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Inhibition of Polymicrobial Biofilm Using Niobium Pentoxide Nanoparticles

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ABBREVIATIONS

$AgNO_3$ – silver nitrate

AgNPs – silver nanoparticles

agr - accessory gene regulator

AHLs – acyl-homoserine lactones

AIPs – autoinducing peptides

AMR – antimicrobial resistance

aPDT – antimicrobial photodynamic therapy

AuNPs – gold nanoparticles

BHI – brain-heart infusion broth

CFU – colony-forming unit

e-DNA – extracellular DNA

EPR - Enhanced Permeability and Retention

EPS – extracellular polymeric substance

HGT – horizontal gene transfer

IR – infrared

MDR – multi-drug resistance

MEMS – micro-electro-machanical system

MVs – membrane vesicles

Nb_2C – niobium carbide

Nb_2O_5 – niobium pentoxide

NPs – nanoparticles

PBS – phosphate buffered saline

PDI – polydispersity index

PET – polyethylene terephtalate

PIA – polysaccharide intercellular adhesin

QS – quorum sensing

ROS – reactive oxygen species

SEM – Scanning Electron Microscopy

TiO_2 – titanium dioxide

UV – ultraviolet

WHO – World Health Organization

XTT – 2.3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

INTRODUCTION

A biofilm is a thin, robust layer of a community of bacteria or other microorganisms grown on a solid surface. Biofilms allow the bacteria to survive hostile environmental conditions against various stressors, such as antibiotic treatment (Shi et al., 2016). Over the years, microorganisms gained antimicrobial resistance, with biofilm being the most important cause, making them extremely difficult to eradicate (Guo et al., 2019). This has given a serious rise in infection numbers and economic loss. Biofilm-related infections are an alarming health problem, causing 65-80% of all infections that are generally persistent and chronic (Macià et al., 2018). Chronic wounds can prolong the healing process with clinical implications, making them a challenge to treat, becoming a significant burden on healthcare systems (Falcone et al., 2022). The globally growing numbers make novel, effective treatments a necessity. Recently, nanomaterials have become a topic of research in medicine, with already proposed strong antimicrobial properties with specific mechanisms of action, such as physical damage or oxidative stress (Xin et al., 2018).

The object of the project is a novel material – niobium pentoxide (Nb_2O_5) nanoparticles – for potential antimicrobial applications. Although limited, research has already been done and confirmed low cytotoxicity, tissue regeneration abilities, and antimicrobial properties of these metal nanoparticles (Dsouki et al., 2014; Capanema et al., 2015; Chen et al., 2022), paving the way for further research. A deeper understanding of the antibacterial mechanisms of Nb_2O_5 is needed, making it an interesting candidate for future investigations.

The **aim** of our study was to evaluate the antimicrobial effects of niobium pentoxide nanoparticles on polymicrobial biofilms, as a potential component in nano-enabled injectable hydrogels for chronic wound treatment.

Objectives:

- To optimize the niobium pentoxide nanoparticle treatment conditions for effective inhibition of polymicrobial biofilm.
- To assess the effects of treatment on metabolic activity, cell viability, and extracellular matrix production in monomicrobial and polymicrobial biofilms.
- To quantify changes in extracellular carbohydrate and protein levels following nanoparticle exposure.
- To visualize possible structural alterations in biofilms resulting from treatment.

1. Literature review

1.1 Biofilm – characteristics, formation, infections and diseases, challenges

1.1.1 Biofilm characteristics

Biofilm is a complex microorganism colony structure embedded in a protective coating called an extracellular polymeric substance (or EPS). It can be metaphorically called “the house of the biofilm cells” if the biofilm is thought of as a “city of microbes” (Watnick, P., and R. Kolter. 2000). EPS has not only the protective function of the biofilm, but also is responsible for biofilm adhesion to surfaces, and microorganism cohesion (Shineh et al., 2023). The most common EPS composition consists of proteins and carbohydrates, in addition to glycoproteins, glycolipids, and, in some cases, extracellular DNA (e-DNA), making it negatively charged and hydrophilic (Flemming et al., 2007; Liu et al., 2024). Microbial biofilms produce EPS, which promotes the sorption of organics, metals, and chemical pollutants and regulates the nutrient composition in bacterial cells (Liu et al., 2024).

1.1.2 Formation and resistance mechanisms of biofilm

Biofilm formation is a complex, dynamic process, often influenced by changed environmental conditions, such as nutrient limitation, oxidative stress, surface roughness, or pH changes (Ghazay et al., 2020). The current biofilm formation model consists of five stages describing the biofilm development process. The first two stages involve reversible and irreversible attachment, respectively. The third stage is biofilm proliferation, followed by maturation as the fourth stage, and finally, biofilm dispersion as the fifth stage (Sauer et al., 2022) (**Figure 1A**). During the first stage, freely swimming planktonic cells reversibly attach to the surface, and the attachment becomes irreversible in the second and third stages. The bacterial cell-produced EPS binds the cells to each other and holds them strongly to the surface. At the fourth stage, the biofilm colony cells divide, undergo adsorption, and cell-cell signaling molecule production and EPS continue to be secreted and then mature. In the last stage, part of the biofilm disperses due to the secretion of extracellular enzymes that partially degrade the EPS, and the dispersed cells restart the biofilm lifecycle (Asare et al., 2022). Quorum sensing (QS), a cell-cell communication process that regulates biochemical and physiological functions, regulates biofilm formation – it is necessary for gene-level regulation and population-level dynamics (Zhang et al., 2020).

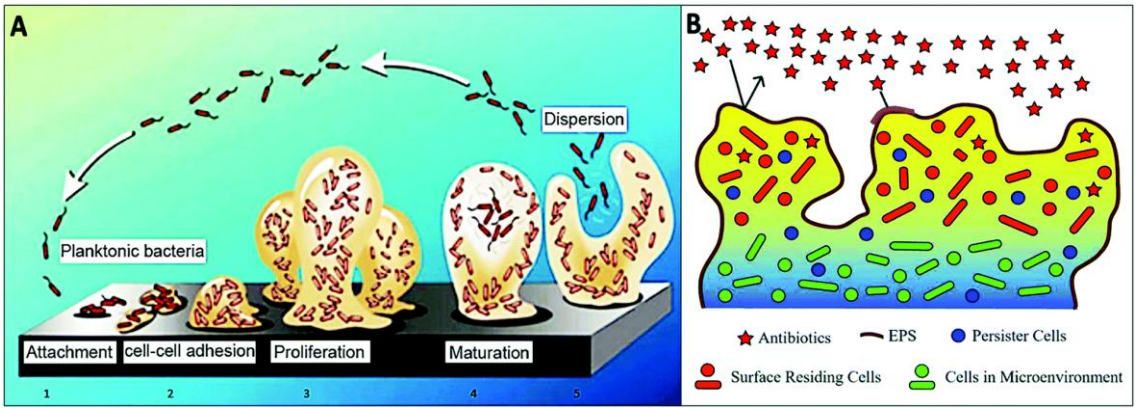

Figure 1. *Biofilm formation stages and antimicrobial resistance mechanisms*. (A) Formation lifecycle of biofilms. Stages: 1 – initial attachment, 2 – irreversible attachment, 3 – colonization, 4 – maturation, 5 – dispersion. (B) Resistance mechanisms of biofilm-embedded microbial cells. EPS resists the penetration of antibiotics and other antimicrobials, which did not freely diffuse through the EPS matrix. Surface residing cells (red) are in contact with the diffused antibiotic. Microenvironmental cells (green) reside at the part where there's less oxygen and nutrients. Persister cells (dark blue) are scattered throughout the whole biofilm. The figure was taken from Asare et al., 2022. Verderosa, Totsika, and Fairfull-Smith. Frontiers in Chemistry, 2017 Elsevier.

In polymicrobial biofilms, QS plays a dynamic and complex role. Polymicrobial biofilms are often more resistant to antibiotics than single-species biofilms (Cui & Kim, 2024); hence, understanding QS in this context is crucial. QS-mediated interactions between different species of bacteria within the polymicrobial biofilm significantly influence their pathogenicity, behavior, and resistance to antibiotic treatment (Cui & Kim, 2024). In polymicrobial biofilm, QS directly regulates biofilm development, offers various QS signal communications, facilitates cooperative behaviors, mediates competitive interactions between different bacterial species, influences horizontal gene transfer (HGT), and impacts persister cell formation (Zhou et al., 2020; Cui & Kim, 2024; Kriswandini et al., 2024). QS directly controls the formation of biofilms and the production of a primary structure of the biofilm, the EPS matrix (Zhou et al., 2020), that acts as a protective barrier and reduces antibiotic penetration (Cui & Kim, 2024). Different bacterial species in polymicrobial biofilm communicate through different QS signals. Gram-negative bacteria (e.g., *E. coli*) commonly use acyl-homoserine lactones, or AHLs, while gram-positive bacteria (e.g., *S. aureus*) typically use autoinducing peptides (AIPs) (Cui & Kim, 2024). Autoinducer 2 (AI-2) is a commonly detected signaling molecule, found in a variety of bacteria, including gram-negative and gram-positive, making it important for interspecies communication (Laganenka, 2018). Laganenka has shown that AI-2, produced by *E. faecalis,* promotes collective behavior of *E. coli* at low densities, enhances *E. coli* autoaggregation, and leads to chemotaxis-dependent coaggregation between the two species. It was also shown that stress resistance has increased in the studied dual-species biofilms. Some bacteria can utilize QS signals, produced by other species, for example, *E. coli* lacks its own AHL synthase,

can respond to exogenous AHLs from other bacteria, which leads to increased cell attachment and EPS production, influencing biofilm formation (Zhou et al., 2020). QS can facilitate cooperative behavior between species in polymicrobial biofilms, such as sharing QS signals (as mentioned above), or modulate each other's QS systems (Cui & Kim, 2024). Cooperation can involve metabolic cross-feeding, for example, Díaz-Pascual et al. proved that when two or more subpopulations coexist, some metabolite produced by one of them may reach the other subpopulation and be utilized by it (Díaz-Pascual et al., 2021). In their research, alanine was shown as a cross-fed metabolite between two spatially segregated *E. coli* subpopulations, aerobically and anaerobically grown cells. This alanine cross-feeding influenced cellular growth and viability in the cross-feeding-dependent region, shaping the overall colony morphology (Díaz-Pascual et al., 2021). QS also mediates competitive interactions within polymicrobial communities. *Staphylococcus aureus* and *Pseudomonas aeruginosa* are the most common microorganisms in chronic wounds. In the literature, it is stated that the interference with one pathogen's QS systems in a co-culture population could modify the other's pathogenicity and antibiotic resistance (O'Brien & Fothergill, 2017). For example, *P. aeruginosa* can modify the composition of microbial community and effectuate the host killing (Korgaonkar et al., 2013); hence, in the co-culture experiments, *P. aeruginosa* reduces *S. aureus* viability (Filkins et al., 2015). Following these insights, Selvan et al. chose to control *S. aureus* virulence, as it has been shown that *P. aeruginosa* virulence factors are increased by many folds when *S. aureus* is present (Selvan et al., 2021). Hence, *S. aureus* was targeted with a previously tested QS inhibitor 2[(Methylamino)methyl]phenol (2-MAMP) in a co-culture with *P. aeruginosa* to inhibit the biofilm, which led to a discovery that *P. aeruginosa* had reduced adherence and virulence in a polymicrobial biofilm when *S. aureus* is targeted (Selvan et al., 2021). An exchange of genetic material, including antibiotic resistance genes, called horizontal gene transfer, occurs within biofilms, and QS can potentially facilitate this by regulating genes involved in competence (DNA uptake) (Cui & Kim, 2024). For instance, *Bacillus subtilis* has a QS system, called ComQXPA, which is involved in controlling sporulation and competence development, which can then facilitate HGT (Cui & Kim, 2024). Lastly, QS signaling takes part in persister cell formation within biofilms. Persister cells are dormant variants that tolerate antibiotics transiently, meaning they can survive the treatment and repopulate once the antibiotic treatment is removed, contributing to recurring infections (Lewis, 2010). This is related to the regulation of genes like (p)ppGpp or alarmone (it functions as a regulator and has influence on transcriptional and metabolic pathways, including those related to lipid, phosphate, and amino-acid metabolism) synthesis by QS. The stringent response shuts down almost all metabolic processes, increasing tolerance to the substratum surface and facilitating adhesion (Roy et al., 2022; Dsouza et al., 2023). The resistance is not only QS-based or genetic, but also metabolic and architectural. Microbial biofilms show spatial and metabolic heterogeneity, which includes

nutrient and oxygen gradients (Jo et al., 2022). These properties contribute to persistence and treatment failure by creating varied susceptibility to antibiotics within biofilms (Jo et al., 2022).

Biofilms reduce the effectiveness of antimicrobial treatments and harbor antimicrobial-resistant subpopulations, challenging the treatment. As visually represented in **Figure 1B**, EPS deactivates most antimicrobials through enzymatic digestion and/or by absorbing them physically; therefore, the antimicrobial treatment efficacy is reduced (Asare et al., 2022). The EPS matrix reduces external stress signals and facilitates horizontal gene transfer. Therefore, microorganisms in the biofilm develop antibiotic tolerance and are protected from the host's immune system through various mechanisms, like decelerating drug penetration through biofilm matrix, dormant cells, and altered microenvironments (Grande et al., 2020).

In addition to antimicrobial treatment tolerance, besides the EPS matrix, biofilms help the pathogens evade the host's immune response using various defense mechanisms and virulence factors. *S. aureus* employs neutrophil-mediated killing, where pore-forming toxins and nucleases disrupt neutrophils and degrade neutrophil extracellular traps (they are used for trapping bacteria for clearance by neutrophils and macrophages) (Ricciardi et al., 2018). *S. aureus* also uses antioxidant defenses, such as the production enzyme superoxide dismutase and the membrane-bound pigment staphyloxanthin that neutralize ROS (Elmesseri et al., 2022). In biofilms, increased accessory gene regulator (*agr*) quorum-sensing system expression further enhances phagocytosis and immune clearance resistance (Scherr et al., 2015).

All in all, the complex biofilm formation, dynamic environment within polymicrobial communities, and a variety of resistance mechanisms prove the urgency of novel antibiofilm strategies.

1.1.3 Challenges caused by biofilms

Biofilms have a variety of pathological manifestations – you can find them in various areas, such as living tissues, water channels or pipes, hospital floors, medical implants, food processing units, and other biotic or abiotic surfaces (Rather et al., 2021). According to the National Institutes of Health (NIH), the cause of 65% microbial and 80% chronic infections is microbial biofilms that infect both medically implanted devices and tissues (Rather et al., 2024). Biofilm infections are problematic because of high resistance toward most common antimicrobial treatments, such as antibiotics, and are associated with high morbidity and mortality, increased hospital treatment cost, and hospital stay time (Asare et al., 2022). **Figure 2** visualizes the current actualities of antimicrobial resistance (AMR) worldwide – from 2 million infections per year containing bacteria resistant to one or more antibiotics in the USA to 1 child death every 9 minutes from an antibiotic-resistant bacterial infection in India.

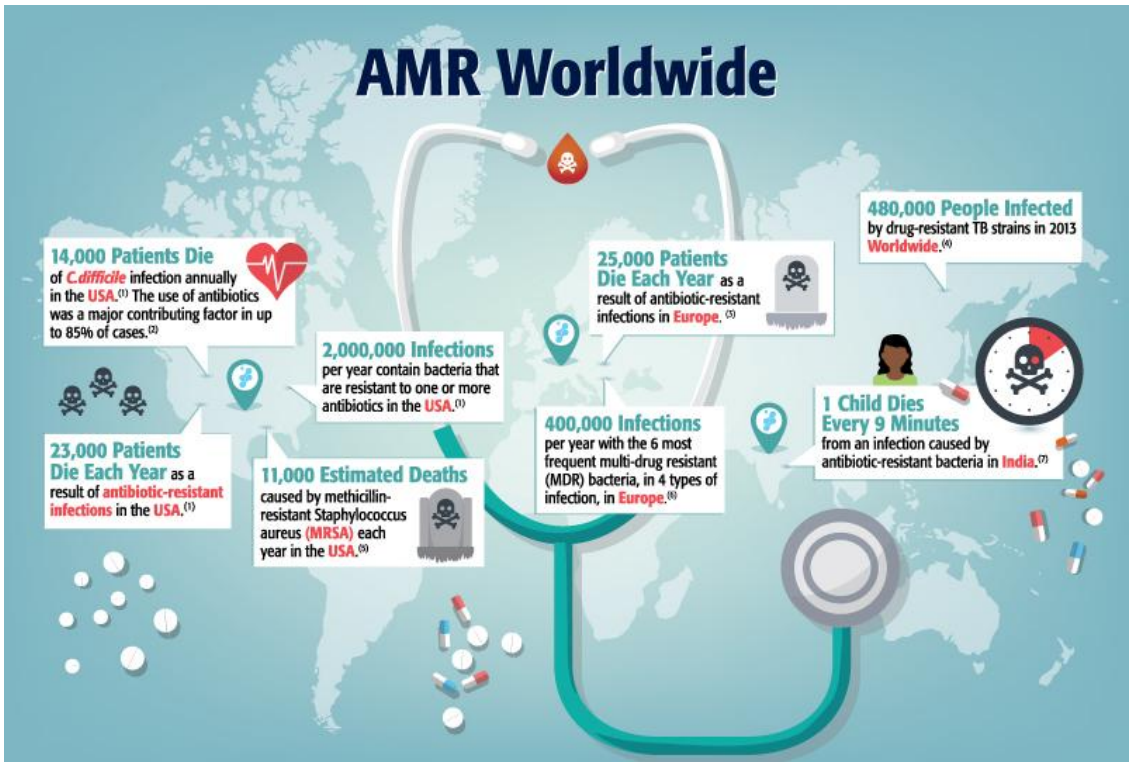

Figure 2. Antimicrobial Resistance Germination (AMR) statistics worldwide over recent years. Source: https://www.biomerieuxconnection.com/2018/07/12/explain-antimicrobial-resistance-friends-family-infographics/.

Biofilm-caused infections can be classified as non-surface-associated and surface-associated (Sauer et al., 2022). Non-surface-associated infections include respiratory tract infections with impaired mucociliary clearance in the host (in individuals with cystic fibrosis in viscous airway mucus), persistent soft tissue infections from diabetes, or impaired lower limb vascularization predisposing to non-healing wounds. Gingivitis and periodontitis are primarily caused by the formation of biofilms in the gingival crevices and on the tooth surfaces, which may be related to polymicrobial synergy and dysbiosis. Oral biofilm formation is very common and caused by bacterial species, such as *Streptococcus* sp. Tooth surface biofilms lead to dental caries (Mosaddad et al., 2019). Chronic infections and persistent inflammation are associated with an increased risk of cancer (Groeger and Meyle, 2019). *Salmonella* can form biofilms on human gallstones, and bile can significantly enhance the biofilm formation. Therefore, it can be a chronic infection source, related to a high chance of developing gallbladder cancer (Walawalkar et al., 2013). Bacteria such as *Escherichia coli* can form biofilms in the host's intestines (Conway and Cohen, 2015).

In contrast, surface-associated infections are often observed in patients with medical devices or implants (Sauer et al., 2022). The use of medical devices can sometimes be associated with

complications, the most common secondary complication being infection from microorganism detachment from biofilm on the medical device, such as catheter-associated biofilms (Donlan, 2001; Nargis et al., 2017). When microorganism biofilms form on the inner surface of catheters in long-term catheterization patients, the infections are prevented using antibiotics, which can cause chronic infections (Delcaru et al., 2016). As bacteria become more resistant to antibiotics, their treatment becomes ineffective, leading to novel treatment investigations, mainly focusing on accurate and fast diagnosis of diseases. When bacteria form biofilms in the host, the infection often becomes untreatable, followed by low-grade inflammation, developing into a chronic state (Hoiby et al., 2015). Chronic infections have a persistent and progressing pathology.

Widely spreading biofilm-related infections pose a substantial threat to society by being difficult to treat, requiring constant financial investments, and leading to increased morbidity and mortality rates globally.

1.2 *Staphylococcus aureus* – characteristics, diseases and infections, treatments

1.2.1 What is *Staphylococcus aureus*? Characteristics, biofilm

Staphylococcus aureus is a gram-positive, facultative anaerobic, nonmotile, non-spore-forming bacterium that can be found on most mammal and bird skin, as well as in the blood, mouth, mammary and intestinal glands, genitourinary, and upper respiratory tracts of infected hosts (Gill et al., 2024). **Figure 3A** shows the visual representation of *S. aureus* planktonic cells, and **Figure 3B** shows *S. aureus* biofilm.

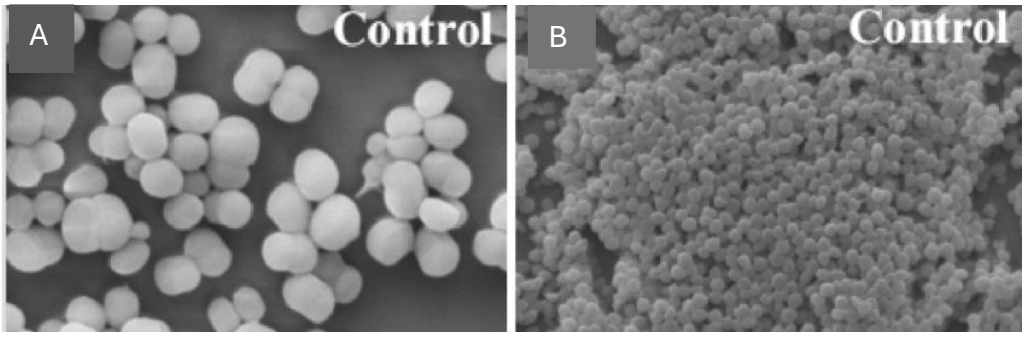

Figure 3. *Scanning electron microscopy (SEM) images of S. aureus*. (A) planktonic cells, (B) biofilm. Sun et al., 2021, *Ultrasonics Sonochemistry*.

S. aureus produces EPS that helps the microbe minimize and resist the effects of antibacterial drugs (Idrees et al., 2021). The biofilm of this bacterium is comprised of 97% water and the organic matter, EPS, and microcolonies. A significant component of EPS is the polysaccharide intercellular adhesin (PIA), acting in the intercellular adhesion of bacterial cells (Reffuveille et al., 2017). Biofilm-associated *S. aureus* cells have been reported to have four different metabolic states – they can be fermentative, dormant (these cells can add to antimicrobial resistance), dead, or growing aerobically (Archer et al., 2011). Microbial biofilm formation is encoded by specific biofilm-associated genes, in *S. aureus* mainly encoded by 12 different genes, i.e., intracellular adhesion (*icaA, B, C,* and *D*), fibrinogen-binding proteins (*fib*) gene, and others. These genes encode different surface proteins to help *S. aureus* adhere to the host, penetrate the host, and colonize it, leading to biofilm formation and virulence (Paharik et al., 2016). An *agr* quorum sensing system regulates the expression of *S. aureus* virulence factors (Rutherford et al., 2012). Although there is much unknown about these *S. aureus*-derived MVs to suppress inflammation, it is crucial to control them since they serve as bacterial-derived molecules carried to the host.

1.2.2 Diseases and infections caused by *Staphylococcus aureus*

Staphylococcus aureus is a primary cause of various invasive human infections, such as endocarditis, pneumonia, bacteremia, and wound infections, leading to mortality, morbidity, and excessive costs of healthcare (Wang et al., 2023). *S. aureus* colonizes and invades host tissues by employing a wide spectrum of secreted and surface-associated virulence factors, evading the host immune response (Wang et al., 2023). *S. aureus* lacks secretion systems of Gram-negative bacteria that transport virulence factors directly into host cells. The bacterium secretes exoproteins to the external environment, where antibodies or enzymes with hydrolytic or proteolytic activities may inactive them through neutralization (Wang et al., 2024).

Pathogenesis in *S. aureus* results from the expression of different virulence factors, such as immunomodulators, exoenzymes, and toxins (Oogai et al., 2011). Toxins protect the bacterium by averting any possible elimination by the host's defense system (Otto, 2014). The pathogenesis involves evading the host's immune system – the immune system of the host encounters the entry into subepidermal tissues or blood, followed by *S. aureus* counterattacks and inactivation of the host's immune system by secreting different proteins, encoded by two immune evasion gene clusters, IEC1 and IEC2 (Kim et al., 2012).

Not only the virulence factors but also the attachment to the host and persistence factors, when regulated by their corresponding genes, are involved in biofilm formation and can cause different infectious diseases. Antimicrobial resistance is becoming a serious threat to human and animal lives

due to the traditional antimicrobial drugs' partial or full efficacy loss. The World Health Organisation's report, issued in the year 2019, reveals 700.000 mortalities each year and 230000 deaths as a direct consequence of resistance to antituberculosis drugs alone (WHO, 2019). *S. aureus* hinders drugs' access to the cells residing inside the biofilm by developing the biofilm to prevent the diffusion of the antimicrobials (Donlan, 2000). As *S. aureus* is a commensal bacterium, it colonizes up to 30% of the human population, from host skin, armpits, nostrils, or groins globally, and many times, it causes very little or no harm at all, however, these niches can sometimes become a primary cause of *S. aureus* infection (Sakr et al., 2018; Zheng et al., 2018).

1.3 *Escherichia coli* – characteristics, diseases and infections, treatment

1.3.1 What is *Escherichia coli*? Characteristics, biofilm

Escherichia coli is a gram-negative, facultative anaerobic, non-sporulating rod-shaped bacterium of the *Enterobacteriaceae* family that is one of the most important pathogens in humans, commonly inhabiting the gastrointestinal tract (Vila et al., 2016). A visual representation of *E. coli* planktonic cells and biofilm is shown in **Figure 4** below.

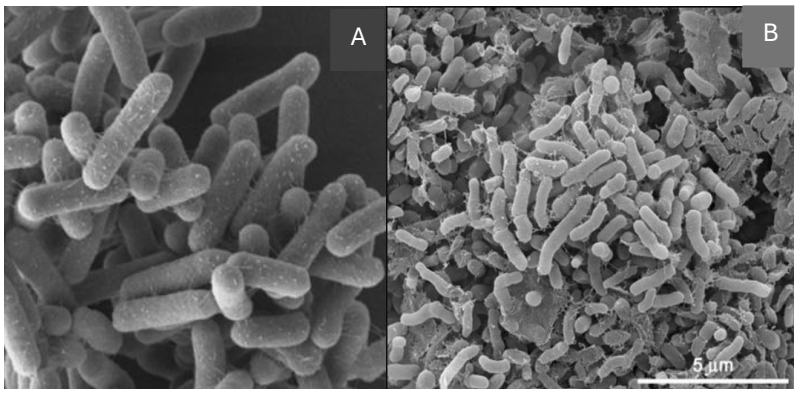

Figure 4. *Scanning Electron Microscopy (SEM) images of Escherichia coli*. (A) *E. coli* plaktonic cells, (B) *E. coli* biofilm. Annous et al, 2009. J Food Si and Apiwatsiri et al., 2021, Frontiers in Vetinary Science.

1.3.2 Diseases and infections caused by *Escherichia coli*

Escherichia coli is not a primary bacterium found in chronic wound infections. It is a commensal bacterium in normal human and animal microbiota. However, some pathogenic strains are responsible for severe bacterial infections, such as gastrointestinal and urinary tract infections, in the later *E.* coli being the most frequent agent in about ~80% of cases with more than 400 million cases reported globally in 2019 (Yang et al., 2022; Mayer et al., 2023). Extraintestinal pathogenic *E.*

coli is one of the most abundant pathogenic *E. coli* species, causing various diseases, including meningitis in neonates, bacteremia, and wound infections as well, when it directly penetrates the broken skin (Mmasoud et al., 2022). This bacterium can acquire virulence factors and mobile genetic elements from related bacteria, leading to different pathogenicity (Harwalkar et al., 2014).

Several extraintestinal infection-causing *E. coli* pathogens have been reported to have developed resistance to every class of antibiotics used for human and animal infection treatment, making them even more difficult to eradicate (Mmasoud et al., 2022). This resistance to many treatments is called multi-drug resistance, or MDR (Mmasoud et al., 2022). This resistance complicates the treatment and increases the number of deaths and disability-adjusted life-years compared with other Europe‘s MDR organisms (De Lastours et al., 2020).

Aware of all the known facts, development of new therapeutic alternatives is needed to control and prevent the virulence and infectivity of pathogenic *E. coli* strains.

1.4 Treatments for bacterial biofilms

1.4.1 Current treatments for biofilm infections

For a long time, the most common method of treating bacterial biofilm was prescription antibiofilm agents, such as drugs (e.g., antibiotics), functional excipients, and other naturally occurring or synthetic antibiofilm molecules. Antibiofilm strategies include inhibiting or preventing new biofilm formation and eradicating or dispersing already existing biofilms (Bhattacharya et al., 2015). Because of antimicrobial resistance, scientists are looking at novel approaches to combat biofilm infections.

Many studies have focused on surface modification techniques to prevent biofilm development or eradicate the biofilm formed on medical devices. A variety of coatings were developed, such as coatings for medical prostheses with metal materials, silver or silver-copper coatings, and coatings with antimicrobial or adhesion-reducing agents, like broad-spectrum antibiotics (chlorhexidine, minocycline) (Zhang et al., 2020). Hydrogels have been used for medical device coatings and have been shown to be effective in combating biofilms due to functional group density, lubricity, and biocompatibility (Norris et al., 2005). Photoactive hydrogels have the ability to facilitate photochemical reactions induced by light and can be applied as therapeutic agents (Straksys et al., 2025). A cationic thiazine dye, methylene blue, can be effective in antimicrobial photodynamic therapy (aPDT), especially for wound healing, because it can generate reactive oxygen species (ROS) upon light activation (Straksys et al., 2025). ROS generation in the wound can eliminate pathogenic

microbes without damaging the surrounding healthy tissue (Nie et al., 2020). Using hydrogel as a topical solution is far superior to traditional hydrogels in preformed pad or sheet form, offering easy application, consistent coverage on irregular wound surfaces, ensuring deeper penetration, sustained release of ROS, making wound healing faster, and the effectiveness against microbes higher (Hamed et al., 2024).

Nanotechnology is also a very promising field to fight against biofilms, as it has been used for nanoparticle production, surface modification, antibiotic carriage, and so on (Li et al., 2019). Nanotools enable traditional antibiotics or novel antimicrobial agents to pass through the biofilm barriers and penetrate the biofilm's deeper layers, killing the cells inside (Galdiero et al., 2019). Metallic nanoparticles enhance the antimicrobial effect of current antibiotics and present their bactericidal activity. They can release metal ions, interacting with cellular components through various pathways of antibiofilm activity.

1.4.2 Alternative approaches for biofilm infection treatment

As our population ages and becomes susceptible, and many bacterial isolates have developed antibiotic resistance, bacterial infections remain a major healthcare challenge. They are responsible for significant morbidity and mortality. Novel strategies to combat biofilm infections have been developed, such as bacteriophage therapy, weak organic acids as antibacterials, photo inactivation, QS inhibition, biofilm metabolic pathway modulation, nanomaterial application as treatment, and so much more. Several of them will be discussed below.

Bacteriophages (phages) are bacteria-infecting viruses that replicate within them and kill the host bacteria by cell lysis. Phage therapy has been used for decades, proving it effective for wound, implant- and catheter-related biofilm infections (Wu et al., 2015). For example, scientists characterized an *S. aureus* phage (AB-SA01), which contained no antibiotic or bacterial virulence resistance genes. Its characteristics met the human user criteria, and the phage was predicted to stay active against multidrug-resistant *S. aureus* strains. Additionally, AB-SA01 killed about 95% of *S. aureus* isolates (Lehman et al., 2019).

QS inhibition could be applied as an effective therapeutic option. Over 10 years ago, it was shown that lung infections of *P. aeruginosa in vivo* were significantly reduced when targeting the QS with synthetic furanones (Wu et al., 2004). In a recent study, scientists have shown that quinic acid, one of the chlorogenic acid metabolites found in plant extracts, significantly inhibited biofilm EPS secretion during biofilm formation and maturity and reduced biofilm formation by regulating core targets in the QS system (Lu et al., 2021).

Lastly, nanotechnology offers numerous advantages for treating biofilm infections. Metal nanoparticles, due to their nanoscale dimensions, possess a high surface area-to-volume ratio, which enhances their interaction with microbial cells. Unlike conventional antibiotics, nanoparticles utilize distinct mechanisms to target biofilms, like ROS formation, metal ion release, DNA damage, protein deactivation (Baptista et al., 2018; Xu et al., 2022). For example, gold nanoparticles (AuNPs) and Au nanoclusters exhibit enzyme-like catalytic activity, resembling peroxidase or glucose-oxidase (He et al., 2013). This catalytic behavior can increase ROS generation, thereby contributing to bacterial cell damage through oxidative stress (Zheng et al., 2017).

These novel methods show great advantages, such as effective biofilm reduction, decreased side effects compared to conventional treatment methods (like antibiotics), and precise targeting. However, potential long-term side effects and cytotoxicity to the human organism require further research.

1.5 Niobium nanoparticle – characteristics, properties, potential applications for wound infection treatments

1.5.1 Niobium nanoparticle characteristics and properties

In this project, we are focusing mainly on the niobium pentoxide nanoparticles. Hence, we are going to further discuss them. Niobium pentoxide is the most thermodynamically stable state in the niobium-oxygen system (Nico et al., 2016). Niobium is one of the transition metal oxides that offer a wide spectrum of potentially useful and highly applicable material properties in many areas, such as antimicrobial (potentially antibiofilm too), non-cytotoxic properties, and biocompatibility (Rani et al., 2014). Nowadays, more comprehensive studies of niobium have been conducted due to the wide application spectrum – especially, thin film and nanostructured Nb_2O_5 have been utilized in solar cells, batteries, other electronic devices such as memristors, as well as in the field of medicine as implant materials due to their antibacterial and good biocompatibility properties in human bodies (Aegerter et al, 2001; Senocak et al., 2021).

The crystal structure of niobium pentoxide is a transparent, air-stable, and water-insoluble solid material with a rather complicated structure. It displays extensive polymorphism (almost 15 polymorphic forms) (Nowak and Ziolek, 1999). **Figure 5** shows both the chemical and crystal structures of Nb_2O_5.

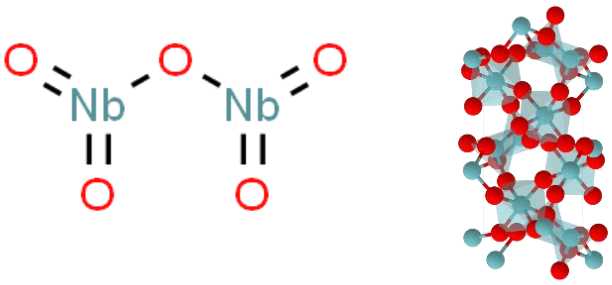

Figure 5. *Niobium pentoxide (Nb_2O_5) structure*. On the left is a chemical structure of Nb_2O_5, and on the right is a crystal structure of Nb_2O_5. Visuals were taken from www.chemsrc.com and https://next-gen.materialsproject.org, accordingly.

The polymorphic form depends on the synthesis method, the crystallization temperature, the nature of the starting material, the present impurities, and interactions with other components (Rani et al., 2014).

Niobium pentoxide has a unique combination of chemical stability, bioactivity, and surface reactivity, making it the most studied niobium form in nanotechnology (Raba et al., 2016). Its ability to form a stable oxide layer enables surface functionalization and enhances biocompatibility, which is crucial for biomedical applications. A broad range of physicochemical, optical, and mechanical properties of niobium have been reported. However, a lot is still underexplored in the biomedical field. Despite limited information, it is known that niobium oxide is a soft, hypoallergenic, non-toxic material, tolerated by the human body (Li et al., 2016). Niobium oxide has been widely investigated as a surface coating material, especially to improve titanium-based alloys used for dental and bone implants. *In vitro* tests showed better cell attachment and growth of niobium oxide than titanium alloys, indicating excellent biocompatibility (Zhang et al., 2015; Borowsi et al., 2023). Cyto- and genotoxicity tests of niobium pentoxide nanoparticles have also been performed both *in vitro* and *in vivo*. Low niobium cytotoxicity and increased bioactivity were observed in calcium silicate-based cements combined with Nb_2O_5 micro and nanoparticles, by comparing the response in four tested cell lines (Mestieri et al., 2017). *In vivo* experiments with Swiss mice treated for 3 and 7 days with a single dose of 3% Nb_2O_5, diluted in PBS, indicated an unprogressive niobium cytotoxicity, and liver cell regeneration 12 days after treatment (Dsouki et al., 2014).

The mentioned properties – niobium's biocompatibility, chemical stability, and potential for surface functionalization – make Nb_2O_5 a promising candidate for biomedical applications. The reported antimicrobial activity and low cytotoxicity support further explorations for niobium as a

novel antibiofilm agent. Both deeper *in vitro* and especially *in vivo* research are necessary, given the rising need for alternative biofilm-associated infection treatments.

1.5.2 Niobium nanoparticle potential application for wound infection treatments

Due to low allergenicity and cytotoxicity, good biocompatibility, and antimicrobial properties, niobium nanoparticles can be applied to develop innovative drug delivery systems or act as a part of therapeutic agents (Khalid et al., 2025). In a recent study, scientists created a delivery platform containing ultrathin niobium carbide (Nb_2C) nanosheets, which exhibit a large specific surface area, offering plenty of anchoring sites for proteins, drugs, or macromolecules, and berberine (BBR), the plant-derived chemical, showing antibacterial, antioxidative, and anticancer, especially metastasis-related protein regulation in the tumor, activities, integrated into the nanosheets (Lin et al., 2023). These nanocomposites were used in combined chemotherapy and photothermal therapy on breast cancer cells, resulting in the efficient elimination of cancer cells and successful metastasis inhibition by regulating the expression of proteins linked with extracellular matrix and epithelial-mesenchymal transition (Lin et al., 2023). Studies have also been based on thermoplasmonic characteristics of rod-shaped, core-shell, and spherical niobium nanoparticles to evaluate if and which type of niobium nanoparticles can potentially target cancer cells (Heidari et al., 2020). To investigate these properties, scientists have simulated synchrotron radiation emission as a function of the beam energy and niobium nanoparticles and discovered that the laser light stimulates the resonance of surface plasmon of the nanoparticles, converting the absorbed energy into heat which destroys tumor tissue near to nanoparticles without hurting sound tissues (Heidari et al., 2020). This makes niobium nanoparticles appropriate for tissue, tumor, and optothermal human cancer cell treatment.

Niobium is a novel nanoparticle, and only a limited number of publications have shown its antimicrobial mechanism. However, some antibacterial mechanisms of other metallic nanoparticles have been shown, leading to the belief that niobium employs similar mechanisms of action. **Figure 6** provides a visual representation of metallic nanoparticle antibacterial mechanisms.

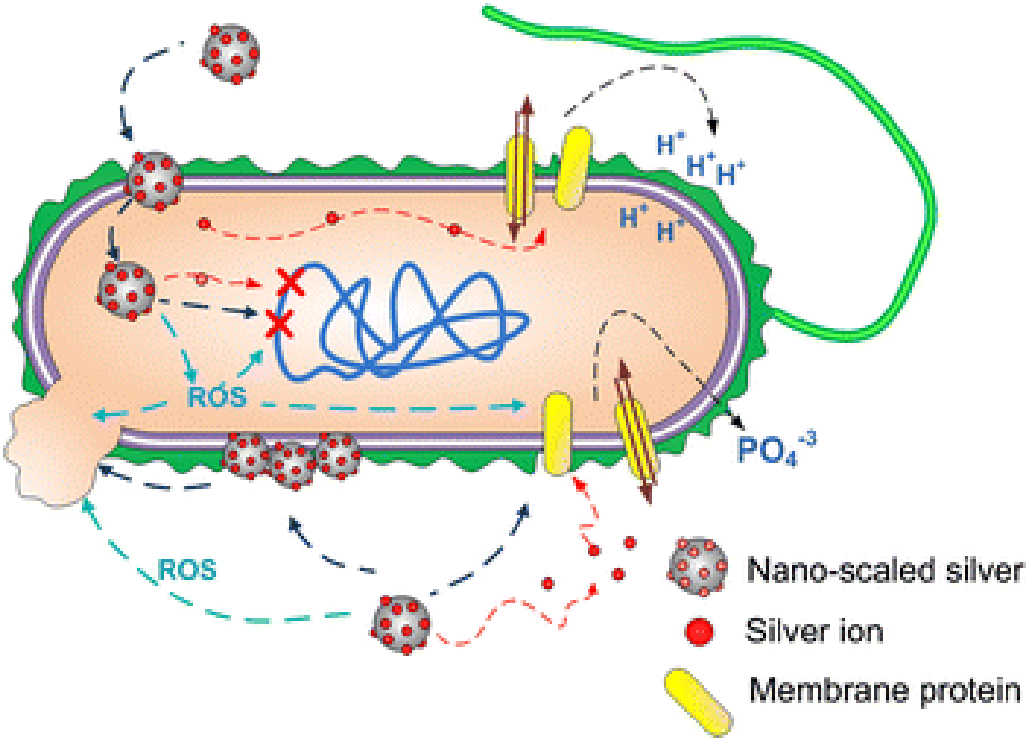

Figure 6. *Antibacterial mechanisms of metallic nanoparticles.* As an example, nano-scaled silver is taken. (1) Silver nanoparticles can release silver ions and generate ROS. (2) Silver nanoparticles may interact with membrane proteins and affect their correct functions. (3) Silver nanoparticles can accumulate in the cell membrane and affect the membrane permeability. (4) Silver nanoparticles can enter the cell, generate ROS, release Ag^{+}, and affect DNA replication. Marambio-Jones & Hoek, Journal of Nanoparticle Research, 2010.

One of the mechanisms of metallic nanoparticle antimicrobial action is causing oxidative damage via the generation of ROS. ROS can disrupt bacterial cells when produced either outside or inside the cell (Wang et al., 2014). Oxidative stress and lipid peroxidation, caused by high ROS concentrations, can damage the cellular membrane and the wall by breaking the peptidoglycan structure, degrading proteins and nucleic acids, and leading to cell death (Ranjan et al., 2016; Jahnke et al., 2016; Ezraty et al., 2017). Metal nanoparticles increase ROS production in bacterial cells, and this effect can cause DNA denaturation by intercalation between pyrimidine and purine bases, leading to altered metabolic and transduction signals, and inhibiting cell growth (Sirelkhatim et al., 2015). As proven before, the most common nanoparticles that produce antibacterial activity mediated by ROS production are zinc oxide, titanium dioxide, and silver (Ranjan et al., 2016; Xu et al., 2012; Xie et al., 2011). For example, we can take this research, where novel biogenic silver nanoparticle-induced ROS inhibit the formation and virulence activities of methicillin-resistant *S. aureus* strain (Hamida et al., 2020). The treatment with silver nanoparticles, as well as silver nitrate ($AgNO_3$), can inhibit the growth of *S. aureus*, causing genotoxicity and denaturation of cellular proteins, as well as apoptotic body formation and cell wall damage (Hamida et al., 2020).

Another mechanism is increased bacterial permeability by accumulating nanoparticles on the bacterial cell membrane. Silver nanoparticles (AgNPs), for example, have antibacterial activity due to their physical interaction with bacterial cell wall peptidoglycans, causing structural changes that increase the membrane's permeability and cause cell death (Thomas et al, 2014). This phenomenon is named Enhanced Permeability and Retention (EPR) and was first reported by Matsamura and Maeda in 1986 (Matsamura et al., 1986). The EPR effect plays a massive role in passive targeting. It assists bulk drug transmission at the target site (Shinde et al., 2022). Passive targeting relies on nanoparticles encapsulating inducible and stable compounds or dyes. In contrast, active targeting relies on antibodies or other biologically active linked molecules, making active targeting fail due to genetic alterations in cancer cells, causing acquired resistance (Lim et al., 2019). The nanoparticle accumulation effect has been tested in targeting tumors. Cancer cells that are far from the blood vessels suffer from a lack of nutrients and proliferate slowly; therefore, under the EPR effect, close and distant cellular populations can be accessed and treated effectively (Ahmadiankia et al., 2019; Deshpande et al., 2020). Photothermal therapy is cancer treatment utilizing photosensitizers accumulating in tumors to eradicate cancer cells, whereas immunotherapy uses activated immune cells to target the tumor cells independently (Zou et al., 2016). Combining immunotherapy and photothermal therapy increases the effectiveness of treatment (Li et al., 2022).

The third known mechanism is metallic ion release from nanoparticles that disrupt DNA replication by depleting intracellular ATP. Right now, there are no mechanisms shown explaining niobium nanoparticle antimicrobial action. However, we can see examples of other nanoparticles, such as AgNPs, hoping that niobium acts similarly. It has been proven that AgNPs may directly damage cell membranes and disrupt DNA replication and ATP production by releasing toxic Ag^{+} ions (Dallas et al., 2011; Sharma et al., 2024). The released Ag^{+} ions can inactivate vital enzymes and affect DNA replication by interacting with thiol groups in proteins (Neal, 2008). This reaction leads to the uncoupling of ATP synthesis from respiration, interferes with the phosphate efflux system, and results in the loss of proton motive force (Marambio-Jones and Hoek, 2010).

Niobium oxide nanoparticles are a promising material that could be applied in a variety of cases, such as drug delivery, diagnostic imaging, antimicrobial treatments, or even cancer therapy. More research is required to provide insights into the mechanisms of action and *in vivo* biocompatibility. Besides that, eco-friendly synthesis methods, surface modification optimization for targeted delivery, and standardization of testing protocols should be explored to provide safe and more effective applications, especially in the medical field. Niobium-based nanomaterials could become next-generation therapeutic and diagnostic tools in medicine.

2. Materials and Methods

2.1 Materials

The following materials were used in the project:

Ethanol absolute (VWR Chemicals BDH), Isopropyl Alcohol (Eurochemicals), Formaldehyde, 37% (Carl Roth GmbH), distilled water, Sulfuric Acid (AppliChem), Sodium Hydroxide (Lerochem), Anthrone, 95% (Acros Organics), Agar-Agar (Carl Roth GmbH), Brain Heart Infusion Broth (BioLab), Congo Red (AppliChem), CHROMagar™ O157 (CHROMagar™), CHROMagar™ Staph aureus (CHROMagar™), Sodium Chloride, 99% (Carl Roth GmbH), Potassium Chloride (VWE Chemicals BDH), di-Sodium Hydrogen Phosphate anhydrous p. A. (AppliChem), Potassium Phosphate monobasic (Sigma Life Science), Pierce™ BCA Protein Assay Kit (Thermo Scientific), MycoLight Bacterial Viability Assay Kit (AAT Bioquest).

2.2 Methods

2.2.1 Bacterial strains and growth conditions

Escherichia coli (K12, DMS498) and *Staphylococcus aureus* (NCTC 11963) were used to evaluate the antimicrobial effect of niobium pentoxide (Nb_2O_5) nanoparticles. *E. coli* and *S. aureus* were grown in brain-heart infusion broth (BHI) (Oxoid, London, UK) at 37 °C and 150 rpm for 16 – 18 hours under aerobic conditions, until the logarithmic growth phase. To harvest, microorganisms were centrifuged for 5 min at 5000 rpm, washed 3 times, and resuspended in a sterile phosphate buffered saline (PBS) pH 7.2 to a final concentration of 1×10^7 cells mL^{-1}.

2.2.2 Microbial biofilm cultivation

Bacterial suspension with a concentration of 1×10^7 cell mL^{-1} was further used for biofilm preparation. The required volume for a chosen sterile tissue culture plate was pipetted to the wells and incubated at 37 °C without shaking for 1.5 hours. After the incubation, the plate was washed three times with PBS, BHI was put into each well, and the plate was left to grow at an orbital shaker for 48 hours at 37 °C, 50 rpm to prepare a mature biofilm. The same conditions were used for polymicrobial biofilm, except that *E. coli* suspension was added first to the plate wells to incubate, followed by 30 min incubation of *S. aureus*. The bacterial ratio used was 2:1, accordingly.

2.2.3 Biofilm treatment

Niobium pentoxide (Nb_2O_5) nanoparticles were synthesized by a colleague, PhD student Muhammad Usman Bajwa, using the hydrodynamic thermal method. The average size was about 130 nm with the polydispersity index (PDI) of 0.0124. The nanoparticle suspension was prepared as such: Nb_2O_5 powder was dispersed in PBS, creating the suspension using ultrasonication. The Bandelin Sonorex Digitec ultrasonic bath's temperature was set to 80 °C, with continuous pulse ultrasonic waves. The solution was sonicated for 1.5 – 2 hours until suspended and milky, then filtered through a sterile Millipore membrane (0.22 μm).

Mature biofilms underwent treatment with niobium nanoparticles at concentrations of 3 g/L, 5 g/L, and 7 g/L for various durations (1, 2, 4, 8, 10, 12, 14, 16, 18, and 24 hours) to optimize the treatment parameters. Based on the results of this optimization, a concentration of 3 g/L with a treatment duration of 24 hours was selected for subsequent experiments. Following treatment, the biofilms were gently rinsed with sterile PBS to eliminate loosely attached bacterial cells and were ready to be used in further experiments.

2.2.4 Metabolic activity assay (XTT)

The XTT (2.3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) cell proliferation assay is a colorimetric test that measures cellular metabolic activity to assess cell viability, proliferation, and potential cytotoxic effects. It was performed in a 96-well tissue-culture plate with mature biofilm treated and untreated with niobium pentoxide nanoparticles using the official protocol from Cell Proliferation Kit II (XTT) (Roche, CH). XTT labeling reagent and Electron-coupling reagent were thawed, and each bottle was thoroughly mixed. Then, the XTT labeling mixture was prepared by mixing the XTT labeling reagent and the Electron-coupling reagent in a ratio of 50:1. 50 μL of XTT labeling mixture was added to each well and incubated at 37 °C for 1.5 – 3 hours. Results were evaluated using Sunrise absorbance microplate reader (Tecan Life Sciences, CH) at 450 nm with the reference wavelength of 620 nm.

2.2.5 Cell viability (CFU)

The colony-forming unit assay (CFU) is a quantification method to verify the bacterial viability. It is done by referring to the number of individual microorganism colonies grown on an agar media plate. The mature biofilm, prepared as described in sections 2.2.2 and 2.2.3 above, is washed with PBS, scraped, and diluted by serial dilutions to a total dilution factor of 10^{-6} for *S. aureus* and 10^{-3} for *E. coli*. 20 μL of diluted samples was spread onto a selective agar plate (CHROMagar™ O157 for *E. coli* and CHROMagar™ Staph aureus for *S. aureus*) and incubated at 37 °C overnight. Colonies were counted, and CFU/mL was calculated to evaluate viable bacterial cells.

2.2.6 Biofilm production assessment (Congo Red staining)

Congo red staining is a method where the dye binds to polysaccharides within the EPS matrix. It was used to quantify the biofilm production of EPS before and after niobium nanoparticle treatment. For this methodology, the biofilms were formed on a 96-well plate and, after the niobium treatment procedure (described above), were incubated with 100 µL of Congo red (0.1% w/v) for 30 minutes at room temperature. The excess dye was washed off, and the bound EPS-Congo red was eluted using ethanol. The amount of EPS was then quantified by measuring the absorbance at 495 nm using a plate reader.

2.2.7 Biofilm visualization using Confocal Spinning Disk Microscopy

This method was performed with the help of a colleague, PhD student Adei Abouhagger. The mature biofilms grown in 24-well tissue plates were stained using the MycoLight Bacterial Viability Assay Kit (AAT Bioquest), which contains a dual-staining method with MycoLight™ Green and propidium iodide to create a 250x stock solution, according to the manufacturer's instructions. The samples were stained by incubating at room temperature with the dye for 25 minutes. The excess dye was removed by gently washing the samples twice with PBS, and they were prepared for imaging.

Stained biofilms were visualized using a Nikon Eclipse Ti2 Confocal Spining Disk Microscope equipped with a 20x objective lens. Fluorescence was detected at an excitation wavelength of 488 nm with emission filters set to 510-530 nm for MycoLight™ Green (live cells) and 600-660 nm for propidium iodide (dead cells). Images were captured using an Andor Zyla sCMOS camera integrated with the DSD2 differential spinning disc system, providing high-resolution and precise fluorescence detection. Z-stack images were acquired at 1 µm intervals to generate three-dimensional reconstructions of the biofilm architecture. Data was acquired using Nikon Elements software, and further image analysis was conducted with ImageJ (Fiji).

2.2.8 Scanning electron microscopy

This method was performed together with dr. Andrius Maneikis. The biofilm was grown and treated as previously described. Initially, the samples were gently rinsed with phosphate-buffered saline (PBS) to remove any non-adherent or planktonic cells. They were then immersed in 2.5% glutaraldehyde prepared in 0.1 M phosphate buffer (pH 7.2) at 4 °C for 1 hour to fix the biofilm. After fixation, the samples were washed three times with 0.1 M phosphate buffer, each for 10 minutes, to remove excess fixative. Following fixation and washing, the samples undergo dehydration through a graded ethanol series. Samples were immersed sequentially in 10%, 20%, 40%, 80%, and finally

100% ethanol, with each step lasting about 10 minutes. The 100% ethanol step is repeated twice to ensure complete dehydration. Once dehydrated, the sample must be dried. After, samples were covered with a thin layer of a few nanometers of chromium to improve their resolution and contrast when observed under a Hitachi scanning electron microscope. The 20 kV accelerating voltage was used in this experiment, which is suitable for imaging samples with a thin chromium coating. It allows for imaging surface features with high resolution and minimal beam-induced damage to the samples.

2.3 EPS quantification

2.3.1 EPS extraction

The biofilm matrix was extracted using chemical treatments with 1 M NaOH, pH 11.0, and 37% formaldehyde to determine the EPS contents. 500 µL of the scraped biofilm was mixed with 37% formaldehyde (1:1 ratio) and incubated at room temperature for 1 hour. 200 µL of 1M NaOH was added to the formaldehyde-containing tubes and left for 3 hours. After incubation, the tubes were centrifuged at 4 °C, 13500 rpm for 1 hour, and the supernatant was filtered through a sterile Millipore membrane (0.22 µm). The filtered supernatant was later used for carbohydrate and protein quantification.

2.3.2 Carbohydrate quantification (Anthrone method)

An Anthrone method was used for carbohydrate analysis. 100 µL of EPS was mixed with 500 µL of absolute ethanol and centrifuged at 4 °C, 13500 rpm for 30 minutes. The supernatant was discarded, and the pellet was left to completely dry in a 90 °C water bath for 15 minutes. A dry pellet was dissolved in 200 µL of PBS and used for Anthrone analysis. Anthrone analysis was performed by mixing the sample and Anthrone reagent (2 g/L) in a 1:4 ratio. Chilled solutions were placed in a 100 °C water bath for 10 minutes and cooled to room temperature. Absorbance was measured at 620 nm using a spectrophotometer with glucose as a standard.

2.3.3 BCA protein assay

The quantification of proteins was accomplished using a Pierce™ BCA Protein Assay Kit (Thermo Scientific). The assay procedure was performed according to the manufacturer's instructions. Working reagent (WR) was prepared by mixing 50 parts of BCA reagent A with 1 part of BCA reagent B (50:1, Reagent A:B). 0.1 mL of each standard and unknown sample replicate was pipetted into an appropriately labeled test tube. 2 mL of the WR was added to each tube and mixed well. Tubes were covered and incubated at 37 °C for 30 minutes. The tubes were cooled to room temperature. The absorbance was measured using the spectrophotometer at 562 nm.

3. Results

3.1 Bacterial biofilm metabolic activity testing

In our research, we have used a novel material, niobium pentoxide (Nb_2O_5), to test its potential antibacterial and antibiofilm properties for possible future applications in treating chronic wounds. Firstly, we wanted to optimize the niobium nanoparticle concentrations and treatment durations to continue our research with the most optimal treatment conditions. XTT tests were performed to evaluate bacterial metabolic activity. This colorimetric assay indirectly measures cellular activity based on a tetrazolium salt reduction by metabolically active cells. If absorbance values decrease, it means metabolic activity has reduced after treatment.

At first, we tested monospecies *S. aureus* biofilm, because it is a predominant bacterium in chronic wound infections. Several treatment conditions were tested – three different concentrations of niobium pentoxide – 3 g/L, 5 g/L, and 7 g/L, and six treatment durations – 1 hour, 2 hours, 4 hours, 8 hours, 18 hours, and 24 hours. As seen in **Figure 7**, with each concentration, the metabolic activity of bacteria has gradually reduced, with a slight uprise at 4-hour (42.77% with 3 g/L; 89.23% with 5 g/L; 46,86% with 7 g/L) and 18-hour (60.12% with 3 g/L; 76.92% with 5 g/L; 82.61% with 7 g/L) timestamps. The highest reduction was at a 24-hour timestamp compared to an untreated control, with 3 g/L reducing metabolic activity to 20.59%, 5 g/L to 32.82%, and 7 g/L to 28.02%. Longer treatments have also been tested, leading to similar results; therefore, the research was continued with 24-hour treatment with Nb_2O_5. As per the concentration, 5 g/L and 7 g/L could be interpreted as more effective than 3 g/L at some time points; however, further experiments were performed with 3 g/L to avoid possible cytotoxicity.

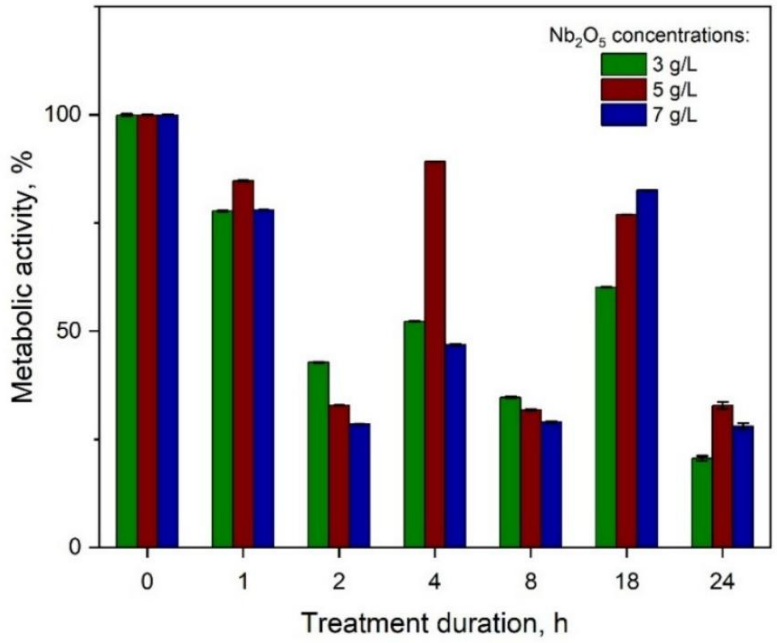

Figure 7. *Metabolic activity of Staphylococcus aureus biofilms, treated with Nb_2O_5 at various concentrations and time points*. Metabolic activity was assessed using the XTT assay. Biofilms were treated with Nb_2O_5 for 1, 2, 4, 8, 18, and 24 hours. Colors represent nanoparticle concentrations: green is 3 g/L, red is 5 g/L, and blue is 7 g/L. Data represent mean ± SD (n = 6).

After optimizing the conditions with *S. aureus* biofilm, we continued testing in polymicrobial *S. aureus* and *E. coli* biofilm, treating with 3 g/L of Nb_2O_5 for 24 hours. The results showed that 65.34% of bacteria were viable (**Figure 8**), proving the therapy effective.

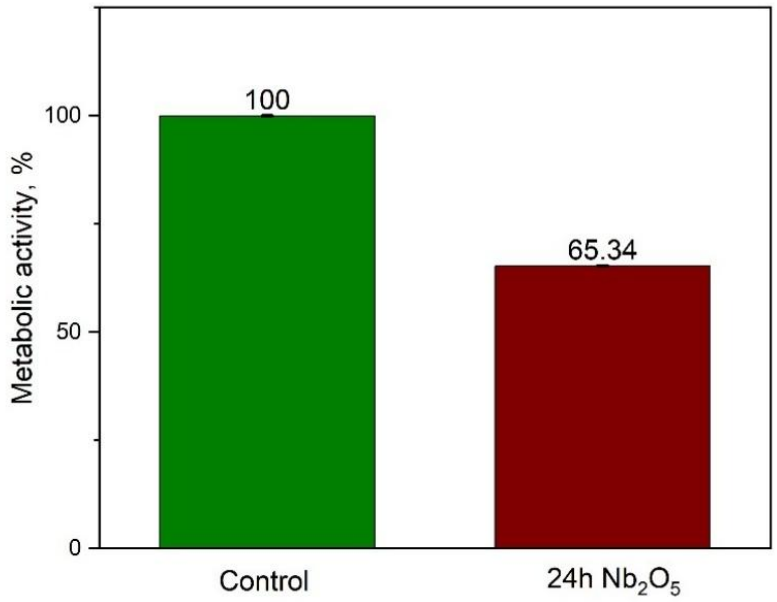

Figure 8. *Metabolic activity testing of polymicrobial Staphylococcus aureus and Escherichia coli biofilms, treated with Nb_2O_5*. Metabolic activity was assessed using the XTT assay. Biofilms were treated with 3 g/L Nb_2O_5 for 24 hours. The treated group is shown in red, and the untreated control is shown in green. Data represent mean ± SD (n = 12).

The optimization of treatment has led to choosing the best conditions, 3 g/L of niobium pentoxide and 24-hour treatment time, for follow-up research with polymicrobial biofilm, including the viability of bacterial cells after nanoparticle treatment.

3.2 Bacterial biofilm viability testing

To evaluate the effectiveness of a potential antimicrobial agent, Nb_2O_5, the colony-forming unit assay was performed by counting viable bacteria grown on selective agar plates after treatment. This method detects only living cells that can reproduce, helping to measure treatment effectiveness directly. Starting with *S. aureus*, 3 g/L of Nb_2O_5 was used, and treatment hours were optimized again. This led to the same results as before, with 24-hour treatment being the most efficient (**Figure 9A**). Percentage reduction ranged from 82.6% in an untreated control up to 99.98% after 24-hour treatment with 3 g/L Nb_2O_5 (**Figure 9B**).

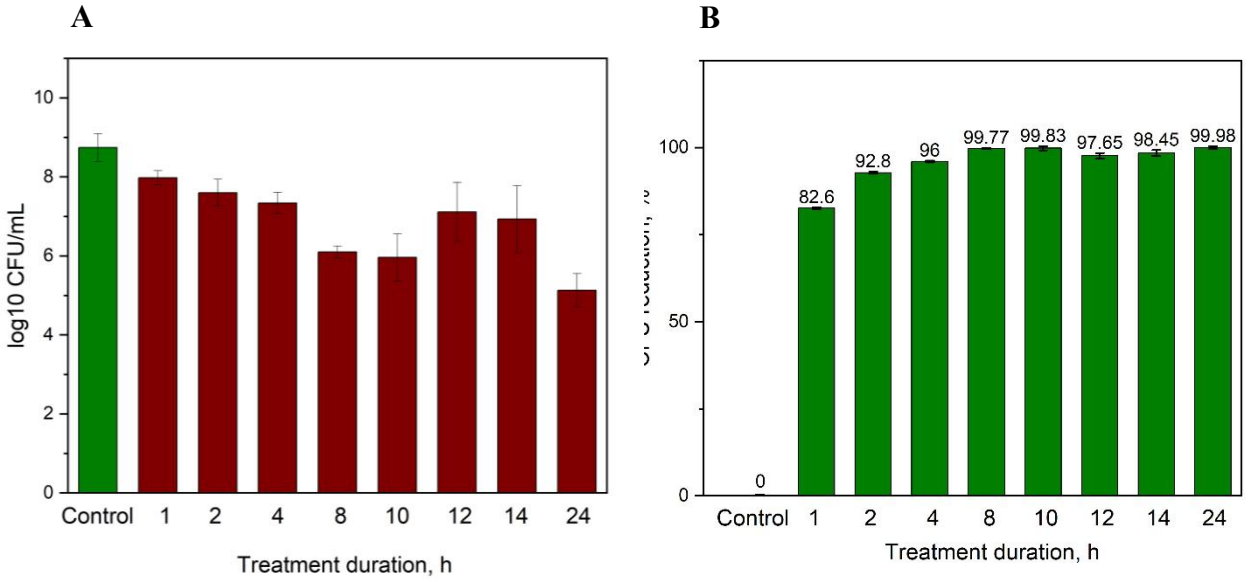

Figure 9. *Bacterial viability evaluation on Nb_2O_5-treated S. aureus biofilm using colony-forming unit (CFU) assay.* (A) Log10 transformed CFU counts of *S. aureus* of untreated and Nb_2O_5-treated biofilms. CFU counts were determined at 0 (untreated control), 1, 2, 4, 8, 10, 12, 14, and 24 hours. (B) Percentage reduction in CFU in *S. aureus* biofilms compared to an untreated control. Graphs illustrate the antimicrobial effect of the treatment. Data represent the mean ± SD of biological replicates (n = 3).

Continuing with polymicrobial biofilm, after several repeated experiments, we noticed that a ratio of 1:1 *E. coli* and *S. aureus*, a standard condition used in various scientific research, resulted in no *E. coli* colonies grown on a selective agar plate.

Therefore, we decided to go with a 2:1 ratio, which has resulted in successful *E. coli* growth. Due to biological variability in biofilm formation and sampling, CFU values showed considerable spread across replicates. Data as mean ± SD based on triplicate plating in **Figure 10**. As seen in the figure, treatment resulted in a complete reduction of *E. coli*. Compared to the control, *S. aureus* viability has also been reduced, although slightly less (**Figure 10A**). In **Figure 10B**, a percentage reduction of colonies is shown. *E. coli* colonies have been 100% eradicated, while S. aureus has been reduced by 99.48%.

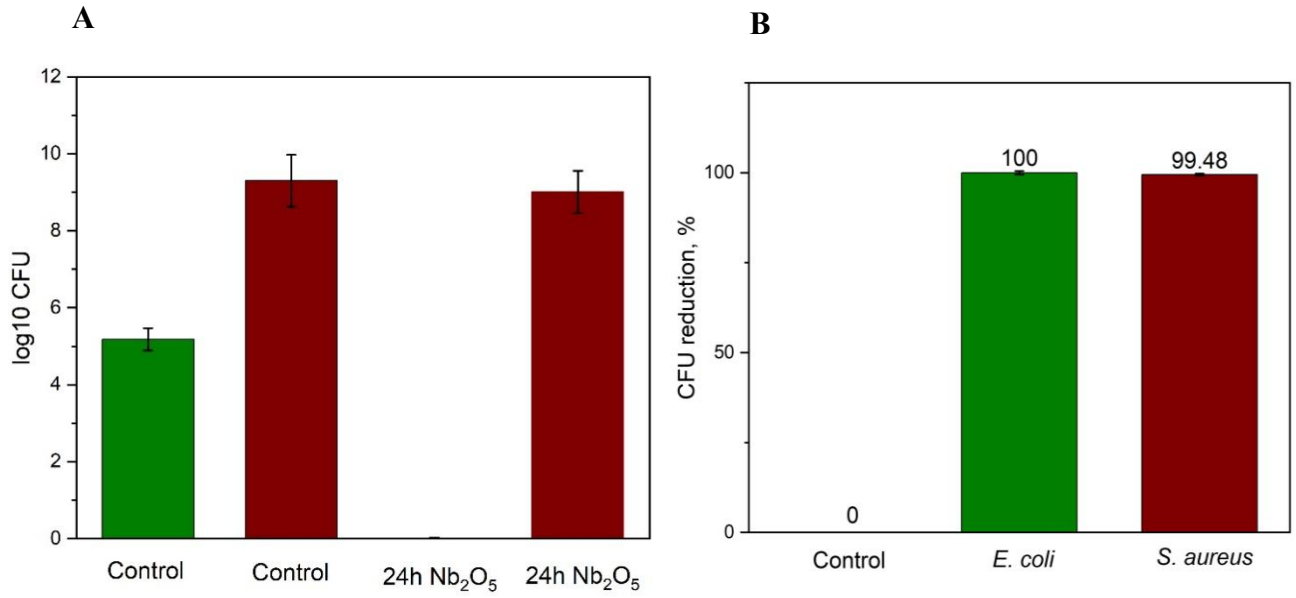

Figure 10. *Bacterial viability evaluation on Nb_2O_5-treated polymicrobial E. coli and S. aureus biofilm using colony-forming unit (CFU) assay.* (A) Log10 transformed CFU counts of *E. coli* and *S. aureus* of untreated and 24-hour Nb_2O_5-treated polymicrobial biofilms. (B) Percentage reduction in CFU in polymicrobial biofilm compared to an untreated control. Green is *E. coli*, red is *S. aureus* percentage reduction. Graphs illustrate the antimicrobial effect of the treatment. Data represent the mean ± SD of biological replicates (n = 3).

The results confirmed that 24-hour treatment with 3 g/L of Nb_2O_5 has effectively reduced the viability of both monomicrobial and polymicrobial biofilms, confirming our hypothesis. Furthermore, we have explored the EPS matrix, evaluating its production, amount, and composition.

3.3 Analysis of biofilm extracellular matrix (EPS)

The extracellular polymeric substance (EPS) matrix is critical to biofilm structure and antimicrobial resistance. Qualitative and quantitative analyses were performed to evaluate how Nb_2O_5 affected EPS production and composition. Congo Red staining was performed to estimate the total matrix production, while EPS after extraction was analyzed for carbohydrate and protein content, using the Anthrone and BCA methods, respectively.

3.3.1 Evaluation of biofilm matrix using Congo Red staining

We used Congo Red staining to evaluate the structural changes in the biofilm matrix after Nb_2O_5 treatment. This dye binds to various EPS matrix components, including polysaccharides and amyloid fibers, providing a great qualitative measurement of biofilm integrity. Staining intensity distinguishes alterations in the overall biofilm matrix in the control and treated samples.

Congo Red staining assay, performed on a polymicrobial biofilm, has shown an 83.17% reduction of total EPS mass, leaving only 16.83% (**Fig. 11**).

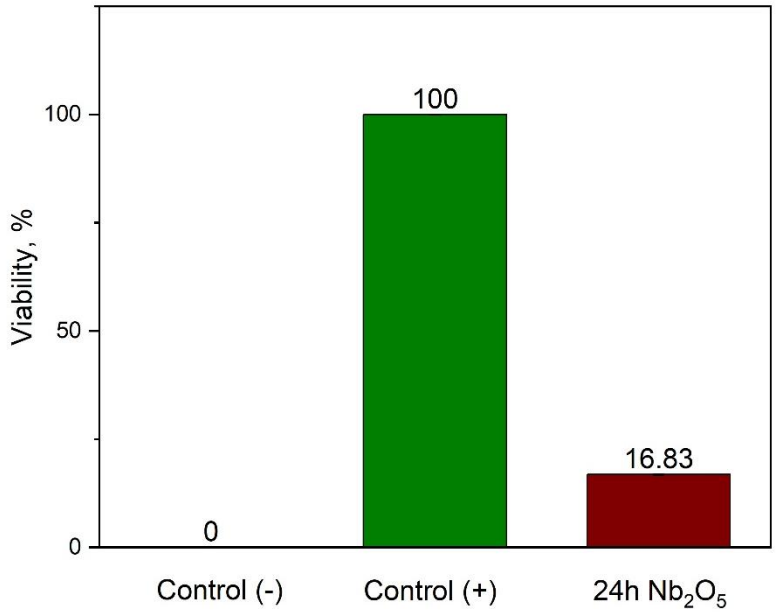

Figure 11. *Congo Red staining of polymicrobial biofilm before and after Nb_2O_5 treatment.* The negative control is Nb_2O_5 mixed with Congo Red dye to evaluate potential chemical interference. Positive, untreated control biofilms (in green) showed intense red coloration, indicating a dense extracellular matrix. In contrast, biofilms treated with Nb_2O_5 nanoparticles for 24 hours exhibited visibly reduced staining intensity, suggesting disruption or reduction of the biofilm matrix. Results were acquired after staining with 0.1% (w/v) Congo red and rinsing with PBS. Results are shown as mean ± SD of biological replicates (n = 12).

These findings lead to a consequent investigation of EPS content to determine whether the treatment affected the carbohydrate and protein amounts in the matrix.

3.3.2 Quantification of EPS components after extraction

To further investigate the effect of Nb_2O_5 treatment on biofilm structure, EPS was extracted from both treated and untreated biofilms. The two essential components of EPS – carbohydrates and proteins – were quantitatively analyzed using Anthrone and BCA assays, respectively. The aim was to determine whether nanoparticle exposure affects the biofilm matrix's main composition, carbohydrates and proteins, which play a critical role in stabilizing the biofilm and providing resilience.

Firstly, the Anthrone method was performed for carbohydrate analysis. It is a colorimetric method, where Anthrone reagent reacts with carbohydrates under sulfuric acid and heat, producing a green-colored complex. Sample absorption is compared to a glucose standard curve, and the exact carbohydrate concentration is measured. The more intense the green color is, the more carbohydrates there are. Compared to the untreated control (15.11 μg/mL), Nb_2O_5-treated samples have shown less carbohydrates (13.57 μg/mL). Results are visualized in **Figure 12**.

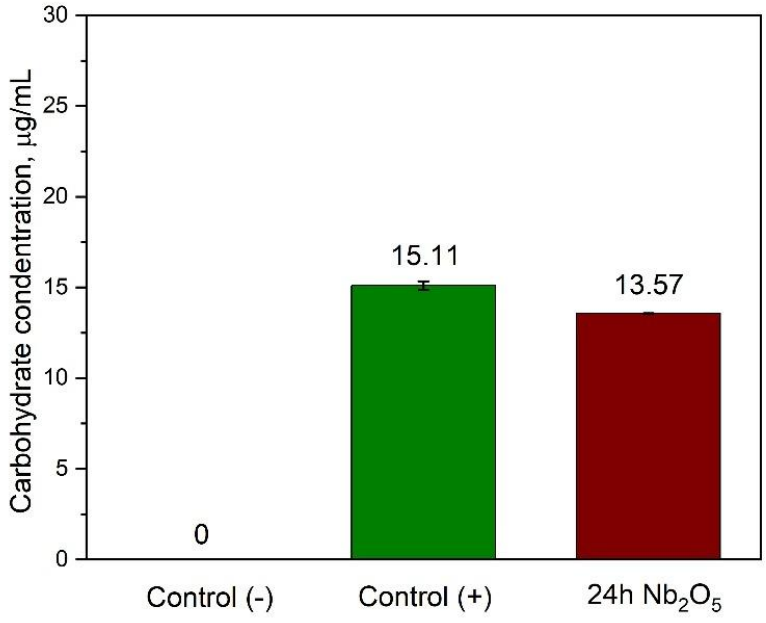

Figure 12. *Carbohydrate content in polymicrobial biofilm EPS measured using the Anthrone assay.* The negative control in black is Nb_2O_5 mixed with Anthrone reagent to account for potential chemical interference. The positive control in green is untreated biofilm with Anthrone reagent, indicating the baseline carbohydrate levels. The treated group in red shows the EPS carbohydrate content after 24-hour exposure to 3 g/L of Nb_2O_5. Results are expressed as mean ± SD from three replicates.

Continuing with the experiments, protein content was evaluated using Pierce™ BCA Protein Assay Kit. In this colorimetric method, proteins react with copper ions, causing them to change form and bind the BCA reagent to the copper. These reactions create a purple color. The more protein is present, the stronger the purple color. The assay has shown that after 24-hour nanoparticle treatment, the protein content in the EPS matrix has increased (174.31 μg/mL in an untreated positive control and 181.46 μg/mL in the treated samples. Results are represented in **Fig. 13**.

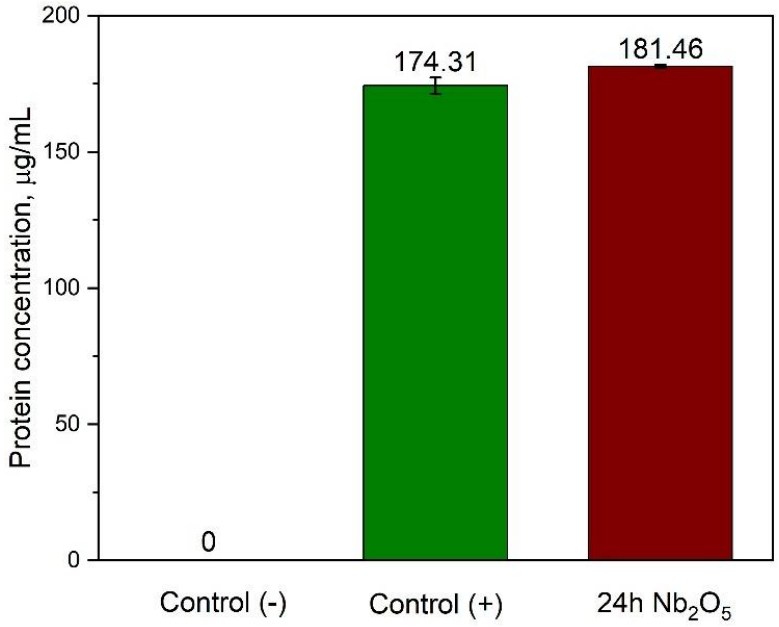

Figure 13, *Protein content measurement in polymicrobial biofilm EPS using the BCA assay*. The negative control includes Nb_2O_5 nanoparticle interference with the BCA reagent. The positive control represents an untreated biofilm mixed with BCA reagent, which indicates baseline protein levels. The treated sample shows the protein content in EPS after 24 hours of exposure to 3 g/L Nb_2O_5. Data are shown as mean ± SD from three replicates.

To better understand the compositional changes in the EPS matrix after Nb_2O_5 treatment, the relative contributions of carbohydrate and protein levels were evaluated compared to the total EPS mass reduction. Congo Red staining indicated an 83.2% reduction in total EPS mass, Anthrone analysis revealed a 10.13% reduction of carbohydrate levels, and BCA assay revealed a 4.1% increase of proteins in the EPS. Results are visualized in **Figure 14** below.

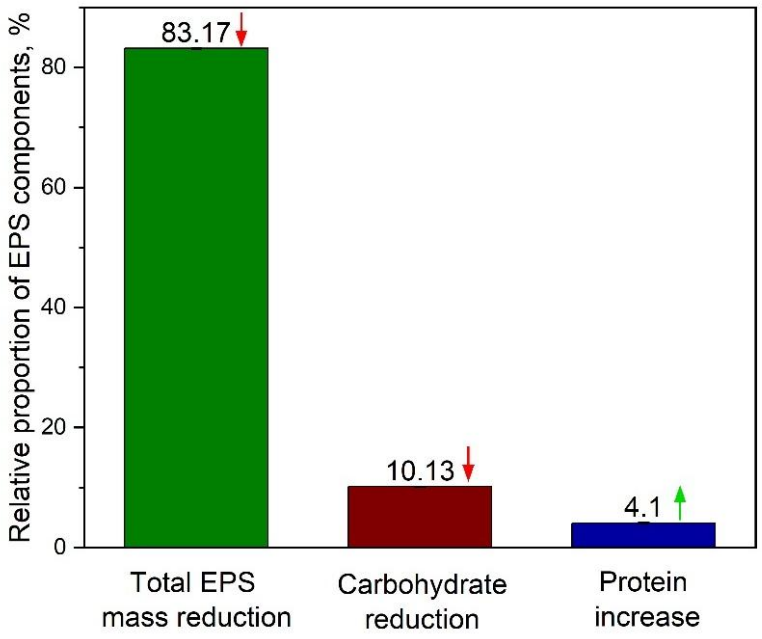

Figure 14. *Relative contribution of EPS components after 24-hour treatment with 3 g/L Nb_2O_5*. In green, total EPS matrix reduction is shown, in red – carbohydrate reduction, and in blue – protein increase. All values are expressed as percentages relative to total EPS content post-treatment.

To summarize, niobium pentoxide treatment led to an observation of decreased overall EPS matrix production, as well as reduced carbohydrate content and increased protein levels, suggesting possible structural disruption and cell lysis or stress responses. These results indicate a measurable impact on the extracellular polymeric substances of the polymicrobial biofilm. The following section explores whether these biochemical changes reflect in the physical structure of the biofilm, as assed through confocal spinning-disk microscopy, as well as scanning electron microscopy.

3.4 Visualization of biofilm structural changes

3.4.1 Visualization by Confocal Spinning Disk Microscopy

Confocal spinning disk microscopy was performed to assess the structural effects of niobium pentoxide nanoparticle treatment on the biofilm. This technique visualizes biofilm architecture and viable and non-viable bacterial cell distribution in three dimensions. Biofilms were stained using live/dead fluorescent staining with MycoLight^{TM}. Images were acquired for untreated and treated biofilms to compare the thickness, density, and cellular viability following Nb_2O_5 exposure.

Visualization revealed that an untreated control contained a smooth, unified layer of biofilm with completely viable cells (**Fig 15A**), whereas niobium pentoxide treatment resulted in a scarce biofilm, with cells scattered unevenly, mostly dead (**Fig. 15B**).

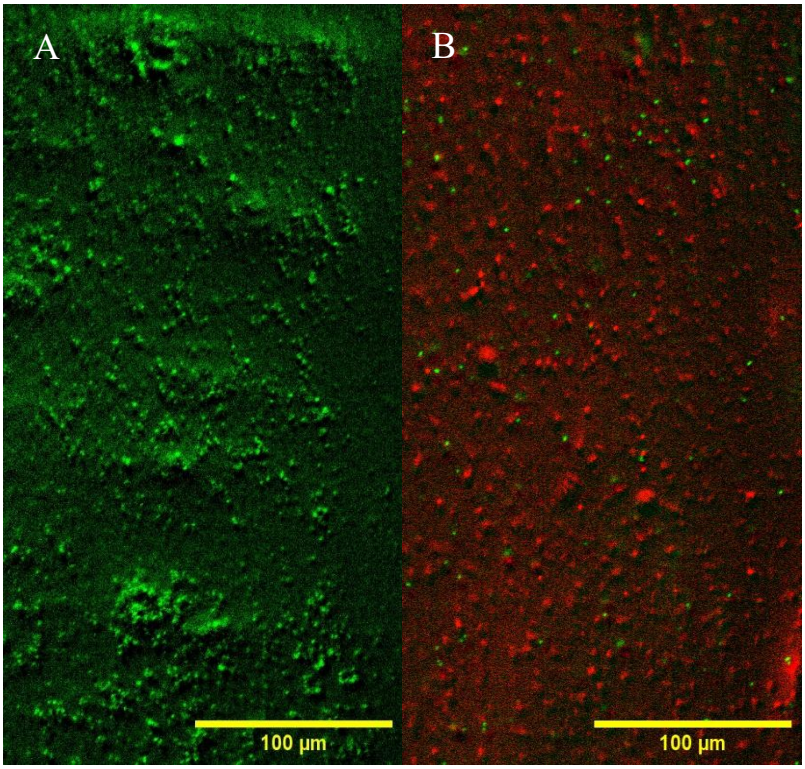

Figure 15. *Confocal spinning disk microscopy visualization of Nb_2O_5-treated biofilms.* (A) shows an untreated polymicrobial biofilm control, whereas (B) shows a 24-hour 3 g/L Nb_2O_5-treated polymicrobial biofilm. Images were analyzed using ImageJ (Fiji). The scale bar represents 100 nm.

Confocal microscopy with fluorescent MycoLight staining revealed a dense, uniform biofilm layer in the control. In contrast, the treated biofilm had sparse, predominantly dead cells, indicating successful bacterial killing after treatment.

3.4.2 Visualization by Scanning Electron Microscopy (SEM)

Scanning electron microscopy, or SEM, was used to observe biofilm surface morphology at high resolution. SEM provides detailed information about biofilm structure, like EPS coverage and bacterial arrangement. We compared untreated and Nb_2O_5-treated biofilms to visually assess structural disruption and potential changes in the surface caused by the treatment.

Visualization revealed a large amount of smooth biofilm in untreated control samples (**Fig. 16A** and **Fig. 16C**). 24-hour Nb_2O_5 treatment effectively reduced the biofilm, with scarce planktonic cells covering the surface (**Fig. 16B** and **Fig. 16D**). **Fig. 16E** shows not only the untreated biofilm but also visualizes the EPS matrix of the biofilm.

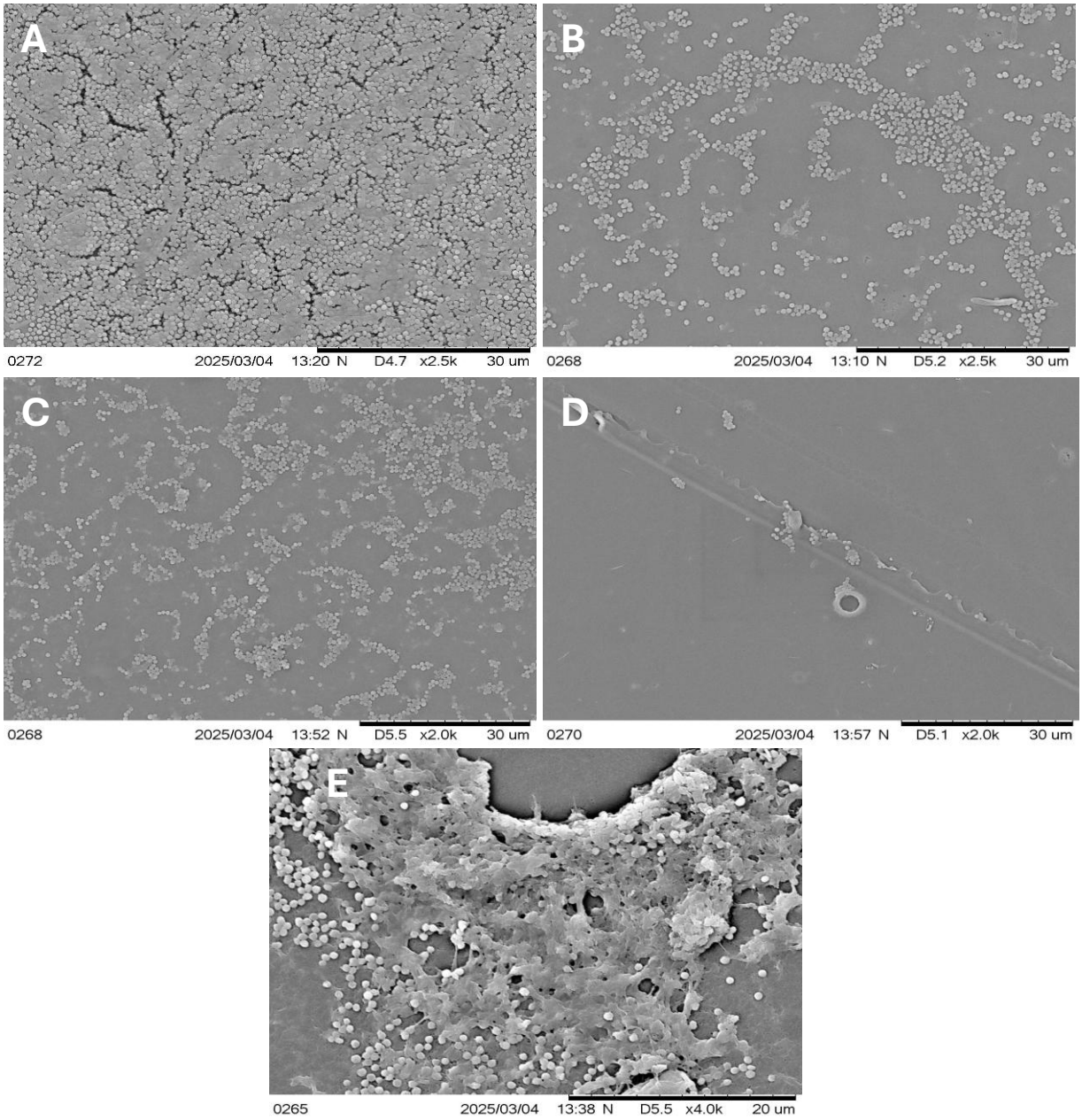

Figure 16. *Scanning Electron Microscopy (SEM) images of biofilms*. (A), (B) show polymicrobial *E. coli* and *S. aureus* biofilms at 2500× magnification, where (C) and (D) show polymicrobial biofilms at 2000× magnification. (A) and (C) represent the untreated control, (B) and (D) show the biofilm after 24-hour treatment with 3 g/L Nb_2O_5. (E) visualizes EPS matrix in the biofilm at 4000× magnification.

Our findings confirm that 24-hour treatment with 3 g/L Nb_2O_5 visibly disrupts the architecture of the biofilm. A reduced surface coverage and a scarce cell distribution indicate it.

Overall, these results suggest that niobium pentoxide acts in different ways against microorganisms. It targets the main components of the biofilm matrix, such as carbohydrates and proteins, leading to a reduction in whole EPS content. Additionally, it inactivates biofilm metabolism, contributing to a decrease in viable bacterial cells.

4. Discussion

This study has investigated the effect of niobium pentoxide (Nb_2O_5) on polymicrobial biofilms composed of *Escherichia coli* and *Staphylococcus aureus*. The optimized conditions of 24-hour treatment with 3 g/L Nb_2O_5 noticeably reduced bacterial metabolic activity by 65.34%, as shown by the XTT assay. CFU assay confirmed a significant viable cell count decrease, reaching 99.46% for *S. aureus* and 100% for *E. coli*. This suggests a differential response between species forming the biofilm. EPS-related analyses, such as Congo Red staining, revealed an 83.17% reduction of total matrix production, leaving only 16,83%. The Anthrone method determined carbohydrate content reduction (from 15.11 µg/mL to 13.57 µg/mL). At the same time, the increase in extracellular matrix protein levels (from 174.31 µg/mL to 181.46 µg/mL) was measured by the BCA assay, most likely due to stress-induced secretion or cell lysis. Structural visualizations were observed via confocal spinning disk microscopy, confirming the increase of dead cells and a disruption of biofilm architecture. Scanning electron microscopy visualized reduced biofilm density and visible surface degradation after treatment. Together, these results provide valuable insight into niobium pentoxide's ability to reduce cell viability and compromise the biofilm's structure.

There are no published studies that reported the effects of Nb_2O_5 nanoparticles on bacterial biofilms, making our research among the first to investigate their potential antimicrobial properties in this context. However, comparisons can be made with other metal or metal oxide nanoparticles, such as silver (Ag), zinc (Zn), or gold (Au) nanoparticles, and even chitosan nanoparticles, which all have shown similar biofilm reduction effects. For instance, AgNPs have been shown to induce the formation of ROS, which can penetrate and damage the bacterial membrane, eradicating various organelles and modulating stress response pathways (Meroni et al., 2020). In a separate study with soil-inhabiting bacteria, AgNPs not only negatively impacted the microbial metabolic activity but also decreased nitrification potential and abundance of bacteria (He et al., 2016). It was also found that NPs could translocate across the *E. coli* cell membrane to the cytoplasm, contributing to microbial toxicity (He et al., 2016).

In addition to reducing the metabolic activity and viability of bacteria, Nb_2O_5 impacted the biofilm's EPS matrix, which is crucial for the biofilm's structural integrity and resistance to external stress. Congo Red staining indicated a reduction in total matrix mass, while extracted EPS analysis revealed decreased carbohydrate and elevated protein levels. These findings suggest weakening of the biofilm structure and possible cell damage or lysis. Similar EPS-destructive effects have been reported with ZnO nanoparticles. The effect of different doses of ZnO nanoparticles was tested on *S. pneumoniae* EPS secretion and resulted in a significant reduction after treatment compared to the untreated controls (Bhattacharyya et al., 2018). The effect of AgNPs on EPS production in *K.*

pneumoniae was also evaluated, resulting in an efficient reduction of EPS compared to controls (Siddique et al., 2020). The decreased carbohydrate levels in the EPS after aluminium oxide (Al_2O_3) nanoparticle treatment have been observed by Muzammil et al. The percentage reduction was found to be 32.55 to 46.72% (Muzammil et al., 2020). Protein level elevation in EPS matrix was also shown in a study with iron oxide nanoparticle (IONPs) treatment on *Halomonas* sp. (Cao et al., 2022). Siddique et al., in addition to reduced EPS levels, have also addressed increased cellular protein leakage due to AgNPs-caused cellular membrane disruption (Siddique et al., 2020). Together, these results support the hypothesis of niobium pentoxide's ability to compromise cell viability, metabolic activity, and the protective biofilm matrix. Decreased carbohydrate content likely reflects disrupted polysaccharide synthesis or degradation of the EPS matrix by Nb_2O_5, which weakens the structural scaffold of the biofilm. In contrast, the increase in protein content may result from bacterial cell lysis or membrane damage, leading to the leakage of intracellular proteins into the extracellular matrix.

Structural analysis was performed using confocal spinning disk and scanning electron microscopy. The aim was to provide visual information about the potential antibiofilm effects of Nb_2O_5 treatment on polymicrobial biofilm. To prepare for confocal microscopy, samples were stained with MycoLight, which revealed a dense, uniform biofilm in the controls, primarily composed of green-fluorescing, or viable cells that formed a continuous, smooth layer on the surface. Unlike the treated biofilms, which lacked this structural cohesion with scattered cells emitting red fluorescence, indicating bacterial death, compromised membrane integrity, and no smooth biofilm architecture. Similar observations have been shown by Tan et al., where the antimicrobial effect of the chitosan nanoparticle, loaded with Deoxyribonuclease I and oxacillin, was tested on *S. aureus* biofilms. Control group revealed a large number of green (live) cell clusters, indicating a mature, multilayer biofilm structure, whereas chitosan-oxacillin treatment resulted in less biofilm mass, thickness, and red (dead) cells. Addition of DNase drastically reduced viable cells, almost completely killing *S. aureus*, and damaging the 3D architecture, which confirms biofilm disruption (Tan et al., 2018). Another research study with Zn nanoparticles caused architectural damage and decreased the biomass of the mature biofilm of the tested bacterial species, one of them being S. aureus, with 23% of the biomass remaining after treatment (Fulindi et al., 2023). Our visualizations accompany the quantitative findings from the XTT and CFU assays, with a visible reduction in both viability and biofilm mass.

SEM microcopy helped further illustrate apparent morphological differences between untreated and treated biofilms. Control samples revealed distinct bacterial shapes, such as cocci and rod-shaped cells, embedded within a thick extracellular matrix. The mature biofilm was tightly packed and covered the whole surface smoothly. However, after Nb_2O_5 treatment, the surface had less bacterial

coverage, with some remaining cells showing deformed morphologies and a disrupted biofilm layer. SEM pictures of *S. aureus* biofilms treated with AgNPs revealed few cells scattered along the surface, an absence in the extracellular matrix, a roughness of the cell surface, and a changed morphology, indicating nanoparticle damage (Ansari et al., 2015). AuNPs have been used to test their efficacy on *P. aeruginosa* biofilms, with SEM showing the reduced number of cells and no bacterial cells with EPS matrix (Ali et al., 2020).

These findings result in a dual-action effect of Nb_2O_5 nanoparticles: dismantling the structural biofilm scaffold and damaging bacterial cell integrity. This adds to the potential of Nb_2O_5 as an effective anti-biofilm agent and is particularly important against resilient polymicrobial communities, which are typically more difficult to eradicate due to their enhanced protective mechanisms. The visual evidence aligns with previous observations of reduced EPS carbohydrate content and shifts in protein profiles, reinforcing the idea of Nb_2O_5 interfering with metabolic activity and biofilm cohesion at multiple levels.

5. Conclusions

1. The 24-hour 3 g/L Nb_2O_5 reduced bacterial metabolic activity by 65.34% in polymicrobial biofilms.
2. Cell viability has reduced to over 90% in monomicrobial and polymicrobial biofilms after 24-hour treatment.
3. The total EPS matrix was reduced by 83.17%, leaving only 16.83% of the EPS mass.
4. The nanoparticle treatment has resulted in the reduction of carbohydrate (from 15.11 μg/mL to 13.57 μg/mL) and an increase in protein (from 174.31 μg/mL to 181.46 μg/mL) levels.
5. Visualization of the biofilm after treatment has revealed structural changes in the architecture.
6. Future investigations should aim to elucidate the specific antimicrobial mechanisms of Nb_2O_5 nanoparticles, such as their potential to induce membrane disruption or oxidative stress in biofilm-associated bacteria.

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Austėja Rudokaitė

Master's Thesis

Inhibition of Polymicrobial Biofilm Using Niobium Pentoxide Nanoparticles

ABSTRACT

Over the years, antimicrobial resistance has increased, with biofilms being the most crucial cause, posing a substantial burden on economic and healthcare systems. Nanomaterials have emerged as a novel treatment for biofilm-caused infections, showing excellent antimicrobial properties and low cytotoxicity. Niobium pentoxide, a metal oxide nanoparticle, exhibits the mentioned properties, making it a promising candidate for further research.

This work aims to analyze the antimicrobial effects of niobium pentoxide nanoparticles on polymicrobial biofilms as a potential component in nano-enabled injectable hydrogels for chronic wound treatments. The goals are: to optimize the niobium pentoxide nanoparticle treatment conditions, assess the treatment effects on metabolic activity, cell viability, extracellular matrix production in both monomicrobial and polymicrobial biofilms, to evaluate the changes in extracellular matrix components, carbohydrate, and protein levels, and to visualize the biofilm structure following nanoparticle exposure.

Biofilms were treated with optimized conditions of 3 g/L niobium pentoxide for 24-hours, following XTT tests for metabolic activity evaluation, CFU tests for cell viability, exracellular polymeric substance extraction for the production evaluation, following with its component amount analysis after treatment, finalizing with the visualization to show the biofilm architecture changes and potential cell disruption.

The results showed effective treatment with niobium pentoxide nanoparticles, confirming the potential antimicrobial properties of polymicrobial biofilms.

VILNIAUS UNIVERSITETAS

GYVYBĖS MOKSLŲ CENTRAS

Austėja Rudokaitė

Magistro baigiamasis darbas

Polimikrobinių Bioplėvelių Inhibicija Naudojant Niobio Pentoksido Nanodaleles

SANTRAUKA

Bėgant metams, atsparumas antimikrobinėms medžiagoms vis didėja. Bioplėvelės yra svarbiausia to priežastis, kelianti daugybę problemų ekonomikai ir sveikatos sistemoms. Nepaisant to, atsiranda inovatyvių metodų bioplėvelių sukeltų infekcijų gydymui, tokių kaip nanomedžiagos, kurios yra mažai citotoksiškos bei turi veiksmingus priešmikrobinius mechanizmus. Niobio pentoksidas, metalo oksido nanodalelės, pasižymi išvardintomis savybėmis ir dėl to kelia stiprų susidomėjimą tolesniems tyrimams.

Mūsų darbo tikslas buvo įvertinti niobio pentoksido nanodalelių antimikrobines savybes prieš polimikrobines bioplėveles, jo potencialiam pritaikymui lėtinių žaizdų gydymui skirtuose injekciniuose hidrogeliuose. Darbo uždaviniai: optimizuoti poveikio niobio pentoksidu sąlygas, įvertinti jo poveikį bakterinių ląstelių metabolizmui, gyvybingumui, tarpląstelinės polimerinės medžiagos kiekiui, angliavandenių ir baltymų lygio pokyčiam po poveikio nanodalelėmis bei vizualizuoti galimus pasikeitimus bioplėvelėse.

Po gydymo optimizacijos, bioplėvelės buvo 24 valandas veikiamos 3 g/L koncentracijos niobio pentoksido nanodalelėmis. Tada buvo atliktas XTT tyrimas metabolizmo pokyčiams įvertinti, CFU testas ląstelių gyvybingumui, tarpląstelinės polimerinės medžiagos atskyrimas ir jos gamybos ląstelėse įvertinimas bei atskirų jos sudedamųjų dalių, angliavandenių ir baltymų, kiekio įvertinimas po poveikio nanodalelėmis. Galiausiai, bioplėvelės buvo vizualizuotos norint parodyti struktūrinius pokyčius.

Rezultatai patvirtino antimikrobinių niobio pentoksido dalelių poveikį prieš polimikrobines bioplėveles.

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