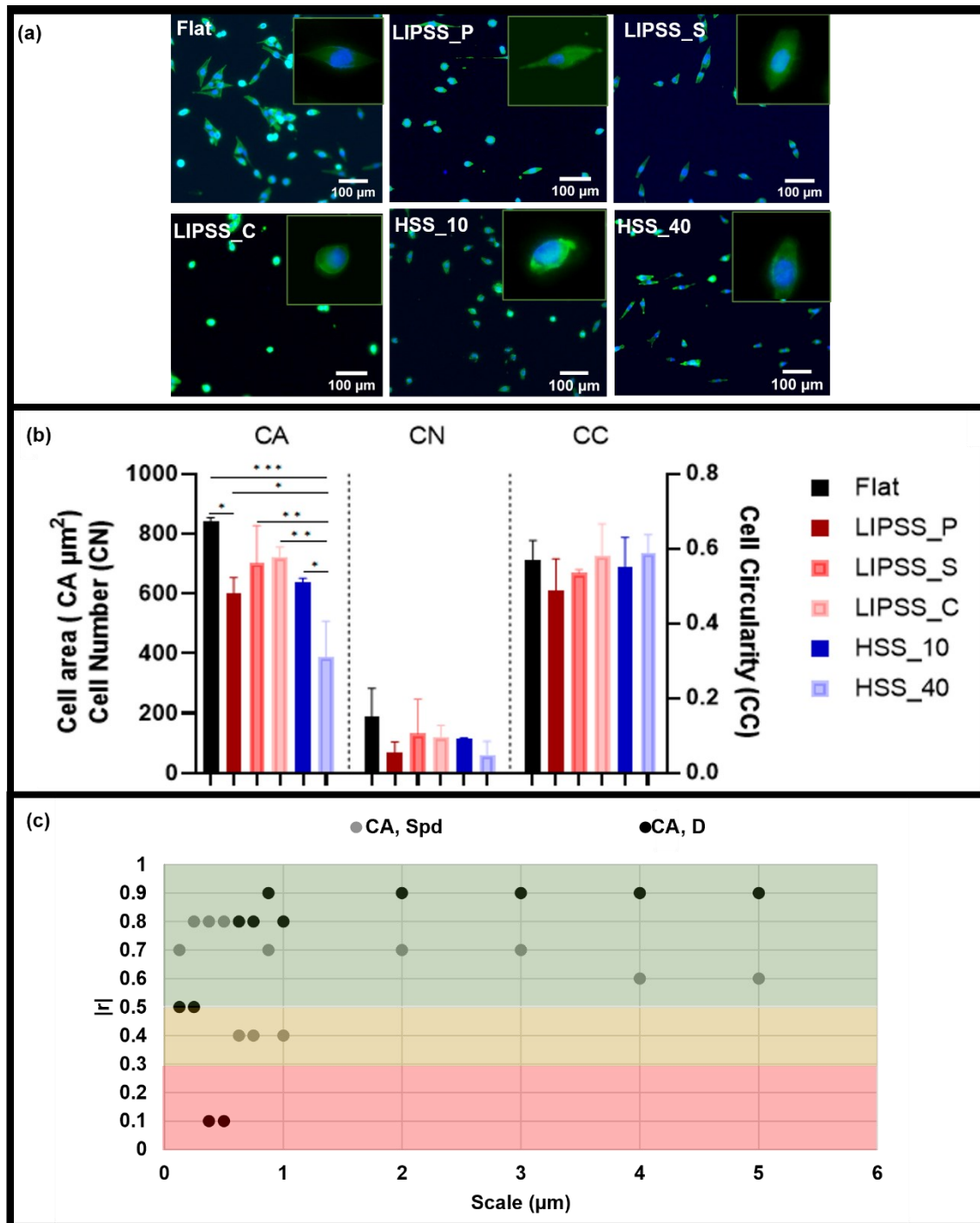


Figure 4-7 (a) Fluorescent microscopy images of MG-63 cells attached to the tested surfaces. (b) The Cell area (CA), cell number (CN) and cell circularity (CC) of the MG-63 cells attached to the tested surfaces. (c) The absolute value of the

correlation factors for the cell area, density of peaks and the fractal dimension across scales. In this case, both correlations are positive.

Additionally, at scales above 0.625 μm the CA highly correlated with the fractal dimension. The S_{pd} correlated well on all analysis scales with CN and D highly correlated with CN above the 0.625 μm . Finally, the CC correlated strongly with D for low scales ($< 3 \mu\text{m}$) with statistical significance at very low scales ($< 0.5 \mu\text{m}$). S_a , S_q , S_{pd} correlated well with CC within the range of scales 0.375 – 0.875 μm . Additionally, Str correlates highly for CC on all scales.

4.3 Discussion

4.3.1 Surface Treatment, Topographical Properties and Surface Chemistry

As anticipated, the LIPSS ridges orientation was seen to depend on the laser polarisation [348]. Since both polarisations P and S were linear, the resulting surface morphology after irradiation was similar. However, for the case of LIPSS_C, the morphology was observed to be slightly different, as nanoripples were distributed irregularly in some areas and showed some alignment patterns in other areas. The surface complexity of LIPSS_C was corroborated by the high fractal dimension D at all evaluation scales. The LIPSS structure period for all the LIPSS surfaces was less than 0.14λ (λ is the laser wavelength, 1030 nm) which indicated that the surfaces could be classed as high-spatial-frequency LIPSS (HSFL) [348]. The nanospheres observed on the LIPSS_P and LIPSS_S surfaces were the result of the redeposition of ablation products [349].

The increase in the laser passes from 10 to 40 for HSS surfaces led to an expected increase in the microfeature height, decrease in the width and an increases slope. The increase in microfeature height was proportional to the number of laser passes. The deep micro-holes observed on HSS_40 resemble single shot crater ablation. Their morphology reflects the energy distribution within the laser beam, which in this case appears to have a distorted Gaussian distribution [350].

Laser structuring can assuredly influence surface chemistry of alloyed materials such as stainless steel due to the preferential ablation of some alloy components and oxidation reactions on the surface layer. Although survey spectra showed that there was generally no significant effect of structuring on the presence of chromium and iron compounds, the main observed difference in the chemical composition of the surface layer is in the fractions of chromium and iron oxides formed on the surface. Particularly, there was a slight increase in the fraction of Cr(OH)_3 , FeCr_2O_4 and Fe^{2+} of Fe_3O_4 for the laser structured surfaces. However, the difference in fraction of Cr(OH)_3 and Fe^{2+} of Fe_3O_4 between the structured surface was very small. The presence of fluoride is similar in all samples. Considering that fluoride was not detected through EDS but only through XPS high resolution scans as FeF_3 , the fluoride is most probably present on the outer most surface and/or in very low concentrations ($< 0.1 \text{ wt } \%$)[351]. According to the XPS results, laser irradiation did not significantly affect the fraction of FeF_3 . The flat and laser structured surfaces were both found to be covered with carbon contamination from the environment. Carbon possesses adsorptive qualities which enrich organic nutrients. This can serve as a bioactive component, which can promote early-stage osseointegration and potentially improve biocompatibility, yet it can also encourage bacterial attachment [352,353]. However, these results do not suggest that the surface chemistry of the samples processed by different laser treatments differed significantly.

4.3.2 Influence of Surface Chemistry on Surface Biological Functionality

The X-ray photoelectron spectroscopy results presented in section 4.2.1 indicate that the structured surfaces exhibit a modest difference in chemical composition compared to the flat surface. The percentage of elemental chromium was lower, while the percentage of chromium oxides was higher in the structured surfaces. Trivalent chromium, which was found on all the test surfaces, is known for its high stability and low toxicity as it is understood to be unable to penetrate biological cell membranes [354]. Nevertheless, the change in the fractions of chromium

oxides between the structured surfaces did not appear to reflect the biological functionalities observed. At first glance, the detection of FeF_3 may raise concerns, but upon further examination, it was apparent that the fraction of FeF_3 was almost consistent across all samples tested and did not exhibit any discernible correlation with biological functionality.

The results thus suggest that surface chemistry is not the primary factor contributing to the reduced bacterial attachment and the changes in the morphology and attachment of MG-63 cells on the structured surfaces.

4.3.3 Influence of Surface Topographical Properties on Surface Energy

It is well-established that the surface topography has a considerable influence on the surface wettability. More particularly, two distinct wetting behaviours for liquid droplets on a rough surface have been defined in the literature: the Wenzel state and the Cassie-Baxter state. The Wenzel state proposes that the droplet fills the surface and its micro/nanofeatures, whereas the Cassie-Baxter model proposed that the droplet cannot fill the surface asperities; instead, air is entrapped between the gaps of the surface features. The contact angle can then be expressed using equation (2-4), in function of the area fraction between the droplet and the solid surface (f_1). Evidently, the wetting behaviour of the droplet on a nanostructured surface will depend on whether or not the droplet is able to overcome the energy barrier and wet the micro-nanofeature gaps. This depends on the topography of the surface, its amplitude and spatial characteristics. Those characteristics can be characterised by a few roughness parameters, mainly amplitude parameters and spatial parameters. The results indicate that, on the roughness scale, with the increase of amplitude parameters such as S_a and S_q and the hybrid parameter S_{dr} , the surface energy decreases, for both types of surfaces.

The results show that, for HSS-type surfaces, the surface energy (SFE) was lower than for LIPSS surfaces with similar roughness and hybrid parameters. For

example, when comparing HSS_10 and LIPSS_C, both of which have similar S_a values, the SFE of HSS_10 was lower than that of LIPSS_C. Similarly, when comparing HSS_40 and LIPSS_C with similar S_{dr} , the SFE of HSS_40 was significantly lower. This difference in SFE can be attributed to the presence of microfeatures on these surfaces. In the case of HSS surfaces, the SFE decreases with the small width and high slope of the microfeature. These variations in the slope and width directly affect the fraction of the solid surface in contact with the droplet, reducing its value. As a result, the contact angle increases, leading to a decrease in the surface energy for HSS surfaces compared to LIPSS surfaces, as predicted by equation (2-4).

However, given the complexity of the factors involved, it is difficult to conclusively attribute these variations in SFE to specific topographical features alone, as originally implied by equation (2-4).

In light of these complexities, it can be said that this study adds to the existing literature by further complicating our understanding of how surface topography influences surface energy. Therefore, it is recommended that future work focus on isolating these variables more distinctly to arrive at a clearer understanding.

4.3.4 Influence of Surface Energy on Bacterial Adhesion

Evidently, the surface energy has an influence on bacterial adhesion. This has been clearly established by the XDLVO theory and further reinforced by the experimental data [355,356]. Furthermore, surface energy affects the bacteria-carrying liquid by influencing how it wets the surface. If a surface possesses a low SFE, the liquid droplet does not spread, thus the interfacial solid/liquid area available for bacteria to adhere is less than that for a high SFE surface as depicted in Figure 4-8 (a). While surface energy (i.e. the sum of the van der Waals attraction, the electrostatic repulsion and the acid-base interaction) plays a role in bacterial adhesion as described by XDLVO [50], the findings here suggest this relationship is not straightforward. Contradictory findings exist in the literature:

Liu and Zhao (2005) [357] found that increasing surface energy led to greater bacterial attachment, while other studies reported the opposite [358].

Surface structuring further complicates this relationship. Although it's known to influence both surface energy and the surface area available for bacterial adhesion, its effects are multifaceted. For instance, the introduction of surface structures could either provide more area for bacterial attachment or render some areas inaccessible to bacteria.

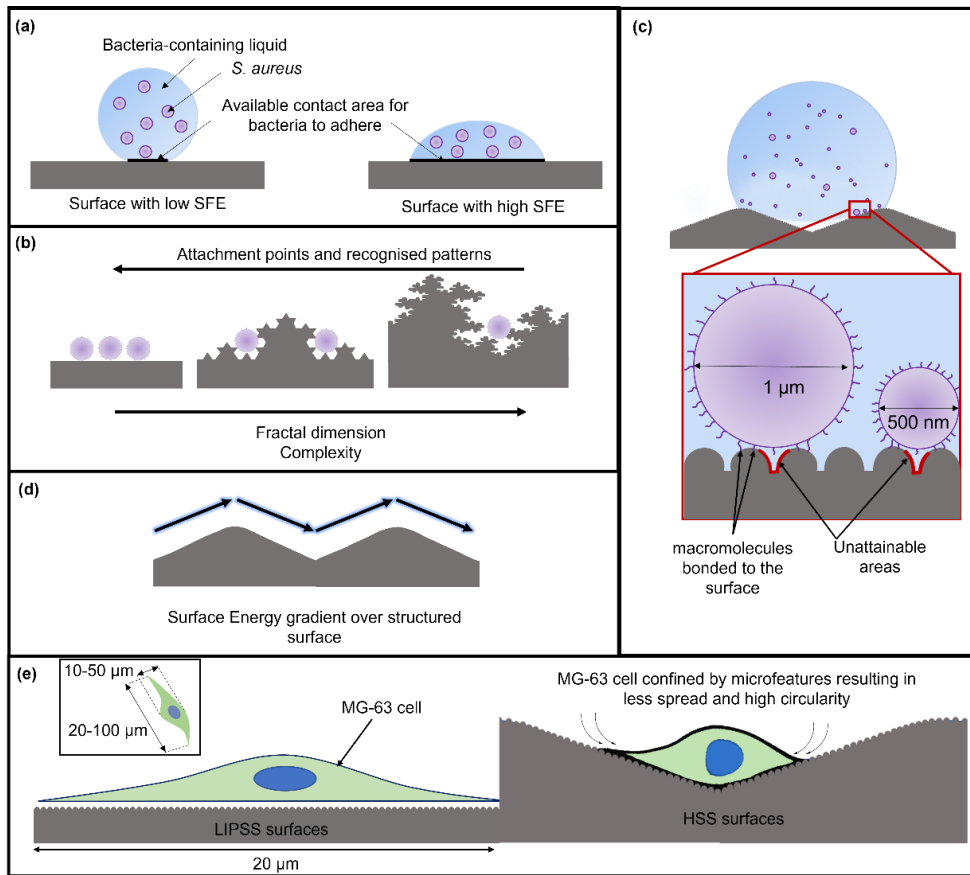


Figure 4-8 (a) The initial effect of surface energy on available solid/liquid contact area for bacteria to attach. (b) The effect of a high fractal dimension or surface complexity on bacterial adhesion. (c) Surface nano-structuring hindering bacterial macromolecule attachment due to the creation of unattainable areas. (d) Surface energy gradient on hierarchically structured surfaces. (e) MG-63 cell spread hindered by microfeature spacing. The inset is showcasing the typical size range for MG-63 cells.

In this particular study, surfaces with low surface energies (in the range of 19.5-32 mJ/m²) did show a general decrease in bacterial attachment compared to high SFE flat surfaces, as documented in Figure 4-6 (c). However, a comprehensive look at structured surfaces revealed an inconclusive correlation between surface energy and bacterial coverage ($r_{\text{SFE,BC}} = 0.30$). This lack of a strong correlation suggests that surface energy might not be the dominant factor in reducing bacterial attachment on structured surfaces.

4.3.5 Influence of Surface Topography on Bacterial Adhesion

The *S. aureus* retention study showed a clear decrease in bacterial coverage on all the structured surfaces compared to the unstructured flat surface. The reduction of bacterial attachment relative to the flat surface was approximately 41 % for LIPSS_P, 48 % for LIPSS_S and 63 % for LIPSS_C. While the reduction was 67 % for HSS_10 and 89 % for HSS_40 surfaces compared to the flat surface.

The dominating wavelength of the structured surfaces correlated strongly (positively) with bacterial coverage ($r_{\text{wavelength,BC}} = 0.67$). Considering that the wavelength of the surfaces ranged between 0.074 and 1 μm , this correlation indicates that bacterial attachment increases when the wavelength of the features increases to values comparable or beyond the bacteria size.

As extensively discussed in chapter 2, there still exists a divide in the literature as to whether roughness correlates positively or negatively with bacterial adhesion. That is mainly due to an oversimplification of the surface topography assessments and lack of comprehensive surface roughness analysis leading to the masking of underlying surface patterns or features as discussed in 2.5.3.

The results of this study do not show any correlation between S_a , S_q on any scale and the bacterial coverage (BC) as can be seen from the Figure 4-6 (e). The parameter S_{tr} correlates (negatively) very well with the bacterial coverage at scales lower than 1 μm . This means that when S_{tr} of a structured surface is high,

BC is low. In other words, anisotropic surface features encourage bacterial attachment while isotropy discourages it. The lack of directional cues on high S_{tr} surface topographies makes it more difficult for the adhesion complexes and proteins to find and bind to their specific binding sites on the surface, reducing the overall adhesion strength, ultimately reducing adhesion [359]. In this study, the fractal dimension was uniquely employed for the first time as an attempt to establish a scalar indicator for complex surfaces, with the aim of understanding their performance against bacteria and MG-63 cells. The results of the fractal dimension analysis over various scales are presented in Figure 4-2 (c).

The analysis (Figure 4-6 (c)) revealed a strong negative correlation between the fractal dimension (D) and bacterial adhesion at scales below 0.5 μm .

A surface with a high degree of complexity (high D) can decrease adhesion sites for bacteria, as depicted in Figure 4-8 (b), making it more difficult for them to establish a foothold on the surface. This in turn disrupts bacterial attachment as is evident in the results. Particularly for *S. aureus*, it has been observed that when they attach to surfaces with low SFE, they use multiple macromolecules that bind weakly to the surface, such as Serine-aspartate repeat-containing protein C [25]. On the other hand, when they attach to high SFE surfaces, they use fewer macromolecules that bind more strongly.

Therefore, for the bacteria to attach to the LIPSS and HSS surfaces, which are surfaces with low SFE, it is likely that *S. aureus* requires attachment of a large number of macromolecules/macromolecule patches of sizes ranging between (0.1 to 0.5) μm [360,361], which can be hindered by the complexity of the surface structure on that scale as illustrated in Figure 4-8 (b, c). Furthermore, if the macromolecules were to bind to the complex surface, the molecules would impart stresses on the bacterium's membrane forcing it to deform which creates an inopportune situation for bacterial adhesion [157]. These results suggest that bacteria do not favour complexity and isotropy for attachment. The results also suggest that the fractal dimension, evaluated at scales comparable to the bacterium and its macromolecule sizes, can be a stronger indicator of bacterial

response to specific surface topographies than the surface energy ($r_{D,BC}$ at low scales $> r_{SFE,BC}$).

Hierarchically structured surfaces have been observed to possess better anti-bacterial properties than LIPSS surfaces, and this may be due to the ability of hierarchical structuring to induce localised surface energy gradients as illustrated in Figure 4-8 (d) [362]. This means that the surface may not be stable for a particular microorganism with a specific surface energy requirement to attach, suggesting that the hierarchical structuring has the potential to prevent bacterial attachment and colonisation. However, it should be noted that these are observations and require further research to confirm their validity. Material residual stresses, though not explored in this study, could also be contributing to the decrease in adhesion on structured surfaces. For instance, Bagherifard et al. [363] have shown that enhanced surface compressive residual stresses in the material surface decreased bacterial attachment on shot peened stainless steel surfaces. Concurrently, some studies have shown that laser processing increases compressive residual stresses [364].

4.3.6 Correlation of Surface Parameters with MG-63 cell Adhesion.

The adherence of MG-63 cells to biomaterials is a multifaceted phenomenon influenced by various factors, ranging across various scales. The examination of the interaction between MG-63 cells and patterned surfaces indicated that the expansion of MG-63 cells, notably the cell area, is substantially impacted by the surface energy, the fractal dimension and the density of peaks. Since the change in surface area was the only cell-bound parameter that changed with statistical significance, all other parameters discussed concerning the cells' morphologies (CN and CC) are considered observational only. The decrease in SFE between the flat and structured surfaces highly correlated with a decrease in cell area ($p_{Flat/LIPSS_P} < 0.05$ & $p_{Flat/HSS_40} < 0.0001$). This finding is in agreement with the literature where high SFE was found to induce spatial osteoblastic cell growth and enhance the area of cells compared to low SFE surfaces [365]. Although the decrease in surface energy between the flat and structured surfaces

corresponded to a decrease in MG-63 osteoblast attachment (CN), it is noteworthy that this decrease was not observed to be statistically significant.

The current results, shown in Figure 4-7(c), suggest that at very low scales ($< 0.375\ \mu\text{m}$) there is a weak positive correlation between the surface complexity and the cell area. However, at high scales ($> 0.5\ \mu\text{m}$) the correlation becomes strong CA indicating that MG-63 cells' spread increases with increasing degrees of surface complexity. Similar results have been presented in the literature where it was shown that random and disordered nanoscale topography enhanced osteo-specific differentiation of human mesenchymal cells [366]. In addition, a positive correlation was found between the number of cells on the surface and the fractal dimension on scales greater than $0.5\ \mu\text{m}$. However, increasing surface complexity had an adverse effect on cell circularity. In particular, it was found that the fractal dimension has a positive correlation with cell circularity on scales smaller than $1\ \mu\text{m}$, with a significant correlation on scales smaller than $0.5\ \mu\text{m}$. This suggests that MG-63 cells tend to attach preferentially to surfaces with high fractal dimension, but their morphology is not favourable on these types of surfaces.

The peak density of small wavelength features ($< 0.25\ \mu\text{m}$) positively correlates with CA, the correlation drops for features with wavelengths between $0.5\ \mu\text{m}$ and $0.875\ \mu\text{m}$, then increases with high wavelengths features ($> 1\ \mu\text{m}$).

As previously mentioned in chapter 2, the cell's extracellular matrix (ECM) macromolecules mediate cell attachment to surfaces. These macromolecules such as fibronectin, vitronectin, laminin and collagen vary widely in size. For instance, collagen fibrils have diameter ranging from 0.05 to $0.3\ \mu\text{m}$, they could also assemble to make larger cable-like collagen fibres that could reach a size of $4\ \mu\text{m}$ in diameter [367]. The hierarchy in surface adhesins explains the correlations found at varying scales, owing that MG-63 cells are affected to different degrees by different wavelength features due to the specific interaction of varying size macromolecules with the surface features. Not only that, but adhesins and ECM components can grow differently depending on the surface

topography. For instance, a study reported that on patterned surfaces (<0.5 feature sizes) MG-63 cells exhibited larger stress fibres and thinner actin filaments compared to flat surfaces [367]. More research is required to understand the preferential attachment of different adhesins to differently sized surface features, and how the surface patterning affects the growth of MG-63 cells' ECM components.

Besides these parameters, microfeatures also affected the spread of MG-63 cells, particularly when the spatial frequency of the microfeatures was similar to the typical size of the cells (diameter: 20-50 μm , length: 20-100 μm) [331,368,369]. This hindered the cell spreading process as illustrated in Figure 4-8 (e), indicating that the presence of microfeatures plays a crucial role in regulating the behaviour of MG-63 cells by controlling their ability to spread and their subsequent morphology.

Generally, nanostructures (LIPSS) did not significantly affect the attachment and morphology of MG-63 cells regardless of the difference in topographical and surface energy parameters (except for LIPSS_P, $p=0.0205$). However, the presence of microfeatures, particularly deep microfeatures (HSS_40) significantly decreased the cell area and observably decreased the number of cells attached.

4.4 Conclusions

This study investigated the effects of femtosecond laser ablation on stainless steel surfaces in creating sub-micron and hierarchical structured surfaces that impact the attachment and adhesion of *S. aureus* NCTC7791 and MG-63 osteoblast-like cells to identify the extent of anti-bacterial effects and whether those effects inadvertently compromise osseointegration. The results showed that all the surfaces fabricated through femtosecond laser ablation, possessing features of dominating wavelengths lower than the investigated bacterium's size and comparable to the size of its surface-adhering macromolecules, effectively reduced the adhesion and attachment of *S. aureus* NCTC7791. Moreover, the hierarchical structured surfaces also had an impact on the attachment and

morphology of MG-63 cells. The investigation of different surface parameters yielded that the fractal dimension and surface texture aspect ratio are most indicative of bacterial attachment on nano/micro-structured surfaces while surface chemistry did not have a significant effect especially when femtosecond laser ablation is concerned. The fractal dimension and the density of peaks significantly influenced the attachment and morphology of MG-63 cells, particularly the cell area. Notably, the presence of deep microfeatures hindered the spread of the cells.

Overall, this study presents strong evidence that laser-induced periodic structured surfaces with sub-micron wavelengths, high fractal dimensions, and high texture aspect ratios (LIPSS_C) are promising for future applications. Specifically, these surfaces demonstrated a substantial reduction in bacterial coverage, showing a 63% decrease compared to flat surfaces, all while not inducing cytotoxic effects on MG-63 cells. The fractal dimension, in particular, emerged as a key indicator of bacterial attachment, underscoring its potential importance in the design of antimicrobial surfaces. However, while these findings are compelling, the role of Surface Free Energy in this context is not yet fully understood and warrants further investigation for a more comprehensive understanding.

5 Deep Reactive Ion Etching: Nature-inspired Nanoscale Silicon structures

5.1 Introduction

In chapter 4, nature-inspired nanostructured and hierarchically structured surfaces were studied mainly for their anti-bacterial ability translating in deterring bacterial adhesion. Another anti-bacterial bacterial strategy inspired by nature was explored in this chapter, based on high aspect ratio nanostructured surfaces. High aspect ratio nanostructured surfaces, particularly nanopillars, have been shown to mechanically damage bacteria leading to their death upon contact [105]. Although the underlying mechanisms have been increasingly investigated in current literature, they remain unclear. This ultimately limits the potential to transform the promising phenomenon into real-world applications. To date, black silicon has been shown to be the most efficient synthetic mechano-bactericidal surface studied, with reported killing efficiencies of up to 97 % for Gram-positive bacteria [102]. Particularly, previous reports have stated the bactericidal efficiency at 3 hours against *S. aureus* to be above 65% [91,261].

The work presented in this chapter entails the fabrication of black silicon by reactive ion etching (SF_6/O_2) of silicon wafers of the (100) and (111) orientations. Herein the surfaces are denoted as Si 100 and Si 111 for unstructured surfaces and bSi 100 and bSi 111 for structured surfaces. Following fabrication, the surfaces were characterised to investigate the mechano-bactericidal effect of these surfaces against *S. aureus* NCTC7791 in light of their topography, surface energy and crystal orientation. Additionally, tests were carried out to assess the cytotoxicity of the fabricated nanostructured surfaces against MG-63 cells.

5.2 Results

5.2.1 Surface Fabrication and Characterisation

Silicon nanostructures denoted as black silicon (bSi) were fabricated on both orientations of silicon through the process of reactive ion etching and are shown by the SEM micrographs presented in Figure 5-1. The surface of bSi 100 was mostly made up of tall pillars with similar widths while bSi 111 was made up of wide tall pillars and clusters of smaller pillars as indicated with the dashed arrows. The surface topography parameters and the surface energy of the fabricated surfaces are shown in Figure 5-2. The nanopillar cap diameter for both surfaces were around 46.1 for bSi 100 and 45.4 for bSi 111. The nanopillar peak-to-peak spacing or pitch was about 281 nm for bSi 100 while for bSi 111 the overall pitch was around 153 nm with a clear divide between short nanopillars pitch of 56 nm and tall nanopillars pitch of 221 nm. The density of peaks, S_{pd} , obtained from AFM measurements was higher for bSi 111 than bSi 100. This was corroborated by nanopillar density calculated through SEM image processing. The developed interfacial area ratio, S_{dr} , was higher for bSi 111 than bSi 100. Finally, the fractal dimension was higher for bSi 100 (2.38) than bSi 111 (2.36).

Considering the surface wettability, all surfaces were found to be hydrophilic (water contact angle between 64° and 83°). An increased surface energy value is apparent for structured surfaces as shown in Figure 5-2 (d) compared to their unstructured counterparts. The average SFE of Si 111 was higher than that of Si 100 and the average SFE of bSi 100 was higher than that of bSi 111. However, these changes in SFE between the different silicon orientations was not found to be significant.

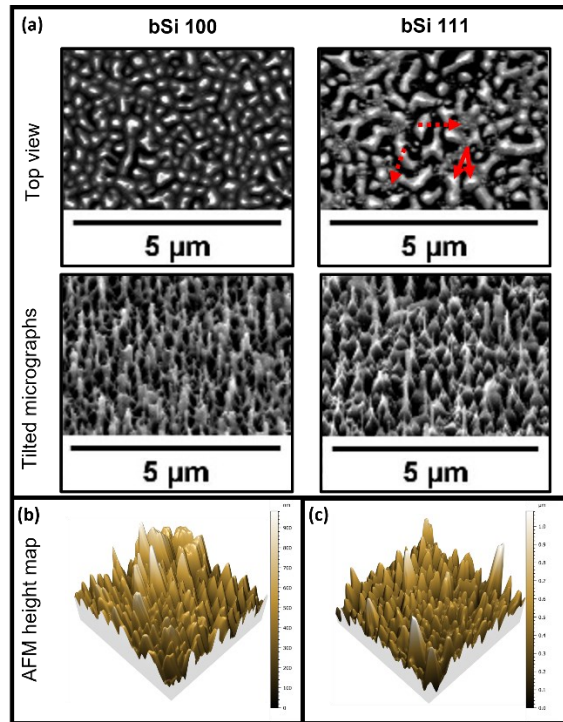


Figure 5-1 (a) SEM micrographs of bSi 100 and bSi 111. Red arrows indicate the tall pillars, dashed red arrows indicate the cluster of small pillars. (b) AFM height maps of bSi 100 and bSi 111 ($5 \times 5 \mu\text{m}^2$).

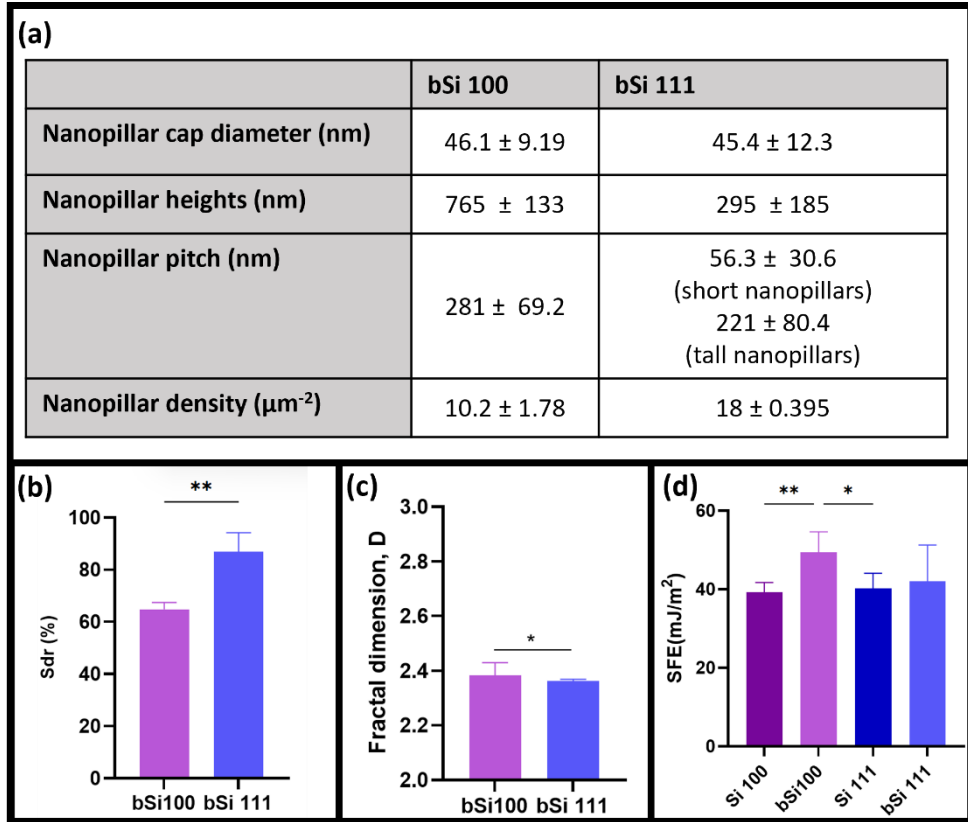


Figure 5-2 (a) parameters calculated from SEM micrographs. (b) The interfacial area ratio, S_{dr} (%). (c) The fractal dimension of the surface, D . and (d) The surface energy, SFE (mJ/m²). The p-value for pairwise comparison between the samples are represented by * ($p < 0.05$), ** ($p < 0.01$).

Figure 5-3 (a, b) shows the configuration of the two types of monocrystalline silicon used in this work. The XRD results presented in Figure 5-3 (c, d) confirmed the orientation of the silicon substrates as the 2θ calculated for 100 and 111 orientations matched the 2θ obtained experimentally (Figure 5-3 (e)).

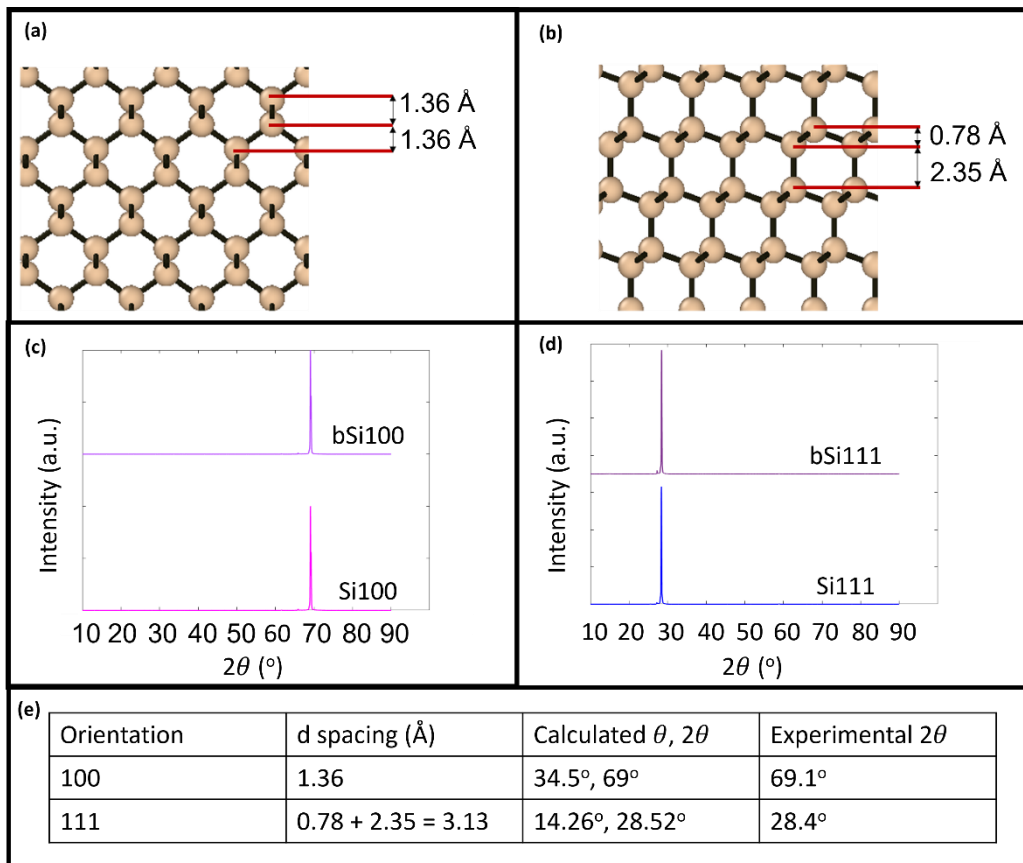


Figure 5-3 The configuration of (a) Si 100 and (b) Si 111 samples. The XRD patterns of (a) Si 100 and bSi 100 and (b) Si 111 and bSi 111. (e) The calculated (Bragg's Law) and experimentally obtained 2θ .

5.2.2 Bacterial Viability and Attachment in Wet Conditions

The bactericidal activity of the surfaces was evaluated after 3 hours of incubation with an *S. aureus* NCTC7791 bacterial suspension in Phosphate buffered saline (PBS) of $OD_{600}=0.09$ using the overlay method [370] as presented in section 3.4.1. As shown in Figure 5-4 (a. b), contrary to initial expectations, it was found that the bacteria on the nanostructured silicon surfaces (> 95 %) remained viable under wet conditions. There was no significant difference observed between the percentage of dead bacteria on the tested surfaces, structured or not.

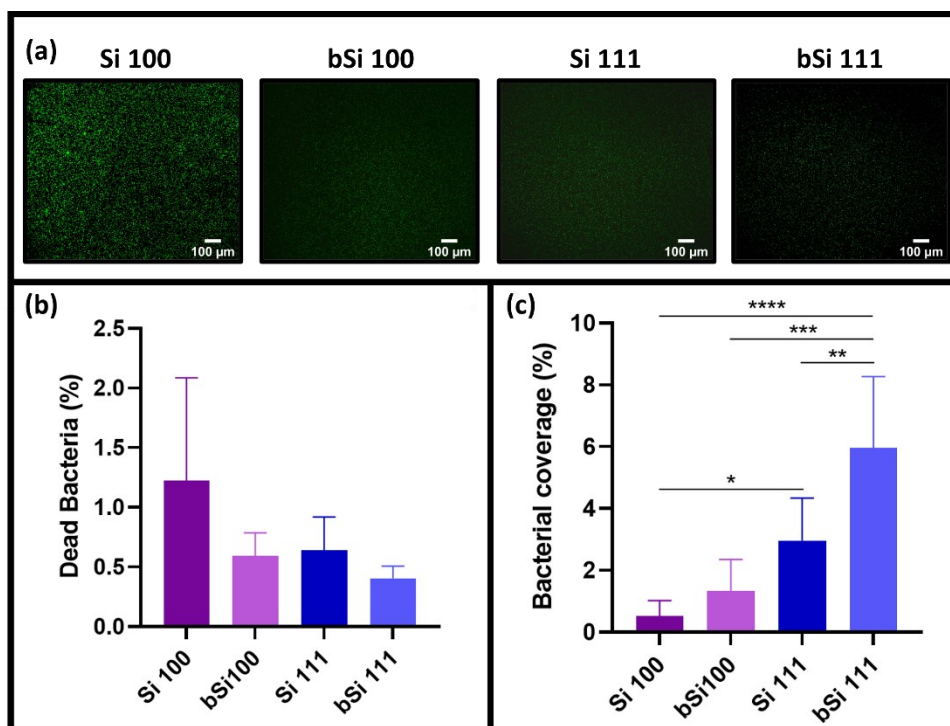


Figure 5-4 (a) Fluorescent microscopy images of *S. aureus* attached to the silicon samples in wet conditions. **(b)** The percentage of dead bacteria on the silicon surfaces in wet conditions. **(c)** The bacterial coverage percentage in wet conditions. The p-value for pairwise comparison between the samples are represented by * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.0005$) and **** ($p < 0.0001$).

The attachment or coverage of bacteria on the surfaces was also studied and is shown in Figure 5-4 (c). The flat surfaces showed lower attachment for both silicon orientations compared to their structured counterparts. The Si 111 had significantly higher bacterial coverage than Si 100, with a similar trend evident for bSi 111 and bSi 100. The highest bacterial attachment was found on surface bSi 111.

5.2.3 Bacterial Viability and Attachment in Dry Conditions

The bactericidal efficiency of the surfaces was also tested for dry conditions and the results are shown in Figure 5-5 (a, b). The percentage of dead bacteria increased for all surfaces in dry conditions. Yet, it remained below 5 %. The bacterial coverage (Figure 5-5 (c)) also decreased while maintaining the trend: unstructured silicon surfaces have less bacterial coverage than their structured

counterparts while silicon in 111 orientation (particularly bSi 111) exhibits higher attachment than silicon in the 100 orientation (particularly bSi 100). Consistently with the wet condition testing, bSi 111 had the highest bacterial coverage.

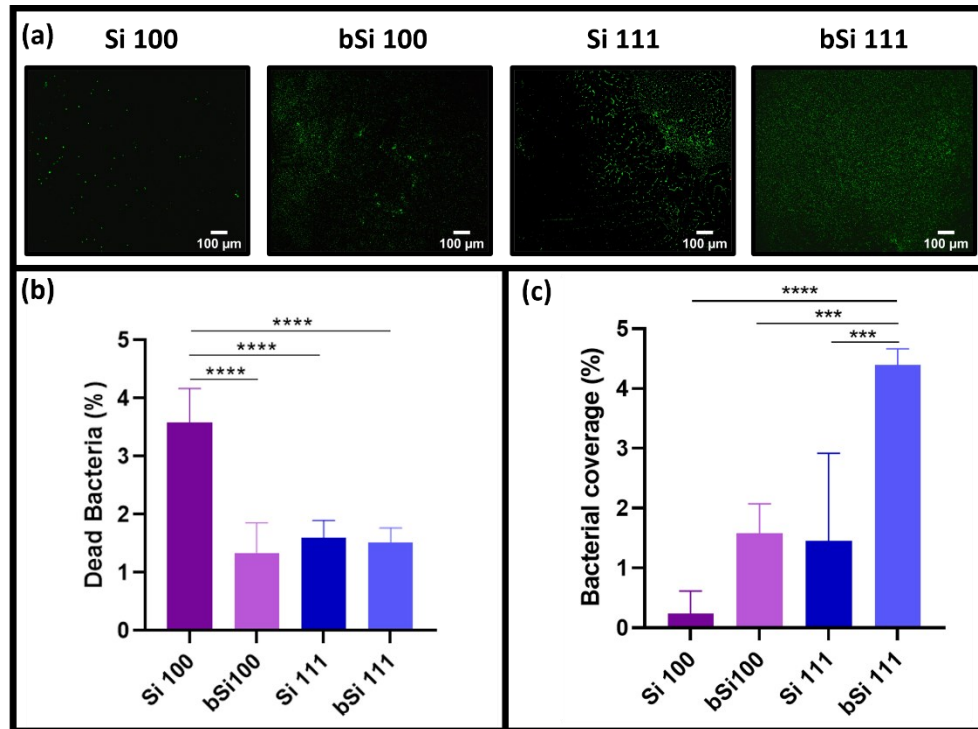


Figure 5-5 (a) Fluorescent microscopy images of *S. aureus* attached to the silicon samples in dry conditions. (b) The percentage of dead bacteria on the silicon surfaces in dry conditions. (c) The bacterial coverage percentage in dry conditions. The p-value for pairwise comparison between the samples are represented by * ($p < 0.05$), ** ($p < 0.01$), * ($p < 0.0005$) and **** ($p < 0.0001$).**

5.2.4 Osteoblast-like Cell Attachment, Viability and Morphology

Figure 5-6 shows the results obtained for MG-63 cell testing. MG-63 cell coverage was studied due to the clustering of cells on some surfaces, sometimes hindering the evaluation of the individual number of cells. The Si 111 surface had the highest MG-63 cell coverage while there was no significant difference between surfaces Si 100, bSi 100 and bSi 111. An observation of the cell circularity analysis indicated that cells on structured surfaces were more circular than on their flat counterparts. Additionally, the cell areas for MG-63 cells on structured

surfaces were smaller than those on the flat surfaces. Finally, the absorbance values at 450 nm of CCK-8 presented in Figure 5-6 (e) show that the absorbance on Si 100 is significantly higher than the absorbance on Si 111, bSi 100 and bSi 111 surfaces, revealing differences in cell viability among the tested surfaces.

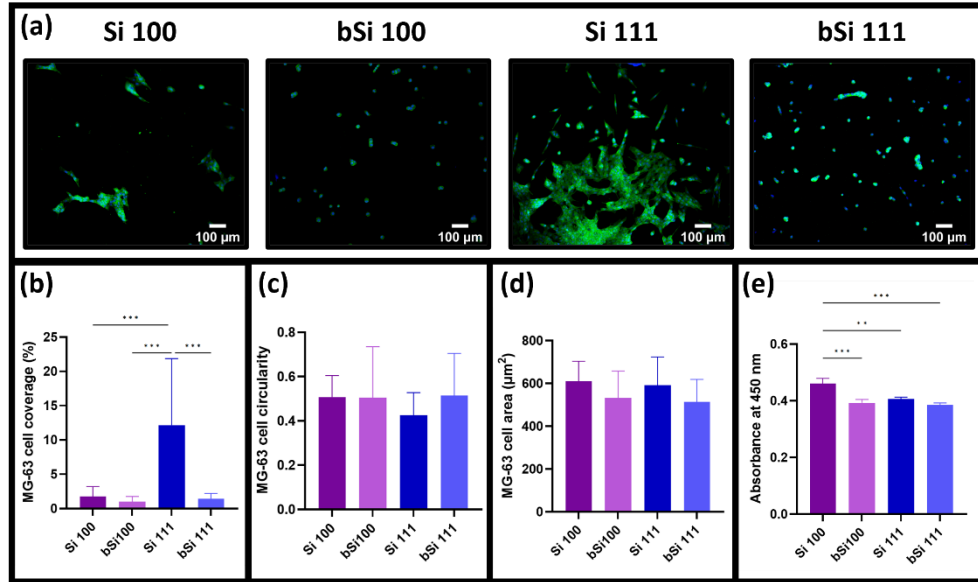


Figure 5-6 (a) Fluorescent microscopy images of MG-63 cells attached to the silicon samples, (b) The percentage of MG-63 cell coverage. (c) The circularity of MG-63 cells. (d) The average cell area of MG-63 cells attached to the surfaces. (e) The absorbance at 450 nm (Reduction of CCK-8) indicating the viability of attached MG-63 cells. The p-value for pairwise comparison between the samples are represented by * ($p < 0.0005$).**

5.3 Discussion

5.3.1 Influence of Crystallographic Orientation on Nanopillar Formation

As presented in section 5.2.1, the nanopillars formed on silicon wafers with 111 orientation showed varied sizes between short and wide nanopillars and taller, narrower ones whereas nanopillars on silicon wafers with 100 orientation showed a more homogenous distribution of tall pillars. This could be explained by a slower etching rate of Si 111 compared to Si 100, because the activation energy required

to remove an atom from the (111) surface is bigger than that required to remove an atom from the (100) surface [371]. This stems from the number of back bonds present on different surface orientations that need breaking to etch material. Si 100 has 2 dangling bonds and 2 back bonds while Si 111 has 1 dangling bond and 3 back bonds.

The XRD spectra after etching remained sharp with no widening or flattening of peaks, indicating that the silicon wafers have retained their crystallinity. However, due to the small size of the nanopillars (below 1 μm) and comparably large interaction depth of the XRD (around 10 μm) [372], any surface amorphous layer that may have formed on the pillars was not fully detectable with XRD. According to the literature, an amorphous oxide layer forms on the surface while the pillars retain their crystallinity [259].

5.3.2 Influence of Nanostructuring on Surface Energy

As discussed in chapter 4, surface energy can be influenced by nanostructuring. Nano-structured silicon, in particular, has previously been found to have a significant impact on surface energy [264]. Depending on the nanostructured surface dimensions such as feature height and spacing between features, the surface energy can either increase or decrease. In this case, the surface energy for the nanostructured surfaces increases, which could be attributed to two aspects: first is the increase in surface area and second is the surface composition:

- The increase in surface area leads to an increase in the number of surface atoms, resulting in a higher energy state compared to a smooth surface.
- The variation in surface composition, although not studied particularly in this work, has been well established in the literature. Due to the nature of the fabrication process, where oxygen ions passivate the silicon substrate, the surface is effectively oxidised hence the silicon content of the surface decreases while the oxygen content increase. This oxidation of silicon has been associated with an increase in the water contact angle [142,264],

also observed in this case (Si 100 = 63.8°, bSi 100 = 82.9°, Si 111 = 67.2°, bSi 111 = 70.4°).

5.3.3 Influence of Nanostructuring on Bacterial Viability and Adhesion

5.3.3.1 Bacterial Viability

Contrary to initial expectations, the results in this work indicated that the bacteria on the nanostructured silicon surfaces (> 95%) remained viable under wet conditions. This is inconsistent with the literature as nanostructured silicon surfaces have been shown to be highly bactericidal [91,102].

Due to the discrepancies with the existing literature, mapping of the surface properties and testing protocols was conducted in order to pinpoint the reason behind the discrepancies. To narrow down the study and be consistent with the test parameters used in the current study, only studies including black silicon surfaces and tests on *S. aureus* after 3 hours of incubation have been considered. Five studies were thus considered, three of which contained variations of surface characteristics and resulting bactericidal efficiencies. This consisted of a total of 11 surface variations.

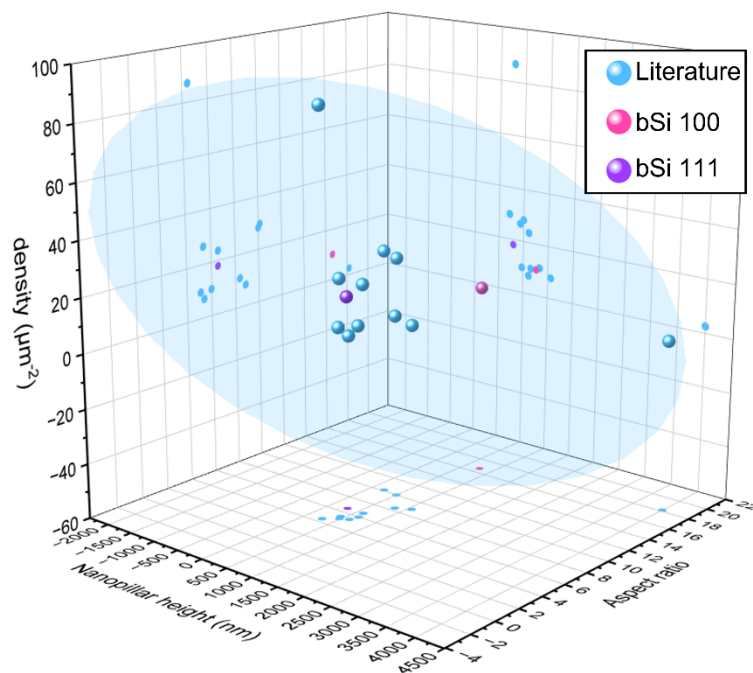


Figure 5-7 Positioning the black silicon fabricated surfaces within the literature, particularly black silicon surfaces with bactericidal efficiencies exceeding 50 % against *S. aureus* after a 3 hour incubation period, matching the current test conditions. The data was extracted from [91,102,142,264,373].

First, the surface characteristics mainly the aspect ratio, the nanopillar height and feature density were mapped for the black silicon nanostructures that possess a bactericidal efficiency above 50 % in Figure 5-7. The characteristics corresponding to the bSi 100 and bSi 111 surface are also mapped. The surfaces fabricated were comparable in geometrical characteristics to the reported mechano-bactericidal surfaces.

Second, the wettability of the effective mechano-bactericidal black silicon surfaces varied widely in literature from superhydrophilic to superhydrophobic, confirming the previously published evidence that the surface wettability is not defining factor for the perceived bactericidal efficiency.

The assessment methods for the different surfaces were then compared. It was found that the medium in which the bacteria were suspended varied between nutrient broth (Oxoid) and PBS between different experiments. In a unique study

conducted by Michalska et al. [203], the mechano-bactericidal efficiency of black silicon was tested as a function of the type of medium used and the type of assessment technique (flow cytometry, plate counting and fluorescent microscopy). It was found that the efficiencies obtained through microscopy were lower when using a rich medium compared to a buffer or particularly PBS. As PBS was used in the current study, the efficiency was expected to be higher compared to studies that have used nutrient broth. However, that was not the case, in fact the efficiency was lower than that of studies using both PBS and nutrient broth. The optical density of the bacterial suspensions used in the tests was also comparable to the optical density used in this study (for 8 out of 11 studied surfaces, the OD was 0.1 while the current study used an OD of 0.09).

Another evident difference between the studies is the different type of *S. aureus* strains employed, notably *S. aureus* CIP 65.8^T and *S. aureus* ATCC 25923. To date, experimental measurements of the stiffness of *S. aureus* bacteria have not covered *S. aureus* CIP 65.8^T, yet it was found that the modulus of elasticity of *S. aureus* ATCC 25923 was in the range of 0.57-1.33 MPa [38,43], comparable to the modulus of elasticity of the *S. aureus* NCTC 7791 strain used in this work (0.95 ± 0.24 MPa). More details about it can be found in the appendix 9A.1.

Finally, the conditions of growth of *S. aureus* in most experiments stated a temperature of 37°C only. In this work, there was additionally 5% CO₂. To date, there are no studies pertaining to the effect of increased CO₂ exposure on bacterial mechanical properties. However, according to research by Persson et al. [374] exposure to CO₂ at 37°C stunts the growth of *S. aureus* bacteria. This would have impacted the results, resulting in an overestimation of the bactericidal effect of the bSi surfaces, which was not the case.

These comparisons in the testing parameters between literature studies and the investigations conducted here eliminate certain discrete parameters (surface architecture, wettability, test parameters such as bacteria, conditions of growth and bacterial load and medium) from being suspected as responsible for the lack of bactericidal activity on the studied black silicon surfaces.

A possible aspect that has been overlooked is the transitional steps that are generally not very well reported. For instance, in these experiments, the samples were washed carefully three times with PBS before staining and imaging. That was done by inserting a pipette tip into the side of each well, flooding the well slowly with 1mL of PBS then pipetting most of the PBS out but ensuring the sample is still immersed in order to be consistent with the “wet conditions” evaluation as is shown in Figure 5-8.

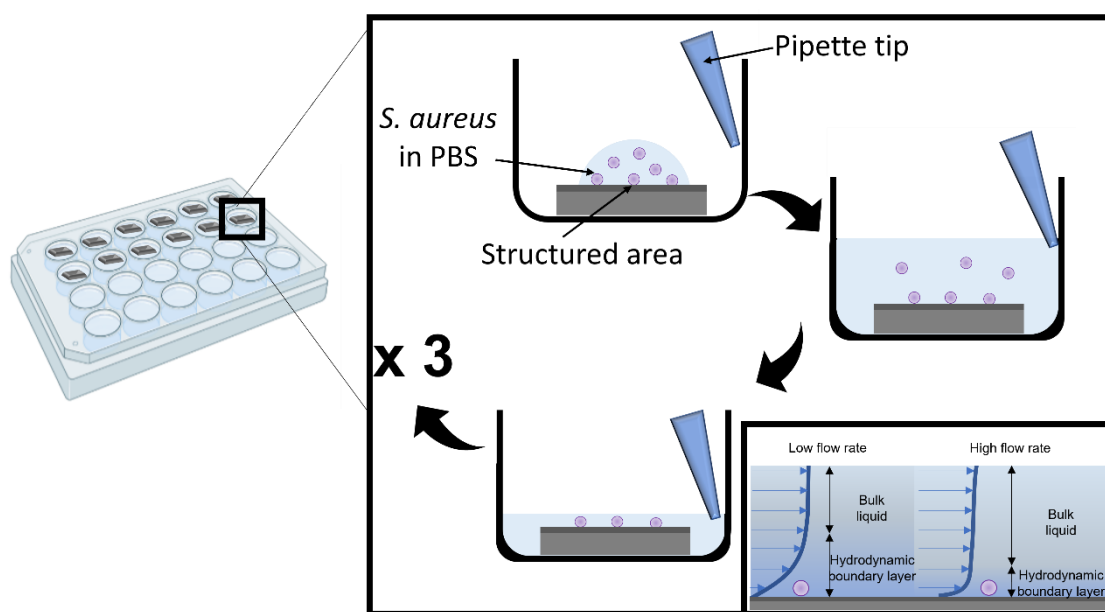


Figure 5-8 The washing technique applied in this study. The inset on the bottom right is an illustration of the effect of the flow rate on the hydrodynamic boundary layer and shear forces attached bacteria are subjected to. Adapted with permission from [45].

A study conducted by Li et al. [208] suggested that a 50 $\mu\text{L}/\text{min}$ flow rate was enough to induce the immediate mechanical killing of 80 % of the *E. coli* bacteria flowing through a microfluidic nanostructured chamber. When the flow rate of the bulk liquid increases, the size of the boundary layer decreases (Figure 5-8 inset), which generates more shear forces for bacteria that are near the substrate surface [45], which could induce their damage. Typically, flow rates induced by washing the samples whether by pipetting or through lab wash bottles [197] are

much higher. However, depending on the specific washing technique, the flow could be slowed before reaching the bacteria on the surface.

Similar results to this study were obtained by Valei et al. [118] where no bactericidal action was found on nano Silicon surfaces (> 24 hours in incubation). In their study, however, the bacteria attached to the surfaces did succumb immediately after the liquid on the surface dried. They concluded that it requires an external force (capillary force in this case) to be exhibited to push the bacteria onto the structures to kill them. In the current findings, even when completely dried, the surfaces did not exhibit any bactericidal activity as was presented in Figure 5-5. The *S. aureus* bacterium, used in this study, is a Gram-positive bacterium made up of several layers; the outer peptidoglycan (20-40 nm thick, Hyperelastic/Viscoelastic), the thin cytoplasmic membrane (<10 nm, Hyperelastic) and the cytoplasm (Viscoelastic). Valiei et al.'s study used the Gram-negative bacterium *P. aeruginosa* which possesses a considerably less stiff cell wall architecture consisting of a thin peptidoglycan layer sandwiched between thin inner and outer membranes. This indicates that the force induced by the evaporation might be just enough to induce the rupture of Gram-negative bacteria but not high enough to have that effect on Gram-positive bacteria. The evaporation rate also would affect this, as it can be linked to the strain rate which in turn impacts hyperelastic/viscoelastic materials' mechanical response. One could argue that at a low evaporation rate, the force required to achieve a certain threshold strain is lower than that in the case of a fast evaporation rate. This means that the lower the evaporation rate, the easier it would be to strain the bacteria to rupture it. In the experiments, the evaporation rate varied between 0.0083 to 0.0196 $\mu\text{L/s}$. Experimenting with different droplet sizes and evaporation rates may provide a better understanding of the role of capillary force in the perceived mechano-bactericidal phenomenon.

5.3.3.2 Bacterial Adhesion

The attachment pattern of *S. aureus* changed significantly between unstructured surface to structured surfaces, and between the silicon orientations for both

structured and unstructured samples. The highest coverage was exhibited by bSi 111 in both dry and wet conditions.

While no significant difference was found in the surface energy between the different silicon orientations, the differences in bacterial attachment point out to a possible role of the net surface charge of the surfaces in mediating interaction with bacteria [375]. Additionally, there might be differences in functional groups formed on the surface which could affect the bacteria-surface interaction [375]. When the surfaces are structured, this effect is exacerbated due to the added significant factor of surface topography change. As explained in the section above, the crystal orientation of the silicon substrate determines the arrangement of atoms on the surface and indeed affects the formation of surface features such as nanopillars. For example, bSi 111 has been found to exhibit a higher density of vertically aligned nanopillars compared to bSi 100, which can provide more attachment points for bacteria. The topographical change not only affects the available attachment points for bacterial adhesion but could also affect protein adsorption [376,377] which in turn affects bacterial adhesion [378].

The fractal dimension, nanopillar density and S_{dr} have shown to be effective topographical indicators of bacterial attachment on nanostructured surfaces. For instance, the surface with high fractal dimension had lower bacterial coverage, resounding the correlation between surface complexity and bacterial coverage discussed in chapter 4. Additionally, high nanopillar density is also corresponding to high bacterial coverage. The number of pillar tips/peaks in a given area are indicative of the attachment points available for the bacteria to attach to, and a higher pillar density indicates that there are more attachment points available which could lead to increase adhesion. S_{dr} also is indicative of the area available for interaction, thus it is logical that a high S_{dr} is also corresponding to a high attachment. The variation in feature heights and sizes also helps the bacteria attach as they might preferentially attach to clusters of short pillars [370] where they would gain more surface area for attachment.

5.3.4 Influence of Nanostructuring on Osteoblast Cell Adhesion and viability

There are a number of reports that have stated that different kinds of mammalian cells can proliferate on nanostructured surfaces, particularly black silicon surfaces [14]. However, there is also a few conflicting reports claiming the opposite [379]. The examination of the MG-63 cells on the tested surfaces indicates that surfaces that have nanofeatures with aspect ratio between 6.56 and 16.63, pitch between 153 nm and 281 nm and feature density of 10-18 μm^{-2} are somewhat negatively affecting the morphology of MG-63 cells. This is evident from the significant decrease in MG-63 cell coverage between unstructured and structured surfaces (especially Si 111 and bSi 111), the increase in circularity and decrease in cell area too. Comparing the two structured surfaces' performance, it seems that in terms of cell circularity, cell area and number of viable cells, the two surfaces do not show much difference. However, bSi 111 shows a higher cell coverage than bSi 100 ($p = 0.99$). Although not statistically significant, this observation could be indicative that MG-63 cells preferably attach to surfaces with lower height features and smaller spacing, allowing MG-63 cells' surface protein to better adsorb. According to a study by *Nguyen et al.* [377] fibronectin, an extracellular matrix protein that plays a key role in the adhesion of MG-63 cells, preferentially adsorbs on the top of black silicon nanopillars. bSi 111 possesses a significantly higher pillar density than bSi 100, thus a higher number of favourable sites for fibronectin and MG-63 cell adhesion.

Interestingly, despite surface Si 111 exhibiting the highest percentage coverage, it did not demonstrate the highest number of viable cells (correlated with absorbance). Instead, Si 100 had the highest viable cell count. A possible explanation for this observation could be that cells on Si 111 tend to spread, thereby increasing their area and contributing to the higher coverage. This suggests that Si 111-oriented silicon surfaces may promote cell spreading, while Si 100-oriented surfaces may stimulate cell proliferation, leading to a higher absorbance (indicating a greater number of viable cells). However, it is essential

to note that this interpretation is speculative and requires further investigation to confirm the underlying mechanisms responsible for these observations.

5.4 Conclusions

Black silicon was fabricated through reactive ion etching on silicon wafers with the 100 and 111 orientations. The resulting nanostructures varied in dimension between the different orientations owing to the lower etching rate of Si 111. Anti-bacterial functionalities (bactericidal and anti-adhesion) were assessed for these surfaces against *S. aureus* NCTC7791. However, no bactericidal effect (< 5 % dead bacteria) was observed under both wet and dry conditions for all surfaces tested. Additionally, no anti-adhesion functionality was detected for the structured surfaces as they had higher bacterial coverage than their unstructured counterparts. MG-63 Osteoblast cells did not get significantly affected in terms of circularity and cell area. However, they preferentially attached to bSi 111 surfaces with high pillar density and low aspect ratio. Both *S. aureus* and MG-63 cells predominantly attached to 111-oriented silicon.

This study calls into question the prevailing assumptions in the existing literature regarding the effectiveness of mechano-bactericidal surfaces in eliminating Gram-positive bacteria, specifically *S. aureus*. It underscores the necessity for standardisation in biological testing and experimental protocols. Furthermore, the findings emphasise the importance of achieving greater control in testing, with particular attention to detailing and reporting transitional steps of the experimental procedure to ensure the reproducibility of the results. In general, the parameter space considered in previous research appears to have been overly simplified, focusing solely on surface architecture parameters. The present study suggests that additional factors related to environmental (test) conditions may also play a significant role in determining the outcomes. Therefore, studies with highly controlled surface features and test conditions are simultaneously required to further the study of mechano-bactericidal surfaces.

6 Prototyping nanofabrication techniques: Contact and non-contact methods

6.1 Introduction

In the previous chapters, relatively large scale manufacturing of nature-inspired nanostructured surfaces was presented and the evaluation of their biological functionalities were conducted. Although some good correlations were found between surface topographical parameters and biological functionality, there were deviations from the literature encountered in the investigations of mechano-bactericidal surfaces. These deviations, in addition to the emerging literature surrounding the field, led to the reinforcement that the interaction between bacterial cells and surfaces with specific nanostructures remains a complex and poorly understood phenomenon. To gain more insight into the mechano-bactericidal effect, there is a need for prototyping techniques that enable the fabrication of surfaces with precise features that can be studied in situ. In this chapter, two different prototyping techniques that can be used for this purpose are being explored. Additionally, a method for studying the effects of these surfaces on bacterial adhesion and growth using AFM tracking is suggested. Through this approach, the aim is to shed more light on the surface-bacteria interaction and pave the way for the development of more effective anti-bacterial surfaces.

6.2 Scanning Probe Lithography Fabricated Nature-Inspired Nano Scale Features on Stainless Steel

6.2.1 Introduction

Mechanical contact milling using the AFM micro probe, otherwise known as scanning probe lithography, has attracted a lot of attention as it can have very good control on the removal of material at the nanometre scale. Materials that have benefitted from the emergence of SPL are mostly polymers and soft metals. To date, mechanical scratching through SPL has not been applied to biomedical

metals. In this chapter, the fabrication of nanostructures with SPL was attempted on stainless steel 316L. The interaction of *S. aureus* bacteria with the nanostructures was monitored over a period of 3 hours through real-time AFM imaging as described in chapter 3.4.2.

6.2.2 Results and discussion

6.2.2.1 Surface Fabrication and Topographical Characterisation

An AFM height map and extracted line scan of the fabricated nanostructures are presented in Figure 6-1 (a, b). The dimensions of the nanostructures fabricated are presented in Figure 6-1 (c). Scanning directions 1 and 2 represent the directions perpendicular to the milling directions. Scanning direction 1 corresponds to the first milling direction, while direction 2 reflects the morphology of the second milling direction after the sample was rotated by 90 degrees and milled again. It can be noticed that in the scan direction 2, there is a significantly visible material pile up. This is due to the increase of machining distance and subsequent wear of the tool. Similar results have been reported in the literature in ultra-precision turning of copper [380]. The height achieved was around 12 nm in both directions, significantly lower than the target feature (200-300 nm). This was due to the limitation in the normal force applicable due to the cantilever stiffness and hardness of the stainless steel. However, performing several scans/passes can be considered in the future in order to fabricate high aspect ratio features as well as exploring a wider range of cantilever stiffnesses. The tool wear remains a problem that might lead to significant changes in the pattern dimensions if several scans are pursued. An emerging concept that has proven to delay tool wear in diamond turning is vibration assisted machining. It has recently been extended to SPL as a SPL tip-based ultrasonic vibration-assisted approach [381]. The cap diameter and pitch between the features could be better optimised with a choice of different tip size.

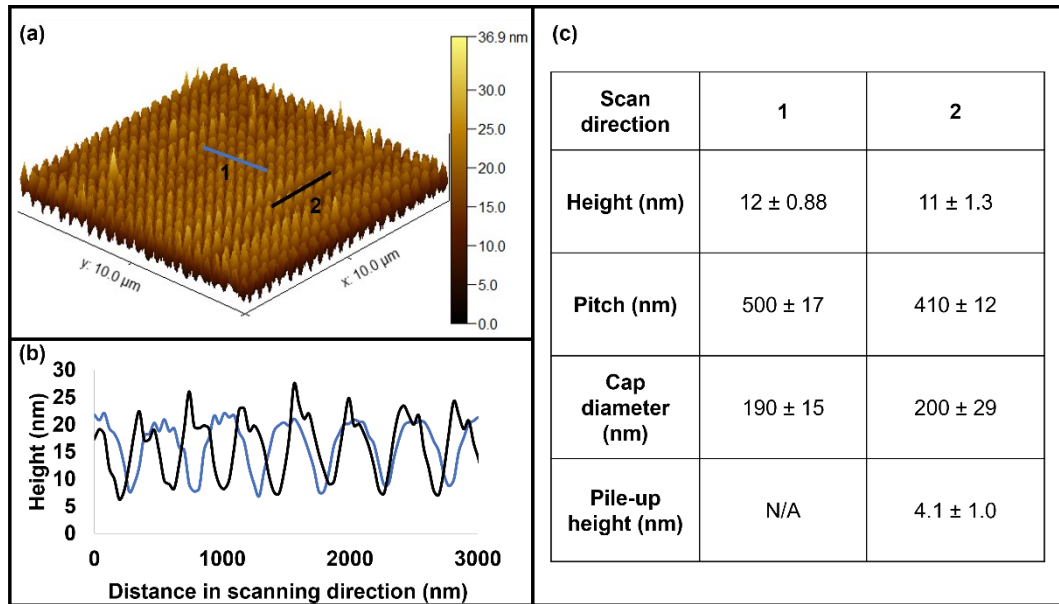


Figure 6-1 (a) AFM height map of the SPL fabricated nanostructures. (b) Profiles of the nanofeatures taken along 2 directions. (c) Nanostructure dimensions extracted from the AFM measurement.

The material removal rate for machining stainless steel using the SPL technique with the parameters applied was found to be around $0.137 \mu\text{m}^3/\text{s}$.

6.2.3 Time-dependant Bacterial Morphology on Modified Surface

The AFM scans of the bacteria on the structured surface are presented in Figure 6-2 (a). As can be observed, after bacteria-surface contact ($t=0$) the bacteria were growing and dividing in what is known as the log phase in the bacterial growth cycle. After 1.5 h, a halt in division was observed (stationary phase) and some bacteria appeared to be wrinkling and sinking into the structures (death/decline phase), which was seen very prominently after 2.5 h as the structures could be identified underneath the bacteria. Some bacteria were observed to have sunk to the point of no recognition. The study on the flat surface yielded very similar observations which led to the insight that the phenomenon observed was not induced by the structures fabricated but it was the natural cycle of the bacterial growth. The line scan extracted from the AFM height maps presented in Figure 6-2 (b) showed the significant difference in bacteria height and morphology

between a live and growing bacterium and dying/dead bacteria. Dead/Dying bacteria appeared to be very shallow and wrinkled compared live bacteria.

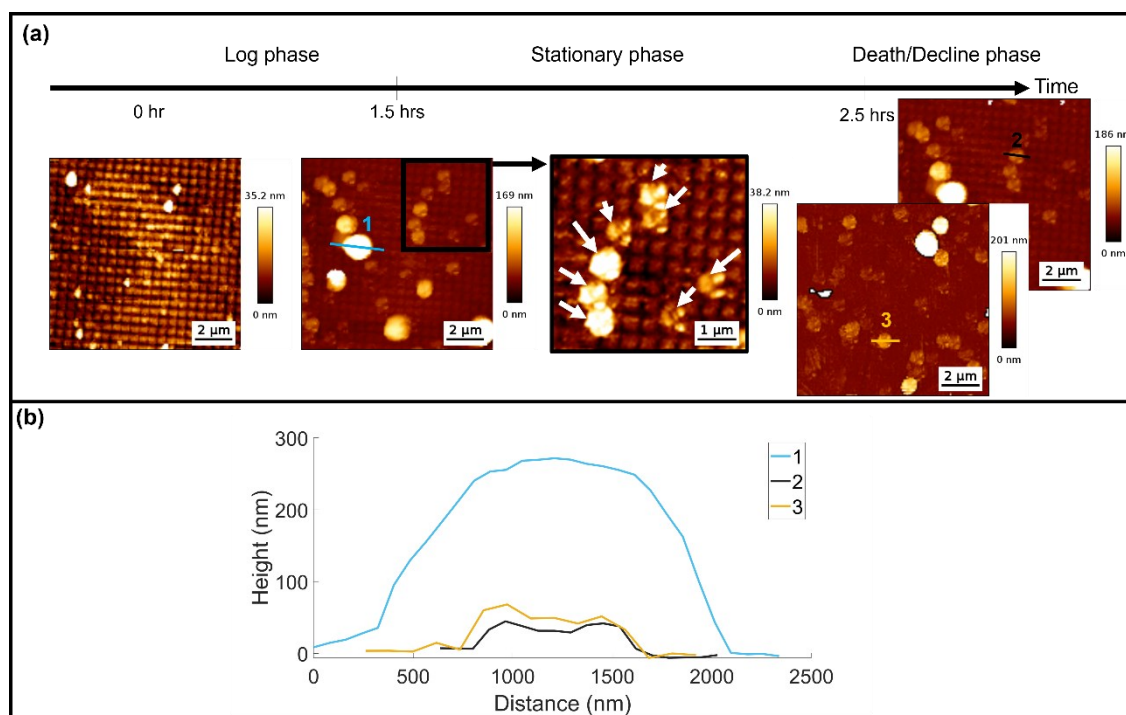


Figure 6-2 (a) Real time AFM imaging of *S. aureus* on SPL-fabricated stainless steel sample. The arrows indicate the wrinkled bacteria. In the death/decline phase, the top scan was taken for the structured area while the bottom scan was taken for the flat area. The lines 1, 2 and 3 are a representation of the (b) profile scan of several bacteria extracted from different growth stages on the flat and structured areas.

The SEM micrographs in Figure 6-3 showed no apparent difference in the adhesion pattern of *S. aureus* between the structured and the flat areas. Since the pattern fabricated was very shallow compared to the bacteria size, it was not surprising that the features did not profoundly affect the bacteria. As stated in chapter 2.3.1.2, one of the most important parameters in anti-bacterial nanostructures is the height of the feature needing to be at least bigger than the sinking depth of the bacteria, which was a limitation in this work.

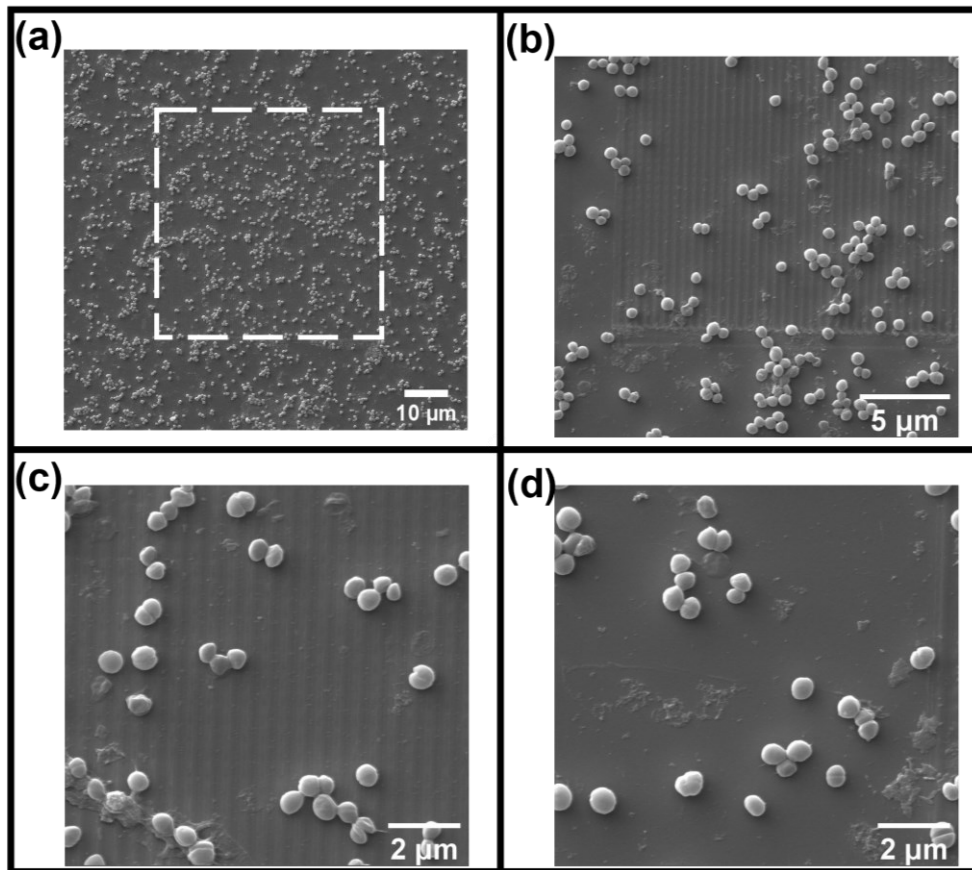


Figure 6-3 SEM micrographs of *S. aureus* on SPL-fabricated sample after 3 hours. (a) overview of the whole surface. The dashed square represents the structured area. (b) A magnified SEM micrograph containing both structured and flat areas. (c) A magnified micrograph of the structured area. (d) A magnified micrograph on the flat area.

6.3 FIB Lithography Fabricated Nature-Inspired Nano Scale Features on Stainless Steel

6.3.1 Introduction

Nanofeature fabrication through FIB lithography provides numerous advantages for flexible prototyping. First, it allows for the use of a wide range of materials (any material that can withstand the high vacuum conditions of the SEM chamber). Second, it grants a high degree of flexibility in the feature geometries achievable through tuning the ion beam current and patterning conditions. Third, it provides

simplicity in term of process operation and monitoring as it is a single-step process that allows for milling monitoring in real time. In this chapter, FIB milling was used as prototyping technique for fabricating a $(50 \times 50) \mu\text{m}^2$ grid of nanostructures on SS316L. The design was based on the dimensions of the nanostructures found on dragonfly's wings, presented in Figure 6-4. The samples were then incubated with *S. aureus* for 3 hours as described in chapter 3.4.1 in an effort to investigate their morphology through SEM imaging.

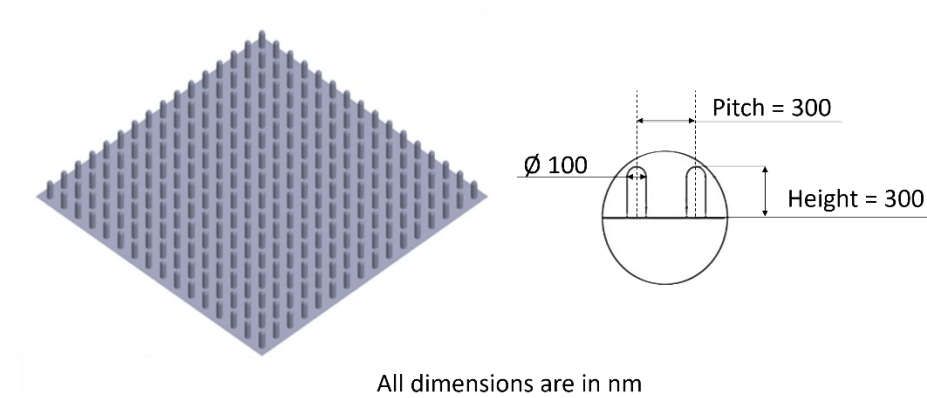


Figure 6-4 The CAD design of the FIB nanostructures based on dragonfly's wings. All the dimensions are presented in nm.

6.3.2 Results and discussion

6.3.2.1 Surface Fabrication and Topographical Characterisation

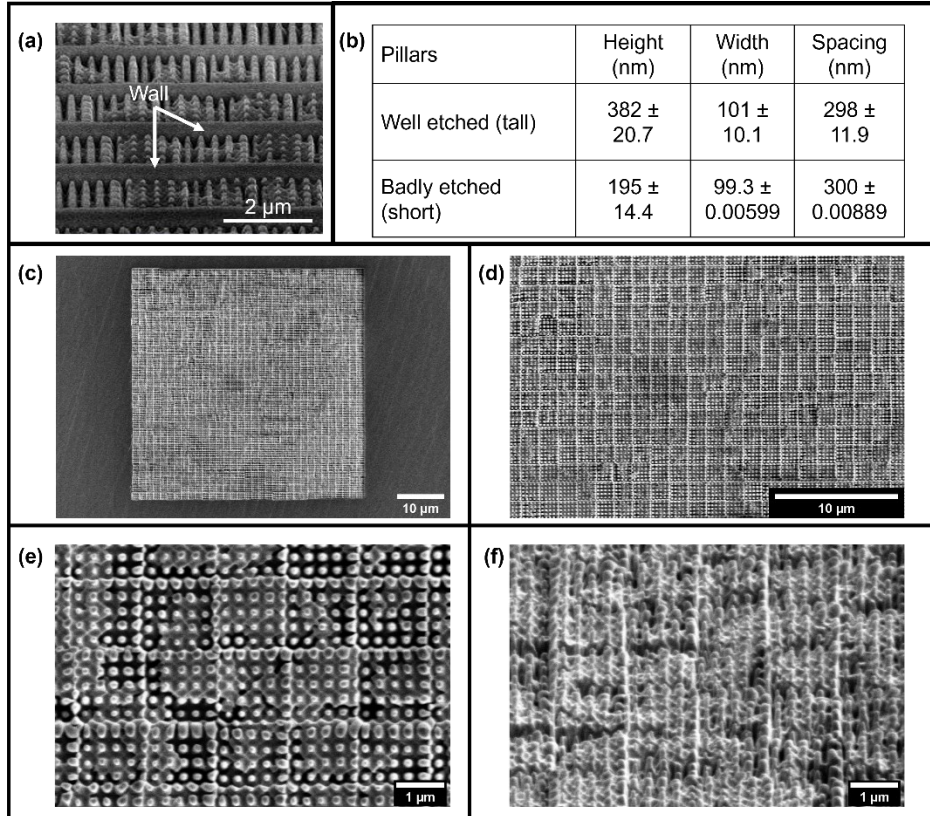


Figure 6-5 (a) Early attempt at FIB milling desired nanostructures. (b) Dimensions of the milled nanostructures. SEM micrographs of the $50 \times 50 \mu\text{m}^2$ milled area at (c) 2.5 kx magnification, (d) 6.5 kx magnification and (e) 25 kx magnification. (f) A tilted view at 25 kx magnification and 52° tilt.

The SEM micrograph in Figure 6-5 (a) is one of the early attempts at milling the nanostructures on large area. It shows a “wall” along the horizontal direction that was a result of the stage position. In later attempts, a datum target was set to reduce this error as described in chapter 3.2.3. The addition of the datum target and automating drift correction by periodic image recognition of the target significantly improved the stitching of the nanoarrays as can be seen in the Figure 6-5 (c-f). From the SEM micrographs in Figure 6-5 (a, c-f), it is clear that the etching or milling rate is changing, most likely due changes in grain orientation where certain grain orientation are etching better than others. The dimensions of

the well etched and “badly” etched structures are presented in Figure 6-5 (b). It has been previously observed that crystal orientation can affect the milling rates of stainless steel [272]. The difference in feature height for stainless steel 316L was found to be around 40 nm between two different grains for features of targeted heights of around 50-100 nm [272]. In the current work, the difference in height between the well etched pillars and the badly etched pillar is around 187 nm for targeted height of 300 nm. Considering a linear trend, the observed height difference of 187 nm for targeted heights of 300 nm is reasonable. Overcoming this anisotropic etching could be pursued by first studying the etching rate of different grain orientations and optimising the etching parameters for specific grain. Second, the patterning script needs to be customised to accommodate for the change in grain orientation. This would need the correlation of EBSD data with FIB-SEM imaging and milling strategy which could be very time consuming.

The fabrication of 33 x 33 arrays with each containing 25 pillars took about 18 hours and 33 min which results in a patterning efficiency of 2.4 s/pillar. Considering the average volume of material removed, the material removal rate was calculated to be around $0.05 \mu\text{m}^3/\text{s}$ ($5 \times 10^{-11} \text{ mm}^3/\text{s}$) which is very well consistent with the literature. In a study by Bhasvar et al.[238], material removal rates for high speed steel ranged between 0.005 and $0.4 \mu\text{m}^3/\text{s}$ for machining trenches of $0.5 \times 0.5 \mu\text{m}^2$. The highest MRR was achieved by increasing the beam current, percentage of overlap between the milling passes and increasing the angle of incidence of the ion beam in relation to the sample. Due to the geometry requirements of the surface fabricated in this work, it was not possible to alter these parameters significantly. For instance, a high incidence angle would create sloped features and if it is excessively high it might over mill adjacent features. The overlap was chosen to be the maximum allowed without over-milling adjacent features. Finally, the beam current is closely related to the beam size and if it were to be increased for higher MRR, the beam size would also increase, compromising the size of the features.

6.3.3 Surface Morphology of *S. aureus* on FIB Milled Nanostructures

The surface morphology of fixed *S. aureus* bacteria on the FIB milled features after a 3 hours incubation period are presented in Figure 6-6.

On the flat area, *S. aureus* appeared as spherical bacteria mostly clustered in grape-like manner as reported in the literature. On the nanostructured area, the bacteria appeared to be attached more individualistically with very few bacteria clustering observed (Figure 6-6 (d)). By comparing Figure 6-6 (b) and Figure 6-6 (c), a higher number of bacteria were seen attached on the flat area (43 vs 15). The morphology of *S. aureus* on the flat and structured areas were very similar as no stretching, wrinkling or any sign of bacterial death was seen. It was apparent then that the nanostructures did not inactivate the *S. aureus* bacteria but alternatively had an anti-biofouling effect as attachment appeared to be reduced on the nanostructured surface. As discussed in chapter 5, the effect of nanostructures of the size ranges applied in this work are within the range that was previously found to be lethal to *S. aureus* in the literature. The lack of bactericidal effect needs to be explored further in order to isolate the underlying causes.

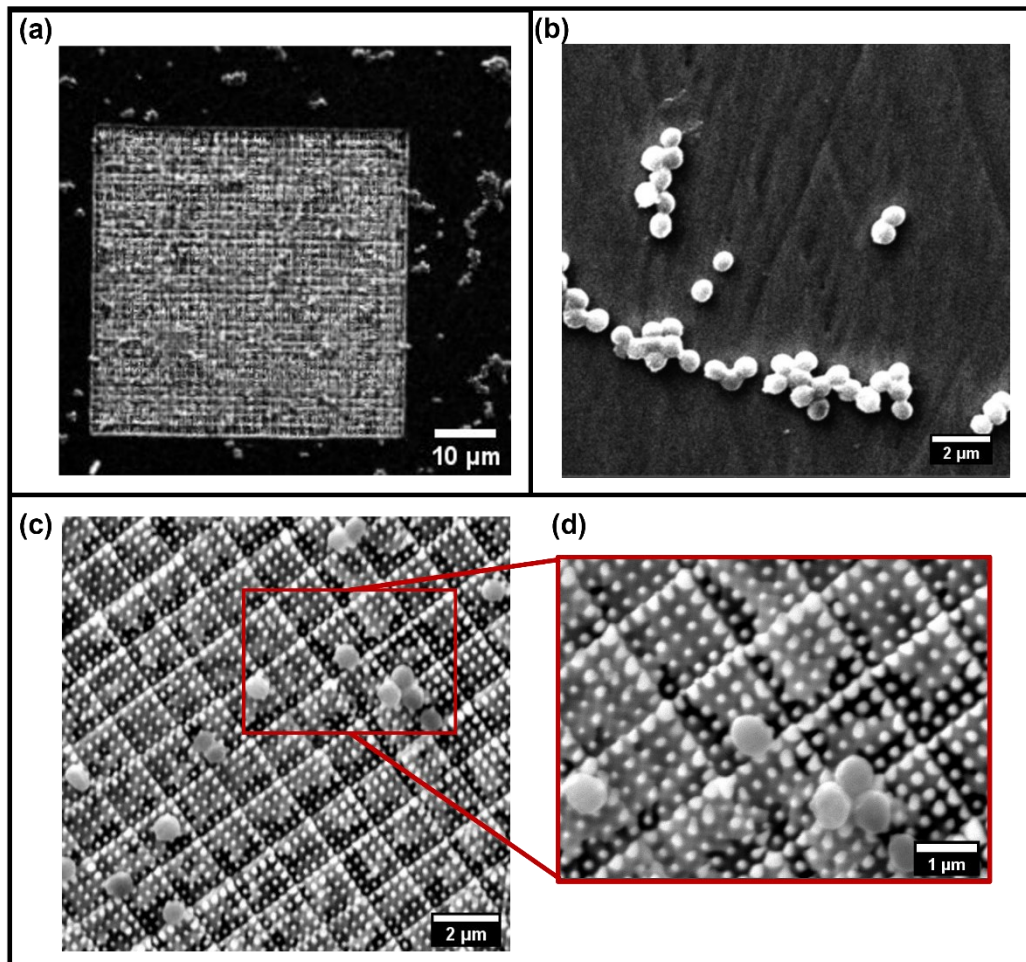


Figure 6-6 SEM micrographs of *S. aureus* on FIB milled sample (a) 1 kx magnification of the sample, (b) 10 kx magnification on the flat area, (c) 10 kx magnification on the nanostructured area and (d) a close-up micrograph (x 40 kx) on clustered bacteria on the nanostructured area.

6.4 Summary

Both SPL and FIB fabrication techniques were used to fabricate nanostructures on stainless steel. Both techniques possess some advantages and drawbacks. For instance, the fabrication of high aspect ratio features proved to be challenging using the SPL technique due to the limitations in cantilever stiffness, not allowing a high enough force input. However, further work in applying several consecutive cuts is worth exploring in addition to exploring a wider range of cantilever

stiffnesses. The material removal rate for SPL is higher than that for FIB milling which makes SPL a quicker method for prototyping.

The response observed between bacteria and the different nanostructures was not evidently different to the response in contact with flat surfaces. The SPL milled features fabricated at this stage were too shallow to affect the response of *S. aureus* significantly. However, surprisingly the nanostructures fabricated in close similarity to dragonfly wings did show an anti-biofouling effect but not a bactericidal effect.

7 Silver Ion Implantation

7.1 Introduction

In chapters 4 to 6, nature-inspired topographies were fabricated, and their anti-bacterial effects investigated as well as the MG-63 attachment and morphology on the topographically-modified surfaces. Although not the focus of this thesis, this section briefly introduces the chemical modification of biomedical surfaces, specifically Lock compression plate implants (LCP), in order to generically compare the two approaches of chemical versus topographical modifications. This is relevant because silver implantation is already a commercially available chemical surface modification technique, and it is important to consider the manufacturing science involved in this context, given that this thesis focuses on various nanomanufacturing methods employed for bacterial eradication. A detailed comparison between these two different approaches is made in chapter 8.

In this brief chapter, the results of a comparative analysis between control LCP and Ag-implanted LCP samples are presented to evaluate the anti-bacterial effectiveness of silver ion implantation. The study investigated the attachment and viability of *S. aureus* bacteria, and the attachment and morphology of MG-63 cells on the surfaces, highlighting the challenges encountered during the analysis due to the freeform shape of the LCP samples. The findings revealed the influence of silver ion implantation on the bacterial attachment, cell morphology and overall anti-bacterial performance.

7.2 Results and Discussion

Silver ion implantation was conducted on 3 different types of implants each having different material composition as outlined in section 3.1.2. The implants are shown before and after implantation in Figure 7-1. These tests were supported by the collaborators based in Spain using their unique sets of facilities. This limited the continuous access of the resources and within those constraints,

the scope of the experimental investigation was confined to stainless steel LCP samples.

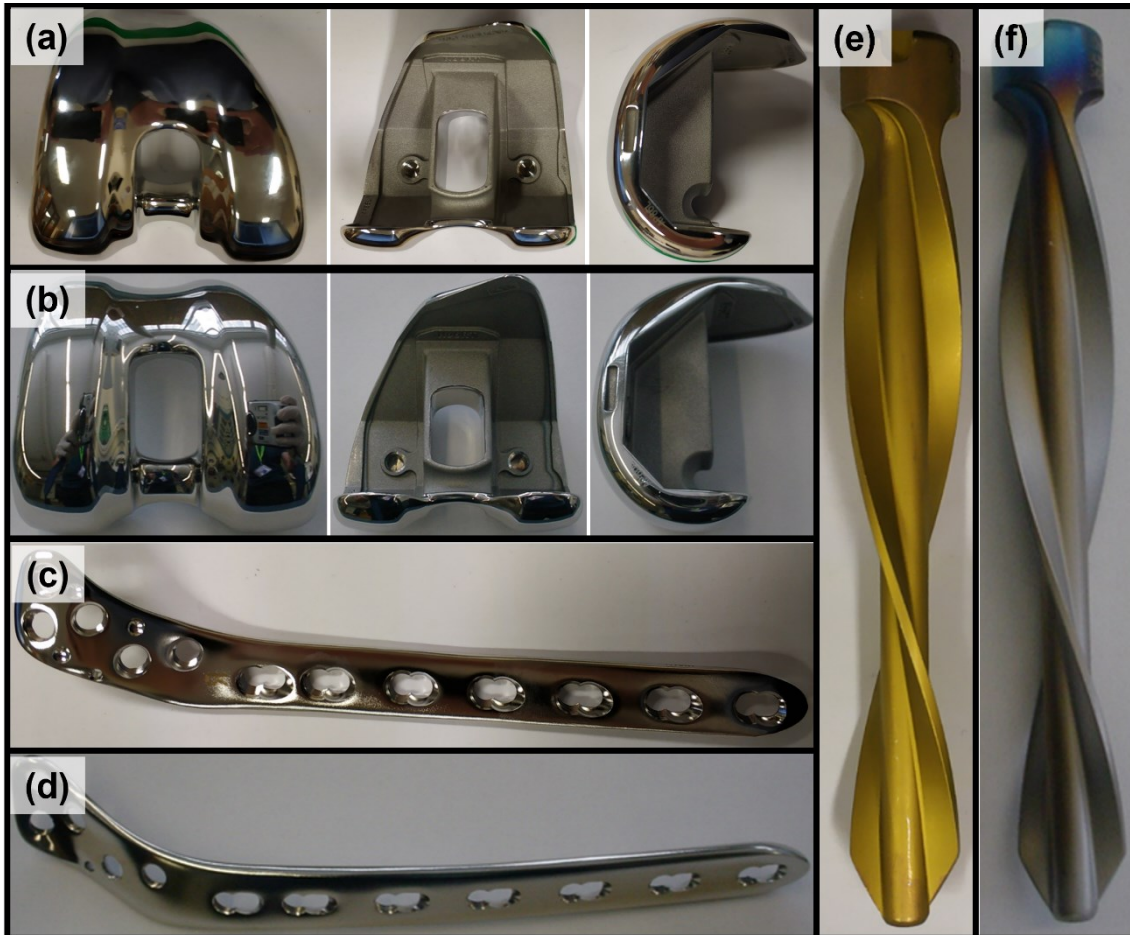


Figure 7-1 Knee replacement femoral component (a) before implantation (b) after implantation. Lock compression plate (c) before implantation and (d) after implantation. Spiral blade (e) before and (f) after implantation.

EDS analysis of the surface of Ag-implanted LCP samples revealed a 0.795 ± 0.148 weight percentage of silver. It was not possible in this instance to conduct detailed and high-resolution chemical analysis of these surfaces. However, Stopping Range of Ions in Matter (SRIM) simulations [382] were applied in order to gain insights into the depth of implantation of silver in the bulk stainless steel material of the LCP samples. SRIM employs Monte Carlo-based simulations to predict ion implantation profiles, considering various factors such as ion energy,

mass, target material and incident angle. Although the software accounts for many physical processes, such as ion stopping, scattering and sputtering, it may not encompass all real-world factors or capture the complexities of experimental setups. Consequently, there could be deviations between the simulation results and the actual outcomes observed in experiments.

Accordingly, the SRIM simulations were conducted by feeding the software details about the composition of the target material, in this case the LCP material as obtained from EDS scans, the ion energy, the implanted material (silver) and the angle of incidence (normal incidence).

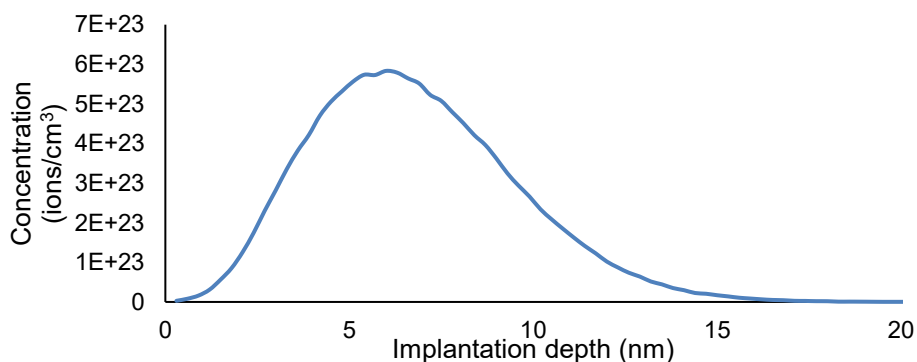


Figure 7-2 Silver ion concentration versus implantation depth

The SRIM simulation results are presented in Figure 7-2. Using these results, the expected range of weight percentage of silver was calculated to be between 1.76 and 8.22 wt% with varying EDS analysis depth between 1 and 5 μm . The actual measured weight percentage of silver through EDS was lower than the expected range. This discrepancy might arise from the inherent limitations in the SRIM simulation, as it offers estimations grounded in theoretical calculations. However, actual experimental conditions may deviate from these calculations, particularly when dealing with intricate freeform surfaces, such as the LCP sample surface. Detailed surface chemical analysis (e.g., XPS or TOF-SIMS, challenging for non-flat samples) is important to consider for future work conducted in this direction in order to provide further insights into the quality of implantation including depth and concentration, while processing freeform complex surfaces.

To extrapolate the testing results from a section of the implant to understand the performance of the full implant, it's essential to consider that the test quantification is per unit area. This allows for a standardised measure that can be applied across the entirety of the implant. However, there are limitations to this approach. Variations in curvature and local roughness gradients can influence the implantation process, potentially resulting in fewer ions being implanted in certain areas. These same variations can also affect the testing process, introducing potential discrepancies when trying to generalise results to the entire implant.

Despite these challenges, this testing technique offers a valuable indication of the implant's functionality in early studies. As research progresses, it's crucial to transition to in vivo testing using full-sized implants as mentioned in future work in the thesis. This ensures a comprehensive evaluation of the implant's performance and safety before it's deemed suitable for human implantation.

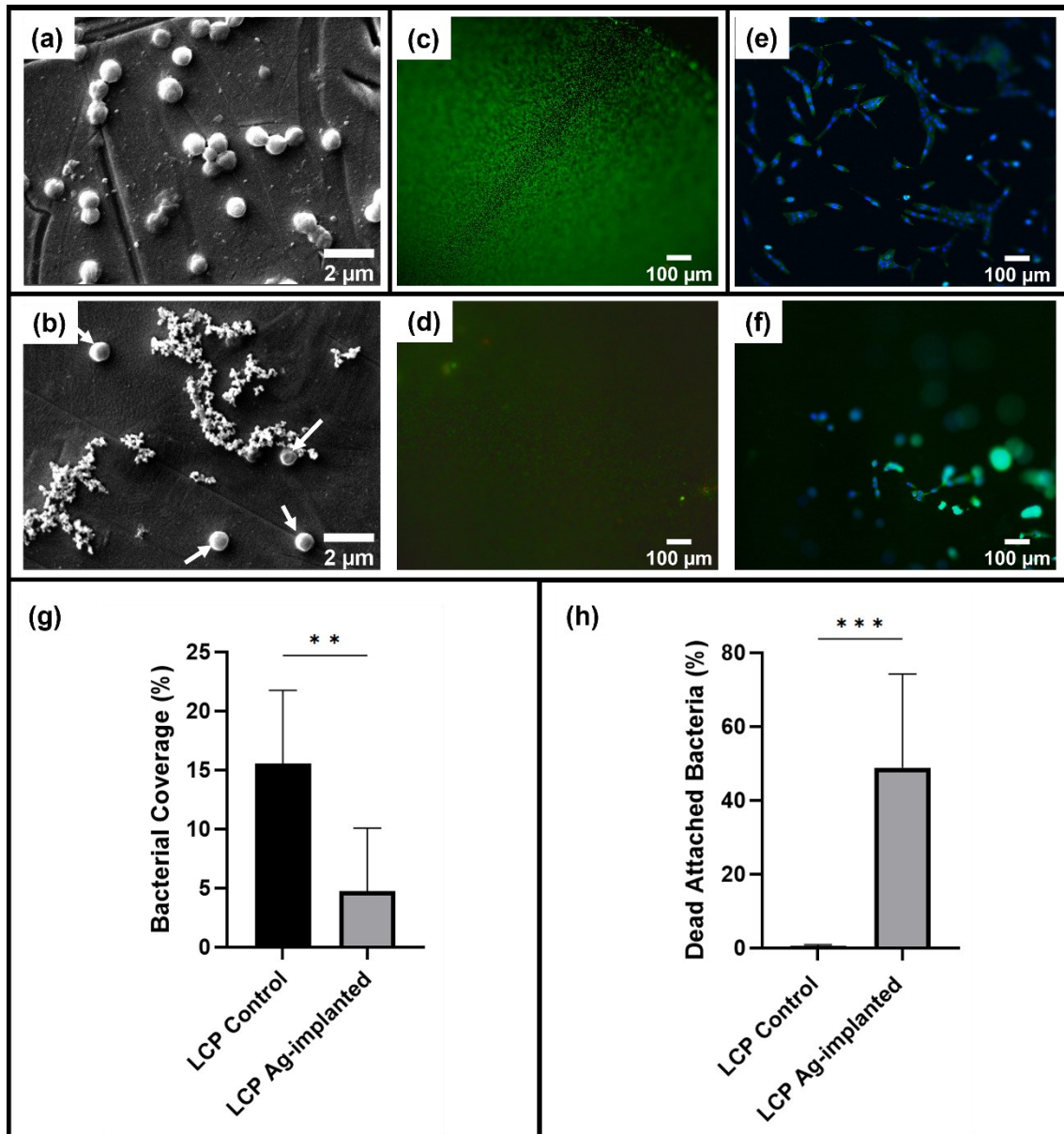


Figure 7-3 SEM micrographs of LCP samples (a) Control and (b) Ag-implanted. Fluorescent microscopy images of *S. aureus* on LCP samples, cell debris can be seen on Ag-implanted samples similarly to [383] (c) control and (d) Ag-implanted. Fluorescent microscopy images of MG-63 cells on LCP samples (e) control and (f) Ag-implanted. (g) The bacterial coverage on both LCP control and Ag-implanted samples. (h) The percentage of dead bacteria attached to the surface of LCP control and Ag-implanted samples. The white arrows indicate the attached *S. aureus* bacteria. The p-value for student's t test comparison between the samples are represented by ** ($p < 0.01$) and *** ($p < 0.001$).

SEM micrographs in Figure 7-3 (a, b) of the control LCP and Ag-implanted LCP show the lower number of *S. aureus* attached to the Ag-implanted surface. The silver implanted LCP samples tested showed significantly reduced bacterial coverage compared to the control LCP while the percentage of dead bacteria on the surface of the LCP was also significantly higher for Ag-implanted LCP samples Figure 7-3 (c, d, g, h). The decreased attachment could be an indication that silver ions actively kill bacteria in the solution, decreasing the number of live bacteria seeking attachment. It has indeed been documented that Ag-ion implantation better acts on free floating bacteria as in this state the Ag⁺ ions can easily collide with the bacteria [27]. It must be noted that due to the freeform surface of the LCP samples, the evaluation using the fluorescent microscope proved to be challenging as some areas of the sample would be out of focus, rendering the analysis of the results a difficult task. In order to quantify the presence of bacteria on the surface, some areas that were in focus in each image were isolated and analysed. The same problem was encountered upon attempting to quantify MG-63 cell attachment and morphology. Due to the large size of the MG-63 cells, the in-focus areas of the images were not enough to confidently quantify the results. However, qualitatively, it can be seen that there is a high attachment of MG-63 cells on control surfaces while there are barely any cells on Ag-implanted LCP samples. Additionally, the cells appeared to have a very rounded morphology on the Ag-implanted LCP samples while they were significantly spreading on the control LCP sample. Fluorescent microscopy images of MG-63 cells attached to the surfaces are presented in Figure 7-3 (e, f).

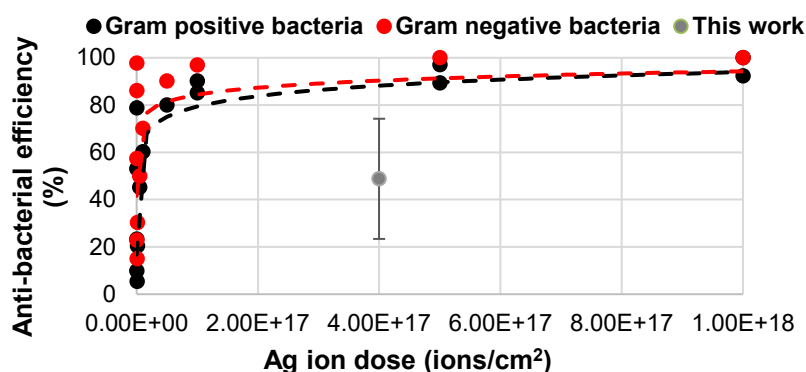


Figure 7-4 The position of the current results within the literature

The mean anti-bacterial efficiency of the tested surfaces is significantly lower than the efficiencies reported in the literature for similar Ag ion dosage (Figure 7-4). It must be noted that the results reported in the literature are done on the flat samples, whereas the results reported here are for freeform surfaces. As seen in the deviation between EDS measurements and the SRIM simulation, the weight percentage of implanted silver is less than that expected for “ideal” conditions of a flat surface. Consequently, this could explain the observed discrepancy in the anti-bacterial efficacy when comparing samples with the same ion dose. The implantation process on intricate surfaces, such as the LCP samples, may result in a lower silver concentration than that reported for flat surfaces in existing literature. This variation in concentration could potentially contribute to the reduced antimicrobial performance observed in the experiments.

8 Overall Discussion, Conclusions and Future Recommendations

8.1 Overall Discussion

The aim of the work was to design, fabricate and characterise sub-micron features to create structured surfaces to mimic natural anti-bacterial strategies stemming from surface topographies of natural surfaces such as the lotus leaf, butterfly wings and dragonfly wings. Two main nature-inspired strategies were thus sought out: the anti-biofouling strategy and the mechano-bactericidal strategy.

The results pertaining to the bacteria-surface interaction showed that the surfaces examined in this work can alter bacterial attachment pattern by either increasing it or decreasing it, with a significant dependence on the surface topography and the surface energy. The comparison of the overall performance of both stainless steel structured surfaces and silicon structured surfaces yielded two main parameters that can be used as good indicators for anti-biofouling across the vastly varying geometries studied. Those are the wavelength of the sub-micron features on the surface and the fractal dimension, with scale dependence.

Correlative analysis of the results from chapters 4, 5 and 6 (where applicable) was conducted. It showed that the dominant wavelength of the features is the biggest indicator of the anti-biofouling effect. Specifically, for *S. aureus*, surfaces with low dominant wavelengths ($< 0.3 \mu\text{m}$) show decreased bacterial attachment. The wavelength (or pitch) was found to correlate strongly with the bacterial coverage ($r = 0.8$, $p < 0.05$). Whereas MG-63 cell circularity showed a strong negative correlation with the surface wavelength, meaning that surfaces with a large dominant wavelength encouraged a healthier cell morphology ($r = -0.857$, $p < 0.05$).

Additionally, the fractal dimension correlated strongly with both the bacterial coverage and MG-63 cell circularity and areas when considering the fractal

dimension at the lower scales ($< 0.625 \mu\text{m}$ for BC and CC and $0.625 - 0.875 \mu\text{m}$ for CA) for femtosecond laser structured surfaces ($-0.821 < r_{BC} < -0.643$, $0.034 < p_{BC} < 0.139$, $0.79 < r_{CC} < 0.89$, $p_{CC} < 0.05$, $0.43 < r_{CA} < 0.64$, $0.139 < p_{CA} < 0.329$).

The results from black silicon studies confirmed the correlative trend between the wavelength, the fractal dimension, and *S. aureus* attachment and MG-63 circularity.

Although feature height and height distribution might play a role in the anti-biofouling effect as a whole due to an increase of attachment point with decrease in height, this parameter is affected by the spacing. Under a certain spacing threshold, the increase area available due to increased feature height is no longer attainable for cells. Indeed, in the current work, the correlation of feature height was found to be moderate ($r = -0.429$, no statistical significance).

This work highlighted the importance of the scale of analysis when studying cell-surface interactions (whether bacteria or MG-63), especially when considering hierarchical complex surfaces, as some correlations could be made on specific scales due to the hierarchy of both the biological surfaces (surface macromolecules with varying sizes) and the hierarchy of the surfaces, particularly when studying intricate multi-scale surfaces such as the femtosecond laser structured surfaces. The scale dependency of surface features on *S. aureus* and MG-63 cells attachment and spread is illustrated in Figure 8-1 and is based on the correlative study of the results obtained from chapters 4,5 and 6.

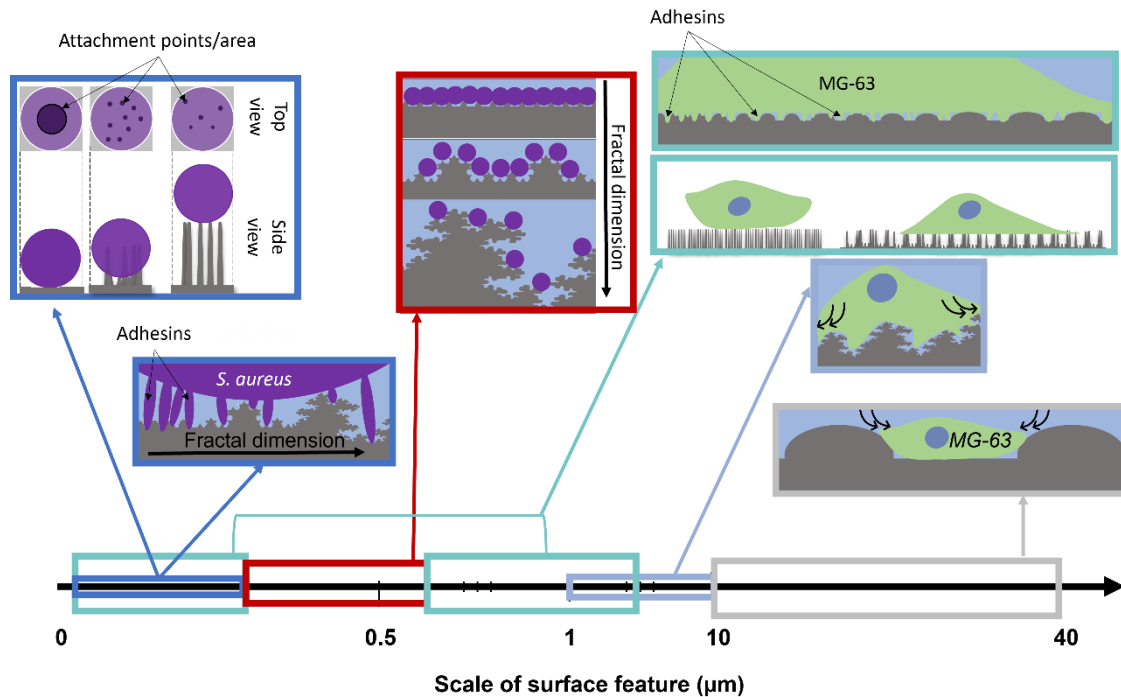


Figure 8-1 Scale dependent interaction of *S. aureus* and MG-63 cells to surface topography

This research did not find convincing evidence of the mechano-bactericidal activity. Evidently, it is known that the mechano-bactericidal effect of nanostructured surfaces is bacteria-specific and that specific features are lethal to some bacteria but not to others. That is mainly understood to be due to the size and mechanical properties of the bacteria in relation to the surface as discussed in section 2.3.1.2. However, in this study a Gram-positive bacterium with known modulus of elasticity was tested against surface features that reportedly are lethal to said bacterium with a similar size and modulus, yet no bactericidal effect was observed.

This observation suggests that the mechano-bactericidal effect of nanostructured surfaces is still an open question and requires further investigation. The conflicting results highlight the urgent need for standardisation of the microbiological tests and experimental protocols as well as the requirements for more research to verify the origin of any observed mechano-bactericidal effect.

This will enable the development of the mechano-bactericidal strategy into a possible application.

The brief investigation into chemical modification through silver implantation of orthopaedic implants demonstrated that silver implantation produced a good level of anti-bacterial (bactericidal) efficiency, albeit with a significant degree of deviation. The variability is attributed to the complex and irregular surface geometry of the implants studied. Additionally, the effect of silver implantation on MG-63 cells was unfavourable, resulting in predominantly rounded morphology and minimal attachment. While the addition of silver presents potential benefits for enhancing implant anti-bacterial properties, its limitations need to be considered. Specifically, pathogens can develop tolerance or even resistance to silver, and it may also have detrimental effects on healthy human cells, as the results in chapter 7 illustrate. Therefore, a cautious approach is recommended when implementing silver implantation for orthopaedic implants.

Comparison between the coverage efficiency (percentage of area with no bacteria attached) is used to compare the three main anti-bacterial strategies evaluated: Anti-biofouling via femtosecond laser ablation, mechano-bactericidal via DRIE, and bactericidal via silver ion implantation. Due to the complexity of comparison of different anti-bacterial mechanisms, the coverage efficiency defined as the percentage of area with no live bacterial attachment, is used as an efficiency indicator. The coverage efficiency does not differentiate between the anti-bacterial mechanisms, it is merely an indicator of the state of a surface.

The coverage efficiencies are presented in Figure 8-2. The anti-biofouling surfaces fabricated through femtosecond laser ablation possesses the highest coverage efficiency between the three techniques as > 99% of the sample surface is not covered by bacteria.

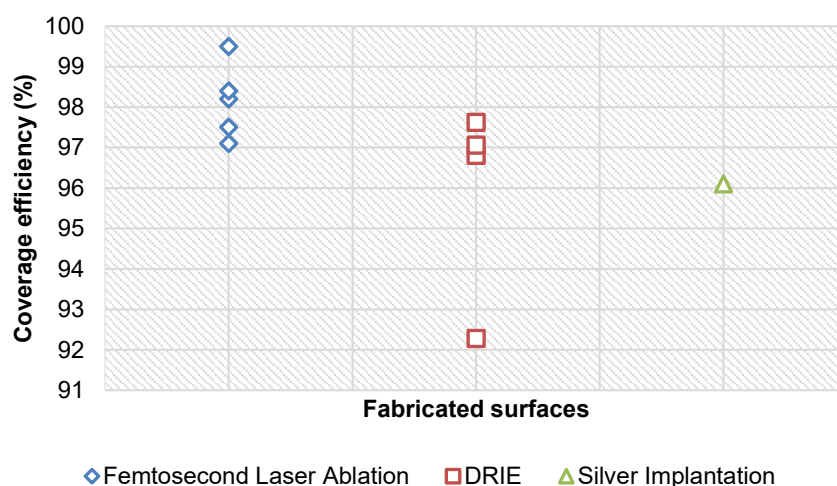


Figure 8-2 Coverage efficiencies of the various fabricated surfaces based on nature-inspired anti-bacterial topographical modifications and chemical modification

Overall, based on the current understanding and the comparison of nature-inspired topographical anti-bacterial modification strategies, the anti-biofouling strategy appears to hold greater promise for practical applications in the near future, while the mechano-bactericidal strategy requires substantial additional research to fully develop its potential. Additionally, when compared with chemical modification through silver implantation, the anti-biofouling strategy still has the upper hand with regards to the efficiency. Moreover, the nature-inspired anti-biofouling strategy proved less detrimental to MG-63 cells, and it does not promote the development of pathogen tolerance or resistance.

One of the overarching goals of this research was to investigate the potential of various nanostructuring techniques to create anti-bacterial surfaces inspired from nature. Table 8-1 depicts the various advantages, limitations and areas of possible advancements for the different manufacturing techniques utilised in this work (including the chemical modification technique). Overall, femtosecond laser ablation was the most promising technique, owing to its unique ability to fabricate versatile features including hierarchical features in a one step process. Rapid advancements in this field are also making the use femtosecond laser ablation for large area processing on industrial scales possible. One such example is the

multipoint consortium, led by IK4-TEKNIKER [384,385], which is utilising multiple beam generation units to significantly improve the productivity of femtosecond laser ablation for large titanium panels for aerospace applications. Additionally, advancements in techniques such as direct laser interference patterning are allowing the fabrication of lower scale features overcoming the limitation of beam size. Moreover, this technique is relatively easily optimisable for different materials, thereby enabling the creation of customised surface topographies on a range of materials. As such, femtosecond laser ablation holds great promise for the fabrication of nanostructured/hierarchical anti-bacterial surfaces. However, due to the nature of the technique, often there is some form of hierarchy present in the fabricated surfaces which could make the investigation of bacteria-surface interactions very complex. Not to mention that it can be costly when looking to prototype nanostructured surfaces for lab-based investigations into bacteria-surface interactions and optimising anti-bacterial nanopatterns. In this case, SPL is a promising technique that can be pursued to create more defined features at low scales, low cost and relatively high speeds (within prototyping techniques). SPL has limitations which were highlighted in this work; however, most can be overcome through specific investigations and further experimentation. Silver ion implantation has demonstrated the ability to process complex orthopaedic implants, such as lock compression plates, spiral blades and knee replacement femoral components. However, the anti-bacterial efficiency of the surfaces modified with this technique showed significant variation, which may require further characterisation to confirm its consistency. Furthermore, the use of chemical agents for implant applications and the possibility of bacterial resistance development must be considered as potential consequences of this approach.

Table 8-1 Table comparing the advantages, limitations and areas of advancement for the different manufacturing techniques explored in this thesis.

Method	Advantages	Limitations/Drawbacks	Areas for advancement
Femtosecond Laser Ablation	<ul style="list-style-type: none"> • Very quick with (MRR ~ $2 \times 10^9 \mu\text{m}^3/\text{s}$). • Easily tuneable to different materials. • Promising technique for freeform processing. • Versatile in feature fabrication with one step fabrication of hierarchical surfaces. 	<ul style="list-style-type: none"> • High equipment and maintenance costs. • Feature sizes limited to minimum achievable beam diameter. • Current technology limited to low aspect ratio features. 	<ul style="list-style-type: none"> • Exploring varied microfeature sizes (micro-scale wavelength) for HSS surfaces to optimise bacterial and osteoblast-like cell response. • Exploring DLIP techniques to overcome feature size limitation.
Deep Reactive Ion Etching	<ul style="list-style-type: none"> • Ability to produce high aspect ratio features. • Could be utilised for large scale production. 	<ul style="list-style-type: none"> • Limited to flat samples. • No specific control on nanofeature dimensions. • Limited to certain materials. 	<ul style="list-style-type: none"> • Exploring different techniques/parameters to pattern a wider range of materials.

	<ul style="list-style-type: none"> Extremely quick (MRR $\sim 8 \times 10^9 \mu\text{m}^3/\text{s}$). 	<ul style="list-style-type: none"> Expensive equipment. Challenging to implement due to process complexity owing to the requirement for specialised equipment and expertise. 	
SPL	<ul style="list-style-type: none"> Quick (MRR $\sim 1.96 \mu\text{m}^3/\text{s}$). Relatively cheap to operate. 	<ul style="list-style-type: none"> Tip wear when machining hard materials. Cantilever stiffness. Most commercial AFM limited in machinable area. 	<ul style="list-style-type: none"> Exploring repeated cuts/passess to create deeper features. Exploring a tapping mode machining technique to delay tip wear (similar concept to vibration assisted machining in SPDT).
FIB Milling	<ul style="list-style-type: none"> Ability to fabricate high aspect ratio features relatively easily. 	<ul style="list-style-type: none"> Slow (MRR $\sim 0.351 \mu\text{m}^3/\text{s}$). Expensive to operate. Anisotropic etching with grain orientation. 	<ul style="list-style-type: none"> Correlation mapping and milling to overcome anisotropic etching. Further optimisation of milling parameters by expanding parameter range through design of experiment.

Silver Ion Implantation	<ul style="list-style-type: none"> • Commercially relevant due to ability to process full scale freeform implants. 	<ul style="list-style-type: none"> • Expensive to purchase and operate. • Deviation in anti-bacterial efficiency. • Toxic to MG-63 cells and possibly others. • Possibility of bacterial resistance. 	<ul style="list-style-type: none"> • Detailed chemical analysis to understand implantation quality. • Bacterial resistance should be further studied as the technique could be considered for external surfaces that are prone to contamination.
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8.2 General Remarks and Conclusions

The primary aim of this thesis was to investigate various anti-bacterial topographical modifications inspired by nature, employing a range of nanofabrication techniques to create sub-micron sized features, and analysing their impact on bacterial and MG-63 cell attachment and viability.

The thesis began with a brief overview of nature-inspired design, the principles behind bacterial attachment to surfaces, and then delved into the background of two nature-inspired topographical modification approaches: the anti-biofouling and mechano-bactericidal approaches. An alternative approach involving chemical surface modification was presented with a focus on silver ion implantation. Its limitations were then discussed along with reasons for preferring topographical modification. The methods to evaluate the anti-bacterial action of anti-bacterial surfaces were subsequently presented.

The literature review then focused on various manufacturing techniques used to engineer nano and micro-scale surface features with a focus on ultrafast laser ablation, deep reactive ion etching, focused ion beam lithography and scanning probe lithography. A review of techniques and parameters used in surface topography evaluation followed. Based on this comprehensive background, a research methodology was developed to address the identified gaps and research questions.

In addressing the research objectives, the study arrived at several pivotal conclusions. For the first objective, which centred on surveying the landscape of various ultra-precision nanomanufacturing technologies, an extensive literature review pinpointed four primary manufacturing methods apt for producing nature-inspired anti-bacterial surfaces. These methods encompass femtosecond laser ablation, deep reactive ion etching, focused ion beam lithography, and scanning probe lithography.

Turning to the second objective, which delved into understanding the biological response of bacteria and bone-like cells to nature-inspired surfaces, the research discerned that only the anti-biofouling mechanism was consistently achieved through nature-inspired surface structuring. This suggests its superior suitability for future applications compared to the mechano-bactericidal mechanism. The study further revealed that the anti-biofouling effect was predominantly steered by surface topography, overshadowing the influences of surface energy or chemistry. A notable observation was the effect of surface crystallographic orientation on both *S. aureus* and MG-63 cell adhesion, with a marked preferential adhesion on 111-oriented silicon surfaces. Moreover, high aspect ratio features within specific ranges did not induce mechano-bactericidal effects on *S. aureus* NCTC7791, a finding that diverges from existing literature, underscoring the need for more nuanced research in this domain.

For the third objective, which aimed to unravel the relationship between the physical dimensions and biological functionality of the fabricated surfaces, the study discerned a scale-dependent relationship. Specifically, for surface feature sizes below 0.625 μm , a positive correlation was observed between the fractal dimension and the anti-bacterial effect. Conversely, for feature sizes either below 0.25 μm or above 0.625 μm , the fractal dimension was found to positively correlate with MG-63 cell spreading. Interestingly, for feature sizes significantly larger than the bacterial size, no such correlation was evident. However, MG-63 cell spreading did show a positive association with surface complexity. When the feature sizes were akin to the MG-63 cell size, the spread of the cells was notably inhibited.

Lastly, in addressing the fourth objective, which sought to evaluate the scalability potential of the developed approaches, femtosecond laser ablation emerged as a promising candidate for commercial fabrication of nature-inspired anti-biofouling surfaces. This was especially evident when juxtaposed with silver

implantation, which, despite its merits, presents concerns like potential toxicity and bacterial resistance. On the research scale, Scanning Probe Lithography (SPL) was identified as a cost-effective and flexible prototyping technique, offering the added advantage of on-machine measurement capabilities.

8.3 Future Recommendations

- **Surface fabrication:** Further investigation into the optimal combination of surface parameters for inhibiting bacterial adhesion and promoting favourable interaction with MG-63 cells could be carried out. Particularly, this could involve the investigation of an optimal microfeature size or size range to accommodate for MG-63 cell spreading while maintaining the anti-bacterial effect of surfaces through hierarchical structuring.

In addition, further studies could explore the link between localised surface energy gradients and biological cell behaviour, as well as investigate the potential influence of residual stresses on biological cell behaviour. Such investigations could help to provide a more comprehensive understanding of the complex interplay between surface topography, chemistry, surface energy and biological responses, and could potentially pave the way for the development of even more effective and targeted strategies for controlling bacterial adhesion and promoting the growth and proliferation of specific cell types. Another potential avenue for future work would be to explore the practical applications of these surfaces in real-world settings. This could involve studying the performance of laser-fabricated surfaces under different environmental conditions, such as exposure to varying levels of contamination, moisture, or temperature.

The research presented in this thesis emphasised the potential of scanning probe lithography (SPL) as an emerging and relatively unexplored technique for structuring surfaces. Moreover, it demonstrated the successful application of SPL in structuring stainless steel, which had

not been previously reported. This study has revealed new opportunities for further investigation in this field. To fully comprehend the scope and limitations of this technique, future research should encompass a comprehensive exploration of fabrication parameters, various cantilever specifications and probe types.

- **Surface characterisation:** While there was an extensive effort at characterising the fabricated surfaces, the surface chemical characterisation was lacking especially for silver implanted samples. Techniques such as XPS and TOF-SIMS can be used to evaluate these surface chemistries however the requirement for a flat surface might hinder the analysis seeing as these surfaces are freeform. The use of X-ray computed tomography, or XCT, to examine in the interior of fabricated surfaces as well as the use of techniques such as neutron diffraction to probe surface residual stresses could be an important direction to analyse the material further.

The discrepancy in scale between the large area evaluated by the sessile drop method for surface energy and the microscale dimensions of bacteria presents an interesting avenue for future research. This could not only clarify some of the existing inconsistencies in the literature but also offer a more nuanced understanding of the findings in this thesis, which did not identify surface energy as a significant factor in anti-biofouling mechanisms.

- **Bacteria studies:** The bacteria incubation period in this work was 1 to 3 hours which fulfilled the specific purpose of this study of understanding the anti-bacterial effect in the initial phases of bacterial attachment. However, longer incubation periods, and measurement of bacterial adhesion at several time periods, are imperative as a next step to understand the longevity of the anti-bacterial effects of the anti-biofouling surfaces produced. Determination of protein adsorption on the structured surface could provide useful insight into the conditioning film that forms. Finally, in

vivo testing needs to be considered at some point during the research as it takes the path towards being a commercialised solution.

Further work needs to be considered focusing on the standardisation of microbiological testing of surfaces, specifically nanostructured surfaces expected to possess mechano-bactericidal properties. This could include standardising bacterial strains used, testing conditions, and evaluation criteria to ensure the reproducibility and comparability of results across different studies.

To standardise the protocols, it is essential to explore various experimental conditions, which can involve a broader range of bacterial strains, different concentrations of bacteria, varying incubation time and conditions, surface preparation, and careful handling of samples during experimental steps, among other parameters. Extending the research methodology to encompass methods like flow cytometry and CFU (where possible) can offer a valuable insight into the effect of nanotopographies on the bacteria tested, as recent research has found [203].

For silver implanted samples, it's important to recognise that the anti-bacterial testing conducted only accounted for bacteria attached to the samples' surface. In future work, tests could be expanded to include the analysis of the incubation medium, especially considering that Ag-ion implantation has been found to better act on free floating bacteria [27]. By expanding the testing to include the incubation medium, it can more accurately determine to what degree the chemical modification method is lethal to bacteria.

- **Mechano-bactericidal surfaces:** In the current study, no mechano-bactericidal effect was observed on black silicon surfaces, necessitating further investigation. The mechano-bactericidal action, as reported in the literature, arises from the mechanical response of bacteria to surface features, making finite element analysis a valuable tool for such investigations. In this work, however, several limitations were encountered, primarily due to the lack of bacteria-specific experimental

data to serve as a foundation for the analysis. Although some previous studies have addressed the FEA topic, the data utilised often relied on numerous assumptions (section 2.3.1.2.1), and lacked bacteria specificity, potentially impacting the accuracy of the results. To advance this research area, it is essential to develop a hyperelastic/viscoelastic material model through comprehensive nano-mechanical experiments. This presents a complex research challenge, as it requires decoupling the material properties of various bacterial layers. In this work, efforts were made to characterise the material properties of *S. aureus* NCTC7791 (see appendix 9A.1), but due to the shallow indentation depth, no distinct critical strain threshold was obtained. Large deformation experiments, extending to the point of failure, are necessary to fully understand the material system's behavior and extract properties that can inform more accurate computational studies.

- **Statistics:** It is important to consider potential limitations of the work, especially considering statistical analysis. One limitation of the present study is that the sample size may not be sufficient to detect small differences in the data. While experiments were repeated multiple times, this may not be enough to capture all variability in the results. Additionally, the use of a one-way ANOVA may not account for all potential sources of variation, such as experimental or environmental factors. Furthermore, the use of observational data in some cases (when statistical significance criteria is not met) may limit the ability to draw definitive conclusions about the results. Therefore, for future work it is recommended that a bigger sample size with a larger number of repeats is encompassed.

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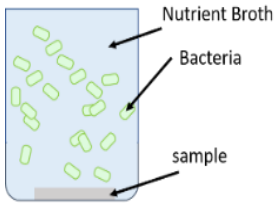
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APPENDICES

A.1 Techniques used to evaluate and investigate anti-bacterial surfaces

Objective	Evaluating the effectiveness of a bactericidal surface against a bacteria strain		
Step 1	Incubation of bacteria on sample		
Methods	Schematic	Limitations/Possible errors	Notes
Immersion (static or dynamic)		Exerting external forces by presence of air bubbles (static) skewing bactericidal efficiency results [198].	<ul style="list-style-type: none"> Temperature of incubation is bacteria strain specific
Drop test (static)		<ul style="list-style-type: none"> Cannot effectively study fate of bacteria after death using this method. 	<ul style="list-style-type: none"> Bactericidal action should appear in first few hours (less than 24 hrs)
Spray (static)		<ul style="list-style-type: none"> Air-bacteria interface exerts external force on bacteria pushing 	<ul style="list-style-type: none"> Repeat tests to see durability of function

		<p>it towards the surface</p> <ul style="list-style-type: none"> • Cannot effectively study fate of bacteria after death using this method 	
Step 2	Evaluating live/dead bacteria		
Techniques/ equipment	Advantages	Limitations/Possible errors	Notes
Colony Forming Units (CFU) assay	Gives quantitative information about viable bacteria attached and their ability to proliferate	<ul style="list-style-type: none"> • Cannot be used when testing small areas. • Time consuming. • Procedure composed of several stages increasing uncertainty [32]. • It can only detect viable bacteria therefore is better 	Can be done by the pour plate method or the surface spread method.

		suited for evaluation of antifouling properties and not bactericidal.	
Microscopy			Microscopy is used in conjunction with staining to quantify live/dead bacteria
Light microscopy	Some techniques were developed to allow real- time observation of bacteria	Cannot be used with opaque substrates	Thoma plate commonly used to count bacteria in conjunction with stains/dyes to differentiate live/dead bacteria
Epifluorescence microscopy	<ul style="list-style-type: none"> Allows differentiation between live and dead bacteria (in conjunction with staining) Fast (suitable for large set of samples) 	<ul style="list-style-type: none"> Limited to macroscopic investigation of bacteria-surface interactions Limited to 2D images 	Used in conjunction with fluorescent bacteria staining agents (most commonly BacLight test kits), quantitative evaluation of live/dead assay done through direct link

Confocal Laser Scanning Microscope (CLSM)	<ul style="list-style-type: none"> • Allows differentiation between live and dead bacteria (in conjunction with staining) • Allows real time observation of samples • Enables cell tracking • Can be used with opaque surfaces • Can produce 3D images of the sample 	Requirement of a CLSM to obtain the requested image quality is expensive.	between number of bacteria and fluorescent signal intensity
Colorimetric assay (Stains & dyes)			
Syto9™	<ul style="list-style-type: none"> • Green, fluorescent dye • Diffuses passively through cellular membranes and binds to DNA of both viable and dead cells. 	It cannot differentiate between live and dead bacterial cells (so it is used in conjunction with dead bacteria staining)	<ul style="list-style-type: none"> • Fluorescent signal intensity • Viable cell staining used in conjunction to dead cell staining is used to

Fluorescein Diacetate (FDA)	Is turned from transparent to fluorescent yellow by viable cells.		quantify anti-bacterial or bactericidal efficiency
Resazurin	<ul style="list-style-type: none"> • Blue colored indicator that can be reduced by viable bacteria and turn pink. • The extent of conversion from blue to pink is a reflection of cell viability. 	Calibration curve needed [387].	e.g. BacLight testing kits contain Syto9™ and PI[386].
Propidium iodide (PI)	<ul style="list-style-type: none"> • Red fluorescent dye • Can only enter cells with disrupted membranes allowing differentiation between live and dead cells [386]. 	Can lead to overestimation of dead bacteria in biofilms due to the presence of extracellular nucleic acids (eNA)[388].	
Methylene blue dye	Stains dead cells		The methylene blue is reduced in viable cells that is why it does not stain them

MTT	Stains live cells only[389].	The mechanism of MTT reduction by bacteria is poorly understood and creates a danger of misinterpretation[389,390].	
Objective	Evaluating how bacteria are dying on mechano-bactericidal surfaces		
Scanning Electron Microscope (SEM)	Reveals morphological features of the bacteria and the substrate	<ul style="list-style-type: none"> • Difficulty in sample preparation. Sample preparation can lead to cell shrinking • Destructive to the samples • Cannot differentiate between live/dead 	It is mostly a qualitative technique because statistically relevant bacteria count would be very time consuming due to its small field view.
SEM-Focused Ion Beam (SEM-FIB)	<ul style="list-style-type: none"> • Can get insight on interaction of structures with bacteria through cross-sectional observation • Can be used for 3D tomography (reconstruction of 		

	cross-sections produced by FIB and imaged with SEM)	bacteria unless morphologically severely damaged (requires an experienced eye)	
Atomic Force Microscope (AFM)	<ul style="list-style-type: none"> • Can simultaneously provide imaging and force spectroscopy. • Probes can be coated with bacteria and interaction forces with the probe and surface can be measured (adhesion of bacteria on surface). • Can be used to measure cell elasticity[109]. 	<ul style="list-style-type: none"> • Destructive deformation of bacteria due to the geometry of the tip, physicochemical treatment in order to firmly anchor the cells to the probe may alter the cell surface properties[391]. • The observation area is very small. 	<ul style="list-style-type: none"> • Can also be used to track cells on surfaces and quantify their detachment cycle[115], contributing to objective 1.

		<ul style="list-style-type: none"> • It is very time consuming especially if used for tracking. 	
Transmission Electron Microscope (TEM)	Can be used to observe the penetration of surface structures inside the bacterial membrane or its deformation.	<ul style="list-style-type: none"> • Samples must be flat sections 100 nanometers or less in thickness[392]. • Difficulty in sample preparation, it can lead to cell shrinking. • Destructive to the samples. 	Microtome mostly used to cut thin TEM samples.
Fourier Transform Infrared Spectroscopy (FT-IR Spectroscopy)	<ul style="list-style-type: none"> • Provides quantitative information about the total biochemical composition of the intact whole microbial cell[393]. 		

	<ul style="list-style-type: none"> Useful in determining cell susceptibility to oxidative stress by correlating them to shift in the IR signals[394]. 		
Protein extraction and proteomic analysis (high performance-reverse phase chromatography with mass spectrometry)	Can be used to identify the increase in specific differentially expressed proteins relating to oxidative stress.	Sample preparation for MS, including spot picking, protein in-gel digestion, target-matrix selection, and contamination[395].	Indirect oxidative stress measurement
ROS-glo™ assay	Bioluminescent assay that measures the level of H ₂ O ₂ ; an oxidative stress marker		Direct oxidative stress measurement

A.2 Comparison of the test conditions of black silicon surfaces

Material	Water contact angle (°)	Efficiency (%)	Medium	volume	Optical density	S. aureus strain	Growth condition	Reference
Black silicon	8	67.87	Oxoid (nutrient broth)	1 mL	0.1	CIP 65.8T	37°C	[102]
	7	66.32						
	7	67.35						
	80	53	Oxoid	5 mL	0.3	CIP 65.8T	37°C	[91]
	8	68	Oxoid	1mL	0.1	CIP 65.8T	37°C	[142]
	12	65						
	10	67						
	130	54	PBS	5 mL	0.1	CIP 65.8T	37°C	[264]
	101	92						
	154	86	PBS	1 mL	0.25	ATCC 25923	37°C	[373]
	82	0.6	PBS	15 µL	0.09	NCTC7791	37°C, 5% CO2	This thesis
	70	0.4						

A.3 Measuring the Elastic Modulus of *S. aureus* NCTC7791

AFM indentations were conducted on *S. aureus* NCTC7791 with the same system as is described in section 3.4.2.

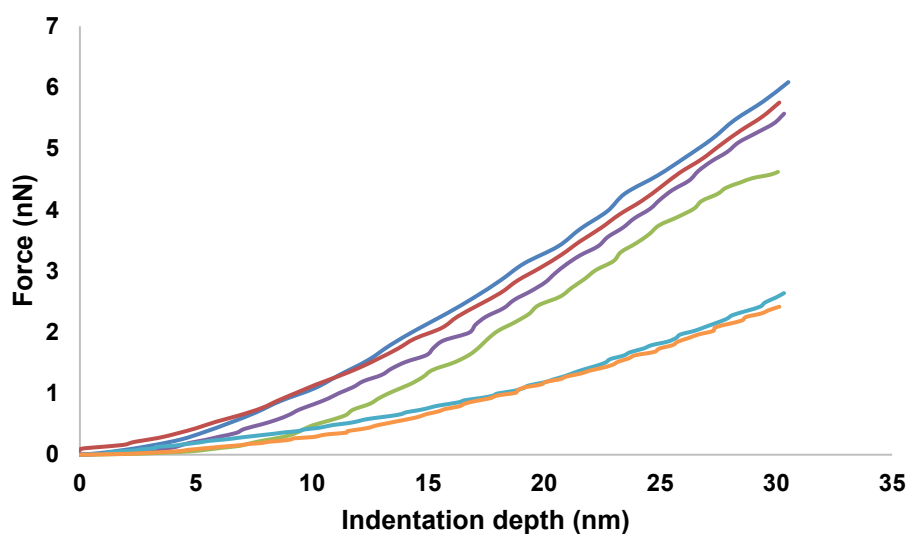
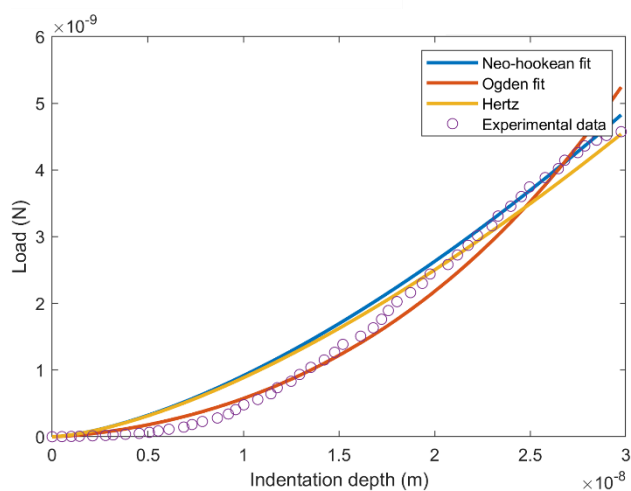


Figure S 1 Force indentation curves for the indentation of *S. aureus* NCTC7791.

The modulus of elasticity of *S. aureus* NCTC7791, calculated through the hertzian model, is 0.95 ± 0.24 MPa.

Two hyperelastic material models were also fitted to the load-indentation curves: the neo-hookean and Ogden models. The neo-hookean model was the best fit between the hertz and Ogden models as can be seen from the least square error below.



Model	Least Square Error
Hertz	1.85E-18
Neo-Hookean	6.30E-20
Ogden	4.45E-15