Nanoparticles in controlling biofilms of pathogenic bacteria

Dissertation

zur Erlangung des Grades

des Doktors der Naturwissenschaften

der Naturwissenschaftlich-Technischen Fakultät

der Universität des Saarlandes

von

Neha Gajanan Bankar

Saarbrücken

2024

Tag des Kolloquiums:	14.03.2025
Dekan:	Prof. Dr.-Ing. Dirk Bähre
Berichterstatter:	Prof. Dr. Claus-Michael Lehr
	Prof. Dr. Martin Empting
Akad. Mitglied:	Dr. Karthik Nuthalapati
Vorsitz:	Prof. Dr. Marc Schneider

Printed and/or published with the support of the German Academic Exchange Service

The present work was conducted from October 2020 to September 2024 under the supervision of Prof. Dr. Claus-Michael Lehr and Dr. Brigitta Loretz at the Helmholtz Institute for Pharmaceutical Research Saarland

"There are no failures– just experiences and your reactions to them."

—Tom Krause

Contents

List of Figures

List of Tables

List of Abbreviations

EPS	-	extracellular polymeric substances
DNA	-	deoxyribonucleic acid
QS	-	quorum sensing
QSI	-	quorum sensing inhibitor
eDNA	-	extracellular DNA
cDNA	-	complementary DNA
RNA	-	ribonucleic acid
DMSO	-	dimethylsulphoxide
CaO_2	-	calcium peroxide
NP	-	nanoparticles
HBSS	-	hanks' balanced salt solution
FTIR	-	fourier transform infrared
AMPs	-	antimicrobial peptides
MTT	-	3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide
HMDS	-	hexamethyldisilazane
SEM	-	scanning electron microscopy
CRISPR	-	clustered regularly interspaced short palindromic repeats
FCS	-	fetal calf serum
DMEM	-	dulbecco's modified eagle medium
RPMI	-	roswell park memorial institute
CLSM	-	confocal laser scanning microscopy
PEG	-	polyethylene glycol
PVA	-	polyvinyl alcohol
LC50	-	lethal concentration 50 %
Sq/ SHS	-	squalenyl hydrogen sulphate
THF	-	tetrahydrofuran
PDI	-	polydispersity index
rpm	-	rotations per minute
RT	-	room temperature

DiI	-	1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine
DLS	-	dynamic light scattering
LNC	-	lipid nanocapsules
CFU	-	colony forming unit
PFH	-	perfluoro hexane
DOTAP	-	1,2-dioleoyl-3-trimethylammonium propane
DOPC	-	1,2-dioleoyl-sn-glycero-3-phosphocholine
HSPC	-	hydrogenated soybean phosphatidylcholine
DSPE	-	1,2-distearoylphosphatidylethanolamine
SA	-	*Staphylococcus aureus*
PA	-	*Pseudomonas aeruginosa*
MIC	-	magnetic induction cycler
qPCR	-	quantitative polymerase chain reaction
MBEC	-	minimum bacterial eradication concentration

॥ श्री ॥

Acknowledgements

I would like to start by expressing my deepest gratitude to everyone who has been a part of my PhD journey. I am truly blessed to have had this incredible opportunity to transform my life personally and professionally. Healthcare has been my passion which inspired me to pursue career in pharmacy. Pursuing PhD has been a rewarding as well as transformative experience, and I am thankful to everyone who supported and guided me throughout this journey.

I am deeply grateful to my supervisor and advisor Prof. Dr. Claus-Michael Lehr for giving me this incredible opportunity to be a part of his team. I feel truly honored that he supported me with the project ideas. His belief in me and my capabilities has been my motivation throughout this journey. Prof. Lehr's constant support, guidance, and encouragement through this journey even during challenging times have been invaluable in my growth as a researcher. I will always value his mentorship and thank him for the confidence he has shown in me.

A very special note of appreciation to my co-supervisor Dr. Brigitta Loretz who has been a great support in both my academic and personal life. She not only helped me structure my project but also helped me through the process of securing scholarship through the DAAD every year. Brigitta has always been more than a supervisor, she has been my friend, my mentor and an excellent teacher. Her mentorship has made a major impact both in research and my personal life.

I would like to extend my gratitude to the Deutscher Akademischer Austauschdienst (DAAD) for supporting my personal expenses through the Research Grants - Doctoral Programmes in Germany (2020/21) scholarship. I am incredibly blessed to be an awardee of this prestigious scholarship. I thank the selection committee for choosing me and believing in my potential to complete my PhD. I am thankful to my contact persons at the DAAD- Mr. Christopher Bock and Mr. Hendrik Weber for their continuous support during the years. I also take this opportunity to extend my appreciation to my previous supervisors Prof. Venkata Vamsi Krishna Venuganti for his invaluable reference and support during the scholarship application, and also to Prof. Swati Biswas and Prof. Akash Chaurasiya.

Furthermore, my gratitude extends to Dr. Cristiane de Souza Carvalho Wodarz, who guided me in the early stages of this journey. Thank you for supporting me during the initial DAAD grant application. I also thank the DDEL office staff, especially Sarah and Karin for their support in making my life easier throughout my time in Saarbrücken. Their organization and assistance with all the documentation have made this journey easier. I am very grateful for their constant keenness to assist whenever I needed their help.

I also express my gratitude to the Lung Club and Particle Club organizers for creating a nice platform for continuous support, discussions, and constructive feedback every week which were crucial in the development of my experiments and the project. With that I extend very special thank you to the DDEL team members, the technicians who are truly the heart of the lab: Pascal, Petra, Tabea, Clara, Annette, and Jana. You people are the backbone of our lab, and I am very grateful for everything you have supported me through and ensured me with the success of this project.

I am equally grateful to my senior colleagues and mentors, the postdocs from our lab: Dr. Thiyagarajan Durairaj, Dr. Robert Richter, Dr. Alberto Hidalgo, Dr. Carla Souza, Dr. Lorenz Latta, and Dr. Nicole Schneider-Daum. Your wisdom and encouragement have been deeply helpful during rectifying any mistakes I did during planning of the research experiments and answering of the research questions.

I also thank my superiors at the Saarland University, Prof. Dr. Andriy Luzhetskyy who has been my second supervisor and provided me with his valuable guidance. Also I am deeply grateful to my collaborators from the University Hospital Clinic, many thanks particularly to Prof. Dr. Matthias Hannig, for granting me access to his lab facilities and providing me his guidance. Also thanks to my supervisor at the clinic Dr. Johanna Dudek for supporting me in experimental techniques, and to Dr. Bashar Reda for his assistance during the experiments. And also to Jessica Schmitt-Bennewart, the dedicated technician at the clinic for her help.

I am also thankful collaborative opportunities provided through scientific exchange within the pharmacy faculties at the Saarland University. Thank you Jun. Prof. Dr. Sangeun Lee, Prof. Dr. Marc Schneider and Prof. Lehr for letting me to be part of the PhD seminar discussions. The exchange of ideas and scientific insights has been incredibly enlightening.

I extend my thanks to Dr. Mathias Müsken for his thoughtful suggestions to the development of experimental techniques in my project. I also thank Prof. Dr. Martin Empting for his support and encouragement and letting me be a part of his work.

My project ideas and thesis would have been incomplete without the guidance and previous works from Dr. Kevin Matha, Tobias Neu, Dr. Duy Khiet, and Dr. Justus Horstmann. Thank you so much for allowing me to continue and build upon your research ideas, these have been integral in progress of my own work here at HIPS.

My PhD journey would not have been levitating without my fellow colleagues and dear friends. Thank you for being my guiding stars and family at HIPS. You have made this time in a new place both comfortable and memorable. Glorjen thank you for being a great friend and also always for helping me refine my presentations and posters. Thanks Mohammed for your constant suggestions and support. Dear Aghiad thank you for being my ranting partner and later for calming the situation down. Sarah and Samy, you both have been truly amazing friends. I would also like to thank Sarah Nasr, Clémentine, and Vesa who were my office buddies, thank you for sharing the space I hope it was not that challenging. Mariza and Benedikt thank you for being such good friends and helping me learn new experimental techniques in the initial days. And also a big shout out to all the Particle Club members Lena, Josch, Yun, Achim, and Max without you we would not have had such scientific fun and engaging discussions.

My PhD journey would be incomplete without thanking my family in Saarbrücken. I extend my heartfelt thanks to Dyuthi, Anjana, Shweta, and Srushti for being my support system. Your friendship and the memories I get to cherish from our travels will be with me always. You girls have been an incredible source of strength and joy to me. I am very thankful to Dr. Vishnu. Thank you for bringing a sense of positivity, ease and comfort during this period of my life.

And now comes the best part. Finally, I take this opportunity to thank my loving family. I would like to dedicate this thesis to my daddy. I miss you so much Daddy. I really wish you were here to celebrate this milestone with me. I cannot thank you enough Aai. I owe so much of my dedication and strength to my mummy. Thank you for being the strongest pillar in my life. Your endless love and sacrifice to make me stand where I am has made me achieve this great milestone. Your steadfast belief in me and my capabilities has forced me to stay strong through every difficulty. You have inspired me to fight through all the problems. How can I not be grateful to my baby brother Abhishek. Bacha, I cannot thank you enough for being the coolest guy. You bring me a lot of strength. Thank you for supporting me and protecting me in ways I cannot imagine. You have really been selfless in taking care of everything back home and have allowed me to pursue further studies.

This list would never be completed if I don't thank you Pratik. I could not be more grateful for your kindness. Thank you so much for helping me and supporting me in countless ways. You being there has made this time so much easier. You truly and simply are flawlessly awesome! I also want to thank the Jambhulkar family who have been an invaluable part of my support system, who have loved me and cherished me.

Priya thank you for being my rock, you simply make it all so much bearable. I cannot be grateful enough to Lila Mom and Firoz Dad who have truly showed me the importance of education and empowerment. Mom and Dad you are my shining stars. Lastly thanks to all my well-wishers and friends, your presence has been my strength. This achievement as much as is mine, also belongs to all of you. I am profoundly grateful and blessed to have your presence in my life.

Kurze Zusammenfassung

Bakterien bilden Biofilme, um sich vor physischen und chemischen Gefahren zu schützen, was die Antibiotikaresistenz erhöht und höhere Dosierungen erfordert. Ziel dieser Studie war die Entwicklung von drei Nanopartikeltypen, um die Wirksamkeit von Antibiotika zu verbessern und die Konzentration bei der Biofilmbeseitigung zu senken. Der Schwerpunkt lag auf Kalziumperoxid-Nanopartikeln in Kombination mit Tobramycin. Diese Partikel setzten Sauerstoff und Kalzium frei, was die Wirkung von Tobramycin verstärkte. Biofilme wurden bei geringeren Antibiotikakonzentrationen beseitigt, bestätigt durch CFU-Assays, Mikroskopie, qPCR und ex vivo-Tests an menschlichen oralen Biofilmen.

Squalenyl-Polymer-Nanopartikel wurden ebenfalls untersucht, wobei die Reproduzierbarkeit und Stabilität durch den Einsatz von Tensiden verbessert wurde. Diese erhöhten auch die Wirksamkeit von Tobramycin und förderten die Biofilmbeseitigung. Lipidnanokapseln wurden verwendet, um größenabhängige Biofilmpenetration und Vancomycin-Freisetzung bei *Staphylococcus aureus*-Biofilmen zu untersuchen. Kleinere Kapseln drangen besser ein, größere transportierten mehr Wirkstoff und verbesserten die Biofilmbeseitigung.

Diese Studie zeigt das Potenzial von Nanopartikeln in der Biofilmbehandlung, insbesondere die vielversprechenden Effekte von Kalziumperoxid-Nanopartikeln bei geringeren Antibiotikakonzentrationen.

Short Summary

Bacteria form biofilms so as to protect themselves from physical and chemical assaults, which include antibiotic resistance where higher doses of antibiotics are needed. The purpose of this study was to come up with three different types of nanoparticles that would enhance the efficacy of antibiotics and decrease the amount of antibiotic needed to eliminate biofilm. The emphasis was on calcium peroxide nanoparticles along with tobramycin. These particles release oxygen and calcium that together had additive effects with tobramycin. Antibiotic concentrations required for biofilm eradication were reduced, as evidenced by colony forming unit assays, microscopy, qPCR and ex vivo assays on human oral biofilms. Squalenyl polymer nanoparticles have also been investigated and the reproducibility and stability of the nanoparticles were enhanced with the addition of surfactants. It also enhanced the efficacy of tobramycin and the dispersion of the biofilm. Lipid nanocapsules were employed in order to assess the ability of the size-dependent delivery of vancomycin and its efficacy against the *Staphylococcus aureus* biofilms. The smaller capsules were more efficient in penetrating through the biofilm while the larger ones released more of the active ingredient and enhanced the removal of the biofilm. This study proves the concept of nanoparticles in the treatment of biofilm and highlighted the efficacy of calcium peroxide nanoparticles at lower antibiotic concentrations.

Introduction

1.1 Bacterial biofilms

Bacterial biofilm infections are one of the most persistent and leading challenge in the clinical settings. The bacteria in biofilms live in a cooperative and well protected setting, which is not the case in the planktonic or free-floating bacteria. Which is one of the reasons why the biofilm bacteria have high survival capabilities. The biofilms are communities of microorganisms which are formed by encasing themselves in a self-produced extracellular polymeric matrix. The biofilm communities can adhere to both biotic and abiotic surfaces which includes human tissues and the medical devices. They are formed as a survival strategy against environmental threats including nutrient deficiency, host immune responses, and the chemical threats like antimicrobial treatments. However, this survival mechanism from bacteria poses a significant challenge, as biofilms are becoming approximately 80% of all microbial infections in humans[1].

Treatment of biofilm infections are representing a significant and complex challenge in modern medicine. Biofilms structurally are complex communities of microorganisms, they embed themselves within a self-produced extracellular polymeric substance (EPS) matrix. This matrix is composed of polysaccharides, proteins, and nucleic acids. The main difficulty in treatment arise as formation of biofilms allows the bacteria to adhere firmly to surfaces. Which creates an environment that in turn enhances their survival and resistance to physical and chemical threats like host immune response and antimicrobial treatments[2].

The applicability of biofilm formation by microorganisms extends beyond their prevalence. Biofilm can not only show phenotypic diversity within their own communities but can also create a robust system capable of fighting environmental stress[3]. Also the EPS composing polysaccharides, proteins, lipids, and extracellular DNA (eDNA) usually act as a shield in protecting the bacteria by preventing the penetration of antimicrobials[4]. Furthermore, bacteria within the biofilms can communicate with each other through quorum sensing which regulates their behavior in response to environmental stressors[5].

The project focuses on understanding of biofilm infections caused by two clinically relevant pathogens like **Pseudomonas aeruginosa** and **Staphylococcus aureus**. These bacterial biofilms cause problems with their virulence, adaptability, and ability to form resistance in various human tissues and medical device settings. *P. aeruginosa* biofilms are a leading cause of morbidity and mortality in patients with cystic fibrosis[6]. While the other bacteria studied in this project *S. aureus* form biofilms, which complicate the treatment of prosthetic joint infections, endocarditis and skin wounds[7].

This study aims to investigate different strategies in addressing biofilm related infections. Here strategies employed had advanced treatment techniques including nanotechnology and biofilm disrupting surfactants. Additionally we have studied the effect of these techniques in the dysbiosis of the **human oral microbiome**. Oral biofilms dysbiosis by Streptococcus mutans strains are a cause in conditions like periodontitis and also cardiovascular diseases. Thus making it important to restore microbial homeostasis in oral health[8, 9].

P. aeruginosa and *S. aureus* bacteria are highly adaptable pathogens that establish niche in biofilm forming environment. These biofilms make it more difficult in treatment and are protected from both host immune defenses and antibiotic therapies which lead to chronic and recurrent infections. Here it was studied about the mechanisms regarding the biofilm formation and the ability of these pathogens

to evade conventional treatments with antibiotics. Also their relevance in infections such as chronic wounds and lung infections[10, 11].

The oral cavity microbiome host a complex microbial community, which essentially maintains oral and systemic health. However when there is an imbalance in this microbiome community, also referred to as dysbiosis can lead to major health issues. Dysbiosis is an infection that occurs when pathogenic bacteria dominate over other symbiotically existent species and can lead to oral diseases like caries and periodontitis[12]. Moreover the dysbiosis in oral microbiome can be linked to systemic diseases such as diabetes and cardiovascular disorders[13]. To solve this problem of microbiome imbalance is crucial and novel strategies including probiotics, prebiotics, and nanotechnologies are employed which offer promising solutions[14].

1.1.1 What Are Biofilms?

Biofilms are usually formed as structured microbial communities embedded in a self-produced extracellular matrix. They adhere to surfaces like the human tissues and also to abiotic materials like catheters and pipelines. The challenge of biofilm formation when the bacteria are thought of isolated planktonic organisms causes the shift in treatment protocol. In biofilms the microorganisms function as a group of communicating and highly adaptable species to their environment through cooperative behavior[15, 16]. The extracellular polymeric substances (EPS) also provides structural integrity, which also retain nutrient and provide protection from hostile conditions which make the biofilms remarkably resilient[17].

Biofilms can exhibit phenotypic and genetic heterogeneity among their constituent community of microorganisms. This diversity can contribute in their robustness which also enables biofilms to exist in challenging environments where the planktonic cells cannot survive[18, 19]. The transition from planktonic growth to biofilm associated growth is due to a significant shift in bacterial physiology. These shifts involve the changes in gene expression, metabolism and surface adherence of the bacteria[20].

The process of biofilm formation is complex and typically follow a series of stages, which start from reversible attachment to further irreversible attachment, later leading to microcolony formation and then the maturation of biofilms, the bacteria can then redisperse[21]. The process basically follows the free-floating planktonic bacteria to adhere loosely on a surface. They then begin the production of EPS, which further is transitioned into a more stable irreversible attachment phase. This transition is a crucial step in the biofilm development and formation of a structured community which later is significant in increasing resistance and affecting its susceptibility to treatment.

The composition of a biofilm is complex and highly adaptive to the cellular communication based on the environment. Biofilms can typically have a three dimensional structure. They have water channels that can facilitate nutrient and waste transport. The EPS usually form the bulk of biofilm matrix which accounts to 90% of its total mass[22]. In brief the EPS consists of-

Polysaccharides: which provide structural integrity and mediate adhesion to surfaces[23].
Proteins: they are enzymatic and structural proteins and are responsible in triggering nutrient acquisition and stress response[24].
Extracellular DNA (eDNA): the eDNA contributes in biofilm cohesion and also facilitate horizontal gene transfer[25].

This extracellular matrix can not only provide physical protection but can also create microenvironments which have gradients in oxygen, pH, and nutrient which can influence the bacterial behavior and survival[26].

1.1.2 Stages of Biofilm Formation

Biofilm formation represent a significant survival strategy for bacteria. They can adapt to diverse environments and can resist adverse conditions. The process as described briefly before is transformation of planktonic bacterial cells into structured and sessile communities which are encapsulated within a self-produced EPS. Biofilm development occurs in a sequential manner. That can be described in five key stages as reversible attachment, irreversible attachment, initial microcolony formation, maturation, and dispersion. These stages are directed by the interaction between bacterial genetic mechanisms, surface characteristics, and environmental factors thus creating a highly adaptable microbial biofilm[21, 22].

Key Stages in Biofilm Formation

1. Reversible Attachment: The initial development in biofilm formation is after the adhesion of planktonic bacterial cells to a surface. This attachment is weak in nature. The attachment to surfaces is governed by the weak forces of attraction like the van der Waals forces and hydrophobic interactions[23]. As this is a reversible attachment the bacteria remain capable to get detached from the surfaces if their development needs exploring a more favorable environment. The properties of the surface and environmental factors play an important role during this stage and can influence the bacterial adherence[24].

2. Irreversible Attachment: The next phase of biofilm development is the irreversible attachment to the surfaces. The bacterial cells get attached to a biotic or abiotic surface which is facilitated with the generation of extracellular polymeric substances. The production of EPS and adhesion to surfaces is dependent on the bacterial cell to cell communication systems. The genetic modifications in the bacterial cells lead to generation of EPS and strengthen the biofilm adhesion to surfaces. This step sets the stage in the developmental cycles of the biofilms[25, 26].

3. Microcolony Formation: After the attachment bacterial cells begin to proliferate and aggregate into microcolonies. In clinical settings these clusters are composed of multiple bacterial species which coexist and interact within the biofilm. In this stage the bacterial cells communicate through quorum sensing (QS) which is a signaling mechanism that enables cells to communicate to regulate the population density and is dependent on regulation of gene expression. QS also facilitates the coordination of functions such as EPS production, nutrient production and acquirement, and defense mechanisms within the biofilms to function as a cohesive community. The formation of micro-colonies is the major reason contributing to the structural complexity observed in mature biofilms[27, 28].

4. Maturation: After the bacteria are attached to surfaces they start forming their own communities. However, in clinical settings the coexistence of various species cause the need of generation of a more complex structure. The biofilms in this stage reach a more complex form of community. The cell signaling between species and within species lead to formation of mature structure. There is generation of channel networks and pores for nutrient distribution and quorum sensing signaling molecules exchange. This structure causes the bacteria to develop resilience towards treatment and increases their chances of survival[26, 27].

5. Dispersion: At this stage the matured bacterial biofilms tend to release the bacteria back in the surrounding environment. This dispersion usually happens due to nutrient depletion and

environmental changes. The separation of bacteria from the biofilms cause the bacteria to return to their planktonic state. The dispersion of biofilm bacteria is responsible for ensuring the survival of bacteria in the surroundings and establish a niche in diverse habitats[28, 29].

Biofilm formation is regulated with the genetic regulation by quorum sensing. This is an integral communication process which enable the bacteria to coordinate the cell to cell talk in maintaining cell density within the biofilms. The quorum sensing signaling happens due to the molecule acyl-homoserine lactone (AHL) which is prominently released by the gram negative bacteria, and oligopeptides released by the gram positive bacteria. The bacterial genes involved in biofilm formation are activated when the bacteria release these quorum sensing molecules in a certain concentration. They are responsible for releasing biofilm virulence factors and the EPS regulation[29, 30]. It is also evident that the QS molecules also facilitate interactions within different species and accordingly influence the biofilm structure, composition and resistance[30, 31].

The formation of biofilms can get significantly influenced based on environmental factors and conditions. The bacteria grow rapidly and form biofilm immediately in environments with higher nutrient concentration. The presence of nutrients influence the biofilm structure and composition, and also changes in metabolic activity of the bacteria[31]. The surface properties such as charge, hydrophobicity and roughness play an important function for the attachment of bacteria. The hydrophobicity of a surface can make the bacterial cell wall adhesion stronger due to interactions between the surfaces. Also the roughness of surface causes the bacteria to create their niche and protect the bacteria from shear forces, making them create colonies in higher surface area[32, 33]. The surfaces which possess a dynamic environment with varied fluid flow and shear forces can impact the biofilm structure and formation. The shear force can affect the biofilm development by either causing increase in nutrient transport or can inhibit the biofilms by dislodgement of weakly attached cells. Thus the biofilms in such environments tend to form firm and robust structures to withstand mechanical stress[32, 33]. The bacterial metabolism and biofilm formation is significantly changed due to the surrounding pH and temperature conditions. In optimal biofilm forming conditions for a specific species they can establish their niche, while for some species extreme surroundings promote dispersion. Bacteria can also promote adaptability based on the surrounding environment to establish their survival[33, 34].

Biofilms are becoming an increasingly difficult clinical infection due to the increased resistance and resilience. This arises due to the dense protective layer barrier which limits penetration of antibiotics and host immune mechanisms and in turn resulting in enhanced survival of bacteria. The antibiotic activity that targets actively dividing cells is significantly hampered due to the slow growth of bacteria in the biofilms. Because of these conditions within the biofilms the embedded bacteria show a 1000 times more tolerance towards antibiotic treatment when compared to their free planktonic counterpart[34].

Biofilm related infectious diseases are prevailing in clinical settings as the biofilms are also formed over abiotic surfaces, which are affecting the medical devices like catheters, implants and prosthetics. As these infections are becoming more challenging to treat by standard antibiotic regimens, these devices are removed by surgical intervention. Thus, the increasing need to change the antibiotic regimens by application of innovative therapeutic strategies like use of disrupting agents of the EPS matrix, employment of nanoparticles and antimicrobial peptides and targeting the QS pathway are under consideration[23, 28].

The biofilm associated difficulties focus on prevention and disruption treatment methods. Here the changes of material used can be antiadhesive in nature. They can possess integral antimicrobial properties to avoid initial microbial adhesion phase. Also coating with silver nanoparticles, hydrophilic

polymers, or quorum-sensing inhibitors have shown promising effect in reduction of biofilm formation on medical devices[23, 31]. The EPS matrix is targeted with enzymes such as dispersin B or DNase that can reduce the integrity within biofilms and with this increase antibiotic efficiency. Treatment strategies involving use of biofilm disrupting agents in combination with antibiotics have offered a promising result in eradication[25, 26]. The quorum sensing pathways within the biofilms can be blocked to prevent virulence and cell talk in biofilm formation. The QS inhibitors of synthetic and natural origin are considered for their antibiofilm potential[29, 30]. Nanoparticles which can deliver antibiotics more effectively within the bacteria and those which possess an intrinsic antimicrobial effect can be a potent strategy used in biofilm infections. Due to their efficiency they used reduced antibiotic concentrations and minimize resistance[32, 33].

The biofilms are complex structures which are formed as a result of the adaptability within the bacteria and their resilience. The biofilm development process can be understood by reversible and irreversible attachment, microcolony formation, maturation, and dispersion of the bacteria. These phases play a critical role in addressing their associated challenges.

1.1.3 Structure of Biofilms

Biofilms are extremely complex and yet continuously developing microbial communities due to which they present a high clinical treatment challenge. The structural complexity contributes to their resilience build up and their survival in harsh environmental conditions to protect themselves from external threats. The biofilms structurally are comprised of microbial cells embedded within a self-produced polymer matrix. This makes it difficult for the antimicrobial agents to penetrate the thick and tight barrier.

Within the biofilms are embedded microbial cells which can belong to a specific group of species or they can be different microorganisms like bacteria, fungi and archaea living together as a community. These microorganisms can contribute differently which provide the biofilm with distinct properties which can increase the biofilm resistance and change the overall structure and functions of the biofilm. This kind of adaptation is the reason biofilms can withstand extreme environmental stressors. The interactions within interspecies microorganisms can create a microecosystem capable of increased survival capabilities due to their cooperation and metabolic changes within the biofilms. These interactions contribute majorly to the biofilms ability to survive in natural habitats like the human tissue[30].

The main composition within the biofilm that majorly affects its structure and function is the extracellular polymeric substance (EPS) that is secreted by the bacteria. This biofilm matrix is viscous and thick with a composition of polysaccharides, protein, nucleic acids and lipids. This matrix has the ability to encapsulate the microbial cells and provide a protective layering giving them a structural integrity within the biofilm. The EPS matrix is responsible for shielding the microbes from external physical and chemical threats including treatments with antimicrobial agents and host immune responses. It is also responsible for nutrient retention and waste disposal. The EPS plays and important role in cell to cell communication through mediating the quorum sensing signaling which coordinates survival of the microbial communities. As the EPS possess such important functions the major focus of treatment strategies involve destruction and disruption of EPS[31, 32].

The biofilms are organized as a three dimensional structure due to its distinct architecture. The microbial colonies align themselves as clusters that vary in size and composition. They aggregate together based on the environmental conditions and form a specific arrangement within the biofilm. These microcolonies are embedded in the EPS matrix that act as a physical structure for stability and

integrity. The microcolonies arrange themselves in a highly organized manner allowing them to make use of the resources completely and provide the biofilms required integrity[33].

The biofilms act as a complex community and form the structures with high intricacy integral for different functions in the biofilms. They possess water filled channels and pores which are responsible for the transport of nutrients and oxygen necessary for the survival of the microorganisms within. They are also responsible for transport of signaling molecules and removal of metabolic waste products. These structural conditions ensure that the cells located in the deep center of the biofilms are less metabolically active than the cells at the periphery. The channels are responsible for biofilms ability to adapt to environmental gradients[34].

The heterogenicity within the biofilms is contributed because of the presence of density variations. The microbial cells distribution within the biofilm EPS creates gradients of chemical and physical properties for the region based on environmental conditions. The outer regions of the biofilms have higher concentration of nutrients and oxygen for optimal microbial growth keeping the bacteria more metabolically active, whereas the inner regions are more anaerobic and nutrient deficient leading to the microbes being dormant and low metabolically active. This adaptions within the biofilms provide them higher chances of survival under adverse conditions[34, 35].

The structural complexity of the biofilms increases the complications during their treatment. The EPS matrix acts as a physical barrier in the treatment of biofilms and avoids penetration of antibiotic within them. The presence of channels results in the exchange of resistance genes and signaling during external stimuli with antibiotics threat in the microbial cells. The dormant and low metabolically active cells pose a difficulty in treatment as the antibiotics targeted for the process of cell division are not treated. These modifications in the biofilms make them increasingly difficult to treat and thus there becomes a necessity for development of innovative therapeutic strategies[36, 37].

The biofilms due to their structure have the ability to survive and adapt to environmental stressors. The EPS matrix due to its content provide protection against pH changes, nutrient deficiency, desiccation and spatially get adjusted based on external stimuli. The cells after sensing treatment with antibiotics enter a dormant state and decrease susceptibility towards treatment. The adaptive ability of the biofilms make them persistent and difficult to treat[36].

Microorganisms within the biofilms also have the capability to provide defensive mechanism and a competition for survival within the species. The generation of metabolites for decreasing the survival of another species is another genetic adaption they undergo. To provide protection from such genetic material the development of antibiotic resistance genes happens. Thus the modifications of the pathogens for their survival in such environment leads to emergence of multidrug resistance. However, for some microbes the metabolites from other species can be beneficial for their survival and act as nutrients. Thus rendering the biofilm community highly adaptive and complex[31, 34].

The biofilms due to their characteristics establish a niche in chronic infection conditions. They are often a difficulty in conditions like cystic fibrosis, chronic wounds and medical device infections. The host immune responses are evaded due to the high adaptations within the biofilms due to the metabolic heterogeneity and EPS matrix production. In cystic fibrosis condition the *Pseudomonas aeruginosa* biofilms form a niche infection and colonize in the lungs. Similarly in medical devices the colonization of infectious pathogens can lead to device failure leading to removal and replacement[35, 38].

1.1.4 Clinical Relevance of Biofilms

The biofilms composition due to the extracellular matrix make them highly resilient and clinically significant. The pathogenic bacteria *Pseudomonas aeruginosa* and *Staphylococcus aureus* particularly predominate the niche in chronic infections and device-associated infections. These bacteria survive in the biofilm state and are becoming a serious challenge in medical settings for treatment[39, 40].

Pseudomonas aeruginosa biofilms are a major threat in the chronic lung infections in cystic fibrosis (CF) patients. The EPS matrix produced by the bacteria is mainly composed of alginate as the polysaccharide that enhances the structural stability and resist immune clearance. The hypoxic environment within the biofilm and the limitation of antibiotic penetration has caused persistent infections due to this bacteria[41]. *Staphylococcus aureus* is a gram positive bacteria, has the capability of adhering to surfaces of devices such as catheters and artificial joints and forms biofilms. The biofilms that are formed by S. aureus contains polysaccharide intercellular adhesin (PIA) that is responsible for cell adhesion as well as cell cohesion within the matrix. Other factors that also contribute to the increased antibiotic resistance include the formation of dormant persister cells that are present in S. aureus biofilms[42].

The biofilms are involved in several types of recalcitrant infections, including periodontitis, endocarditis, chronic wounds and lung infections of CF patients. The biofilms of *Escherichia coli* are the common cause of recurrent UTIs, while the biofilms of *Candida albicans* frequently colonize mucosal surfaces and medical devices. Therefore these biofilms basically require longer treatments with the use of antibiotics, some surgical interventions, or replacements of infected devices, all seriously burdening the patient and healthcare system[43].

The microbial dysbiosis especially within the oral cavity biofilms causes difficulties in overall health. There is a usual population of more than 700 species of microbes usually within the human mouth. This multispecies environment maintain oral health normally in their natural state and balance. Poor hygiene, together with diet, produces imbalance that encourages many of the diseases related to the formation of biofilms: dental caries and periodontitis are examples. The acid production by biofilms dominated by *Streptococcus mutans* leads to enamel demineralization. Whereas, *Porphyromonas gingivalis* has been the cause of inflammatory responses that promote periodontitis. Oral dysbiosis is also been linked with systemic diseases like diabetes and cardiovascular conditions, thus suggesting its wider clinical relevance[44, 45].

Biofilms are one of the major causes of device associated infections which are a major cause of healthcare associated infections. Catheters, prosthetic joints and heart valves are some of the examples of devices that are at high risk of biofilm formation. These biofilms are very difficult to eradicate and when they are formed these devices have to be removed and the patient placed on antimicrobial therapy for a long time. For instance, biofilms on urinary catheters cause catheter-associated urinary tract infections (CAUTIs) which are difficult to treat and may recur frequently[46].

Biofilms possess a number of characteristics that allow for their survival like antibiotic resistance. Biofilms have the ability of being 1000 times more resistant to antibiotics as compared to the planktonic bacteria. Some that of are the involved mechanisms include; decrease antibiotic penetration, low metabolic rate and existence of dormant persister cells[47].

Biofilms are associated with quorum sensing which is a process of regulation of gene expression depending on the density of the bacterial population. This co-ordination is used in biofilm formation and drug resistance[48]. Biofilm infections are a major challenge to treat. About 60-80% of them are a result of longer hospital stay. This includes surgical procedures and continuous administration of antibiotics which leads to the development of antibiotic resistance. For instance, due to biofilm forming pathogens results in increased length of ICU stay and high mortality[49, 50].

Current research is centered on finding new ways of dealing with biofilm related infections. New strategies comprise of; anti-biofilm agents, such as compounds that interfere with biofilm formation and EPS synthesis, like the quorum sensing inhibitors, are being developed[51]. There are device coatings, anti-biofilm coatings that contain antimicrobial peptides or silver nanoparticles which has the potential to prevent the biofilm formation on the medical devices[52]. Physically breaking or disruption techniques of biofilms are of increasing importance, by the use of ultrasound and antimicrobial photodynamic effect therapy[53].

Novel delivery systems, such as lipid nanocapsules and calcium peroxide systems have been found to effectively deliver oxygen and antimicrobial agents to the biofilms thus enhancing the treatment outcome[54]. Biofilms are a major cause of the occurrence of chronic infections and antibiotic resistance especially in the areas which involve the use of devices in the clinical settings. Their versatility and durability call for a multiple strategy to prevent and tackle them, which proves the importance of research and development[55, 56, 57].

1.1.5 Pathogens Prominent in Biofilm Formation

Biofilm formation is one of the most important features of pathogens and it plays a crucial role in the development of chronic and persistence infections. Out of the numerous biofilm forming pathogens, *Pseudomonas aeruginosa* and *Staphylococcus aureus* are the most important ones due to their potential to produce highly organized biofilms and cause disease. To create efficient therapeutic approaches it is crucial to know the dynamics of these pathogens.

Pseudomonas aeruginosa is a versatile and often persistent pathogen. *Pseudomonas aeruginosa* is gram negative, opportunistic and very well known for its production of biofilms. This pathogen is especially dangerous for patients with impaired immune systems, including those who suffer from cystic fibrosis, where it causes chronic infections that are very hard to treat. With regards to CF, *P. aeruginosa* produces biofilms in the lungs leading to more destruction of the respiratory structures. These biofilms are dense and city like structures which are mainly composed of extracellular polymeric substances (EPS) which include; polysaccharides, proteins and nucleic acids. The EPS provides protection to the bacteria from the host immune responses as well as antimicrobial agents[58, 59].

Biofilm formation in *P. aeruginosa* is a complex process consisting of several steps which include; adhesive any to surface, formation of microcolonies, development of mature biofilm and dispersion. This process is made possible by quorum sensing which is a way through gene expression which are depending on the density of bacterial cells population. This gene expression regulates the formation of biofilms and also the switch over from the planktonic to the biofilm mode of growth. It has been found that quorum sensing inhibitors can be potential therapeutic agents[60-66].

Clinically *P. aeruginosa* biofilms are linked with recurrent infections. In cystic fibrosis, biofilms in the host's lung environment are resistant to antibiotics and hosts' immune system and further cause chronic colonization. This is because of the appearance of "persister" cells that are dormant bacteria which are present in the biofilm and are highly resistant to antibiotics. These persisters are the main cause of reinfection after the treatment since they are not affected by the antibiotics[61, 63]. Besides CF, *P. aeruginosa* is also involved in chronic wound infections that hinders the healing and sustain inflammation. This is due to the biofilm's capacity to escape host's defense mechanisms and the action of antimicrobial agents thus making these infections challenging[67].

Biofilm forming pathogen *Staphylococcus aureus* is also an important biofilm producer and has a very involving large prosthetic clinical devices significance. The biofilm infections acquired in hospitals are chronic in nature. The infections on clinical devices like catheters and especially artificial prosthetic

joints as well as tissues can further lead to conditions like endocarditis and osteomyelitis[68, 69]. The *S. aureus* biofilms are rather complex and the heterogeneity of biofilm's increases resistance to antibiotics. S. aureus biofilms are regarded as some of the most dangerous since they cause the most complications. The biofilms act on the surface of the devices and when they are implanted, they are usually removed through surgery since they are not easily treatable. For instance, in prosthetic joint infections the biofilm persists can cause device failure, inflammation and therefore more surgical interventions. In a similar manner biofilm infections could be life-threatening if they pass in the bloodstream and cause intravascular infections[69, 70].

Other than device related infections, *S. aureus* biofilms have been associated with chronic wound infections. In such cases the biofilms prevent the infiltration of immune cells and antibiotic penetration thus prolonging the infection and the healing processes. The stability of these biofilms clearly indicates the necessity of the development of new treatment strategies including biofilm disruptors and enhanced delivery systems of antimicrobial agents[68].

Besides *Pseudomonas aeruginosa* and *Staphylococcus aureus*, several other pathogens are capable of forming biofilms and therefore cause persistent infections and therapeutic problems. *E. coli* is a gram-negative bacillus which is the predominant cause of UTIs and has been known to colonize and form biofilms on bladder tissues and catheters. These biofilms help *E. coli* to escape from immune system and antibiotic treatments, which results in recurrence of infections[64]. *Enterococcus faecalis* is one of the most important pathogens that cause endocarditis and UTIs; *E. faecalis* produces biofilms which increase its pathogenicity. Especially, their biofilms are highly resistant to antibiotics, which creates a problem for the management of infections caused by this organism[64].

Klebsiella pneumoniae is a hospital acquired pathogen; it has been known to form biofilms on devices such as ventilators and cough assistive devices as well as in the respiratory tract. The stability of these biofilms therefore facilitates the spread of the pathogen in the hospital environment thereby causing multidrug resistant infections[65].

Biofilm related infections always require a longer period of treatment with antibiotics, and sometimes require more invasive approaches. For instance, biofilm associated infections of devices are commonly associated with device related complications such as endocarditis and osteomyelitis; these may require the removal of the device, which is burdensome to the patient[66]. Biofilm related infections are also associated with higher morbidity and mortality as well. Patients with such infections remain sick for longer periods, at a high risk of complications, and with reduced quality of life. In chronic infections, the biofilms are spread throughout the tissues and organs, which results in tissue damage, systemic inflammation and spread of the pathogens to other tissues[64, 65].

From the healthcare standpoint, biofilm-associated infections are a major source of expenses. Longer stays in the hospital, more surgical procedures, and the requirement for unique therapeutic strategies put a drain on the financial resources of health facilities. Also, the increasing incidence of multidrug-resistant pathogens in biofilms calls for the discovery of new antimicrobial agents and novel therapeutic strategies[66, 67].

1.1.6 State-of-the-Art Treatment Strategies for Biofilms

Biofilm is one of the major problems in clinical medicine because it is very difficult to treat due to the antimicrobial therapy resistance. These infections are characterized by the formation of a biofilm that is a matrix of extracellular polymeric substance (EPS) that protects the bacteria from antibiotics and immune system eradication. Biofilm related infections are not easily eradicated hence there is need to develop new strategies for treatment. There are several potential approaches that are being

investigated which could prevent biofilm development, increase the permeability of antimicrobials and thus eliminate these biofilms.

One promising avenue include the use of AMPs. These are natural compounds that are antimicrobial peptides and are known to have a wide spectrum against bacteria including those that form biofilms. While the EPS matrix hinders the penetration of conventional antibiotics, AMPs are capable of breaking through the bacterial membrane and targeting the biofilm cells. It also minimizes the chances of the bacteria becoming resistant to it. AMPs are current research interests as to whether they can be used as single agents or in combination with the current antibiotics to combat biofilms; the dual action of disrupting the biofilm matrix and killing the bacteria. This approach has the potential of dealing with the multidrug resistant biofilms and thus provide a way of finding new and efficient treatments[71].

There is another approach which is based on the use of quorum sensing inhibitors (QSIs). Bacteria in biofilms communicate with each other using quorum sensing which is a regulatory mechanism that governs biofilm formation, biofilm development and biofilm dormancy. The QSIs act on these signaling pathways thereby preventing the formation of biofilms and encouraging dispersal of the bacteria. Quorum sensing inhibitors also inhibit the expression of virulence factors and the synthesis of EPS; it has been shown that the addition of QSIs to antibiotics can greatly improve the effectiveness of standard therapies. This symbiotic approach solves the problems associated with the use of antibiotics in eradicating biofilm forming pathogens such as *Pseudomonas aeruginosa* and shows how QSIs can be useful in clinical practice[72, 73].

There are other techniques which are enzymatic therapies in the management of biofilm. The EPS matrix which includes polysaccharides, proteins, eDNA, DNases, proteases and acts as a physical barrier against antibiotics. Polysaccharide hydrolases are enzymes that attack these components with the aim of dissolving the biofilm matrix. For instance, DNases are enzymes that are capable of breaking down eDNA that is an essential component in the formation of biofilms while proteases and polysaccharide hydrolases attack on proteins and carbohydrates framework disrupting respectively. Therefore these enzymes can greatly improve the ability of antibiotics to penetrate through the biofilm and increase the antibiotics potential as an adjuvant in the treatment of chronic infections[74].

These include infections from medical devices which lead to biofilm formation on surfaces which has lead to the creation of anti-biofilm coatings. Silver based coatings are one of the most well known methods for reducing biofilm formation and the antimicrobial properties of silver ions makes it difficult for the bacteria to adhere and grow on surfaces of catheters and stents etc. Likewise, the use of antibiotic impregnated materials ensures that the antimicrobial agents are released slowly thus covering the device surface and preventing bacterial adhesion. However, the issue of antibiotic exposure and the risk of developing resistance, calls for some level of precaution when using them. On the other hand, the responsive polymer coatings provide a better approach since the release of antimicrobial agents is dependent on the presence of bacteria or other environmental stimuli. These smart coatings are thus effective in infection control through a proactive approach that also helps to reduce antibiotic consumption and increase the durability of the device[75–77].

Not only have there been chemical ways of approaching biofilms, but there have also been physical ways of breaking them down. Ultrasound is one example which uses high frequency sound waves to create cavitation bubbles that mechanically disrupt the biofilm matrix. This technique enhances the efficiency of antibiotics in penetrating and destroying the biofilm and is a non-invasive way of dealing with biofilm infections. Another therapeutic approach that has been gaining increasing attention in recent years is photodynamic therapy (PDT). This form of treatment involves the use of photosensitizing agents which are triggered by light to produce reactive oxygen species (ROS). These ROS lead to oxidative destruction of the biofilm and results in the breakdown of the biofilm matrix.

There is also the possibility of using electric fields that affect the bacterial membrane in biofilm and increase permeability of antibiotics in the EPS matrix. All these physical methods along with the other therapies are also useful in combating biofilm formation as they expand the arsenal of strategies that are available[78–80].

The use of combination therapies has been seen to be a very effective approach in overcoming the biofilms' heterogeneity. The use of antibiotics with different mechanisms of action can lead to synergistic effects therefore increasing the efficiency of the treatment. This way not only the bacterial killing is increased but the chances of resistance are also reduced to a great extent as multiple pathways are targeted at the same time. Furthermore, the use of antimicrobial peptides along with the conventional antibiotics has been found to be more effective against biofilms. AMPs break down the biofilm matrix, thus enabling the antibiotics to reach the bacteria with better efficiency; antibiotics eliminate bacterial metabolism. This approach shows how a combined therapeutic approach is necessary to combat the various problems that are associated with biofilm infections[81, 82].

New technologies such as nanotechnology is also changing the face of biofilm control. Nanoparticles present a highly versatile system for the controlled and targeted administration of antimicrobial agents at the site of biofilms. This is because of their small size through which they can navigate through the EPS matrix and since they can be functionalized on the surface, they can be targeted. Furthermore, nanoparticles can affect various kinds of bacterial strains and help in eradication of biofilms. These nanoparticles can create controlled release of antibiotics. These nanoparticles include metal based, polymeric, lipidic nanoparticles for delivery of therapeutic agents. Thus they enhance the efficiency of antimicrobial treatments[83].

Among the novel strategies, gene-editing techniques such as CRISPR-Cas systems are also gaining importance in the management of biofilms. This revolutionary technique enables the manipulation of specific genetic targets that are involved in the process of biofilm development and spread. Thus, CRISPR-based therapies can target and disrupt the genes responsible for the formation and preservation of biofilm bacteria while leaving the other health-promoting microorganisms unharmed. However, this technology is in the early stages of development and thus is only applicable in a research setting; nonetheless, it provides a potential way to create highly targeted and efficient therapies for persistent infections[84].

Immune modulation is another emerging concept that is being explored in the current scenario. In order to enhance the host immune response, the investigators are trying to enhance the body's ability to recognize and get rid of biofilm associated bacteria. Vaccines that contain biofilm antigens and immune stimulants are being created with the intention of enhancing the host's immune response. Thus, this approach expands the attention from direct attack on bacteria to the support of the immune system, which can be considered as the complementary approach to conventional antimicrobial treatments[85].

Bacteriophages or phages that are used for targeting and eliminating bacteria in body is also gaining popularity as an accurate approach for biofilm control. Due to their ability to infect the EPS matrix, replicate inside the bacterial cells and produce enzymes that dissolve biofilms bacteriophages are highly effective against them. Their targeting of specific bacterial strains does not affect the natural flora as greatly as antibiotics do, which is why they are considered as potential substitutes for antibiotics. There is a growing interest in the use of phage therapy for the treatment of multidrug-resistant infections and as an adjunct to conventional therapies[86].

Biofilm infection is a major problem in healthcare since it is very difficult to eradicate and is the cause of persistent and device-related infections. The increasing knowledge on biofilm biology has emerged various treatment options such as antimicrobial peptides and quorum sensing inhibitors, enzymatic

therapies, and anti-biofilm coatings. Physical disruption methods, combination therapies and other advanced technologies such as nanotechnology and CRISPR-Cas systems have also been used to combat biofilms. Immune modulation and phage therapy are also introduced in this review to stress on the need for a combined approach for biofilm control. Further research and development are necessary to enhance these strategies and to implement them in the clinical setting. All these approaches that have been discussed are focused on the management of biofilm infections, are effective in the treatment of the infections due to the fact that they address the biofilm in its wholeness[71–91].

1.1.7 Infections Caused by Pseudomonas aeruginosa

After becoming adhered to a surface the bacterium is often in a biofilm, this is a community of microorganisms that is surrounded by an extracellular polymeric substance. This is an important characteristic of *P. aeruginosa* as it is a major cause of chronic infections where the bacteria can survive on surfaces due to its biofilm forming ability. Using adhesion molecules that promote binding to host cells and abiotic surfaces like medical devices is another strategy. These adhesion factors including pili, flagella and surface proteins enable *P. aeruginosa* to set on different surfaces like the epithelial tissues as well as indwelling devices such as catheters and ventilators[92, 93].

Besides the biofilm formation, *P. aeruginosa* are involved in large number of tissue toxins destruction and enzymes for avoidance of host's defense mechanisms. Exotoxin A is one of the most lethal toxins produced by the bacterium that inhibits protein synthesis in the host cells thereby causing apoptosis. Some of the enzymes include elastase and alkaline protease which break down the host tissue structure thus allowing the bacteria to infect and colonize the area[93]. These virulence factors not only increase the bacteria's potential to cause infections but also are responsible for the complications and persistence of infections it causes.

Among the most important clinical manifestations of *P. aeruginosa* infections, one can identify chronic respiratory disease, which is often observed in patients with cystic fibrosis (CF) or chronic obstructive pulmonary disease (COPD). In the case of CF patients, the bacterial biofilm works in the favorable environment of the thick mucus lining the airways and establishes colonization. This results in the bacteria's ability to remain in the lungs and cause chronic inflammation that in turn results to progressive pulmonary complications and worsening of the lung function. Although there have been new strategies in the management of CF, the presence of *P. aeruginosa* in the airways is still a complication due to the antibiotic resistance and the inability of antibiotics to penetrate biofilms. The study also shows that the airway microbiome in CF patients is dysbiose hence promoting the pathogen's growth making it difficult to treat respiratory infections caused by this bacterium[94].

Some of the other important infections include UTIs caused by *P. aeruginosa* which is a serious problem especially in patients with indwelling catheters or anatomical abnormalities of the urinary tract. These infections are most often acquired in the hospital, and the bacteria's capability of colonizing and form biofilms on catheters also plays a role in its persistence and the development of antimicrobial resistance. Biofilm related UTIs are difficult to treat and may necessitate the removal of the catheter and a duration course of combination antibiotic therapy. This shows that there is still a need for new strategies that can prevent or break down biofilms and enhance the treatment of these types of infections[95].

In addition to causing pneumonia, *P. aeruginosa* is also involved in skin and soft tissue infections including burns and wounds. In burn wounds the bacterium is a fast starter and acts on damaged tissues thus causing complications that include inflammation, necrosis and delayed healing. This type of infection that is associated with *P. aeruginosa* in burn wounds is difficult to manage because of the

pathogen's capacity to escape from the immune system and from antibiotics' effects. In the same manner, in the chronic wounds including diabetic ulcers *P. aeruginosa* forms biofilms which hinders the healing process, perpetuates inflammation and enhances the likelihood of developing systemic infections[95].

There are also bone and joint infections including osteomyelitis and septic arthritis through which *P. aeruginosa* causes problems. These infections are usually complications of trauma or surgical replacement procedures and such are as especially joint frequent in immunocompromised patients or those with other diseases. This is due to the bacteria's capability of establishing biofilms on orthopedic implants thus making the infection hard to treat requiring surgery along with antibiotics. The effect of these infections on the patient's mobility and the quality of life show the importance of *P. aeruginosa* in musculoskeletal infections[96].

One of the most fatal complications of P. aeruginosa infections is sepsis which is a severe response of the body to an infection, manifested by inflammation and failure of many organs. In the hospitals *P. aeruginosa* is one of the most frequent pathogens that induce sepsis especially in the patients located in the ICUs. This is because the bacterium is capable of spreading through the blood stream and to other parts of the body which makes it very virulent. Sepsis due to *P. aeruginosa* is fatal and this is because the pathogen is usually resistant to many of the antibiotics used in the initial treatment. This makes the management of septic patients with *P. aeruginosa* infections to be challenging and this requires early diagnosis, intensive management and close observation[97].

The management of *P. aeruginosa* infections is challenging due to the efficiency of this organism in developing resistance. This pathogen has several intrinsic mechanisms that provide it with resistance to many antibiotics such β-lactams, as aminoglycosides and fluoroquinolones through the impermeable outer membrane and efflux pumps. In addition, *P. aeruginosa* has the capacity to develop antimicrobial resistance through the genetic exchange, which leads to the appearance of the multidrug resistant strains. Such mechanisms may require the use of the combination therapy and newer antimicrobial agents, and this may enhance the costs of treatment and the incidence of adverse events[98].

The risk of *P. aeruginosa* infections is high especially in the immunocompromised patients including the cancer patients, organ transplant recipients and those in the intensive care units. Such patients are at high risks of developing severe infections because of their compromised immune systems and frequent hospital admissions and interventions. Due to its prevalence in the healthcare settings and its capacity to take advantage of the host's compromised status, *P. aeruginosa* is one of the most frequently isolated pathogens in nosocomial infections[99].

From the economic angle, the infections caused by *P. aeruginosa* lead to the excessive spending of the healthcare budgets. Extended hospitalization, more surgeries and the application of sophisticated modalities are some of the factors that increase health costs. Also, high mortality rates that are linked to life threatening conditions like sepsis also emphasize the importance of infection control and antimicrobial stewardship. Therefore, it is important to implement measures that will help in the prevention of *P. aeruginosa* in the healthcare facilities in order to decrease the economic as well as clinical burden[100].

Pseudomonas aeruginosa is a very versatile gram negative rod which is an opportunistic pathogen that causes many different types of infections especially in patients with compromised immune systems and intensive care unit patients. This including its biofilm formation, secretion of various virulence factors as well as its antimicrobial resistance makes the treatment of infections caused by the organism difficult and calls for more research on new therapeutic approaches. To create new ways of preventing

and dealing with infections caused by this very difficult organism it is crucial to understand the processes that this bacterium uses to cause disease and to develop resistance.

1.1.8 Anaerobic Environments in Biofilms

Biofilms are the complex communities of microorganisms which are capable of attaching to the interfaces and are surrounded by the matrix of their own extracellular origin. One of the most important characteristics that can be attributed to many biofilms including those that are associated with chronic infections is the ability of these biofilms to create anaerobic conditions within their interior. These anaerobic zones have a marked effect on the biofilm's virulence, its response to treatment and its ability to survive in the clinical environment. It is therefore important to have a proper insight into these conditions in order to come up with proper therapeutic interventions for controlling biofilm related infections.

This means that biofilms operate in anaerobic conditions mainly because of oxygen depletion by the metabolic processes of microorganisms in the outer layers and reduced oxygen transport to the biofilm due to the presence of the biofilm matrix. This leads to different oxygen levels with the peripheral parts of the biofilm being more oxygenated while the central parts become anoxic or even aerotolerant. These gradients give rise to a territorial hierarchy that allows for the colonization of a wide range of microorganisms of which some are obligate and some are facultative anaerobes. Anaerobic bacteria that are present in biofilms are also important in increasing the biofilms' resistance as these organisms are capable of growing in low oxygen conditions and use other metabolic pathways[101, 102].

In response to the anaerobic conditions, the microorganisms in anaerobic biofilms make a number of important alterations to their metabolism. As such, anaerobic bacteria have adapted the ability to produce energy through fermentation or anaerobic respiration; in the absence of oxygen, bacteria create energy and waste products such as organic acids or alcohols that can affect the biofilm environment. Such metabolic changes allow the bacteria to persist in conditions that would otherwise be detrimental, help to cement the biofilm architecture, and make it difficult to break up or eliminate the biofilm[103].

The anaerobic microenvironments that exist in biofilms are particularly important in the context of chronic infections. These biofilms are commonly associated with infections which occur in low oxygen conditions such as in periodontal pockets, chronic wounds and some of the intra-abdominal infections. In these localities, the anaerobic conditions offer the following benefits to the biofilm-associated bacteria. First, it increases the resistance to antimicrobial agents. This is because antibiotics that affect the dividing cells have less efficacy on the non-dividing or dormant bacteria present in the anaerobic parts of the biofilm. Also, the antibiotics' distribution is hindered by the extracellular matrix thus providing protection to the bacteria within[104].

The biofilm form also enables bacteria to evade the host immune response. This feature is due to the fact that the biofilm can create an environment of reduced oxygen which in turn affects the function of certain immune cells like the neutrophils and macrophages that require oxygen for maximum function. This reduced immune response enables the bacteria to survive in the host tissues thereby maintaining the chronic nature of biofilm related infections[104].

Among the major problems that can be identified when treating anaerobic biofilms, the most crucial one is the issue of treatment resistance. Conventional antibiotics are not very effective in eradicating anaerobic biofilms; therefore, increased amounts of antibiotics or longer periods of treatment are required, which may cause toxicity and other side effects. Such therapies that involve the anaerobes

use for of instance antibiotics metronidazole that together act with on agents that break the biofilm matrix have been found to be efficacious. But such measures are not always successful and may lead to the creation of resistance as well[105].

There are current techniques that are being used in order to deal with the problems that are associated with anaerobic biofilms. Oxygen-releasing agents for instance are designed to combat hypoxia within biofilms, thus limiting the opportunity for anaerobic bacteria to thrive and potentially increasing the effectiveness of oxygen-dependent antibiotics. Photodynamic therapy is also an emerging technique in which reactive oxygen species are generated through the interaction of light and certain compounds to destroy biofilm including the anaerobic zone. Furthermore, there is current development of biofilm-disrupting enzymes such as DNases or proteases that dissolve the extracellular matrix and enhance the penetration of antibiotics and bacterial killing[105].

Certain infections that are linked to anaerobic biofilms show how much they are relevant to the practice. In the oral region, it is possible to find biofilms that contain anaerobic bacteria such as *Porphyromonas gingivalis* and this is common in periodontal disease. These bacteria live in anaerobic environment of periodontal pockets where they cause inflammation, tissue degradation bone and loss. This shows that these biofilms are still remaining after conventional treatment therefore there is need to come up with targeted therapies that will be against anaerobic biofilms[106].

In the case of chronic wounds, the anaerobic bacteria *Bacteroides fragilis* is the predominant bacterium that develops biofilms in the hypoxic tissues. These infections are very challenging to treat as the anaerobic environment favors the persistence and even the development of resistance by the bacteria. This highlights the need for effective biofilm management as anaerobic biofilms are often associated with delayed healing of chronic wounds and an increased chance of systemic infection[107].

Intra-abdominal infections which are common in postsurgical cases or trauma also involve anaerobic biofilms. Some of the organisms that are commonly involved are *Clostridium perfringens* which grows in the micro-aerophilic environment that is created by necrotic or ischemic tissue. These biofilms not only make the treatment difficult but also increase the mortality and morbidity rate in the patients affected by them[108].

Among the various mechanisms of resistance that are encountered in clinical practice, the ones that are associated with anaerobic biofilms are the most challenging ones. Besides, the metabolic dormancy and the protective roles of biofilm matrix, these anaerobic bacteria are also capable of intrinsic resistance to some antibiotics like aminoglycosides which are active only in aerobic conditions. This leads to the need to use the combination therapy or adjunctive therapy thus increasing the treatment burden and the costs[101].

The creation of new therapeutic strategies is very important in overcoming the problems raised by anaerobic biofilms. Such approaches that attack the metabolic processes of the anaerobic bacteria or break down the biofilm matrix are very encouraging. For instance, current research is being conducted to identify the small molecules that can prevent the formation of biofilms or help the antibiotics to diffuse through the extracellular matrix. It will be important to know the functions of oxygen gradients and metabolic regulations in biofilms in order to develop efficient measures of dealing with these persistent microbial colonies[103].

Anoxia is a major factor that can be associated with biofilms and this is a very important factor that affects the persistence, as well as the resistance of biofilms to treatments as well as their role in chronic infections. These hypoxic conditions present barriers to conventional therapeutic interventions hence calling for new approaches to biofilm formation and bacterial killing. It is therefore possible to identify

the characteristics of anaerobic biofilms, including their metabolic adaptations and the protective matrix, that can help researchers to find new ways of treating biofilm-related infections.

1.2 Dysbiosis in the Oral Microbiome

The oral microbiome refers to the microbial population that inhabits the oral cavity and has recently been recognized as a vital organ that contributes to oral and general health. This community of microorganisms forms a sophisticated ecosystem which has a mutual relationship with the host and is in a fine tune homeostasis for health. Dysbiosis which is the destruction of this balance has been associated with various oral and systemic diseases. Knowledge on the characteristics, roles and changes of the oral microbiome together with the effects of dysbiosis offers a deeper management of the conditions associated with it.

The oral cavity is considered to be one of the most complex microbial habitats in the human body, where more than 200 different species of bacteria and other microorganisms are present. These microbes are found in different sites such as the tongue, teeth, gingival sulcus and in the saliva and are exposed to different microenvironments. The oral microbiome is complex and consists of a number of bacterial species including those that are present in the tongue, teeth, gingival sulcus and saliva; the composition of the biofilm is modulated by diet, genetic background, age, oral hygiene and other environmental factors thus displaying inter subject variability[109].

The oral microbiome has many functions of which the most important ones are described below. The major responsibility of the microbiome is to provide protection against pathogenic microorganisms. By means of competition and synthesis of antimicrobial compounds the normal microflora counteracts the adhesion microorganisms[110]. The invasion of pathogenic interactions of the oral microbiome and the processes it carries out such as health degradation[111]. In addition, dietary components regulates oral microbiome by production of short chain fatty acids that are crucial in the response of oral and systemic immunity to both the normal flora and the pathogens. This regulation is to ensure that there is balance of the tissue and to avoid inflammation[112].

Dysbiosis of the oral microbiome occurs when there is an imbalance between the helpful and harmful microorganisms in the mouth. The causes of dysbiosis include; poor oral hygiene, diet, systemic diseases and the wrong use of antibiotics. The improper oral hygiene practices lead to the buildup of dental plaque which is a perfect medium for the growth of pathogenic bacteria including *Porphyromonas gingivalis* and *Fusobacterium nucleatum* for instance[113]. Carbohydrate-rich diets also worsen this ratio since they provide a suitable environment for the proliferation of the acidogenic and aciduric bacteria for instance *Streptococcus mutans* and lead to diseases like caries[114]. Other diseases of the body also affect the balance of microorganisms since they alter the immune responses and the metabolism of the tissues[115].

Antibiotic overuse therefore contributes to the disruption of the oral microbiome since it not only eradicates pathogenic organisms but also the beneficial ones thereby changing the microbial balance to favor the opportunistic pathogens[116]. This is a clear indication that the use of antimicrobials should be prescribed only when necessary.

Dysbiosis has far reaching implications. In the local sense it is involved in the formation of caries, periodontal disease and halitosis. Dental caries is caused by an increase in the number of acid producing bacteria that dissolve the tooth structure. Periodontal disease is a chronic inflammatory disease which occurs due to dysbiosis and presence of pathogens such as *Aggregatibacter actinomycetemcomitans*[117]. Halitosis or bad breath is a common oral condition that is usually as a result of sulfur compounds that are produced by certain bacteria when the oral environment is

dysbiotic[118]. Systemically, the dysbiosis in the oral cavity is associated with the cardiovascular diseases, diabetes, respiratory infections and adverse pregnancy outcomes. The inflammation of the biofilms in the mouth may lead to the shedding of bacteria and their products into the blood stream thus causing systemic inflammation and products atherosclerosis[119]. Moreover, oral microbiome dysbiosis as well as inflammatory responses may worsen such conditions as diabetes and respiratory diseases[120].

There are growing evidence that oral health is interconnected with the general health through oral dysbiosis. For instance, chronic periodontitis has been linked with cardiovascular diseases because of the spread of inflammatory mediators and microbial products through the bloodstream[121]. In a similar manner, dysbiosis has been associated with diabetes as the inflammation response that is characteristic of periodontal disease can itself lead to increased insulin resistance[122].

There are also emerging findings that show that oral dysbiosis may be connected with cancer. Bacteria such as *Fusobacterium nucleatum* has been linked with carcinogenesis as it is believed to enhance inflammation, DNA damage and modulates the host's immune response[123]. This association is also observed in oral cancers and other GI cancers which shows that dysbiosis in the mouth has systemic effects.

To prevent and manage dysbiosis, one has to use a number of strategies that consist of oral hygiene and dietary changes as well as specific therapeutic approaches. Therefore, brushing, flossing and cleaning of teeth by a dentist every now and then helps in reducing the buildup of plaque and thus helps in balancing the microbial flora of the mouth[124]. The intake of sugars should also be minimized and the intake of foods that help in the growth of good bacteria in the mouth such as fermented products with probiotics can also be of benefit[125].

There are certain probiotic supplements for oral health which have been found to be effective in maintaining proper the microbial population. These probiotics contain microorganisms that promote the growth of the helpful bacteria and at the same time inhibit the growth of the harmful bacteria thus minimizing the chances of dysbiosis and other diseases[126]. Antimicrobial agents, including mouth rinses and topical treatments, can be applied to control infections on specific areas. Nevertheless, such treatments should not eradicate the natural microflora in order to prevent the formation of another disorder[127].

There are novel strategies with nanotechnology to manage dysbiosis. There are nanoparticles like calcium peroxide and squalenyl derivatives that have been found to target biofilms efficiently and increase the effectiveness of standard antimicrobials. These particles possess inherent antimicrobial potential which also helps in enhancing the efficacy of antibiotics in eradicating biofilms thus leading to better therapeutic results[128].

Use of latest techniques including next generation sequencing and bioinformatics has enhanced the knowledge of oral microbiome to a great extent. These tools make it possible to analyze microorganisms present in a community, their roles and interactions, and therefore shed light on the processes that cause dysbiosis and diseases associated with it[129]. The fields like the personalized medicine and microbiome transplantation are growing rapidly, which provides hopes of creating specific management strategies to cater to the differences in the microbiome makeup.

There are however some challenges that are still unsolved in the understanding and control of the oral microbiome. This is a problem because there is no way to set up diagnostic and management guidelines that can be applied to each patient. Also, the effects of interventions such as the probiotics and the antimicrobial agents that were evaluated, require further investigation in the long run to establish the efficacy and safety. Multi-disciplinary research works in the fields of microbiology,

immunology and bioengineering are needed to solve such issues. For instance, in the design of antimicrobial therapy on normal flora, nanotechnology can be employed. Also, the manipulation of the microbiome through alterations in diet and the use of probiotics has the potential of being an efficient way of promoting oral health and preventing diseases that are as a result of dysbiosis.

The oral microbiome plays an essential role in the overall health and it does not only affect the oral cavity but also the rest of the body. Dysbiosis threatens this equilibrium and results in various diseases which demonstrate the interdependence of microbial and host systems. Some of the preventive measures that are effective in preventing dysbiosis include; proper oral hygiene, diet changes and the use of probiotics. New approaches in intervention such as nanotechnology based therapies provide new perspectives in managing dysbiosis as well as improving the therapeutic management.

1.3 Nanoparticles in biofilm treatment

1.3.1 Benefits in nanoparticle in biofilm context

Nanoparticles have been recognized as a viable way of combating biofilm related infections that are otherwise very hard to treat because of their antibiotic resistance. Biofilms are communities of bacterial cells that are encapsulated in a matrix of bacterial products; this biofilm provides a protective shield against host and immune antimicrobial responses agents including antibiotics. This protection leads to an increase in the bacteria's tolerance levels thus making biofilm related infections very hard to treat. Due to their small size and special physiochemical characteristic, nanoparticles have been proved to be effective in overcoming the above mentioned limitations and enhancing the antibiotic therapy.

Among the advantages of nanoparticles in biofilm treatment, the most important one is that they can get through the biofilm matrix more efficiently than the conventional antibiotics. Due to their small size which is usually between 1 and 100 nm, nanoparticles can move freely throughout the extracellular matrix and reach the bacteria present in the biofilm. This increased penetration leads to enhanced delivery of the drugs and targeted effects thus increasing the efficiency of eliminating biofilm-associated bacteria.

Nanoparticles can also increase the efficacy of antibiotics in numerous ways. For example, nanoparticles can act as vectors for antimicrobial agents as well as enhance the stability and bioavailability of the agents and hence achieve high local concentrations at the infection site. This can be very helpful when dealing with infections that are caused by antibiotic resistant bacteria. Due to the ability of the nanoparticles to carry the antibiotics, the effective concentration of antibiotics at the site of infection can be maintained at therapeutic levels in the bloodstream for longer durations and thus reduce the incidence of resistance and the need for high doses that may lead to toxicity.

Also, nanoparticles are capable of having direct antimicrobial actions through mechanisms for instance formation of reactive oxygen species that leads to damage of bacterial cells. There are some nanoparticles such as calcium peroxide or silver nanoparticles which have inherent antibacterial activity that can break the cell membrane or affect the cellular activities. When administered along with antibiotics, the nanoparticles are able to enhance the effects of the antibiotics known and as produce synergistic effect. This synergy is particularly helpful in treating biofilm associated infections since the dual exposure of nanoparticles and antibiotics can break up the biofilms and increase the sensitivity of the bacteria to the antibiotics.

Also, nanoparticles influence the bacterial resistance mechanisms, for instance, efflux pumps or biofilm formation genes. Some of the genes that are regulated in the negative sense by nanoparticles include those that are involved in biofilm formation or the oxidative stress response; this makes the bacteria to be more sensitive to antibiotics. This effect does not only develop resistance but enhance the effectiveness of drugs. Nanoparticles are effective in treating of the biofilms through various mechanisms that include; increasing antibiotic penetration, protecting drugs from degradation, generating ROS and altering genes and bacterial phenotypes. Their capability to enhance the effects of antibiotics makes nanoparticles a potential solution for managing the infections caused by biofilms especially those that are resistant to multiple antibiotics.

1.3.2 Calcium peroxide nanoparticles to generate oxygen

Calcium peroxide (CaO_2) is an inorganic compound which has recently been found to have the capability of decomposing in aqueous solutions to release oxygen. This property has made it useful in many areas such as medical treatments, dental uses and environmental restorations among others. When calcium peroxide is in nanoparticle form, it enhances some features that are useful in dealing with problems that are associated with biofilm related infections. Biofilms are compact communities of microorganisms that are surrounded by often an contain extracellular anaerobic matrix, zones. The oxygen releasing properties of calcium peroxide nanoparticles have therefore been seen as a potential way of dealing with these hard to eradicate biofilms.

Calcium peroxide is a white, non-hygroscopic, low-soluble powder that does not decompose at normal conditions. When it comes in close contact with water it dissolves and releases oxygen in a regulated way as shown below:

$$CaO_2 + 2H_2O \rightarrow Ca(OH)_2 + O_2\uparrow$$

This gradual release ensures oxygen release over a period of time thereby making calcium peroxide very useful where oxygen is a requirement and where there is little or no oxygen available. When made as nanoparticles calcium peroxide has enhanced solubility, reactivity and surface area which enables it to release oxygen at faster rates and also to have better contact with biological targets. These have positive impacts on biofilm erosion and therapy as well. The use of calcium peroxide nanoparticles to produce an aerobic atmosphere can alter the balance of microorganisms in biofilms and thus contribute to the complete removal of the anaerobic bacteria and improve the treatment results[130].

In the context of biofilms, calcium peroxide nanoparticles have the following advantages: Biofilms can develop and exist in hypoxic or even anaerobic environment which makes the bacteria not to be affected by antibiotics or the immune system. The oxygen released by calcium peroxide goes a long way in altering these conditions thereby affecting the biofilm's structure and functionality. Calcium peroxide has been extensively used in the management of wounds. Oxygen is an essential component in the body since it is used in cellular processes like angiogenesis, collagen formation and immune response among others. In the wound care products calcium peroxide nanoparticles release oxygen to the wound site thus helping in the healing of the hypoxic areas of the wound and at the same time reducing the risks of anaerobic bacterial infection. For instance, calcium peroxide loaded hydrogels have been used to treat wounds as they help in the reduction of the bacterial load and support tissue formation[131]. This is especially so in the case of chronic wounds which are known to contain biofilms that hinder the healing process.

Dental biofilms are a common problem in endodontic infections in which anaerobic bacteria are the predominant microorganisms that grow in the root in canal endodontics system. Calcium peroxide nanoparticles have oxygen generating capacity that helps in eliminating the microorganisms that thrive

in an anaerobic environment such as the root canal. The current literature shows that calcium peroxide is effective in eliminating bacteria in root canals, improving the disinfection process and therefore the treatment results. Furthermore, it is non-toxic, biocompatible and has sustained oxygen release making it suitable for use in delicate oral soft tissues[132].

Calcium peroxide nanoparticles are used in oral hygiene products like tooth pastes and mouth washes which shows the flexibility of the product. The oral hygiene products use the oxygen releasing property of calcium peroxide to eliminate the anaerobic bacteria that causes halitosis and other oral diseases. Oxygen interferes with the synthesis of volatile sulfur compounds by anaerobes and therefore enhances the breath odor and minimizes the biofilm accumulation in the oral cavity[133].

Not only is calcium peroxide used in medical field, it also has potential to be applied in environmental remediation, including the treatment of biofilms in industrial and environmental contexts. The oxygen generated from calcium peroxide enhances the growth of aerobic microorganisms that break down organic matter and other biofilm-associated hazards. For example, calcium peroxide has been applied in the treatment of oil spills and other hydrocarbon contaminated sites for the enhancement of biodegradation of the hydrocarbons[134]. It also aligns with the concept of sustainability thus making it valuable since it supports natural recovery mechanisms without the addition of toxic substances[135].

This is because with the help of nanotechnology the performance of calcium peroxide has been enhanced. Nanostructured calcium peroxide has improved solubility and thus its reactivity and therefore it is capable of releasing oxygen efficiently and in a controlled manner. This is particularly important in biofilm because targeted and sustained oxygen release can be used to disrupt the biofilm matrix and facilitate the action of antimicrobials. For instance nanoparticles are capable of reaching into the depths of biofilms, thus changing the anaerobic environment and the microbial metabolism[136].

There are studies that have been conducted to reveal the synergistic effects of using calcium peroxide nanoparticles along with other antimicrobial agents or biofilm dispersants. The following are the combinations that enhance the effectiveness of calcium peroxide in managing the various aspects of biofilm's polymicrobial tolerance. For instance, the combination of calcium peroxide and antimicrobial peptides has been reported to increase the potency of biofilms removal and prevention of recurrence[137]. Such combination therapies are especially important in the clinical practice especially when dealing with the issues of multidrug resistant biofilms.

New controlled delivery systems have been designed to manage the oxygen release from calcium peroxide nanoparticles. These systems provide a constant oxygen supply over time and thus supports aerobic conditions in the specific areas. In wound management, such formulations have been depicted to enhance the process of healing by regulating oxygen delivery to the ischemic tissues. In the environmental aspect, controlled release systems help in sustaining the effectiveness of bioremediation in the long run as the aerobic conditions are favorable for pollutant degradation[138].

One of the greatest difficulties in treating biofilms is the capability antimicrobial of and calcium oxidative peroxide stress nanoparticles resistance. The breaking down biofilms and improving the antimicrobial effects solves this problem. These nanoparticles release oxygen which breaks down the shielding role of biofilm matrix, increases the sensitivity of bacteria to antibiotics and facilitates the treatment of chronic infections.

1.3.3 Enhancing Antibiotic Delivery with Squalenyl Hydrogen Sulfate Nanoparticles

Pseudomonas aeruginosa is a gram-negative opportunistic pathogen which is difficult to treat in the chronic infections because of its biofilm forming capability. These biofilms provide a protective matrix where the bacterial communities are isolated from the antibiotics and the host's immune system hence reducing the therapeutic effects and encouraging the development of the antibiotic resistance. In situations such as cystic fibrosis, recurrent and persistent biofilm-associated infections are common and may become life threatening because of associated complications and lack of effective therapeutic options[139]. Tobramycin is an aminoglycoside antibiotic that is commonly employed for the treatment of infections caused by *P. aeruginosa*; however, it is not very effective in such a scenario because it cannot easily cross the biofilms and the bacteria has developed mechanisms to resist it[140]. Such challenges highlight the necessity for the development of new drug delivery systems that can breach these barriers and enhance the treatment efficiency.

Squalenyl hydrogen sulfate (SHS), another amphiphilic molecule with self-organizing properties has emerged as a possible answer to these challenges. SHS nanoparticles have the potential to incorporate the therapeutic agents such as tobramycin and quorum sensing inhibitors (QSIs) with enhanced drug stability[141]. This dual nature is advantageous for the function as a drug carrier since the lipophilic agents like QSIs can be incorporated in the lipid part while the hydrophilic part enhances the water solubility and stability of the system in the body fluids[142]. Hence, SHS nanoparticles are very versatile in the sense that they can be used to deliver a variety of therapeutics especially for biofilm related infections[143-146].

SHS nanoparticles are effective in addressing biofilm problems by using the following mechanisms. The nanometric dimension allows the particle to infiltrate the compact matrix of the biofilm and reach the bacteria. Also, they interact with both the hydrophilic and hydrophobic parts of the matrix since they are amphiphilic and thus ensure a constant release of the drug at the infection site. The encapsulation of therapeutic agents with SHS nanoparticles prevents them from degradation by enzymes and rapid clearance from the site of action hence sustained release is achieved. This is especially important for QSIs for instance, they need to be constantly effective in interfering with QS, a bacterial function that is essential for biofilm formation and stability[147-149].

Dr. Duy-Khiet Ho has contributed to the development of applying SHS nanoparticles in treatment of biofilm related infections. His work on the synthesis and characterization of amphiphilic squalenyl derivatives provided the necessary fundamentals for developing these materials as multi-functional drug carriers. In his study, Synthesis and Biopharmaceutical Characterization of Amphiphilic Squalenyl Derivative-Based Versatile Drug Delivery Platform, Ho explained how SHS nanoparticles could solve problems like drug instability in the bloodstream, low solubility of hydrophobic drugs and the inability of conventional drugs to penetrate biofilms. SHS nanoparticles were observed to increase the solubility of the drug, control the drug release and protect the encapsulated molecules thus enhancing the stability and bioavailability of the therapeutic agents[144].

In his subsequent work, the authors then developed SHS nanoparticles for the simultaneous delivery of tobramycin and an alkylquinoline QSI to overcome the biofilm infections of *P. aeruginosa* in burns. In this approach, Ho aimed at enhancing the delivery of tobramycin and QSIs to combat biofilm persistence and antibiotic resistance. The QSIs targeted the quorum sensing pathways which caused the dispersal of the biofilm and made the bacteria susceptible to tobramycin. The use of SHS nanoparticles in the encapsulation of tobramycin helped in achieving high concentrations of the drug at the site of infection while at the same reducing the toxicity that is seen when the drug is used

systemically. The following advantages over free drug combinations were observed; improved biofilm infiltration, decrease in bacterial number and enhanced antifungal effects[145].

Furthermore, in a collaboration with Christian Schütz, Ho presented the research on "A New PqsR Inverse Agonist Potentiates Tobramycin Efficacy to Eradicate *Pseudomonas aeruginosa* Biofilms" which demonstrated the symbiotic interactions between a new QSIs and antibiotics. This study revealed the development of a PqsR inverse agonist that interfered with the quorum sensing regulation thus, compromising the biofilm structure and increasing the permeability of tobramycin. The co-administration of these agents using SHS nanoparticles for delivery enhanced the eradication of biofilm and reduced the bacterial numbers regardless of the low antibiotic concentrations used. Not only did this synergistic effect enhance the therapeutic effects but also provided a solution to the major problem of antibiotic resistance[146].

SHS nanoparticles are known to have a rather short half-life and are not very stable when exposed to physiological conditions which is a major concern as to their further development. This behavior makes SHS nanoparticles to clump hence hampering their therapeutic effectiveness and bioavailability. To this end, polysorbates and Pluronic copolymers have been added to SHS to counter this limitation. These surfactants keep the nanoparticles from clustering and this is important in maintaining the size distribution and stability of the nanoparticles in the biological fluids. This stability also helps to enhance the shelf life of nanoparticles as well as provide a constant therapeutic effect in vivo[150-156].

Dr. Ho has played a great role in proving the relevance of such stabilization techniques. In his research, he has used surfactants in SHS to enhance the dispersion of nanoparticles, prevent their aggregation and enhance the efficiency of drug delivery. This way the nanoparticles are protected and maintain their functional properties when stored or circulated in the body thus enhancing their therapeutic utility[150, 157].

It is therefore important that the methods used in this study are extended and developed to be used by others. The current literature provided by Dr. Ho offers a step-by-step guide on how to synthesize and characterize SHS nanoparticles as well as how to optimize the hydrophilic-lipophilic balance, stabilize the nanoparticles and assess their efficacy on in vitro and in vivo models. It will be crucial for anyone to try to replicate and improve these processes in order to achieve reproducibility and scalability of SHS-based drug delivery systems for future clinical uses as well[144, 158].

The development of SHS nanoparticles for the management of biofilm related infections is a revolutionary leap in the treatment of bacterial resistance and persistent infections. The current study led by Dr. Duy-Khiet Ho has demonstrated the flexibility and effectiveness of these nanoparticles in circumventing biofilm hurdles thus offering a new approach to antibiotic and QSI delivery. With the improvement in the stability of SHS nanoparticles by using surfactants and enhancing the synthesis of the nanoparticles, the reproducibility of the study can be enhanced and the therapeutic application of this new platform can be extended.

1.3.4 Lipid nanocapsules for gram-positive bacterial treatment

The appearance of the antibiotic resistant Gram positive bacteria is a serious danger to the worldwide health. These pathogens, for instance *Staphylococcus aureus* and *Enterococcus faecalis*, are known to cause very severe infections and are life threatening especially in immunocompromised patients, and those with prosthetic devices such as artificial joints. Traditional antibiotics are not very effective against these organisms because of their ability to form biofilms and because these bacteria become resistant rather easily. To meet these challenges, lipid nanocapsules have been identified as a potential drug delivery system with improved antibiotic efficacy, stability and targeting. These novel carriers

have the capacity of transforming the management of Gram positive bacterial infections by dodging numerous barriers that are associated with conventional therapies.

Gram positive bacteria are known to have thick peptidoglycan cell wall as one of their characteristics which enhances the cell wall's rigidity and provides protection against stress factors such as antibiotics. This structural feature along with their capability to form biofilms- a matrix that houses the bacterial communities makes the Gram positive organisms difficult to treat with conventional approaches. Biofilm related infections are challenging to manage as the biofilm itself provides a shield that hinders the access of antibiotics and therefore the bacteria can exist in a quiescent state that is relatively unaffected by antimicrobials[159].

Lipid nanocapsules are the current solution to these problems. Here, antibiotics are conjugated to a lipid-based vector which improves the stability of the drug, increases its ability to penetrate biofilms, and delivers the drug to the bacterial cells. This approach not only enhances the therapeutic of effect antibiotics but also minimizes the adverse effects on the whole body and the potential for resistance[160]. Lipid nanocapsules are emulsions which contain a lipid core and are coated with a monomolecular layer of surfactants or phospholipids. This design is suitable for the encapsulation of hydrophilic as well as hydrophobic drugs and thus they are suitable for the delivery of a large number of antibiotics.

The lipid core which includes triglycerides, phospholipids or fatty acids. This holds the antibiotics and drugs in the core and allows slow degradation therefore helps in controlled and sustained release at the site of infection[160]. The lipid core is surrounded by a layer of surfactants or phospholipids that help the nanocapsules to be dispersed in the body fluids as well as to recognize the bacterial membranes. This surfactant layer may also contain targeting ligands for increasing selectivity towards Gram positive bacteria[161]. The size of lipid nanocapsules is usually between 50 to 200 nm and this size is suitable for the nanocapsules to penetrate through biofilms and engage with bacteria. Not only does this structure increase the efficiency of the delivery of the drug but it also reduces any side effects that may occur in the body making the treatment more effective[161].

The efficiency of lipid nanocapsules in the treatment of Gram-positive bacterial infections is based on the following mechanisms. First, these nanocapsules are able to cross the biofilms, which is very important due to the protective functions of biofilms during the course of infections caused by *S. aureus* and *E. faecalis*. Due to their small size and amphiphilic character lipid nanocapsules are capable of penetrating through a thick biofilm matrix and reaching the bacterial cells which are imbedded in it[162].

Secondly, targeting ligands coating increases the lipid selectivity from nanocarriers and can be functionalized for drug delivery. This targeted approach helps in reducing the delivered antibiotic[163]. Third, encapsulation within lipid nanocapsules increases the stability and also the pharmacokinetics profile of antibiotics. The managed launch of the drug permits for constant antibacterial action and helps to keep up the right concentrations within the physique for longer durations. This characteristic is quite important in the treatment of persistent infections as it minimizes the number of doses to be given and increases the patients' cooperation[164].

Based on numerous research works, lipid nanocapsules have been found to have a high possibility of enhancing the treatment of Gram-positive bacterial infections. In vitro experiments have demonstrated that the antibiotic loaded lipid nanocapsules including vancomycin and linezolid have increased bactericidal effect against *S. aureus* and *E. faecalis*. The findings of these studies indicate that the encapsulated antibiotics are able to cross the biofilms more efficiently resulting in the increased reduction in bacterial viability. For instance, lipid nanocapsules have been found to release

high levels of vancomycin into biofilms and thus produce better antibacterial results than the conventional formulations[165].

Using animal models, the group has also been able to support the effectiveness of lipid nanocapsules in vivo. The research has also shown that these nanocarriers enhance the clearance of bacteria from infected tissues than the standard antimicrobial therapy. Besides, the lipid nanocapsules have also been found to help in the treatment by shortening the duration of the therapy and decreasing the side effects on the systemic circulation, thus underlining their clinical applicability[166].

Furthermore, the use of antibiotics can be improved through the delivery of antibiotics using lipid nanocapsules to specific sites of infection. This is particularly beneficial in the management of form Gram-positive of infections drug since delivery this system minimizes the chances of having subtherapeutic drug levels that promote the development of resistance as seen with conventional doses[167].

Although lipid nanocapsules have numerous advantages, they need further advancement for their efficient use in clinical practice. Another important aspect that needs attention is formulation optimization. Improving the stability and the biocompatibility of the lipid nanocapsules by varying the composition of the lipid core and the surfactants will be crucial for their scalability and reproducibility as well[168].

Another approach is the use of lipid nanocapsules in combination therapies. The use of multiple antibiotics or the use of antibiotics alongside adjuvants such as biofilm inhibitors or quorum sensing inhibitors could potentially enhance the spectrum of activity and the therapeutic outcome against the multidrug resistant Gram positive organisms[169].

Also, there is a need for clinical trials to assess the risk, absorption, distribution and the general use of lipid nanocapsules in human beings. Although the preclinical research has been quite encouraging the success of such findings in the clinical trials cannot be established unless tried on patients with varied medical history[170].

Lipid nanocapsules are one of the most promising innovations in the current struggle against Gram-positive bacterial infections. The improvement in the delivery and therefore the effectiveness of the antibiotics that these nanocarriers provide solves most of the problems that are associated with traditional treatments. These include their ability to cross the biofilms, enhanced drug targeting and the ability to maintain the antibiotic activity, which makes them useful in the control of persistent infections from pathogens such as *S. aureus* and *E. faecalis*.

With the advancement of the research, the application of lipid nanocapsules in delivering combination therapies and minimizing the development of antibiotic resistance shows the significance of these nanocarriers in the current medical practice. However, there are still some issues regarding formulation and how to move from the laboratory works to the clinical practice, but lipid nanocapsules are a potential way to enhance the efficacy of the treatments, and provide the necessary amount of drugs to the infected area, which is advantageous in the treatment of Gram-positive bacterial infections.

Aim of the Thesis

The overall goal of this thesis was to develop and analyze the nanoparticle platforms that can help with the issues related to the biofilm-associated infections. Some of the challenges that have been identified include; hypoxic conditions, limited uptake of antibiotics into the biofilm matrix, and stability of the delivery systems. The work aimed at improving the effectiveness of the antibiotics as well as decreasing the amounts that are needed to be used, leading to the development of better cures that do not have severe side effects and resistance problems. Three types of nanoparticles were considered: Calcium peroxide nanoparticles, Squalenyl hydrogen sulfate nanoparticles and Lipid nanocapsules, which aimed at addressing various elements of biofilm treatment.

Calcium Peroxide Nanoparticles: Addressing Hypoxia and Oral Dysbiosis

The research question of this thesis was to discover how calcium peroxide nanoparticles (CaO_2 NPs) evolve and can be used to treat biofilms with particular emphasis on Pseudomonas aeruginosa biofilms. The research hypothesis of this study was that CaO_2 NPs could break down the hypoxic regions in biofilms and release oxygen and reactive oxygen species (ROS) in a controlled manner and thus, dissolve the biofilm structure and increase the susceptibility of biofilm to antimicrobial agents. It was also predicted that the calcium ions that would be released from these nanoparticles would have a twofold role; it would increase the biofilm dispersal and at the same time, would help the entry of tobramycin into the bacterial cells through calcium dependent cellular processes.

The main objective was to determine whether the simultaneous processes of oxygen release and calcium ion supply could increase the effectiveness of tobramycin against biofilms. This was done using biofilms of different ages including 48 h and 72 h biofilms which represented two different levels of biofilm development and potential for resistance. The combined use of CaO_2 NPs and tobramycin at increasing concentrations was evaluated in order to check if the nanoparticles could help in reducing the amount of antibiotic needed for biofilm eradication while enhancing the antibiotic effect.

The strategies involved in treatment with calcium chloride in order to determine the impact of calcium ions on the system and the use of oxygen loaded perfluorohexane liposomes to assess the effect of oxygen delivery apart from ROS production. Thus, the approach used in this study helped to disentangle the effects of oxygen, and calcium ions released by CaO_2 NPs on biofilm and the viability of bacteria by using a comparative analysis.

This research is significant due to the unique characteristics of calcium peroxide, a compound that reacts with water to release oxygen. When engineered as nanoparticles, calcium peroxide enhances solubility, reactivity and surface area through which oxygen release is effective even in dense biofilms. In biofilms, oxygen released from these nanoparticles alters the anaerobic conditions which are protective of bacteria against antibiotics more thus sensitive making to them treatment. Also, the ROS that are formed may also help in disrupting the structure of the biofilm and also affect the bacterial cells leading to enhancement of the antibiotic actions of the antibiotics that are co-administered.

The future use of this work is not limited to the treatment of biofilm related infections in the respiratory system. Calcium peroxide nanoparticles can also be effectively used in the treatment of oral biofilms which are known to cause diseases such as periodontitis and dental caries. In such situations, the regulation of oxygen levels and ROS output might help to balance the microbial flora, thus minimizing the imbalance and the consequent disease. Therefore, due to the ability of CaO_2 NPs to both, disrupt biofilms and increase the antimicrobial effect, they are a very promising solution to the problem of biofilm's resistance and antibiotic resistance.

This research is significant in adding to the current database of nanotechnology based strategies for biofilm control as it shows the role that CaO_2 NPs can play in improving the efficacy of treatment through the delivery of oxygen and calcium in a targeted manner. Through a structured analysis of the effects and a comparison with other oxygen and calcium delivery systems, this work provides a basis for future research and enhancement of CaO_2 nanoparticle based therapies(see Figure 1, 2).

Squalenyl Hydrogen Sulfate Nanoparticles: Stability and Biofilm Penetration

Following the work of Dr. Duy-Khiet Ho, this project aimed at developing amphiphilic polymer nanoparticles from squalenyl hydrogen sulfate (SHS) to improve the effectiveness of the therapy. Dr. Ho's study proved that SHS nanoparticles have the capability of being used in drug delivery system for the co-delivery of antibiotics for instance tobramycin and QSIs to treat P. aeruginosa biofilm infections. However, some issues like the reproducibility of the results and the stability of colloids were highlighted, which affected the nanoparticles' stability and performance. The primary aim of this project was to enhance the effectiveness of SHS nanoparticles in treating biofilm infections by addressing the challenges identified in Dr. Ho's research. This included the enhancement of the nanoparticle platform to enhance the stability, drug loading efficiency, consistency and the ability of the nanoparticles to penetrate the biofilm. The other key aim was to assess the efficacy of nanoparticle based drug delivery in the prevention/eradication of biofilm and thus, patient outcome (see Figure 3).

In order to overcome these limitations, this study also examined the potential of using surfactants in the formulations of SHS nanoparticles. The hypothesis was that the aggregation could be prevented with the help of surfactants to increase the colloidal stability of the nanoparticles and to ensure their functional integrity under different conditions. Surfactants were also expected to enhance the biofilm penetration as they are amphiphilic in nature and can interact with the hydrophobic as well as hydrophilic parts of the biofilm matrix. This property was expected to enhance the delivery of antibiotics such as tobramycin to the biofilms since the production of the biofilms is usually associated with reduced antibiotic efficacy.

In order to check whether the stabilizing agents such as polysorbates and vinyl polymers helped in preventing aggregation or not and to ensure that the modifications made on the nanoparticles were indeed to maintain the nanoparticles within the desired size. The efficacy study was conducted to encapsulate the antibiotics and to develop a stable and controlled release system.

This project was quite relevant in the context of *Pseudomonas aeruginosa* which is a Gram negative bacteria that is capable of forming biofilms. These biofilms provide a shield for the bacteria making it difficult for the antibiotics to penetrate through and thus reducing the therapeutic effect. When it comes to tobramycin's effectiveness in such environments it is usually reduced due to lack of tissue penetration and bacterial resistance. Due to their self-assembly and encapsulation capability for both lipophilic and hydrophilic molecules, SHS nanoparticles are believed to be a potential answer to these issues.

The study also established that the addition of surfactants not only improved the stability of SHS nanoparticles but also enhanced their efficiency in penetrating through biofilms. From the work of Dr. Ho, this development has taken a step further to optimize the composition and the possible application of SHS nanoparticles in the management of biofilm related infections.

Lipid Nanocapsules: Size-Dependent Biofilm Penetration

The second side project was on lipid nanocapsules (LNCs) as a potential vector for biofilm penetration and an efficient vehicle for antibiotic delivery, and the effect of particle size on such that processes are was available investigated. These nanoparticles were chosen because of their accurate, well-

characterized, controlled and reproducible fabrication methods, tunable size, and also their capability to load both water soluble and water insoluble drugs. This flexibility of LNCs made them a perfect candidate for antibiotics such as vancomycin, a very important drug used in the treatment of Gram positive bacteria and which has a difficulty in penetrating into biofilms when given in its free form.

The research hypothesis predicted that smaller LNCs would show better biofilm invasion than the larger ones. This hypothesis was made on the basis that smaller particles would have less steric hindrance and better diffusivity to engage with the biofilm matrix. For this purpose, *Staphylococcus aureus* was selected as a model organism because it is a Gram-positive bacterium that is known to form biofilms and is commonly associated with implant-related infections. The present study highlighted the importance of biofilms in reducing the effectiveness of antibiotics through providing a physical and chemical shield that protects the enclosed bacterial cells from the antibiotic action.

Vancomycin was chosen as the encapsulated drug due to its ability to treat *S. aureus* infections and the fact that it only provides a certain level of effectiveness against biofilm related infections due to poor tissue penetration. The experimental design consisted of the preparation and characterization of LNCs of different sizes and the assessment of their interactions with *S. aureus* biofilms in order to analyze the effect of particle size on the penetration and antibiotic efficacy. The study aimed at determining the best LNC size that would enhance the therapeutic effects, the hypothesis being that increased drug delivery within the biofilm would be achieved by using small nanoparticles thus enhancing the treatment outcome.

The general goal of this work was to assess the possibility of LNCs as a novel carrier to navigate the issues of Gram positive bacterial infections with particular emphasis on the biofilm forming ones. Antibacterial resistance among Gram positive organisms is a growing concern with potentially life threatening consequences in immunocompromised patients and those with prosthetic devices. These bacteria are known to have thick cell walls made of peptidoglycan and are also very efficient at forming biofilms which makes them difficult to treat with conventional antibiotics. Following the work of Dr. Matha and Tobias where LNCs were developed and fine tuned, this study aimed at determining whether LNCs could enhance the stability, biofilm diffusivity and therapeutic effectiveness of the encapsulated antibiotics.

Nano systems were prepared with lipid core anchored by other components like Labrafac, Kolliphor and Span 80. These components were selected based on their compatibility and their ability to prepare stable nanoparticles with reproducible quality. The size of LNCs is in the range of nanometers (nm) that is between 25 and 100 nm and this size is appropriate to go through the biofilm matrix and release the antibiotics in close proximity to the bacterial cells that are covered by the extracellular polymeric substance. It was expected that the encapsulation of vancomycin into LNCs would improve the drug's therapeutic index through sustaining the effects of the drug, controlling the release profile and maintaining high levels at the infection site. In this way, it was intended to address some of the problems associated with the use of free drug forms including toxicity and side effects (see Figure 4).

This project focused on the issue of biofilm associated *S. aureus* infections which is one of the most common type of infections which are recurrent and implant related. This is due to the fact that biofilms reduce the effectiveness of treatments since they have a matrix that prevents the diffusion of drugs and therefore the bacterial communities persist. Due to the amphiphilic nature and the ability to control of the LNCs, size it was expected that their use would enhance the delivery of antibiotics with the assumption that smaller LNCs would enhance the penetration and distribution of the antibiotics within the biofilms. The outcomes of this study are anticipated to help in the development of the optimal LNC formulation for managing the biofilm-associated infections as well as the enhancement of the use of LNCs as a novel drug delivery system.

Therefore, the purpose of this thesis was to identify ways of improving the nanoparticle-based treatment of the biofilm-related infections. The work was developed based on hypotheses that stemmed from the capacity of nanoparticles to break through biofilm barriers, enhance the delivery of antibiotics and thus, overcome the limitations to treatment. Calcium peroxide nanoparticles were developed with an aim of reducing hypoxia and increasing the efficacy of antibiotics while also having the potential to treat oral dysbiosis. Squalenyl hydrogen sulfate nanoparticles were also developed to be more stable and to provide better reproducibility with enhanced ability to penetrate biofilms. Lipid nanocapsules were also assessed for their ability to penetrate and eradiate Gram positive bacterial biofilms based on size and drug delivery system. All these approaches offer a base for new and more rational therapeutic management of biofilm related infections.

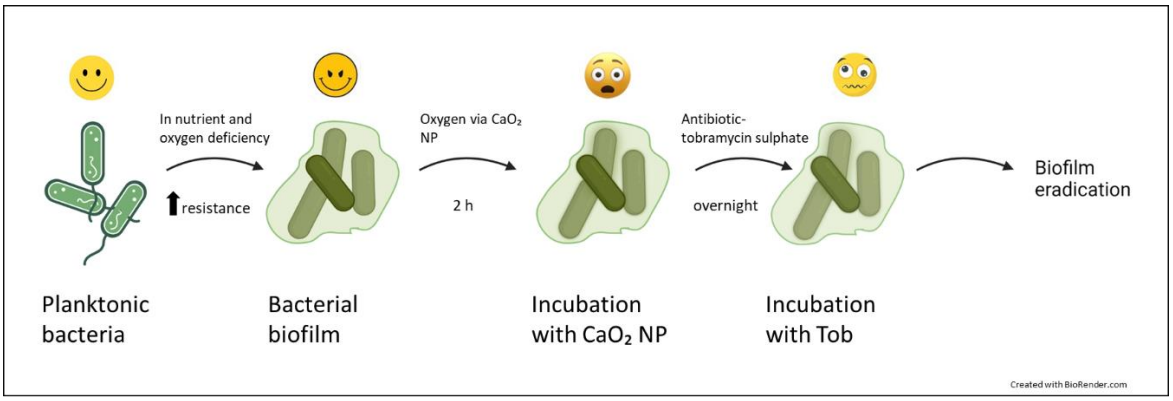


Figure 1 Schematic representation of hypothesis in treatment of in vitro Pseudomonas aeruginosa PAO1 biofilms with calcium peroxide nanoparticles and tobramycin

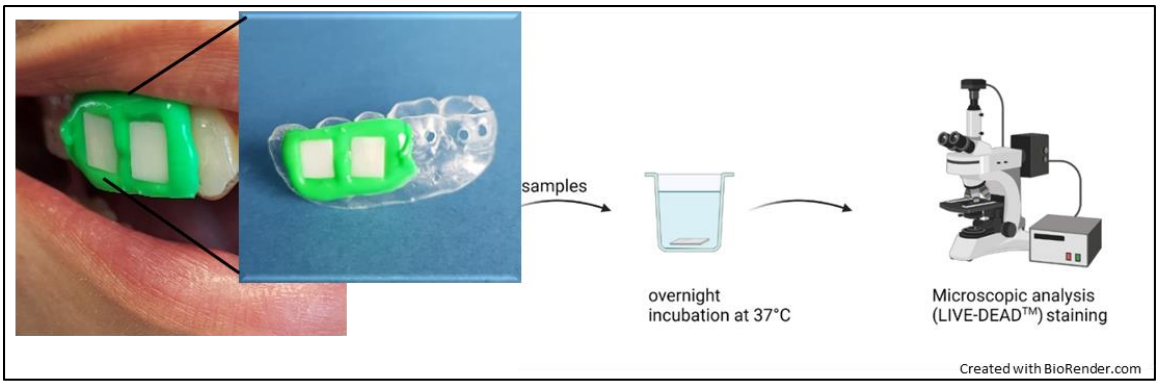


Figure 2 Schematic representation of hypothesis in treatment scheme of in situ grown oral biofilms from human volunteers- treated ex situ (images adapted from Bankar N. et al. [176])

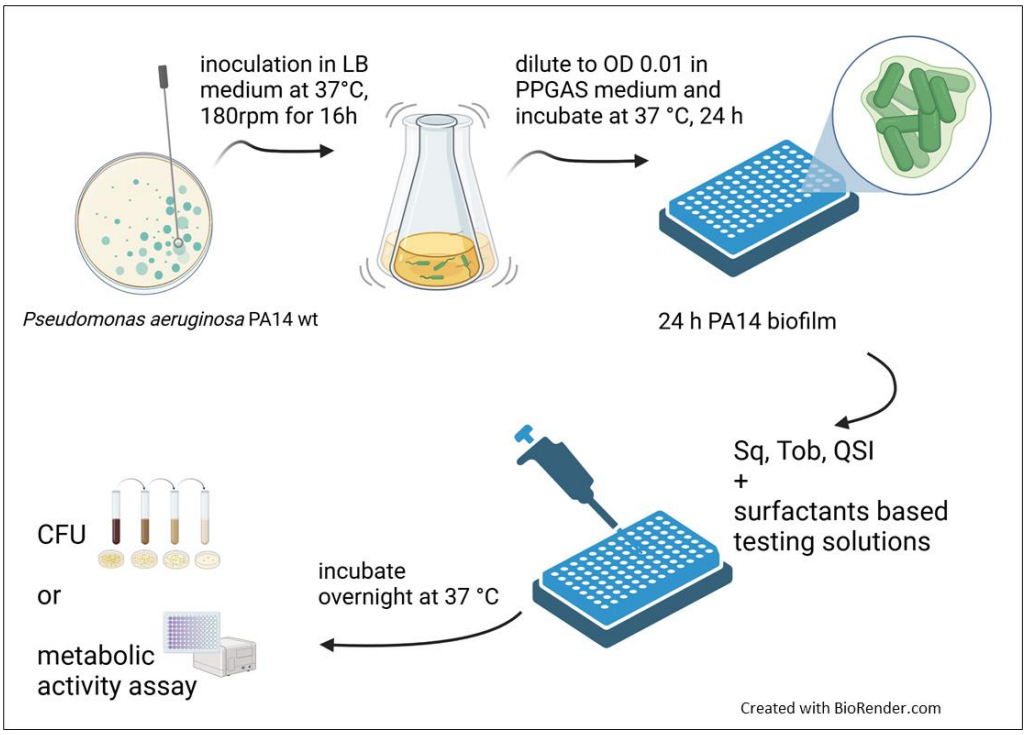


Figure 3 Schematic representation of hypothesis for treatment scheme of squalenyl hydrogen sulphate, tobramycin, QSI containing nanoparticles on in vitro Pseudomonas aeruginosa PA14 biofilms

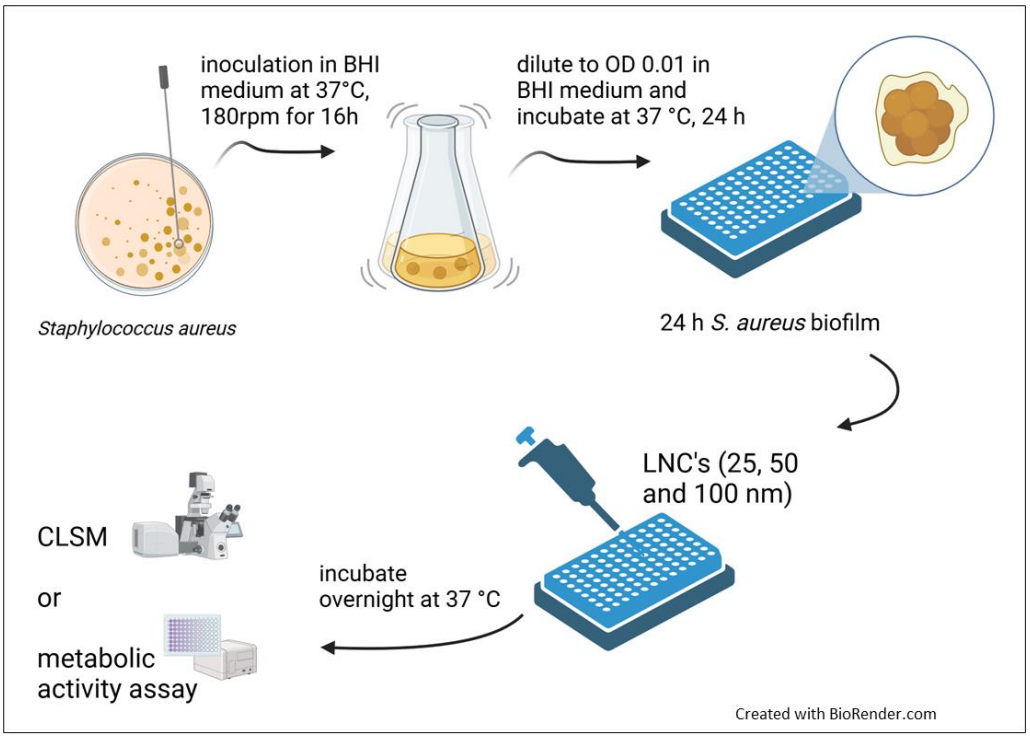


Figure 4 Schematic representation of hypothesis for treatment scheme of LNC's on in vitro S. aureus biofilms

Methodology, results and discussion

3.1 Particle preparation and characterization

3.1.1 Calcium peroxide nanoparticles

- **Material**

Calcium chloride obtained from BDH® prolabo VWR chemicals (Darmstadt, Germany), ammonia solution, 25 % obtained from Suprapur®, Supelco (Merck KGaA, Darmstadt Germany), and hydrogen peroxide, 35 wt % solution in water was obtained from Acros Organics™. Fetal calf serum (FCS), trypsin, RPMI 1640 cell culture medium, DMEM (low glucose, GlutaMAX™ Supplement, pyruvate) (Gibco™) cell culture medium, SYBR green real-time qPCR master mix (SYBR MM), RNAProtect® bacteria reagent Qiagen, were all brought from Thermo Fisher Scientific (Darmstadt, Germany), tobramycin sulphate (salt), PEG 200 (P3015), polyvinylpyrrolidine (PVP, mol wt 10,000), polyacrylamide (PAA, mol wt 8000), glutaraldehyde 25 % solution in water, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT), paraformaldehyde, luria bertani (LB) broth and agar, hexamethyldisilazane (HMDS), PBS (Dulbecco's Phosphate buffer saline), NaOH pellets, ethanol (99.95 % *v/v*) were all brought from Sigma-Aldrich (Merck KGaA Darmstadt, Germany), BreathSeal foils were brought from Greiner Bio-One (Frickenhausen, Germany), paraformaldehyde 16 % aqueous solution EM Grade (Electron Microscopy Sciences), qScriber™ cDNA synthesis kit from highQu, RNeasy® Micro Kit from Qiagen, TURBO™ DNase kit, Live/Dead® BacLight™ Bacterial Viability kit L7012 and L7007 were all brought from Invitrogen, Thermo Fisher Scientific (Darmstadt, Germany). Poly-lactic-co-glycolic-acid (Resomer RG 503 H) was received from Evonik Industries (Darmstadt, Germany), the average particle size as determined by dynamic light scattering (DLS), Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, U.K.) was 231 ± 46 nm, PDI < 0.3.

Cell procurement

A549 cells (human lung adenocarcinoma cells) (ATCC) were obtained from the American Type Culture Collection. Gingival fibroblast cells- human origin were obtained as immortalized cells from patients. *P. aeruginosa* PAO1 (DSM No. 22644, DSMZ, Braunschweig, Germany) was used.

- **Method of preparation**

Optimization of size

- Preparation of calcium peroxide nanoparticles (CaO_2 NP) with PEG 200
  To optimize the size and calcium peroxide nanoparticles were prepared from the method adapted from Sheng et al. and Rastinfard et al.[171, 172]. 3 g calcium chloride was dissolved in 30 mL distilled water, which was further mixed with 15 mL ammonia solution and 120 mL PEG 200. In this mixture, under stirring at 800 rpm with a speed of 100 µL/min 35 % hydrogen peroxide was added dropwise. The precipitation of these particles was performed by addition of sodium hydroxide solution (pH 13), until the product pH was 11.5[173]. To separate the precipitate, the product was filtered using a filtration assembly with vacuum pump, for faster precipitation. Later the precipitate to remove excess PEG, was washed twice with water and filtered. The particles were kept in hot air oven at 50 ˚C for drying.
- The method described above was further modified for faster separation of calcium peroxide nanoparticles. Here, 1 g calcium chloride was dissolved in 10 mL distilled water. Which was further mixed with 5 mL ammonia solution and 40 mL PEG 200. In this method the stirring speed was kept at 750 rpm, and the addition of hydrogen peroxide was performed at a speed of 50 µL/min. The

washing after precipitation involved washing twice with sodium hydroxide and once with water. Here to separate the precipitate, centrifugation at 7417 g for 7 min was performed.

- The method was further changed to remove excess washing steps of the precipitate, and was washed only once with sodium hydroxide and once with water.
- Freeze drying was performed for separation of particles, and drying of the particles. Which was further modified by addition of cryoprotectants like mannitol and PVP (mol. wt. 10,000).
- The method of drying of the precipitated particles was further modified to using vacuum oven at 60 ˚C overnight and 80 ˚C for 2-3 h.
- For further optimization of oxygen release the washing of particles was done using ethanol.
- Calcium peroxide nanoparticles with PAA
  The preparation method was adapted from above-described methods, with the change of surfactant stabilizer to PAA.
- Calcium peroxide nanoparticles with PVP
  The preparation method was further adapted to addition of surfactant stabilizer PVP.
- Calcium peroxide nanoparticles with PEG 400
  The method used with PEG 200 as surfactant stabilizer was further modified by using PEG 400, the stirring speed was decreased to 650 rpm. And the centrifugation method was modified to 5000 rpm.

Optimization of preparation temperature

Further a comparison between surfactant stabilizer PEG 200 and PEG 400 was performed and the method was finally modified as described below,

To prepare calcium peroxide nanoparticles, 16 mL PEG 200 and 2 mL ammonia solution were added to a stirred solution of 0.4 g calcium chloride in distilled water (~2 mL); this mixture was heated at 70 °C. Under continuous heating and stirring conditions of 750 rpm, addition of 2 mL H_2O_2 at a rate of 50 µL/min was performed. To precipitate the nanoparticles, pre-heated at 50 °C, 0.1 M NaOH solution was added till the mixture pH was 13. Precipitate was immediately centrifuged at 5000 rpm for 5 min and supernatant was discarded. To remove excess PEG200 residue, precipitate was washed three times with 10 mL NaOH solution: distilled water (1:1); centrifuged each time at 5000 rpm for 2 min. The product was dried in a vacuum oven at 80 °C, 0 mbar for 3 h.

Optimization of oxygen release

The above prepared nanoparticles were coated with polymers like PVP (K30), pluronic, and chitosan. The dried nanoparticles were dispersed in ethanol and added dropwise (60 µL/min) to solutions of polymers. A ratio of 1:3 was used between particles and polymer solutions. An appropriate solvent was used to dissolve the polymers (pH 3.5 acetic acid for chitosan, and water for PVP and pluronic).

Spray-drying of the nanoparticles mixed with the polymer solutions was performed using a Büchi-90 nano spray dryer (Flawil, Switzerland), the mixture was spray-dried under the following conditions (gas fow 112 L/min, frequency 122 kHz, inlet temperature 87 °C, outlet temperature 35 °C, pump 30%, spray 80%, pressure 37–38 mbar, and room humidity 20–30%).

After preparation of the particles, they were characterized for size, morphological characteristics, oxygen and peroxide release, cyto-toxicity, FTIR spectral analysis.

- **Method of characterization**

For determination of size

Calcium peroxide nanoparticles were dispersed in ethanol and the size was initially analyzed on the Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, U.K.) using laser scattering. The particles were diluted at different ratios of 1:100, 1:1000 for automatic attenuator setting between 5-7.

In the later, size was determined using the Horiba LA-950V2 Laser Scattering Particle Size Distribution Analyzer (Retsch GmbH, Haan, Germany). The particles were dispersed in ethanol and sonicated using an ultrasonic probe Sonicator S-250D model (Branson Ultrasonics, USA) with 10 % amplification intensity for 20-30 s.

For morphological analysis

The morphology and size of particles was determined using the scanning electron microscopy (SEM). Calcium peroxide nanoparticles were dispersed in ethanol, a double-sided carbon tape was placed on the SEM stubs and a glass-shard was placed on it. A drop of ethanol dispersed particles was placed on the glass and this was allowed to dry completely. After the sample was dried, gold sputtering was performed in QUORUM Q150R ES, (Pfungstadt, Germany) pumped coater under vacuum under the program QT timed gold. Images in the EVO HD15 microscope (Zeiss, Oberkochen, Germany) were obtained.

For oxygen release analysis

The oxygen release from the particles was analyzed using the dissolved oxygen meter (sensor- InLab OptiOx, Mettler-Toledo AG, Analytical, Schwerzenbach, Switzerland)[174]. For determination of the dissolved oxygen of water, 20 mg of particles were initially placed in a small beaker which was supplemented with 20 mL distilled water and the oxygen concentration of the water was determined at several time points for 2-3 h. The method was later modified by using deoxygenated water, prepared by bubbling nitrogen for 2-3 min. The oxygen concentration of deoxygenated water at the beginning of the experiment was measured to be around < 1 mg/L.

The method was further developed to avoid interference from environmental oxygen, here with the help of R-300 Rotavapor (Büchi, Flawil, Switzerland), samples were placed in a 2-necked round bottom flask and at 32 rpm and 150 m bar vacuum, oxygen release was measured at several time points over a period of 4 h.

For peroxide release analysis

The intermediate peroxide release from calcium peroxide nanoparticles was determined using Pierce™ quantitative peroxide assay kit. A calibration curve for hydrogen peroxide between the concentrations 7.5 to 90 µM/mL was generated. The concentration of peroxide released from calcium peroxide nanoparticles was calculated based on the absorbance observed at 595 nm.

For cyto-toxicity assay

Cyto-toxicity of calcium peroxide nanoparticles was determined on A549 cells and gingival fibroblast cells.

The effect of CaO_2 NP on A549 cells, MTT assay was performed. 10,000 cells per well were cultured at 37 °C in a 5 % CO_2 incubator in RPMI 1640 medium containing 10 % FCS for 3 days in a 96 well plate. 100 µL samples with increasing concentration of CaO_2 NP (8, 16, 32, 48, 60, 72, 96, 108 µg/mL) were placed on cells in HBSS buffer, after 4 h cells were washed and treated with MTT reagent (450 µg/mL) for 3 h. DMSO was added to each well and absorbance at 550 nm was recorded. Analysis of controls with 1 % Triton-X in HBSS for complete cell death as positive control, and only HBSS buffer for 100 % viability of cells as negative control were also performed.

Later similarly this effect was analyzed on gingival fibroblast cells. 10,000 cells/ well were cultured at 37 °C in a 5 % CO_2 incubator in DMEM medium (low glucose, GlutaMAX™ Supplement, pyruvate) containing 10 % FCS for 2 days in a 96 well plate. 100 µL samples with increasing concentration of CaO_2 NP (1, 8, 16, 32, 64, 96, 128, 1000 µg/mL) in HBSS buffer, after 4 h cells were washed and treated with MTT reagent at concentration 450 µg/mL for 3 h. DMSO was used to dissolve the formazan and absorbance at 550 nm was recorded. Analysis of positive controls with 1 % Triton-X in HBSS for complete cell death, and negative controls with only HBSS buffer for 100 % viability of cells were performed. The % cell viability was measured based on the following equation[176],

$$\%\ cell\ viability = \frac{A_{sample} - A_{positive\ control}}{A_{negative\ control} - A_{positive\ control}}$$

For Fourier Transform Infrared Spectroscopy (FTIR) spectral analysis

The particles were analyzed with FTIR spectral analysis. Here, particle powder was placed on sample holder on the crystal of FTIR (Vertex 70 Frontier Optical FTIR Spectrometer, PerkinElmer, Hamburg, Germany) and surface molecular bond structures were determined with respective transmittances analyzed from 4000 to 650 wave numbers.

- **Results**

Size determination

The initial size determination for calcium peroxide nanoparticles was performed using Zetasizer Nano-ZS. Particle batches prepared using different drying methods, and surfactants were dispersed in ethanol and at dilution where the attenuator was adjusted automatically between 5 and 7 were measured. The measurement was performed in triplicates and the average of the three readings was considered for average size determination.

It was observed that the Z-average in size determination (see Table 1) was between 200 to 5000 nm, also having a high polydispersity index ≥ 0.5. It could be due to higher degree of aggregation observed due to electrostatic attraction in calcium containing particles[175].

Batch	Method	Z-ave (nm)	PdI
1	Vacuum filtered	582	0.5
2	Lyophilized nanoparticles	4969	1.0
3	Vacuum oven dried	354	0.2
4	PEG 400 as surfactant	1677	0.5
5	Heating reaction mixer	370	0.1

Table 1 Size determination of CaO_2 NP by Zetasizer Nano-ZS

It was observed that the particles prepared using PEG 200 and method involving heat stabilizing the reaction mixture produced particles with lesser aggregation. However, the size determination using Zetasizer did not provide conclusive results between similar batches.

The size was observed under microscope, the nanoparticles were aggregated, which was measured in the Zetasizer and observed as greater particle size.

The size determination was also performed using the Horiba LA-950V2 Laser Scattering Particle Size Distribution Analyzer. The samples were diluted in ethanol and sonicated before analyzing on the instrument.

Batch	Diameter (10 %) nm	Diameter (50 %) nm	Diameter (90 %) nm
1	75	112	168
2	78	122	191

3	72	106	160

Table 2 Size determination of CaO_2 NP by Horiba LA-950V2 Laser Scattering Particle Size Distribution Analyzer

When the calcium peroxide nanoparticles were prepared using PEG 200 as the surfactant, and the reaction mixture was heated to make the degree of precipitation and aggregation lower[174], the nanoparticles were better stabilized. When analyzed for size, median size of the calcium peroxide nanoparticles was found to be around 120 nm. The particles were further analyzed for morphological characteristics using the SEM- microscopical analysis.

Morphological characterization

The morphological characterization of calcium peroxide nanoparticles was conducted using the scanning electron microscopy. It was observed that the particles were spherical and aggregated. Different batches prepared with different method of preparation were observed under the microscope, but were observed as aggregates of smaller particles.

It was observed that when particles were diluted at a ratio more than 1:1000 in ethanol, it dissolved the PEG 200 and caused higher aggregation of particles and salt or polymer deposits (see Figure 5c).

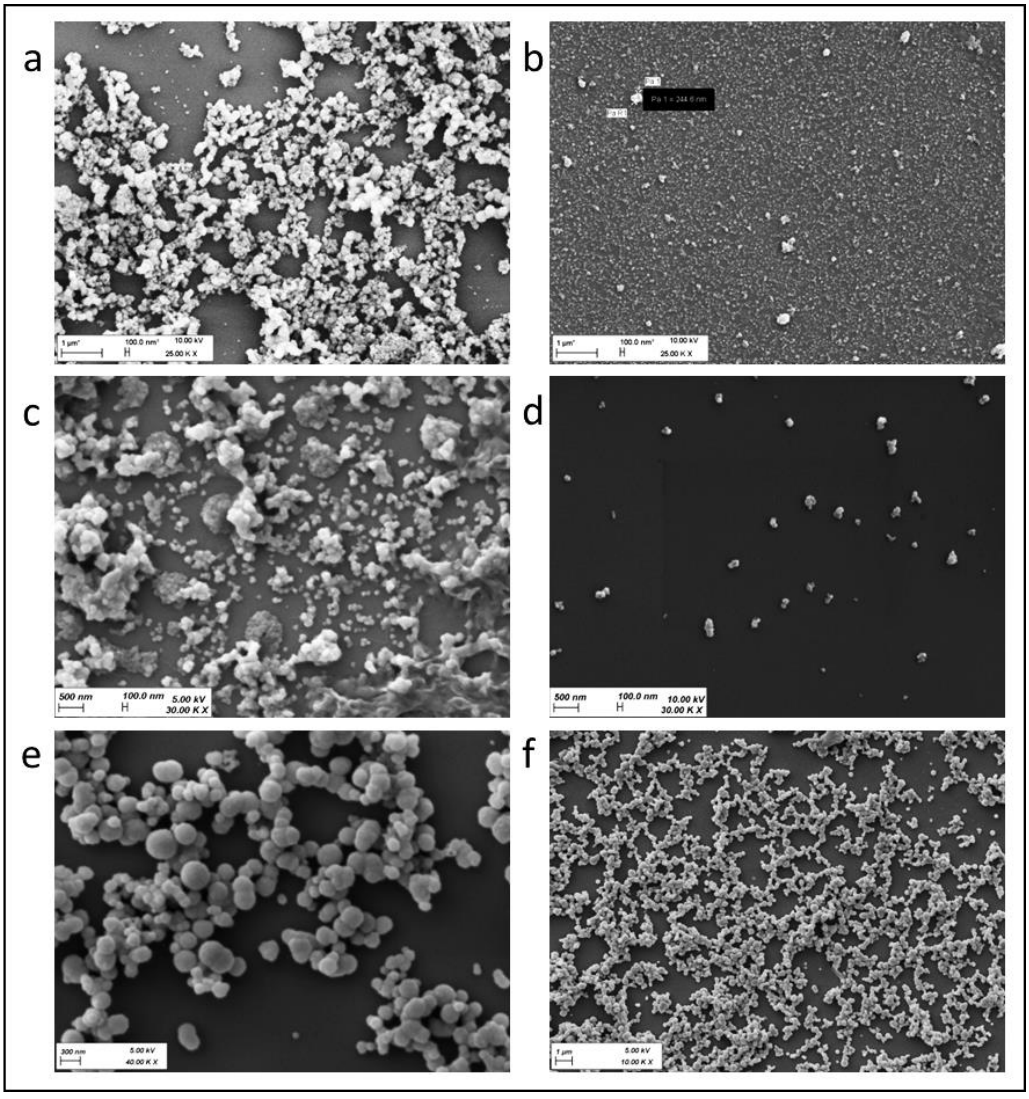


Figure 5 SEM morphological analysis of calcium peroxide nanoparticles, (a-f) representation of different batches of particles (adapted from Bankar N. et al. [176])

It was observed from (see Figure 5a) that the particles dried using vacuum oven when over-washed were spherical, separated and smaller in size. However, the particles prepared after heating the reaction mixture (see Figure 5d) were smaller aggregates and the particle size of the aggregates was around 120 nm.

Oxygen release studies

The calcium peroxide particles released oxygen upon contact with water, which was measured by the dissolved oxygen meter. It was observed that when the oxygen release was measured without deoxygenation of water, the maximum oxygen increase observed was 13 mg/L (see Figure 6).

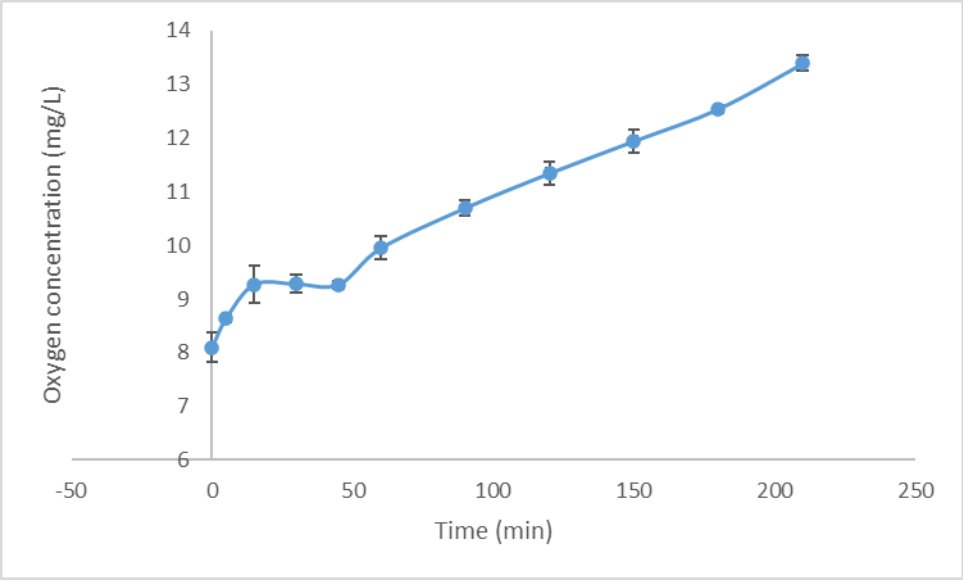


Figure 6 Oxygen release from calcium peroxide nanoparticles (without deoxygenation of water)

The method was optimized further using rotary evaporator for oxygen measurement. Nitrogen bubbling removed the dissolved oxygen from water and the initial oxygen concentration for the release studies was adjusted below 1 mg/L. It was observed that the oxygen release from the calcium peroxide nanoparticles was nearly 9 mg/L over a period of 4 h (see Figure 7). Additional experiments were conducted to observe the oxygen concentration changes using a non-oxygen releasing material such as the powder of polymer PLGA. The PLGA polymer after dissolving in water were not able to increase the oxygen concentration of water above 2 mg/L and thus was considered as baseline for these experiments.

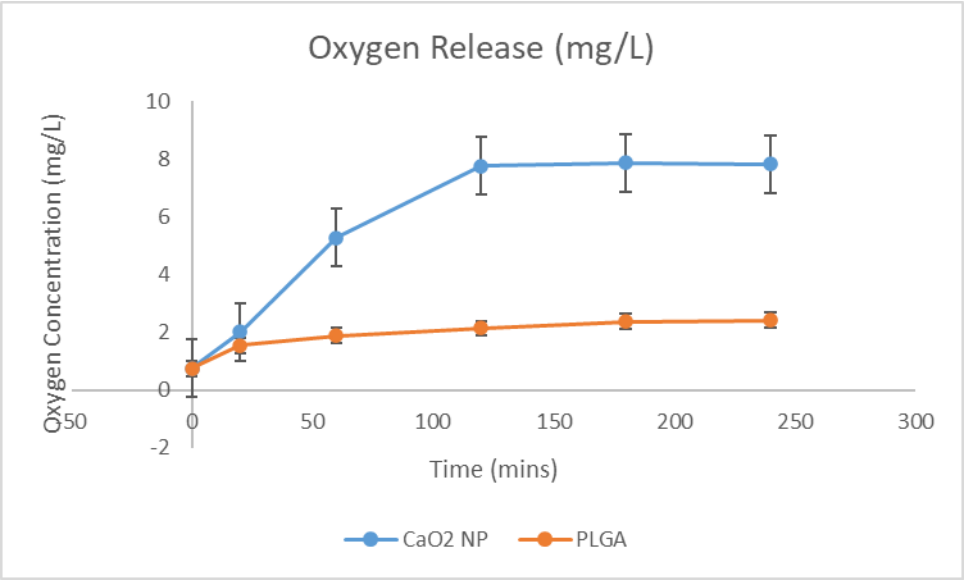


Figure 7 Oxygen release studies of calcium peroxide nanoparticles, compared with a non-oxygen releasing polymer PLGA (dissolved in water)

The non-oxygen releasing control showed a plateau release of oxygen, with oxygen concentration of around 2 mg/L. However, the calcium peroxide nanoparticles increased the oxygen concentration gradually for 2 h, and reached a plateau at the concentration of 8 ± 1 mg/L (see Figure 7).

Peroxide release studies

Hydrogen peroxide at different concentrations were analyzed for absorbance after reaction with the Pierce™ quantitative peroxide measurement kit. The calibration curve for this was plotted (see Figure 8). And an equation for slope determination was obtained. From this equation the intermediate peroxide released from calcium peroxide nanoparticles was measured.

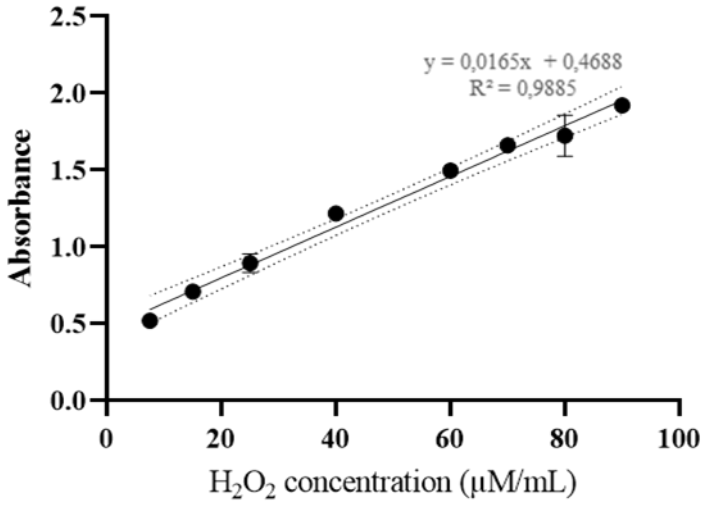


Figure 8 Calibration curve of hydrogen peroxide for determination of peroxide release from calcium peroxide nanoparticles (adapted from Bankar N. et al.[176])

After calculations it was observed that the calcium peroxide nanoparticles at the concentration of 50 µg/mL, were able to release 49 ± 20 µM/mL hydrogen peroxide.

Cyto-toxicity assay

The calcium peroxide nanoparticles were observed for biocompatibility and cyto-toxicity. They were initially tested on the A549 cells.

MTT assay was used to determine the lethal concentration of the calcium peroxide nanoparticles. It was observed that the cytotoxicity was concentration dependent. The particles tested on 72 h grown A549 cells, showed the lethal concentration 50 % at around 60 µg/mL concentration. The concentration below 60 µg/mL were considered for further experiments on bacteria.

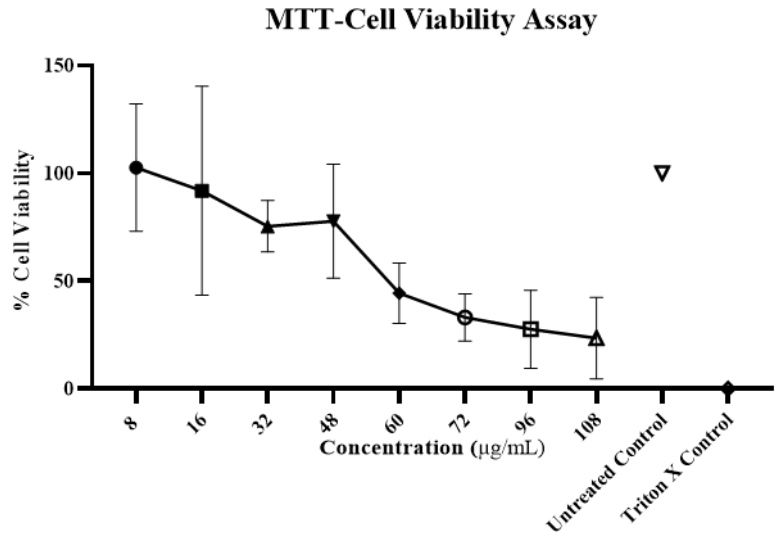


Figure 9 Cyto-toxicity studies by MTT assay performed on A549 cells cultured for 72 h

Further experimental studies, resulted in studying the biocompatibility of the calcium peroxide nanoparticles on gingival fibroblast cells. The cyto-toxic effects were concentration dependent. When tested on 48 h grown fibroblast cells the lethal concentration 50 % was observed to be around 16 µg/mL.

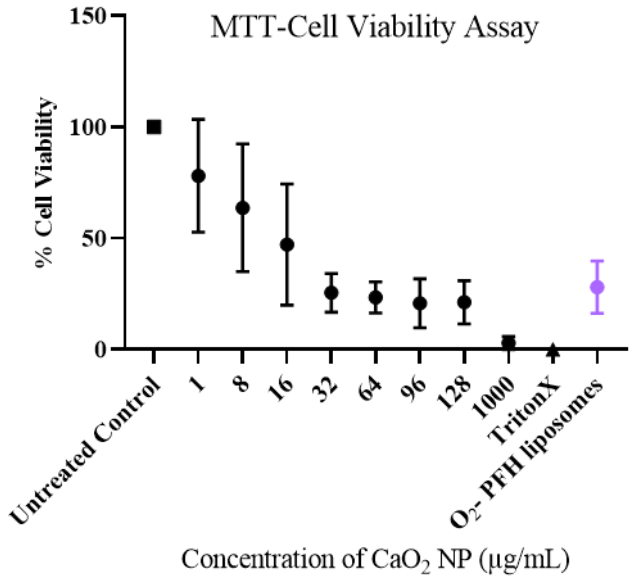


Figure 10 Cyto-toxicity studies by MTT assay performed on gingival fibroblast cells cultured for 48 h (adapted from Bankar N. et al.[176])

It was observed that the cyto-toxicity was concentration dependent, and depending on the cell line and time for which cells were cultured, the toxic effect was higher on gingival fibroblast cells.

FTIR analysis

FTIR analysis was performed on the calcium peroxide nanoparticles for surface group characterization. The salt calcium chloride used in preparation of calcium peroxide nanoparticles, was observed for stretches in functional groups and compared with the nanoparticles.

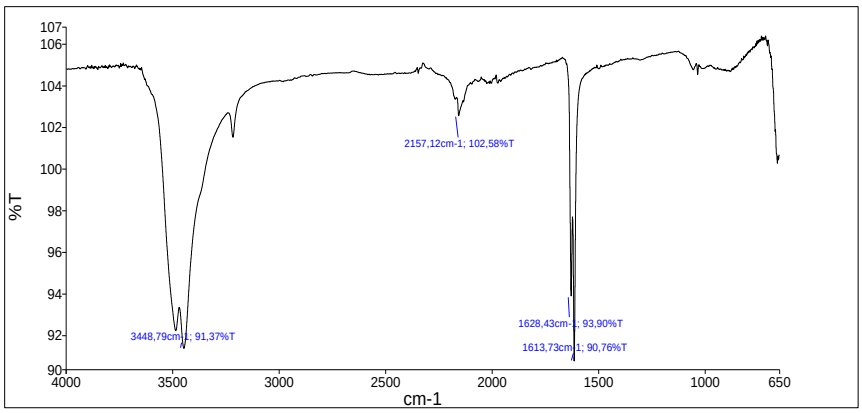


Figure 11 FTIR spectra for calcium chloride

The particles were observed for the shift in -OH stretch. Both calcium chloride, and the by-product of calcium peroxide nanoparticles after reaction with water- calcium hydroxide had the -OH stretch between wavenumbers 3700-3100 cm^{-1}. The formation of calcium peroxide was confirmed using this analysis.

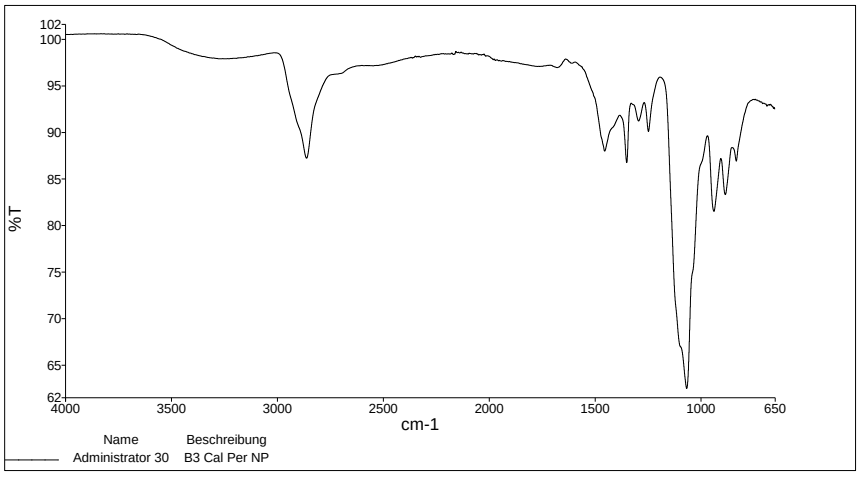


Figure 12 FTIR spectra for calcium peroxide nanoparticles

- **Discussion**

Nanoparticle Size and Aggregation

The size and the aggregation behavior of the calcium peroxide nanoparticles (CaO_2 NP) very are important in determining the effectiveness of the nanoparticles in biomedical applications. The results of this study showed that the particle size was highly variable depending on the preparation method as well as the type of surfactant used. The Zetasizer analysis also showed very high polydispersity indices (≥0.5) and polydispersity of 200-5000 nm which points to a high level of aggregation. This is in

line with previous research works which have pointed out that calcium based nanoparticles are prone to aggregation because of the electrostatic forces of attraction[177].

The laser scattering particle size distribution analysis gave more constant results and the median particle size was about 120 nm when heat stabilization and PEG 200 were used. These findings highlight the need to use surfactants and control the reaction conditions to prevent aggregation, which is in line with the conclusions made by Zhang et al. (2018)[178], who stressed that surfactants are crucial for the stability of colloidal systems.

Morphological Stability

In the SEM analysis, the morphology of the nanoparticles was observed to vary considerably depending on the method of preparation. Particles obtained after vacuum oven drying presented a spherical shape and had minimal aggregation especially when over washing was avoided while those that were heat stabilized had small aggregates. The above findings are consistent with Guo et al. (2017)[179],and the stabilization work done with surfactants enhances nanoparticles morphology. It was also found that when particles were dispersed in high ethanol dilutions, especially due to PEG dissolution, aggregation was observed. Hence underlining the importance of balancing solvent interactions for the purpose of preserving particle structure during processing.

Functional Characteristics

- **Oxygen Release**

The oxygen-releasing capacity of CaO_2 NP is very important for breaking through hypoxic barriers and this is especially so in biofilm communities. Our results showed that oxygen release was maximal at 9 mg/L for about 4 hours and this was similar to what Song et al. (2021)[180] reported for oxygen-releasing nanocarriers. This was also in line with the ability of CaO_2 NP to release oxygen steadily, which is an important aspect in dealing with the oxygen gradients that are associated with biofilms.

- **Peroxide Release**

In this study, the controlled release of hydrogen peroxide was also detected which is another aspect that can be added to the list of therapeutic effects of CaO_2 nanoparticles (49 ± 20 µM/mL at 50 µg/mL). It is important to note that hydrogen peroxide is an effective oxidative antimicrobial stress agent on microbial cells. The same results were described by Liu et al. (2020)[181] that the reactive oxygen species and peroxide release jointly hinder the structure of biofilm.

Cytotoxicity

In MTT assays it was observed that CaO_2 nanoparticles are toxic to the cells in a concentration dependent manner and the LC50 values were found to be 60 µg/mL for A549 cells and 16 µg/mL for gingival fibroblasts. This is consistent with a study by Rao et al. (2019)[182], they stated that although CaO_2 NP has the potential to act as oxygen releasing agents, the cytotoxicity of the particles cannot be overlooked and therefore dose dependent optimization is required. The difference in sensitivity of the two cell lines highlights the fact that the concentration of nanoparticles needs to be appropriate for the particular treatment that is required.

Surface Chemistry and Structural Integrity

FTIR analysis also showed the formation of calcium peroxide and its transformation into calcium hydroxide after interacting with water. The characteristic –OH stretch for both the raw materials and the nanoparticles is in line with the structural transformations stated by Kim et al. (2016) who employed the same or similar analytical techniques to monitor the functional group transformations

when synthesizing nanoparticles as mentioned earlier[183]. Therefore, we can say that our synthesis process is well established and there is a basis to make other modifications that may improve the functional performance.

This study enhances the knowledge on CaO_2 nanoparticles in order to solve major problems of size control, aggregation, and function. Most of the existing research has been based on the therapeutic use of oxygen releasing particles[171, 172]; however, this work provides a detailed analysis of the preparation techniques and the characteristics of the nanoparticles.

- **Summary**

The development of calcium peroxide nanoparticles and the characterization of these nanoparticles in this study offers a strong base for their use in biomedical and antimicrobial therapy. Findings include:

Optimized Preparation Techniques: PEG 200 and heat stabilization effectively minimized particle aggregation, resulting in median particle sizes of approximately 120 nm.

Enhanced Morphological Stability: SEM analysis showed spherical particles and reduced aggregation when vacuum oven drying and heat-stabilizing methods were employed.

Oxygen capacity: Oxygen release peaked at 9 mg/L over four hours, while peroxide release for the particles at 50 µg/mL was about 49 ± 20 µM/mL. This showed that the nanoparticles have the potential to counter act hypoxia and biofilm related resistance mechanisms.

Tailored Cytotoxicity Profiles: The cytotoxicity was found to be dose dependent and the LC50 values showed that there is a need to be very careful with the dosage depending on the type of cells.

Surface Characterization: The FTIR analysis also confirmed the formation of calcium peroxide as the functional groups exhibited shifts that were expected during the synthesis of the product.

Hence, this study also demonstrates that CaO_2 nanoparticles are capable of overcoming hypoxia as well as biofilm formation. From the development of the preparation techniques and the assessment of their properties, this work provides significant information for their potential application in biomedical therapy.

3.1.2 Squalenyl hydrogen sulphate nanoparticles

- **Materials**

Squalenyl hydrogen sulphate, quorum sensing inhibitor 1121 (QSI) were all synthesized in-house. Tobramycin, Mowiol (PVA mol. wt. ~31000), tween 80, tetrahydrofuran (THF), ammonium chloride, potassium chloride, tris hydrochloride, glucose, tryptone and magnesium sulphate were all purchased from Sigma-Aldrich (Merck KGaA Darmstadt, Germany).

Pseudomonas aeruginosa PA14 was purchased from DSMZ, strain 19882.

- **Method of preparation**

The squalenyl hydrogen sulphate (Sq) nanoparticles were prepared with electrostatic interaction with tobramycin. Particles were either prepared using quorum sensing inhibitor (QSI) loading in the hydrophobic core of the particles or unloaded nanoparticles, and the method was adapted and modified as mentioned in Ho D-K et al.[151].

Modifications were made in the preparation technique in terms of instrumentation used, syringe pump with a speed after trials was used at 50 µL/min. The organic phase was prepared by dissolving 700 µg QSI and 7 mg Sq in 700 µL THF. This was added dropwise to the aqueous phase containing 3 mg tobramycin dissolved in 10 mL water. The stirring speed was kept at 750 rpm. To avoid coagulation of hydrophobic components modifications were made in terms of the evaporation technique for THF and addition of surfactants for formulation stability.

With the initial technique, THF was removed from the formulation using rotary evaporator. However, due to precipitation of hydrophobic contents, this method was modified further by evaporating the THF overnight, under stirring at 100 rpm.

Surfactants were added for formulation stability, both hydrophilic and hydrophobic stabilizers were used. Hydrophilic surfactants included PVA (0.01, 0.05, 0.1 % w/v) and egg albumin (0.4 % w/v), they were prepared by mixing in the aqueous phase of the formulation. Hydrophobic surfactants included use of tween 80 (1, 0.2, 0.1 % v/v), they were prepared by mixing in the organic phase of the formulation.

- **Method of characterization**

Sq nanoparticles were dispersed in distilled water and the size, polydispersity index (PDI) was analyzed in the Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, U.K.) using laser scattering. The nanoparticles were diluted at ratios of 1:100, 1:1000 for automatic attenuator setting between 5-7.

Sq nanoparticles were characterized for surface charge and the zeta potential was measured using Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, U.K.). Same dilution as used for particle size determination was used in zeta potential analysis.

- **Results**

The method involved using rotary evaporator for evaporation of THF from the formulation, but it caused separation of hydrophobic phase. Thus, this step was further modified and the evaporation of THF was performed under slow stirring at 100 rpm overnight. It was observed that the slow evaporation resulted in nanoparticle formation.

When nanoparticles were prepared without using surfactants, there was precipitation of hydrophobic components and the formulation was not stable for long. Thus, various surfactants were tried during

formulation preparation. Hydrophilic and hydrophobic surfactants were analyzed for particle formation and particle stability. The particle size and zeta potential was measured for several particles (see Table 3).

Surfactant	Particle Size (nm), PDI		Zeta Potential (mV)	
	Sq-Tob	QSI-Sq-Tob	Sq-Tob	QSI-Sq-Tob
PVA (0.05 %)	242, 0.051	218, 0.056	-5.71	-3.26
PVA (0.1 %)	186, 0.032	209, 0.062	-3.41	0.30
Tween 80 (1 %)	78, 0.15	23, 0.13	-7.22	-4.88
Tween 80 (0.1 %)	178, 0.172	282, 0.373	-16.3	-22
Tween 80 (0.2 %)	145, 0.15	170, 0.212	-14.1	-18.2
Albumin (0.4 %)	150, 0.067	144, 0.071	-7	-12.1

Table 3 Particle size and surface potential determination of Sq nanoparticles prepared using different surfactants

It was observed that the particles were not stable under longer storage conditions. Thus, they were used for in vitro experiments on PA14 biofilm within 72 h of preparation. The formulation containing Tween 80 as surfactant showed potential in size and surface charge for further experiments.

- **Discussion**

The synthesis of squalenyl hydrogen sulfate (Sq) nanoparticles with tobramycin and quorum sensing inhibitor (QSI) loading was done through electrostatic interactions thus providing a new strategy for the development of antimicrobial nanoparticles for biofilms. The method used in this study included the use of different surfactants and the optimization of the solvent evaporation process in the formulation of nanoparticles. The findings highlight the role of particle size and charge in the stability of nanoparticles and their efficacy as drug carriers with emphasis on infections caused by biofilms.

Surfactants are an important component in the formulation of nanoparticles as they can influence the size and stability of the nanoparticles to a certain extent. Hydrophilic surfactants like PVA and egg albumin were used in the synthesis of nanoparticles to prevent aggregation of the nanoparticles. The findings of this study are in conformity with previous research works which established that the use of PVA at different concentrations of 0.05% to 0.1% produced small and stable nanoparticles[184]. The surfactants keep the particles from sticking together by binding to the surface of the particles and thus preventing them from clumping together and settling at the bottom of the container when dispersed in water. In our study, the PVA based formulations had the particle size between 186 to 242 nm with a low PDI which showed that PVA was efficient in stabilizing the nanoparticles.

Other additives such as Tween 80, a hydrophobic surfactant, was also used to improve the formation of nanoparticles. From the results obtained here, it was seen that the addition of Tween 80 at concentrations of 1% and 0.2% brought down the particle size to 78 nm thus enhancing the stability of the formulation. This result is in consistent with Chai et al.[185] who proved that Tween 80 could be used to stabilize nanoparticles the especially case in of lipophilic drugs. The particle size of the Tween 80 treated nanoparticles is also in line with the proposition that hydrophobic surfactants can alter the surface energy of nanoparticles thus preventing aggregation and enhancing dispersion.

Surfactant choice also determines the size and the charge of the nanoparticles. The zeta potential measurements in this study showed that the nanoparticles prepared with Tween 80 had negative surface charge from -7.22 to -16. The negative charge is very important in order to increase the stability of nanoparticles the and to decrease their chances of aggregation[186]. Surfactants such as Tween 80

also reduce the surface tension and create electrostatic forces of repulsion which is very vital in preventing aggregation, increasing the stability of the nanoparticles over time[187].

The nanoparticles were developed in a way that they would carry the QSI and tobramycin at the same time. This made the nanoparticles to have a negative charge and this helped in the incorporation of the positively tobramycin. The use of two or more therapeutic agents in the same nanoparticle has been a subject of study to achieve the synergistic effects against microorganisms. For example, Zhou et al. also observed similar results when a combination of both the loaded systems was used for the biofilm dispersion and eradication of the MDR bacteria[188, 189].

The findings in this study are in line with the hypothesis that the conjugation of QSI with tobramycin in the same nanoparticle can improve the antibiofilm potential. The nanoparticles were stable for the duration of the 72 h experiment after formulation and had appropriate drug loading, with the formulation that contained Tween 80 being of particular interest for future in vitro testing. This synergistic interaction of QSI and tobramycin may improve the ability of the nanoparticles to penetrate biofilms and inhibit the growth of bacteria, which has been observed in other nanoparticle based antibiotic delivery systems[190].

Despite the proper creation and identification of the nanoparticles, we noticed that the stability of the nanoparticles was not good and was seen to reduce with time especially if the nanoparticles were not stabilized with surfactants. This is because Zhao et al. pointed out that aggregation of nanoparticles is a major problem, particularly for hydrophobic drug carrying nanoparticles that easily settle at wrong conditions as we have seen in storage[191]. This is evident from the results where nanoparticle aggregation was observed in formulations that did not contain surfactants and also in formulations that were stored for longer periods. The following factors need to be considered to enhance the shelf life of the nanoparticle formulations include the storage conditions as well as the concentration of the surfactants. Further research can also be done to find out the possibility of using other stabilizers or storing the nanoparticles under different conditions to improve the stability of the nanoparticles.

The efficiency of nanoparticles in breaking down biofilms is another key aspect that defines the antibacterial performance of the nanoparticles. It has been well documented that biofilms are highly resistant to conventional antibiotics and using nanoparticles to target biofilms is a new approach. A recent study by Badran et al.[192] has revealed that nanoparticles are capable of reaching biofilms and increasing the delivery of drugs to biofilm-associated bacteria thus making antibiotics more effective. The formulation that we have developed which includes QSI along with tobramycin has the potential to achieve this as it also targets biofilm's resistance mechanisms by increasing the penetration and suppressing the quorum sensing pathways. The findings of this study are in agreement with the study done by Lian and colleagues where it was seen that the nanoparticles with dual functionalization (antibiotic and QSI) had better biofilm dispersal than the single agent nanoparticles[193].

- **Summary**

In conclusion, the squalenyl hydrogen sulfate nanoparticles loaded with tobramycin and quorum sensing inhibitor seem to offer a viable solution to the problem of biofilm related infections. The choice of surfactants as well as the process of formulation is very important in the formation of these nanoparticles and their stability. All these findings support the notion that the type of surfactant used has a great impact on the size, zeta potential and stability of the nanoparticles. In addition, the co-therapeutic strategy with tobramycin and QSI has the possibility to improve the antibiofilm activity and antibiotic efficacy. Nevertheless, concerns regarding the stability of nanoparticles over time suggest that there is still a need to improve the formulation as well as storage conditions. About the next steps, it would be possible to work on the enhancement of the stability of these formulations and the assessment of their efficacy in vitro for the intended use.

3.1.3 Lipid nanocapsules

- **Materials**

Labrafac™ lipophile WL 1349 was received as a free sample from Gattefossé Deutschland GmbH. Kolliphor, span 80, vancomycin, brain heart infusion (BHI) agar and broth medium, and sodium chloride were all purchased from Sigma-Aldrich (Merck KGaA Darmstadt, Germany). Dil Stain (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate ('DiI'; DiIC18(3))) was purchased from Invitrogen, Thermo Fisher Scientific (Darmstadt, Germany).

Staphylococcus aureus MSSA (methicillin resistant) was purchased from ATCC, strain 25904.

- **Method of preparation**

The lipid nanocapsules were prepared by phase inversion using temperature. The components as described in Table 4 were weighed in proportions to prepare sizes of nanocapsules in between 25, 50 and 100 nm. Three heating and cooling cycles of temperatures between 45 and 75 ˚C were performed at 500 rpm to prepare the formulation, and the final phase inversion of the last cycle was performed at 60 ˚C using Milli Q water for quenching. The formulation was stirred for another 2 h at 500 rpm and was stored in refrigerator before use.

Component	Quantity (mg)		
	25 nm LNC	50 nm LNC	100 nm LNC
Labrafac	600	1116.8	1800
Kolliphor	1800	916.8	950
Span 80	300	450	300
Milli Q water	1300	1516.8	950
NaCl	54	54	54
Quench Milli Q water	2000	2000	2000

Table 4 Preparation of lipid nanocapsules- variability of components for different sizes

Loading of the lipid nanocapsules with vancomycin at a concentration of 1.5 mg/mL was performed by mixing the drug within the formulation components. The drug loaded LNC's were tested for activity on SA biofilms. Effect from size of LNC's on SA biofilm was observed using confocal laser scanning microscopy. The LNC's were loaded with 0.1 % (w/w) DiI for different size LNC's 25, 50 and 100 nm.

The particle size and PDI of LNC's was measured by dispersing them in distilled water, and analyzed in the Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, U.K.) using laser scattering. The nanoparticles were diluted 1:100, 1:1000 times and measured at automatic attenuator setting between 5-7.

- **Results**

The particle size of LNC's was found to be 25 ± 7 nm, with a PDI of 0.02 ± 0.04; 50 ± 3 nm, with a PDI of 0.03 ± 0.05; and 100 ± 5 nm, with a PDI of 0.01 ± 0.03. The loaded particles were in the same size range as of the free unloaded LNC's.

- **Discussion**

The research work aimed at developing lipid nanocapsules of different sizes including 25 nm, 50 nm and 100 nm and biofilm. The characteristics of LNCs depending on their size were analyzed with regard

to their efficiency in breaking biofilms with the focus on the size matter for *S. aureus* biofilm invasion and drug release.

The size of the LNCs was measured with dynamic light scattering (DLS) and the results showed that the LNCs had a size within the range of 25-100 nm which is suitable for efficient drug delivery and had a polydispersity index (PDI) that was not more than 0.2. This is consistent with the previous research works that have been conducted to support the fact that the size and distribution of nanoparticles plays a crucial role in the uptake and cellular interactions of drug delivery systems[194]. For instance, small sized nanoparticles such as 25 nm are able to easily infiltrate through biofilms while larger particles of 100 nm may provide stability and may also have longer half life in the circulation as evidenced by Raghunandanan et al.[195]. The results of the size and PDI show that the phase inversion method used for the preparation of nanoparticles was effective in the generation of nanoparticles with a controlled size distribution.

The incorporation of vancomycin into the LNCs was effective with the size range of the nanoparticles being unchanged from the unloaded nanoparticles. The use of DiI dye for labeling of the LNCs established that the nanoparticles were indeed part of the formulation and that the nanoparticles were appropriate for the analysis of biofilm by confocal laser scanning microscopy (CLSM). This approach has been employed extensively in the analysis of bacterial biofilms as it allows for the observation of the uptake and localization of the nanoparticles in the biofilm matrix in real time[196].

The use of LNCs as a delivery system for vancomycin is most effective in the treatment of biofilm and related infections due to its ability to penetrate them[197]. The enhanced delivery of vancomycin to the bacterial cells that are present in biofilms highlights the possibility of using phase inversion LNCs preparation method. There is also a possibility of large-scale production, which is critical for the preparation of clinical dosage forms.

- **Summary**

In this study, the possibility of forming lipid nanocapsules (LNCs) of different sizes, including 25 nm, 50 nm, and 100 nm, to deliver vancomycin to *Staphylococcus aureus* biofilms was investigated. Phase inversion temperature technique was employed for the preparation of LNCs and the prepared nanoparticles were in the range of 25-100 nm. The LNCs had the potential of disrupting biofilms and this may be related to the size which would affect the efficiency of drug delivery. The current outcomes indicate that LNCs may have the potential of improving the antibiotic treatment of biofilm associated infections.

3.2 In vitro analysis

3.2.1 Calcium peroxide nanoparticles

- **Viability analysis on PAO1 biofilms**

Pseudomonas aeruginosa PAO1 was inoculated in LB medium for 16 h under shaking at 180 rpm. On the next day bacterial suspension was centrifuged at 5000 rpm for 10 min at 4 ˚C. After discarding the supernatant, the bacterial pellet was redispersed in sterile PBS. OD was measured and the bacterial suspension was further diluted in M63 minimal medium to the OD of 0.01, which corresponded to a CFU of $1*10^7$/mL of bacteria. This was further incubated at 37 ˚C without shaking, in 96 well plate for 48 and 72 h for biofilm formation. The 96 well plate was covered with a BreathSeal foil. The biofilms due to their maturity were differently complex. They were further tested with different concentrations of calcium peroxide nanoparticles, in combination to tobramycin sulphate in a preliminary checkerboard assay. Calcium peroxide nanoparticles in the concentration of 16 and 32 µg/mL were used for further testing. Tobramycin sulphate was used at increasing concentrations of 64, 128, 256 µg/mL. The combination was tested on 48 and 72 h PAO1 biofilms resp. The treatment was incubated overnight with biofilms and CFU assay was performed to observe bacterial viability.

Several control testing solutions containing calcium chloride, O_2-PFH liposomes and antibiotic like colistin were tested on the PAO1 biofilms.

The calcium peroxide nanoparticles released calcium and oxygen after contact with water. Calcium chloride was tested at concentration of 16 and 32 µg/mL as used for calcium peroxide nanoparticles. Tobramycin sulphate was used in combination at concentrations of 64, 128 and 256 µg/mL.

The O_2-PFH liposomes were prepared as oxygen releasing counter-part of the calcium peroxide nanoparticles. Liposomes were prepared from DOTAP:DOPE:HSPC:DSPE-PEG2000:Chol used at molar ratios of 10:20:40:10:20 to get a total of 30 mg content[198]. This was dissolved in 10 mL chloroform and liposomes were formulated using thin film hydration technique. Chloroform was evaporated by using the rotary evaporator at 100 rpm and 390 m bar vacuum at 37 °C (R-300 Rotavapor (Büchi, Flawil, Switzerland)). Later liposomes were obtained by addition of 10 mL MilliQ water mixing the phases by sonication in a bath sonicator for 10 min with shaking. To dissolve excess oxygen in the liposomes, 1.2 mL PFH was added to the liposomes and this was sonicated with ultrasonic probe Sonicator S-250D model (Branson Ultrasonics, USA) at 10 % intensity for 15 min on an ice bath. And oxygen was loaded by bubbling air for several minutes and oxygen concentration was checked before using it in experiments which was around 9 mg/L. The particle size of liposomes was determined using Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, U.K.) and was around 262 ± 40 nm, PDI < 0.1. The O_2-PFH liposomes were tested in the concentrations of 36 and 300 µg/mL, with the combination of 256 µg/mL tobramycin sulphate on 72 h PAO1 biofilm.

The combination of calcium peroxide nanoparticles with tobramycin which is an intracellularly acting antibiotic, was further changed using colistin, an antibiotic acting through cellular membrane and calcium channel competitor. Colistin was used in the concentrations of 8, 16 and 32 µg/mL, in combination to 32 µg/mL calcium peroxide nanoparticles on 72 h PAO1 biofilm. The bacterial viability after treatment was observed using the CFU assay.

- **Morphological analysis of biofilms**

Bacterial biofilms were observed for morphological characteristics using the scanning electron microscopy (SEM). The method used for mounting the samples for SEM studies initially involved the

treatment of samples with calcium peroxide nanoparticles in combination with tobramycin sulphate and several other controls. The method developed for the ease of processing the samples further for imaging was modified several times. The initial method development involved growing the biofilms in 48 well plates and processing them. However, this needed a further modification for easing the imaging by using sterilized hermatic sealing lids (used in DSC (Differential Scanning Calorimetry) analysis) placed in 24 well plate for biofilm growth. After the regular treatment scheme was followed, samples were fixed using 4 % paraformaldehyde solution in PBS for 2 h at room temperature. These samples were then treated with a series of ethanol diluted in PBS. The samples were processed with 30, 40, 50, 60, 70, 80, 90, 95 (twice), and 100 % ethanol for 10 min each time and the supernatant was discarded. A complete dehydration of the biofilm was performed with a final treatment of HMDS for 10 min. After discarding the supernatant, the biofilms were dried overnight at room temperature. On the next day, lids containing dried biofilms were placed on SEM stubs with a double-sided black carbon tape. To obtain the SEM images, gold sputtering in argon atmosphere vacuum was done in QUORUM Q150R ES, (Pfungstadt, Germany). Later images were taken using EVO HD15 microscope (Zeiss, Oberkochen, Germany).

PAO1 biofilms were analyzed using crystal violet staining technique for estimating the thickness and density within biofilms and were observed microscopical after staining. The treated biofilms were exposed to 0.1 % crystal violet stain for 15 min in dark.

- **Microscopical qualitative viability assay**

Samples were analyzed microscopically for viability using the live/dead bacterial viability stain. The standard BacLight™ Live/Dead® Staining Kit L7012 and L7007 were used for the purpose of this assay.

In the initial protocol 72 h PAO1 biofilms were grown in an 8 well microscopic slide, and was treated with several combinations with tobramycin sulphate and nanoparticles. The treated biofilms were then stained using the L7012 staining kit, the component A and component B of the kit were used in same ratios and same volumes for biofilm staining. A 1:1 ratio between the components, and nearly 2 µL staining solution was used for the assay.

Due to variability within the samples, modifications in the protocol were made to report comparable results. Here, L7007 component A of the staining kit was used as it had similar quantities of the live stain SYTO9 and dead stain propidium iodide. During the initial analysis, same volume of staining solution was used within treated samples. The fluorescence intensity varied between the samples. However, after considerations of the biomass remained after treatment with antibiotic, the volume was adjusted based on the biomass and type of the sample. The samples with higher biomass were stained with 2.5 µL staining solution. And the samples with lower biomass were stained using 1.5 µL stain. After the stain was added, the slide was kept in dark for 15 min at room temperature. Later samples were analyzed using fluorescence microscopy Leica DMi8 Confocal laser scanning microscope (Leica, Mannheim, Germany). Settings used to observe samples under the microscope involved using a 10x objective (HC PL Fluotar 10X/0.3 Dry, Leica, Germany) and images analyzed with LASX software from Leica Application Suite X.

- **Gene expression studies**

The genetic expression of bacteria after treatment with the several samples studied above was analyzed. For expression analysis bacterial RNA was isolated and after cDNA synthesis the gene expression was determined using qPCR analysis.

To obtain and isolate higher quantities of RNA the method was modified for RNA isolation. Initially the treated samples were mixed with equivalent amount of bacteria RNA protect reagent and vortexed for

10 min at RT. This mixture was then centrifuged for 10min at 12000g at 4°C to separate undissolved material. Supernatant was further stored at -80°C for 2h before using processing with RNA isolation and purification. RNA was isolated by treating the above mixture with 80 µL mixture of 100 µL 1-Bromo-3-chloropropane per mL of Tri reagent used. This was then vortexed for 15s, then was allowed to stand at RT for 15 min, then was centrifuged at 12000g for 15min at 4°C; after centrifugation the upper aqueous phase containing RNA was isolated. The isolated RNA was further purified following RNeasy Micro Kit including on-column DNA digestion. For elution of RNA 28 µl RNAse free water was used. The concentration of purified RNA was analyzed using NanoQuant Plate™ in a plate reader (CytoSparc Tecan Instruments, Männedorf, Switzerland).

Later this method was modified further for isolation of RNA from treated samples (total volume 400 µL, 48 well plate); equivalent amount of RNAProtect® bacteria reagent was mixed with the samples and vortexed for 10 min at room temperature. This mixture was centrifuged for 10 min at 5000 g at 4 °C, the supernatant was discarded and pellet was kept at -80 °C for 2 h before using it for RNA isolation and purification. RNA was extracted by addition of 96 µL of Tris-EDTA (20mg/mL) buffer and 4 µL lysozyme to the pellet, which was vortexed for 15 min. To this mixture 350 µL of RLT buffer supplemented with 1 % ß-mercaptoethanol was mixed and incubated at -70 °C for 1 h. After thawing the sample, it was further loaded onto QIAshredder column, then was centrifuged at 12000 g for 2 min at 4 °C. Further the flow through containing RNA was isolated and mixed with equivalent amounts of ethanol. RNA was purified following RNeasy® Micro Kit instructions, which included on-column DNA digestion. For elution of RNA 28 µL RNAse free water was added to the flow through. To completely remove DNA contamination an additional step of DNA digestion using 1 µL TURBO™ DNase reagent for 1 h, at room temperature. Finally, the concentration of purified RNA was measured using NanoQuant Plate™ in the plate reader (CytoSparc Tecan Instruments, Männedorf, Switzerland).

Further the isolated RNA was converted to cDNA using qScriber™ cDNA Synthesis Kit protocol, in the Thermal Cycler (from Applied Biosystems). Here, the RNA concentration was kept between 150-300 ng/per 20µl cDNA reaction and incubation temperature of 45°C for 30 min. After the reaction was ended, enzymes were deactivated at 85 °C for 10 min. The synthesized cDNA was further stored at -20 °C. The cDNA was then diluted to make 50 µL and further used in qPCR analysis. qPCR was conducted using Mic qPCR Cycler (Bio Molecular Systems). For PCR analysis primer-sets for genes including hcnA, pqsA, phzA, rpoS, mexG, rpoD, rhlR, mvfR, nadB, algD, and gyrA were mixed with SYBR Mastermix and tested for cycle number (C_q values). gyrA gene was used as the control gene for the regulation calculations. As residual DNA contamination could not be entirely avoided, a –RT control was additionally run for each reaction in parallel.

3.2.2 Squalenyl hydrogen sulfate nanoparticles

- **Viability analysis on in vitro PA14 biofilms**

Sq nanoparticles with and without the encapsulated QSI as described before[151], were tested for in vitro efficacy on *Pseudomonas aeruginosa* PA14 biofilms. PA14 was inoculated in LB broth medium for 16 h at 180 rpm and 37 ˚C. Sterile PPGAS medium was prepared by mixing of NH_4Cl (0.02 M), KCl (0.02 M), Tris-HCl, (0.12 M), glucose (0.5%, w/v), tryptone (1%, w/v), and $MgSO_4$ (0.0016 M), adjusted to pH 7.2.

Later PA14 biofilm with an initial OD of 0.01 was cultivated for 24 h in the PPGAS medium and the test solutions were analyzed for bacterial biofilm eradication. Test solutions involved the serial dilutions of nanoparticles (Sq-Tob, QSI-Sq-Tob); physical mixtures of Sq, QSI, and Tob; and tobramycin with or without the surfactant were used in the experiments. The test solutions were serially diluted to make the concentration of tobramycin between 200, 100, 50, 25, 12.5, 6.25, 3.12, and 1.56 µg/mL. The

dilutions were tested on 24 h biofilm overnight and the bacterial viability was assessed using CFU counting assay and presto-blue metabolic assay.

3.2.3 Lipid nanocapsules

- **Microscopical analysis**

The LNC's were prepared and loaded with a laser sensitive dye DiI, and the effect between sizes of 25, 50, and 100 nm on biofilm penetrability of the particles based on size was studied. *S. aureus* biofilm was cultivated for 24 h, for which the SA was inoculated at 37 ˚C and 180 rpm for 16 h. It was diluted to 0.01 OD and the biofilm was cultured in 96 well plate for 24 h at 37 ˚C. The DiI loaded LNC's (10 µL) were placed on the surface of the cultured SA biofilm (100 µL) for 15 min in dark at room temperature. This was performed using far red laser at the excitation wavelengths between 600 – 800 nm. Sample analysis was performed using fluorescence microscopy Leica DMi8 Confocal laser scanning microscope (Leica, Mannheim, Germany). Samples were observed under the microscope using a 10x objective (HC PL Fluotar 10X/0.3 Dry, Leica, Germany) and images were analyzed with LASX software from Leica Application Suite X.

LNC's loaded with vancomycin (1.5 mg/mL), were tested on SA 24 h biofilm. The test solution was serially diluted to make the final concentration of vancomycin from 750, 375, 187.5, 93.7, 46.8, 23.4, 11.7, 5.8 µg/mL. The dilutions were incubated with the biofilm overnight and the bacterial viability was assessed using the presto-blue reagent and the metabolic activity within the bacteria was assayed.

Results and discussion: Calcium peroxide nanoparticles

- **Viability analysis on PAO1 biofilms**

Calcium peroxide nanoparticles were used in combination to tobramycin and tested on differently matured pre-grown PAO1 biofilms. From preliminary studies it was observed that depending on the biofilm maturity the concentration of calcium peroxide nanoparticles was increased to observed similar effect of biofilm eradication in conjugation to tobramycin sulfate.

For the 48 h grown PAO1 biofilm, a lower concentration of 16 µg/mL showed biofilm eradication when used in combination to 256 µg/mL tobramycin sulfate (see Figure 13a). However, when only the calcium counterpart i.e. calcium chloride was used in the combination of tobramycin sulfate the effect on biofilm eradication decreased and a bacterial viability of nearly 0.5 log CFU/mL was observed.

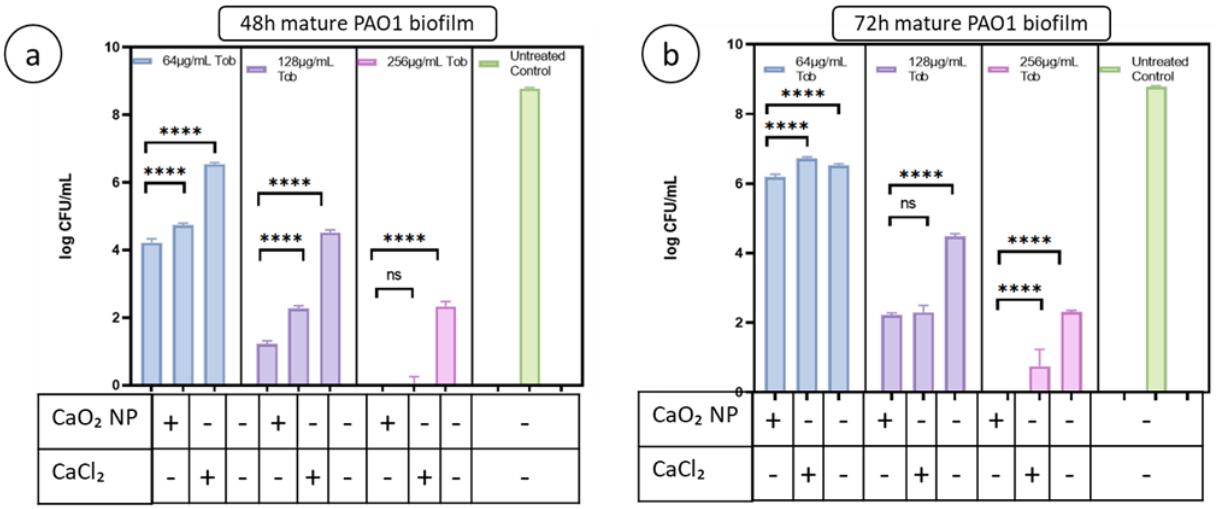


*Figure 13 MBEC assay for in-vitro P. aeruginosa PAO1 48h and 72h matured biofilm: Comparison between 16 µg and 32 µg CaO_2 NP containing both Ca^{+2} and O_2; 16 µg and 32 µg $CaCl_2$ containing Ca^{+2}; with antibiotic Tobramycin at different concentrations respectively (a) CFU counting for viability analysis- Decrease in viability observed at combination treatment with Tobramycin, Complete biofilm eradication at 16 µg CaO_2 NP+ 256 µg/mL Tobramycin (c) CFU counting for viability analysis- Decrease in viability observed at combination treatment with Tobramycin, Complete biofilm eradication at 32 µg CaO_2 NP+ 256 µg/mL Tobramycin. Experimental analysis performed using One-Way ANOVA: Experiment*replicates= atleast 3*3; p:0,05; ****= 0,0001.*

Similarly, for 72 h grown PAO1 biofilm, the combinations with calcium peroxide nanoparticles and tobramycin sulfate were tested. At increasing concentration of tobramycin sulphate the biofilm eradication was higher in combination treatments as compared to tobramycin alone. However, as the biofilm was more mature it was observed that it required 32 µg/mL calcium peroxide nanoparticles concentration in combination to 256 µg/mL tobramycin sulfate for biofilm eradication. The effect observed from calcium peroxide nanoparticles stemmed from the released oxygen and calcium, whereas in case of the calcium counterpart; calcium chloride this effect was significantly reduced and the bacterial viability was nearly 1.5 log CFU/mL (see Figure 13b).

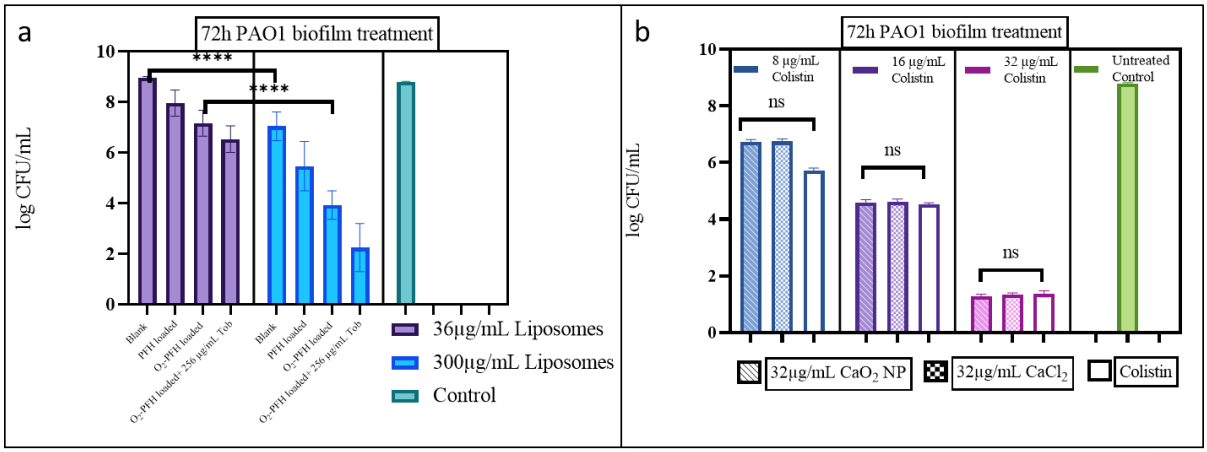


*Figure 14 MBEC CFU counting- viability assay for in-vitro 72 h PAO1 biofilm treatment (a) Treatment with 36 and 300 µg/mL blank, PFH loaded, O_2- PFH loaded liposomes, O_2- PFH loaded liposomes with 256 µg/mL Tob. Decrease in bacterial viability at higher concentrations, 2.24 log CFU/mL at O_2- PFH loaded liposomes with 256 µg/mL Tob combination, (b) 32 µg/mL CaO_2 NP, 32 µg/mL $CaCl_2$; with antibiotic Colistin at 8, 16 and 32 µg/mL concentrations; nearly 1 log CFU/mL increased bacterial viability in combination treatment of 32 µg/mL CaO_2 NP/ $CaCl_2$ with 8 µg/mL Colistin as compared to 8 µg/mL Colistin alone; however at higher concentrations of colistin the effect is negligible in combination treatments. Experimental analysis performed using One-Way ANOVA with Tukey's post hoc test. N*n= 3*3, ****p<0.0001.*

Several other controls were analyzed on 72 h PAO1 biofilm, to observe the effect from calcium peroxide nanoparticles. As they release oxygen, the oxygen counterpart used were the O_2-PFH loaded liposomes, containing PFH which dissolved more oxygen in the core of the liposomes. It was observed that, the combination of O_2-PFH loaded liposomes with 256 µg/mL tobramycin sulfate increased the bacterial death within the biofilms by 0.5 log CFU/mL, than tobramycin alone (see Figure 14a).

To observe the effect from calcium, a calcium channel competitor- colistin was used in combination with calcium peroxide. It was observed that the colistin at higher concentrations, nullified the effect observed in earlier experiments from both calcium peroxide nanoparticles and calcium chloride (see Figure 14b).

- **Morphological analysis of biofilms**

To observe the morphological characteristics of the treated biofilms, 72 h PAO1 biofilm after treatment was processed and observed under SEM microscope. It was observed that the bacteria after treatment with calcium containing solutions showed a rough surface morphology (see Figure 15c-e). It was observed that for treated biofilms, the biomass significantly reduced in comparison to the untreated control. For the biofilm treated with calcium peroxide nanoparticles there was hole generation within the biofilm possibly due to peroxide effect (see Figure 15e). The combination treatment of calcium peroxide nanoparticles with tobramycin sulfate showed a significantly reduced flat biomass and bacterial envelopes on the surface (see Figure 15f).

The bacterial biofilm was also observed under light microscope, using the crystal violet staining. Similar, effect as observed with SEM was seen in the results. However, higher aggregation was observed for the samples treated with calcium as compared to the untreated control (see Figure 16a, c-d). The samples treated with tobramycin alone or in combination showed a reduced biomass (see Figure 16b, e-f).

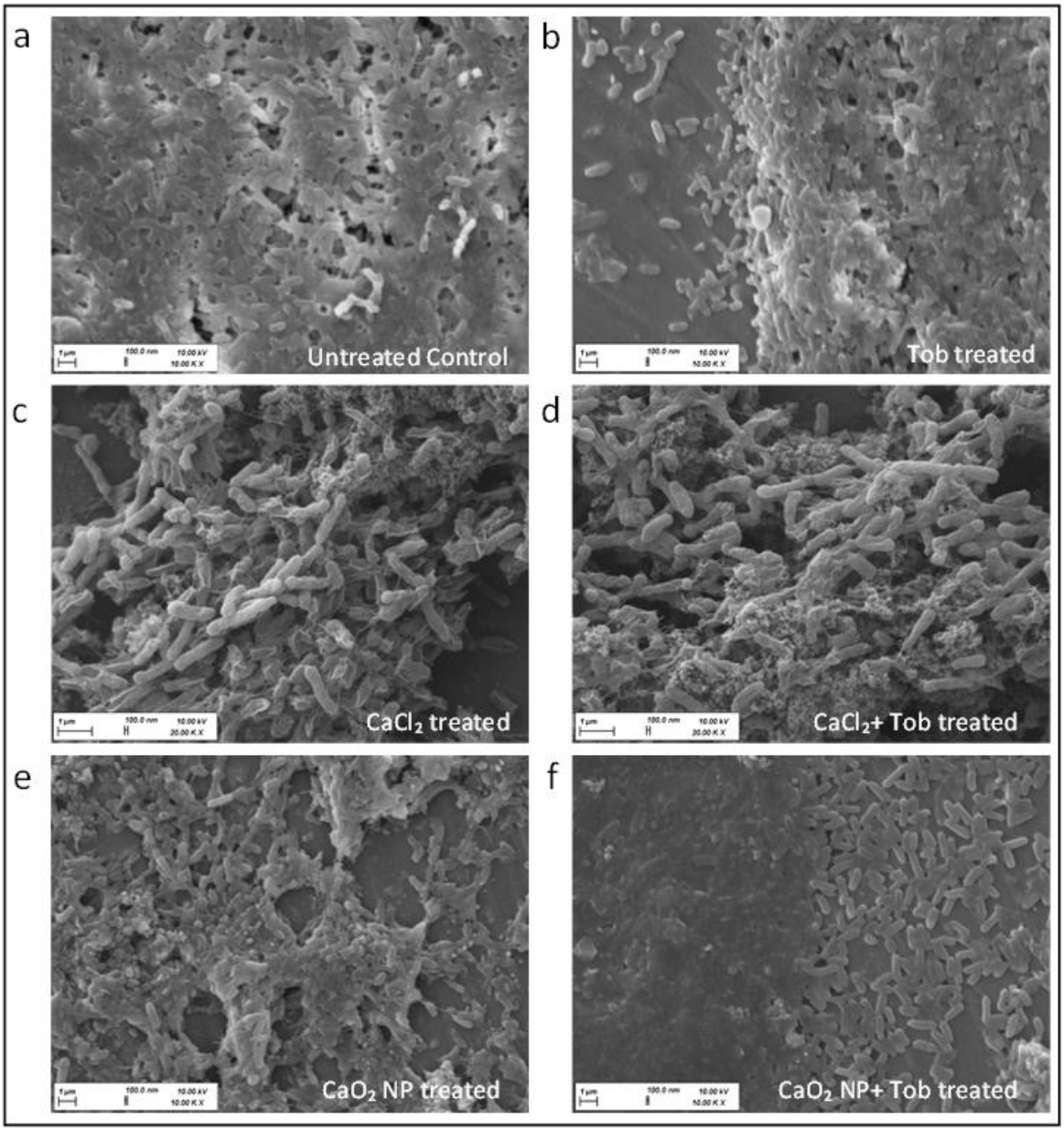


Figure 15 Morphological analysis of extracellular biofilm matrix for treated in-vitro 72 h old PAO1 biofilm by SEM; a) Untreated Control, (b) 256 µg/mL Tob treated, (c) 32 µg/mL $CaCl_2$ treated (d) 32 µg/mL $CaCl_2$ and 256 µg/mL Tob co-treated (e) 32 µg/mL CaO_2 NP treated (f) 32 µg/mL CaO_2 NP and 256 µg/mL Tob co-treated. The biomass for co-treated biofilms with CaO_2 NP and Tob was significantly lower. (adapted from Bankar N. et al. [176])

- **Microscopical qualitative viability assay**

It was observed from initial trials that when L7012 bacterial viability kit was used in staining of the biofilms, as the ratio of SYTO9 dye and propidium iodide dye was not equally mixed; also, that the biomass within the biofilms differed, the stain was unevenly distributed. The representative images (see Figure 17) indicate that the stain for tobramycin treated biofilm showed stronger green signal than untreated biofilm, possibly due to the tightness, thickness within the biofilms. However, the co-treated biofilm had reduced signal and biomass.

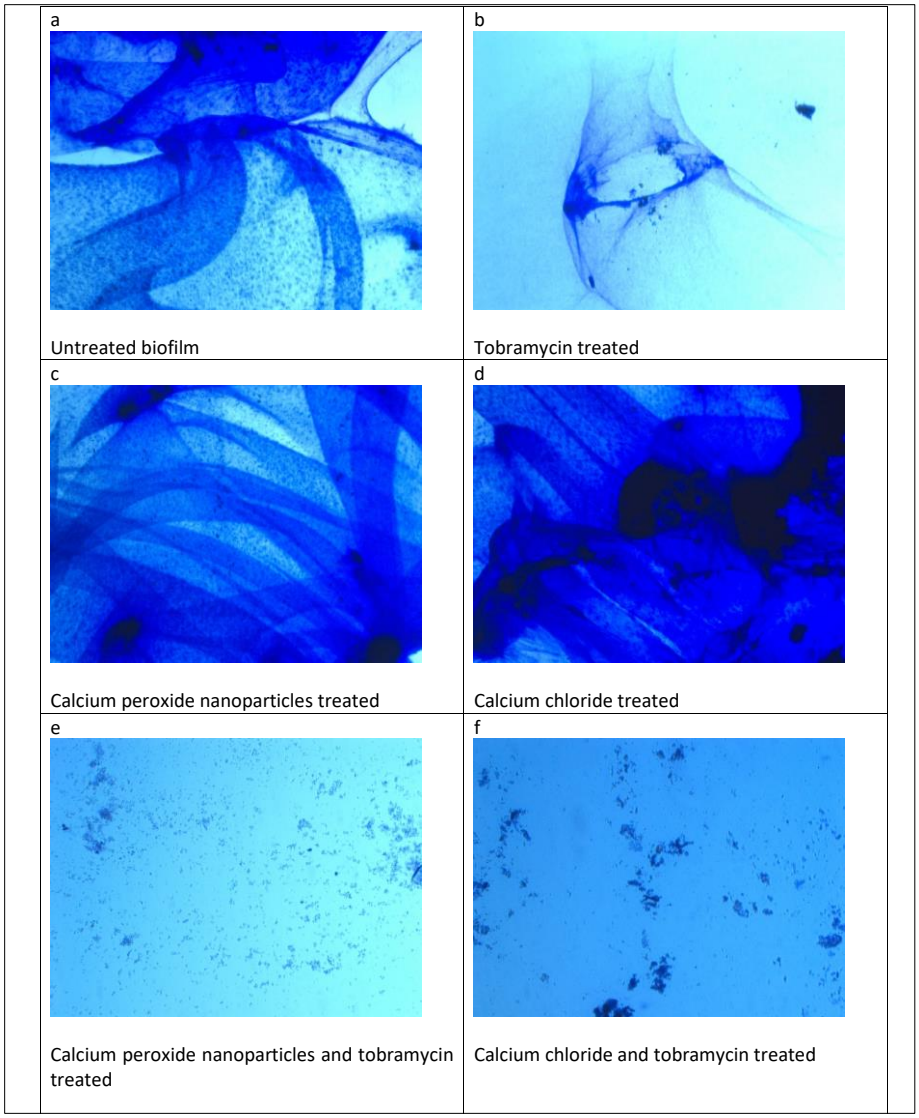


Figure 16 Morphological analysis of extracellular biofilm matrix for treated in vitro 72 h old PAO1 biofilm by crystal violet assay; a) Untreated Control, (b) 256 µg/mL Tob treated, (c) 32 µg/mL CaO_2 NP treated, (d) 32 µg/mL $CaCl_2$ treated, (e) 32 µg/mL CaO_2 NP and 256 µg/mL Tob co-treated, (f) 32 µg/mL $CaCl_2$ and 256 µg/mL Tob co-treated. The biomass for co-treated biofilms with CaO_2 NP and Tob was significantly lower.

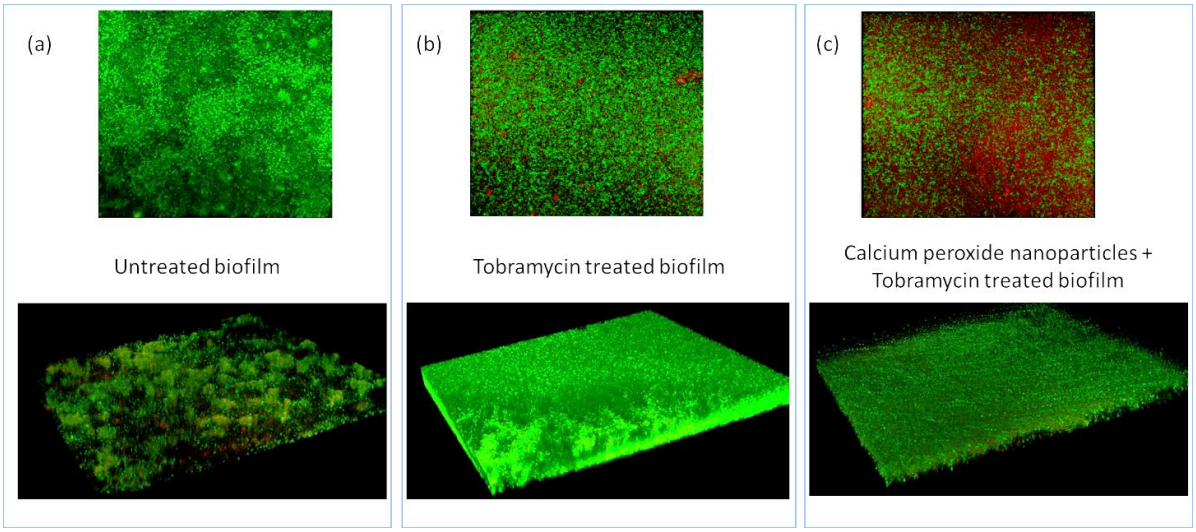


Figure 17 Microscopical analysis for treated in vitro 72h PAO1 biofilm viability analysis- Confocal Laser Scanning Microscopic (CLSM) analysis, using BacLight™ Live-Dead™ staining, live bacteria indicated by green signal and dead bacteria indicated by red fluorescence signal. Overlay of red and green fluorescence in the represented images observed under 10X objective. (a) Untreated Control, (b) 256µg/mL Tob, (c) 32µg/mL CaO_2 NP and 256µg/mL Tob.

In the later, the method was modified and L7007 bacterial viability assay kit was used; which had the SYTO9 and propidium iodide stains equally distributed. It was observed that when the volume of stain was increased in the untreated and only calcium treated samples, the fluorescence intensity was equivalent to the tobramycin treated samples.

To measure the fluorescence and observe the bacterial viability, green fluorescence was indicative of the surface for measurement of the z-stack of the biofilm. The live bacteria indicated by green fluorescence was distributed variably along the center and corner of the wells. It was observed that the untreated, calcium chloride and calcium peroxide nanoparticles treated samples the biofilm mass was nearly 50 µm in thickness, whereas the samples treated with tobramycin and calcium chloride with tobramycin were 30 µm in thickness (see Figure 18). However, the calcium peroxide and tobramycin co-treated sample showed a thickness > 50 µm, with the maximum red fluorescence observed from dead bacteria.

When the biofilms were observed in 2 D format, it was noted that the biofilm treated with calcium peroxide nanoparticles showed generation of pores (see Figure 19e), possibly due to the peroxide effect. It was also observed that samples treated with calcium (see Figure 18c,e and 19c,e) showed more aggregation and tightness within the biofilm, indicated with the intensified green fluorescence.

The red fluorescence indicating the dead bacteria was highest in the samples treated with calcium peroxide nanoparticles and tobramycin co-treated, compared to calcium chloride and tobramycin co-treated samples or the tobramycin alone treated samples.

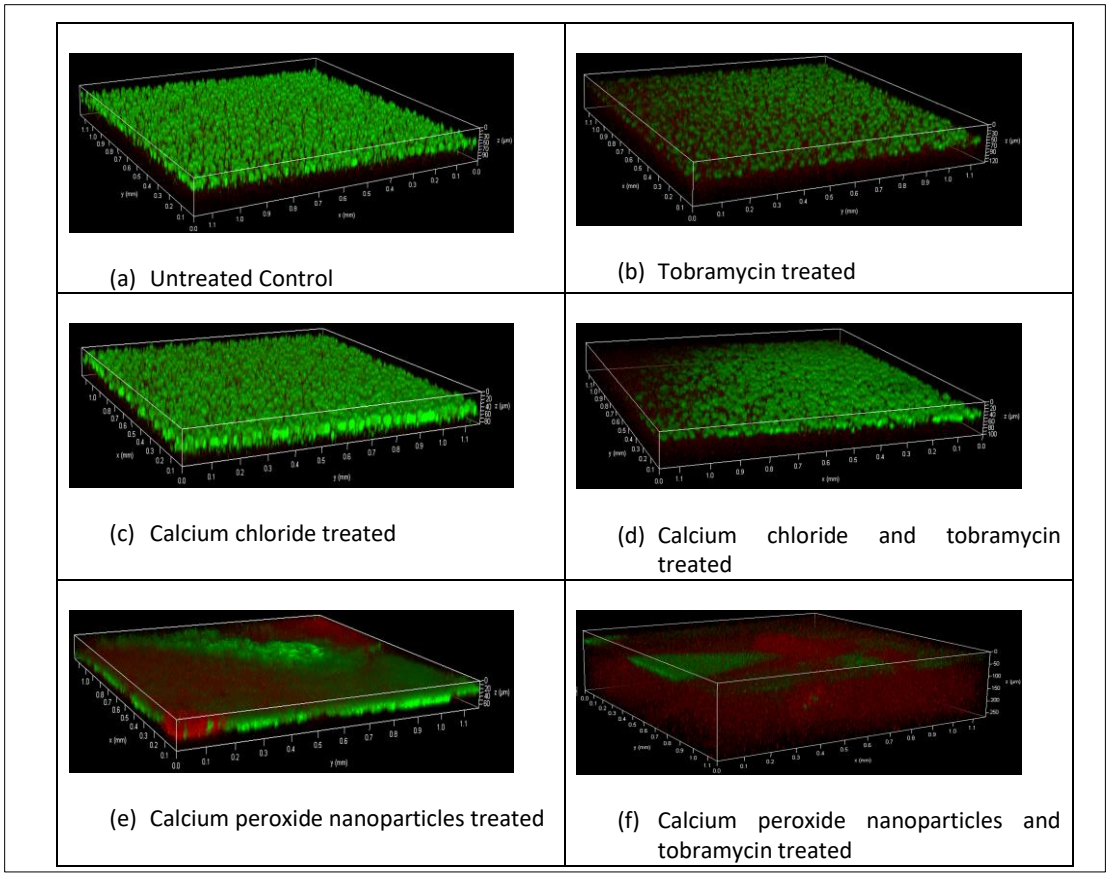


Figure 18 Microscopical analysis for treated in-vitro 72h PAO1 biofilm viability analysis- Confocal Laser Scanning Microscopic (CLSM) analysis 3 D images, using BacLight™ Live-Dead™ staining, live bacteria indicated by green fluorescence signal and dead bacteria indicated by red fluorescence signal. Overlay of red and green fluorescence in the represented images observed under 10X objective. (a) Untreated Control, (b) 256µg/mL Tob, (c) 32µg/mL $CaCl_2$ (d) 32µg/mL $CaCl_2$ and 256µg/mL Tob (e) 32µg/mL CaO_2 NP (f) 32µg/mL CaO_2 NP and 256µg/mL Tob. (adapted from Bankar N. et al. [176])

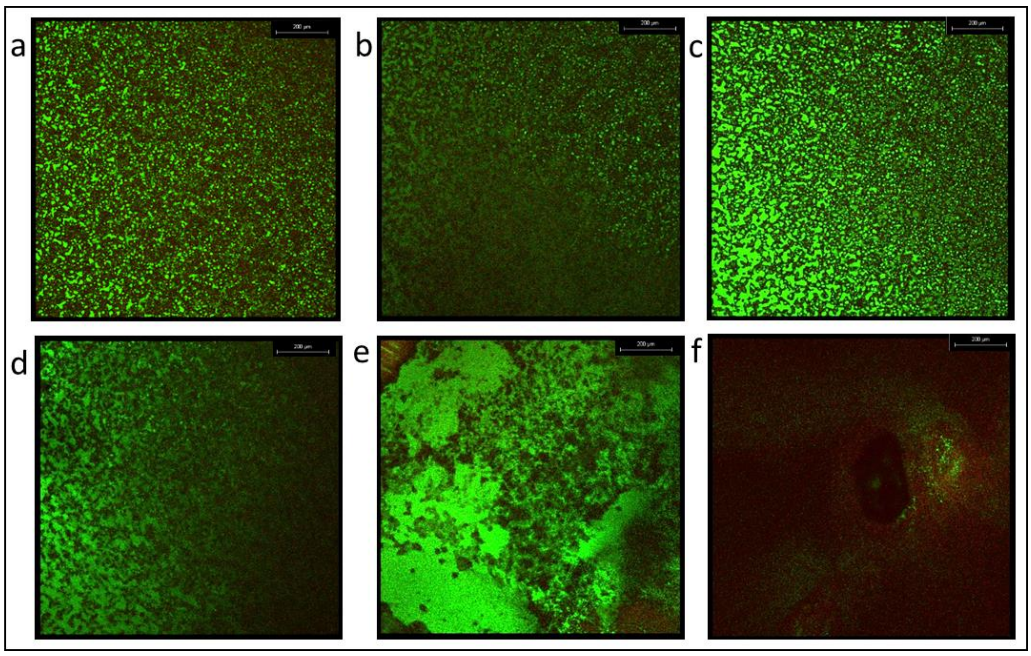


Figure 19 Microscopical analysis for treated in-vitro 72h PAO1 biofilm viability analysis- Confocal Laser Scanning Microscopic (CLSM) analysis 2 D images, using BacLight™ Live-Dead™ staining, live bacteria

indicated by green fluorescence signal and dead bacteria indicated by red fluorescence signal. Overlay of red and green fluorescence in the represented images observed under 10X objective. (a) Untreated Control, (b) 256µg/mL Tob, (c) 32µg/mL $CaCl_2$ (d) 32µg/mL $CaCl_2$ and 256µg/mL Tob (e) 32µg/mL CaO_2 NP (f) 32µg/mL CaO_2 NP and 256µg/mL To . (adapted from Bankar N. et al. [176])

The images (2D and 3D) obtained from CLSM were analyzed for live-dead bacterial percentage, which were analyzed for red and green stain signal using the ImageJ software. After plotting the graph, it was observed that the % dead bacteria were higher in calcium peroxide and tobramycin co-treated sample as compared to other samples (see Figure 20). However, the untreated control also showed nearly 10 % of red fluorescence. The samples treated with tobramycin sulphate and calcium chloride with tobramycin sulphate combination showed nearly similar bacterial death, which was interestingly comparable to the calcium peroxide nanoparticles treated sample.

- **Gene expression studies**

When bacteria form biofilms, the changes in their genetic expression are observed. Here, these changes in genetic expression were analyzed in response to the different treatments in PAO1 biofilm. At higher treatment concentrations of Tob and CaO_2 NP or $CaCl_2$, the RNA isolation process was unable to generate enough quantities for further gene analysis. Therefore, we chose a lower concentration of the co-treatment where an analyzable quantity of RNA was isolated. Here, samples with 32 µg CaO_2 NP and $CaCl_2$, with Tob at 64 µg/mL were selected to study the genetic variations due to treatments. We selected few genes considering the treatment strategy. Several genes regulating virulence in *Pseudomonas* were analyzed including hcnA, pqsA, phzA, mvfR, rhlR, rpoS. hcnA operon is a subunit of the Hydrogen Cyanide Synthase (HCN) responsible in anaerobic respiration and virulence generation during multi-bacterial infections; the gene expression upregulates for hcnA during micro-aerobic conditions[199]. Pyocyanin is a phenazine produced by *Pseudomonas* as a virulence factor to establish its niche, responsible for depletion of antioxidants and increasing antibiotic resistance; encoded by the phzA operon[200].

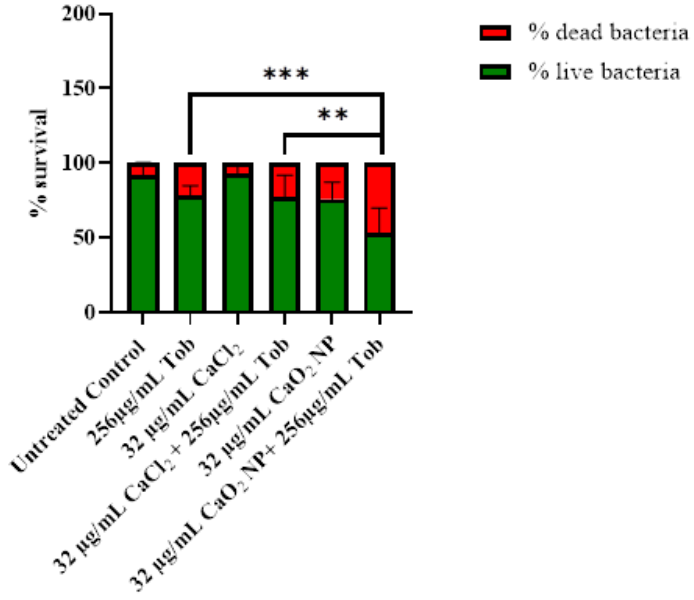


Figure 20 Graphical representation of % dead and % live bacteria, calculated with ImageJ

The quorum sensing involved for cell-to-cell communication in *Pseudomonas* biofilm, and in regulation of cell density involves the expression of 4-hydroxy-2-alkylquinolines (HAQs) by the pqsA operons[201]. Similarly, mvfR is involved in *Pseudomonas* Quinolone signaling (PQS), a quorum sensing path

responsible in synthesis of HAQs and in generation of several compounds like elastases, pyocyanin in the biofilm responsible for virulence generation[202]. In the *Pseudomonas* quorum sensing involving the homoserine lactones rhlR is responsible for the regulation of virulence related to the rhamnolipid generation[203]. The sigma factors rpoD and rpoS are involved in *Pseudomonas* quorum sensing at different phases of growth cycle, the earlier involved during stationary phase extracellular virulence factors generation, while the latter is involved in transcription of several housekeeping genes[204]. The efflux pump protein mexG encoded by mexGHI-opmD operon responsible in antimicrobial resitance was also studied for its expression[205]. Under carbon limiting conditions, the extracellular respiration with Nicotinamide Adenine Dinucleotide hydrogen is governed with the nadB operons[206]. In the biofilm formation, algD is one of the genes required in the production of alginate[207]. gyrA is the DNA gyrase genome replication housekeeping gene, it is considered stable during treatments so is used as the reference gene in the study[208]. After selection of the above genes we performed the RNA isolation and PCR studies for gene expression analysis. The expression was compared to untreated control samples and expression in fold change was noted. Further the fold change was calculated and plotted based on the geometric mean and the following observations were made (see Figure 21).

It was observed that the expression analysis showed upregulation of several genes with calcium containing treatments. The upregulation of hcnA, phzA, mexG, rhlR which are responsible for virulence in *Pseudomonas* were activated due to the treatment with CaO_2 NP or $CaCl_2$ at 2.1, 14.8, 6.8, 2.5 and 5.1, 27.4, 147.2, 8.3 folds respectively. The results indicated that only calcium treatment increased the virulence much higher compared to CaO_2 NP and Tob. When CaO_2 NP or $CaCl_2$ were in combination treatment with Tob the expression of hcnA, phzA, mexG, rhlR was downregulated when compared to single treatment. The efflux gene mexG was upregulated in treatment with Tob by 2 folds. When PAO1 biofilm samples were in treatment with Tob combinations, the gene expression for rpoD and algD were upregulated responsible for immune response and virulence from *Pseudomonas*. The upregulation was observed by 2.4, 1.5 and 1.6 folds in Tob, CaO_2 NP+ Tob, $CaCl_2$+ Tob respectively for algD. However, in the case of rpoD it was observed that expression for Tob treatment was upregulated by 1.3 folds whereas downregulated in combination treatments CaO_2 NP+ Tob, $CaCl_2$+ Tob by 0.7 and 0.6 folds respectively. In the combination treatment of CaO_2 NP+ Tob almost every gene showed a downregulation compared to the untreated control, indicating that the combination treatment helped in decreasing the virulence and quorum sensing of *Pseudomonas* biofilms.

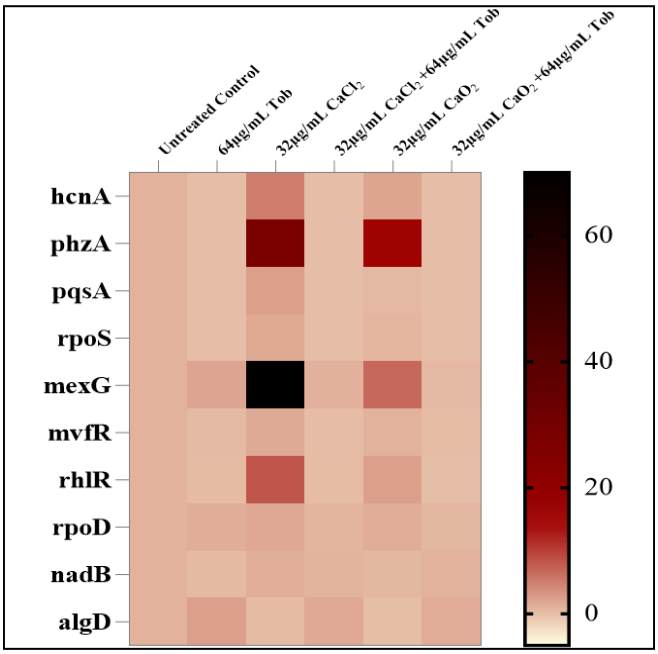


Figure 21 Gene expression analysis of 72h PAO1 biofilm in response to treatments. Expression for 32µg/mL CaO_2 NP and $CaCl_2$, with Tob at 64µg/mL were compared to the Untreated control. qPCR performed for selected genes. Analysis performed with gyrA as reference gene. Gene expression measured with differences in geometric mean for fold change of Untreated control and samples. Values were normalized considering a 10 cycle difference in the +RT and –RT control. N=3, n≥3. (adapted from Bankar N. et al. [176])

- **Discussion**

The combined descriptions detail the investigation of calcium peroxide nanoparticles (CaO_2 NP) as a potentiating agent with antibiotics, particularly tobramycin sulfate, for the treatment of *Pseudomonas aeruginosa* PAO1 biofilms. The studies focus on the effects of the treatments on biofilms, cell viability and the genetic responses of the cells to the treatments. According to the findings, CaO_2 NPs, through oxygen and calcium release, increase the antibiotic efficacy, decreases biofilm biomass and regulates the expression of genes that are involved in virulence.

Biofilm eradication was assessed at maturity (48 h and 72 h biofilms) to observe the effect of CaO_2 NP concentration when used along with tobramycin. The 48 hours biofilms were completely eradicated with the use of 16 µg/ mL CaO_2 NPs and 256 µg/ mL tobramycin while to eradicate the 72 hours biofilms the concentration of CaO_2 NPs required was 32 µg/ mL. The ineffectiveness of calcium chloride ($CaCl_2$) in producing similar results shows the importance of oxygen release in the process of biofilm disruption. This could be due to the fact that ROS increases the efficiency of tobramycin in penetrating through the biofilm and exerting its effect in agreement with earlier findings that point to oxidative stress as a means of biofilm eradication[209, 210].

CLSM microscopic analysis confirmed the changes in biofilms structurally after treatment. Treated biofilms with CaO_2 NP and tobramycin showed significant red fluorescence indicating bacterial death. The CaO_2 NPs also caused compaction and aggregation of the biofilms because of calcium interaction while the peroxide part was responsible for breaking down of the biofilm matrix thus creating pores. These findings are in consistent with previous researches which has been done on the role of calcium and oxygen in biofilm dispersion[211].

Gene expression analyses showed that biofilm treatment affects the pathways of virulence and quorum sensing. Only calcium (CaO_2 NP or $CaCl_2$) treatment enhanced the expression of genes that are related to virulence, such as hcnA, phzA, rhlR, and mexG. The overexpression of phzA which encodes for pyocyanin and rhlR which regulates rhamnolipid biosynthesis shows the biofilm's response to calcium in terms of stress. However, the combined use of CaO_2 NPs with tobramycin reversed these effects and led to the overall suppression of virulence and quorum sensing genes such as pqsA and mvfR. This reduction in the expression of virulence genes proves that the combination therapy can be effective in combating biofilm related antibiotic resistance[212].

Surprisingly, the efflux pump gene mexG which is known to be involved in resistance antimicrobial was found to be upregulated when tobramycin was used alone which shows the potential for resistance. But the expression of this gene was downregulated in the combination treatments thus suggesting that CaO_2 NP inhibit resistance when used along with antibiotics.

The gene rpoD which is involved in biofilm growth regulation was found to be down regulated in CaO_2 NP and tobramycin combinations thus establishing the additive effect of this treatment in inhibiting the biofilm formation and virulence. These results are consistent with the studies which have demonstrated that oxidative and calcium based treatments can interfere with the quorum sensing mechanisms and increase the antibiotic susceptibility[213].

From the above morphological, viability and gene expression results, it can be inferred that CaO_2 NPs can be a potential adjuvant to improve the efficacy of antibiotics against biofilm related infections. The function of CaO_2 NPs as oxygen releasing particles and calcium supplement also helps in the breakdown of biofilm matrix as well as the regulation of bacterial virulence genes. This suggests that calcium alone could lead to the expression of virulence genes hence the need for combination therapies to avoid worsening of bacterial resistance or pathogenicity.

- **Summary**

From the findings of this study, calcium peroxide nanoparticles (CaO_2 NPs) have been identified to have adjuvant properties in the treatment of *P. aeruginosa* biofilms-

Enhanced Biofilm Disruption: CaO_2 NPs in combination with tobramycin achieved complete biofilm eradication at lower concentrations than tobramycin alone. The oxygen released from CaO_2 NPs enhanced oxidative stress and disrupted biofilm matrix integrity

Gene Expression Modulation: Combination treatments of CaO_2 NP and tobramycin downregulated genes associated with virulence and quorum sensing (*hcnA, phzA, pqsA, rhlR*), whereas calcium alone upregulated the gene expression indicating that there was necessity of combination of CaO_2 NPs with antibiotics.

Reduced Resistance Development: The efflux pump gene *mexG* was less upregulated in combination treatments which suggested the suppression of resistance mechanisms.

The present work reveals that CaO_2 NPs hold a lot of potential in improving the antibiotic efficacy and therapeutics of biofilm related infections. More investigations are, however, needed to establish the best dosage of nanoparticles and to evaluate their potential use in clinical practice.

Results and discussion: Squalenyl hydrogen sulfate nanoparticles

- **Viability analysis on PA14 biofilms**

To prepare the Sq-Tob nanoparticles several surfactants were used in preparation. Initially to study the effect of Sq, Tob and QSI alone and in combination without preparation of particle on the PA14 24 h biofilm, they were dissolved in DMSO and tested. Control with DMSO was also tested and the viability was then compared with untreated control, and measured using CFU assay.

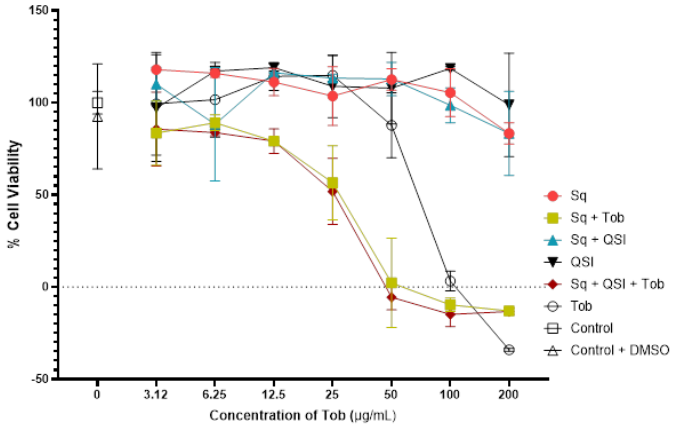


Figure 22 Physical mixtures of Sq, Tob and QSI for treatment on PA14 24 h biofilm

The physical mixture of Sq-Tob and Sq-QSI-Tob showed similar effect on the bacterial viability and the QSI did not increase bacterial eradication. The comparison between Sq-Tob and Tob showed that the Sq-Tob combination was more effective by 70 % to reduce bacterial viability as compared to Tob alone even in a physical mixture at 50 µg/mL concentration of Tob (see Figure 22).

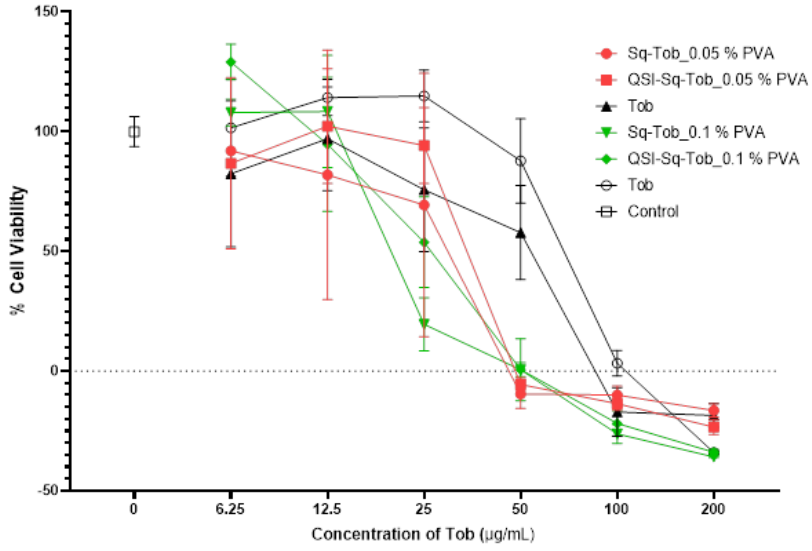


Figure 23 Sq and Tob nanoparticles prepared with or without QSI loading and 0.05 or 0.1 % PVA as surfactant, and Tob alone treatment on PA14 24 h biofilm

The Sq-Tob nanoparticles with or without QSI loading, were initially prepared in combination with aqueous phase surfactant PVA. They were tested immediately on the biofilms after preparation, to avoid precipitation issues. It was observed that at concentrations below 25 µg/mL of Tob, the surfactant used at 0.1 % PVA were more effective in reducing bacterial load as compared to when the surfactant was used at 0.05 % (see Figure 23).

However, the Sq-Tob and Sq-Tob-QSI nanoparticles prepared with PVA at both at 0.05 and 0.1 % showed enhanced bacterial biofilm eradication at and above 50 µg/mL Tob concentration. The effect from these nanoparticles was 50 % more pronounced in bacterial biofilm eradication than Tob alone at 50 µg/mL concentration of Tob (see Figure 23).

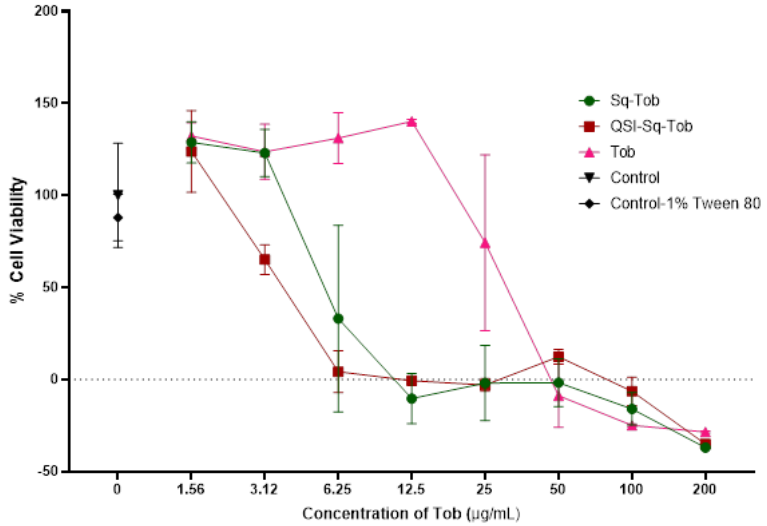


Figure 24 Sq and Tob nanoparticles prepared with or without QSI loading and 1 % Tween 80 as surfactant, and Tob alone treatment on PA14 24 h biofilm

It was observed in the later experiments that when organic phase surfactant was used at 0.2 and 1 % Tween 80 and tested immediately on the 24 h PA14 biofilms, the formulation showed enhanced activity in biofilm eradication.

When 1 % Tween 80 was used in the formulation, the Sq-QSI-Tob particle showed enhanced biofilm eradication when compared to Sq-Tob and Tob alone. The 1 % Tween 80 was also tested on the untreated control, which had little effect of bacterial viability as compared to control alone. The Sq-Tob particles in presence of Tween 80 with or without QSI were active in bacterial biofilm eradication at and above 12.5 µg/mL (see Figure 24).

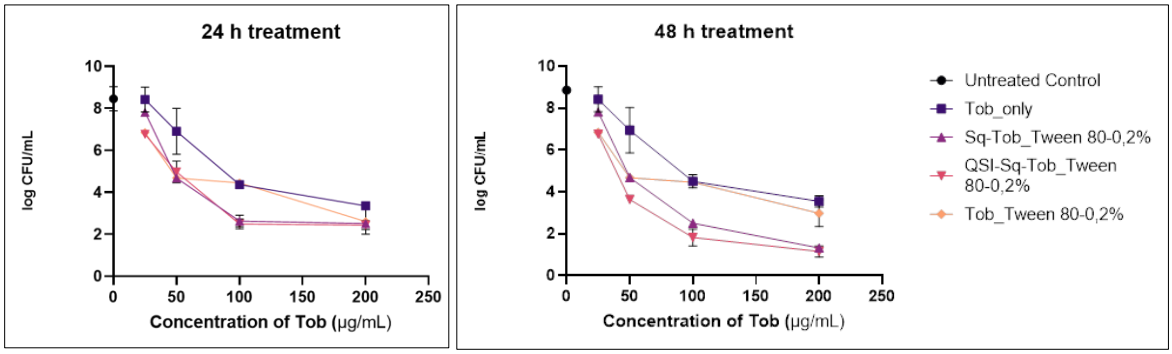


Figure 25 Sq and Tob nanoparticles prepared with or without QSI loading and 0.2 % Tween 80 as surfactant, and Tob alone treatment for 24 and 48 h on PA14 24 h biofilm

Initial results were measured using metabolic assay, later however, CFU assay was used for bacterial viability assessment. However, due to expected toxic effect on human cells, the Tween 80 concentration was reduced to 0.2 %. The test solutions were incubated on the biofilm for 24 and 48 h. The initial results of 24 h treatment showed that Sq-Tob and Sq-QSI-Tob particles had similar effect on bacterial viability. However, the physical mixture of Tob and 0.2 % Tween 80 had almost similar effect

on the bacterial viability. Tween 80 alone also had effect on bacterial viability, and enhanced the Tob bacterial eradication activity (see Figure 25). However, the treatment for 48 h showed that this effect from Tween 80 alone was reduced and the bacterial viability was not affected when the treatment was for longer time. It was observed however, that the Sq-Tob and Sq-QSI-Tob particles retained their bacterial killing activity in the longer treatment (see Figure 25). The bacterial viability was lower than 2 log CFU/mL for samples treated with Sq-Tob and Sq-QSI-Tob, where the Tob concentration was at and above 100 µg/mL.

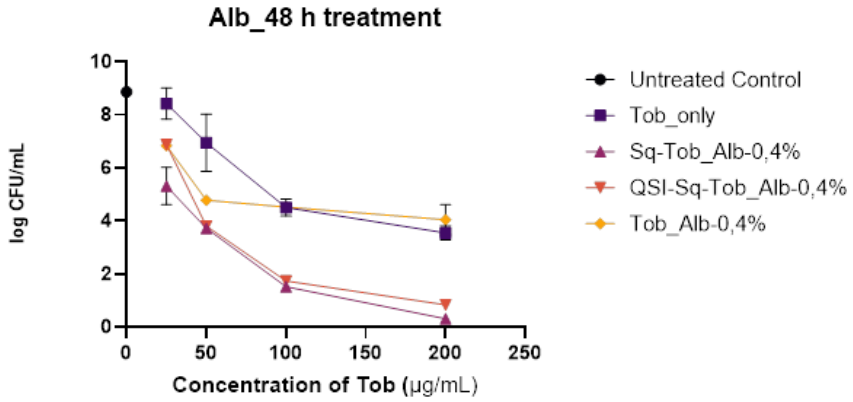


Figure 26 Sq and Tob nanoparticles prepared with or without QSI loading and 0.4 % albumin as surfactant, and Tob alone treatment for 48 h on PA14 24 h biofilm

Later experiments were conducted with albumin as a surfactant. It was observed that due to particle stability, they were tested immediately on the biofilms and the treatments were kept for 48 h on the biofilms. When 0.4 % albumin was used in physical mixture to Tob it did not affect the bacterial killing effect as that of Tob alone. However, the particles from Sq-Tob and Sq-QSI-Tob with albumin had not greater difference in the bacterial killing. The bacterial load decreased below 2 log CFU/mL and then to eradication at and above 100 µg/mL concentration of Tob (see Figure 26). However, it was observed that at 200 µg/mL concentration of Tob, the Sq-Tob particles with albumin were more effective in bacterial biofilm eradication than Sq-Tob particles with Tween 80.

- **Discussion**

The present study aims at assessing the effectiveness of squalene-tobramycin (Sq-Tob) nanoparticles, with or without quorum-sensing inhibitors (QSIs) against Pseudomonas aeruginosa PA14 biofilms. To formulate the nanoparticles and to assess the effectiveness of different surfactants such as PVA, Tween 80 and albumin in enhancing the biofilm eradication was also done. The tests with physical mixtures showed that the combination of Sq with Tob was more effective in the decreasing bacterial load than Tob alone. However, the addition of QSI to the combination did not enhance the effect, suggesting that the synergy was mainly due to the interaction between Sq and Tob[214].

The biofilm eradication was carried out using nanoparticles where PVA was used as a surfactant and the biofilm eradication was found to be dose dependent. The 0.1% formulations PVA containing was found to be more effective as compared to the formulations containing 0.05% PVA at concentrations of Tob below 25 µg/ mL. Both the concentrations of PVA were found to enhance the activity at concentrations of ≥50 µg/mL as compared to Tob alone. This can be attributed to PVA which is believed to help in stabilizing nanoparticles thus enhancing the delivery of the drug to the biofilm matrix[215].

The formulations with Tween 80 were the most effective in the process of biofilm eradication especially when used at the concentration of 1%. However, since there is a problem of toxicity, the experiments were conducted again with 0.2% Tween 80. At first, the antibacterial effect of Tween 80

only was observed to be significant but it reduced when used for extended duration (48 hours). On the other hand, Sq-Tob and Sq-QSI-Tob nanoparticles did not lose their activity thus indicating that Tween 80 increases the delivery of nanoparticles but does not enhance the bactericidal potential of the antibiotics once alone for a long period. These findings are in consistent with the work that has been done by earlier researchers in which they have demonstrated the use of Tween 80 in enhancing the solubility and penetration of drugs through biofilms[216].

Albumin-based Sq-Tob nanoparticles showed better antibiofilm activity at higher concentrations of Tob (≥100 µg/mL) where the bacteria were completely killed at 200 µg/mL Tob. Albumin may also help in the stabilization of the nanoparticles and also help in the gradual release of the drug over time thus enhancing the effectiveness of the nanoparticles in the treatment especially over longer durations. The above findings are consistent with the previous works which state that albumin is a stabilizer of nanoparticles that targets the biofilms[217].

The QSIs in combination with Sq-Tob nanoparticles did not significantly improve the bacterial killing as compared to the Sq-Tob nanoparticles alone. The QSIs could inhibit quorum-sensing pathways and can reduce biofilm virulence and resilience[218]. However the results indicated a more dominant role of the Sq-Tob activity in biofilm disruption than quorum-sensing inhibition.

For the several surfactant types used in preparation of Sq-Tob and Sq-QSI-Tob nanoparticles it was demonstrated that the bactericidal activity sustained over 48 hours. There was an achievement of reduction of bacterial load to below 2 log CFU/mL. The prolonged effect for biofilm reduction indicates the potential of Sq-Tob nanoparticles in biofilm-targeted treatment. Surfactants used in stabilization of nanoparticles, particularly those using albumin and Tween 80 showed enhanced efficacy, emphasizing the importance of surfactant selection in optimizing therapeutic outcomes.

- **Summary**

From the present study it is observed that Sq-Tob nanoparticles, which are synthesized with the help of various surfactants, can be effective in eliminating P. aeruginosa PA14 biofilms. Conclusions are as follows:

Nanoparticle Efficacy: Sq-Tob nanoparticles were more potent in reducing the bacterial viability as compared to Tob alone, which was effective in eradicating biofilm at relatively low concentrations.
Surfactant Optimization:
PVA: Effective stabilization of particles with higher concentrations (0.1%)
Tween 80: Enhanced biofilm penetration, however the toxicity concerns limit prolonged use.
Albumin: Complete biofilm eradication observed at 200 µg/mL.
QSI Role: The QSIs did not improve bacterial killing
Prolonged Activity: Sq-Tob nanoparticles showed prolonged bacterial killing effects

These findings support the notion that surfactant-based Sq-Tob nanoparticles are a novel therapeutic approach for management of biofilm related infections. Further research can also be done to identify the synergistic effects of QSIs and the compatibility of the surfactants for future clinical use.

Results and discussion: Lipid nanocapsules

- **Microscopical analysis**

The LNC's were prepared in different sizes of 25, 50, and 100 nm loaded with DiI dye. The dye was responsible for red fluorescence of the particles, and they were incubated on 24 h SA biofilm. The biofilms were imaged and the depth of fluorescence signal was observed (see Figure 27).

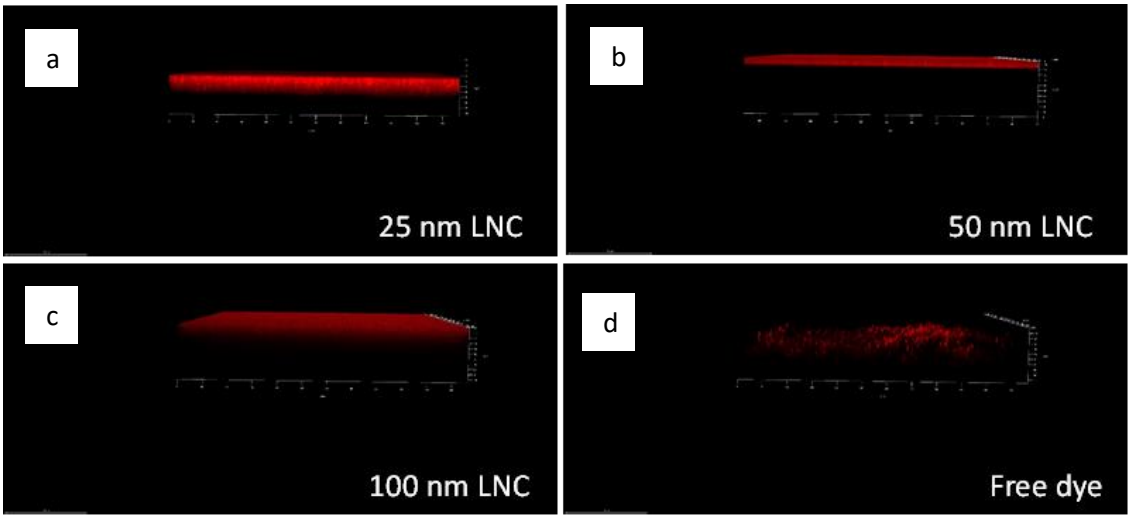


Figure 27 Confocal laser scanning microscopy (CLSM) of LNC's sized 25, 50, and 100 nm loaded with DiI dye, and free dye, observed as red fluorescence tested on S. aureus biofilm

For observing the depth of the signal from the LNC's, the images were zoomed in (see Figure 28). It was observed that the LNC's of size 25 nm were penetrated to a depth of 40 µm within the SA biofilm with a greater fluorescence intensity compared to 50 and 100 nm. The LNC's having the size of 50 nm, showed a biofilm penetration of nearly 20 µm; and the LNC's with 100 nm showed the biofilm penetration of 20 µm with lower fluorescence intensity than observed for the 50 nm particles.

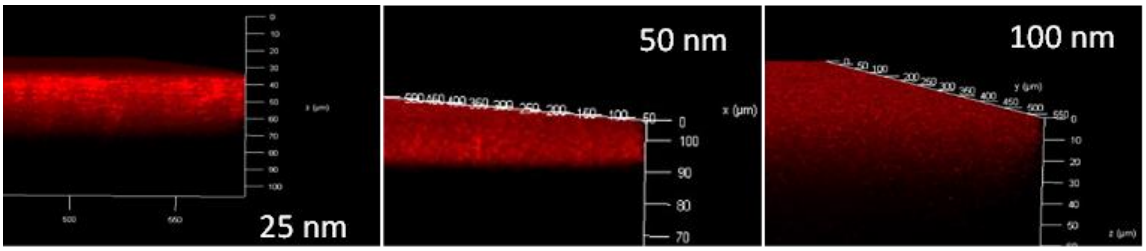


Figure 28 Confocal laser scanning microscopy (CLSM) of LNC's sized 25, 50, and 100 nm loaded with DiI dye, observed as red fluorescence tested on S. aureus biofilm (zoomed from Figure 27)

Later the LNC's were loaded with 1.5 mg/mL vancomycin irrespective of their sizes and tested on the 24 h SA biofilm. It was observed that (see Figure 29) the LNC's with 100 nm size were able to decrease the bacterial viability more than the 50 nm and 25 nm LNC's. At vancomycin concentration of 750 µg/mL the biofilms were eradicated in cases of the LNC's; whereas when compared with the free drug showed 80 % more viability of bacteria.

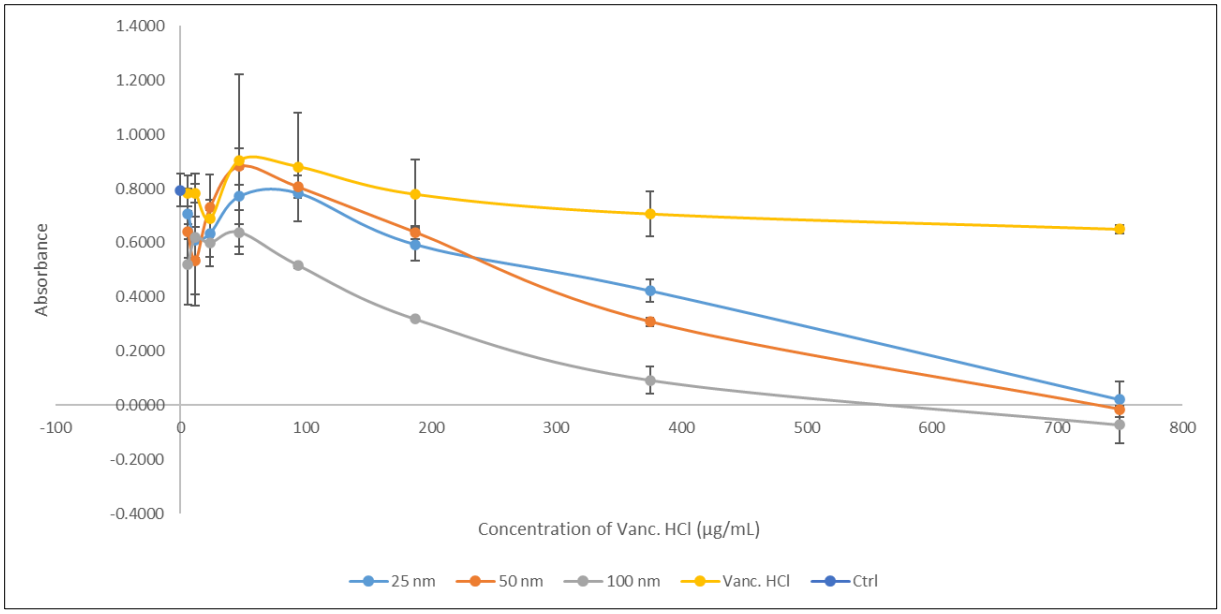


Figure 29 MBEC assay- vancomycin loaded LNC's of size 25, 50, and 100 nm tested on 24 h S. aureus biofilm

- **Discussion**

From the study, it is evident that the size of lipid nanocapsule (LNC) affects the ability of the biofilm penetration and bacterial killing of *Staphylococcus aureus* (SA) biofilms. The results also show that the increase in the size of the nanoparticles leads to the increase of the antibacterial activity while the small nanoparticles are able to penetrate more deeply into the biofilms.

The LNCs of 25 nm size was found to have the best biofilm penetration with the depth of 40 µm and the highest intensity of fluorescence as observed by CLSM. On the other hand, the LNCs of 50 and 100 nm sizes penetrated less efficiently with the biofilm invasion depth of around 20 µm only. The smaller size nanoparticles penetrate biofilms more easily as they are not as bulky and have better diffusivity to travel through the EPS matrix[219, 220].

When incubated with vancomycin, the 100 nm LNCs was found to be more effective in killing bacteria and eliminating biofilm than the smaller particles where complete biofilm eradication was observed only at a concentration of 750 µg/ml. This could be as a result of their enhanced drug carrying capacity which makes them efficient even for their shallower invasive abilities. Free vancomycin was also found to be less effective as can be seen from the 1.8 fold higher bacterial viability observed for the LNC formulations. The results are in agreement with research works that have shown that encapsulation of the drug in nanoparticles improves the delivery and retention of the drug at the biofilm surface, thus, overcoming the diffusion limitations and protection of the drug from enzymatic degradation by the biofilm matrix[221,222].

The variation in the performance of LNCs according to the size of the particles highlights the importance of achieving a proper balance in the design of nanoparticles, in terms of their ability to penetrate biofilms and their drug carrying capacity. It has been suggested that smaller particles are more effective in penetrating through biofilms while larger particles are more effective in delivering higher doses of drugs to specific areas.

- **Summary**

Penetration Depth: The LNCs of 25 nm size were the most effective in terms of the penetration depth into SA biofilms which was observed to be 40 µm and this had the highest fluorescence intensity as compared to 50 nm and 100 nm LNCs that only penetrated to a depth of 20 µm.

Antibacterial conjugated Activity: The 100 nm LNCs were found to completely eradicate biofilms at a vancomycin concentration of 750 µg/ml which was much effective than free vancomycin. This could be because smaller LNCs had a lower drug loading capacity.

Optimization: The findings of this study also provide evidence of the fact that there is a balance between the biofilm penetration and the drug carrying capacity of the nanoparticles in the form of LNCs which shows that LNC of specific sizes are effective in treating infections caused by biofilms.

3.3 Ex vivo analysis of calcium peroxide nanoparticles

- **Method**

In-situ grown human oral biofilms were treated *ex-situ*. The protocol[223, 224] adapted involved formation of biofilms *in-situ* (in oral cavity) of two volunteers. Enamel specimens were made from bovine incisor teeth. Enamel slab surfaces (ca. 3 x 4 x 1.5 mm) were prepared by wet grinding polish, with a final grain size of 2500 (Buehler, Düsseldorf, Germany) and they were cleaned, purified before oral exposure. Further these specimens were fixed on individual splints (Duran, SCHEU-DENTAL GmbH, Iserlohn, Germany) with the help of silicone impression material (PRESIDENT light body, Coltène/Whaledent GmbH + Co. KG, Langenau, Germany). For biofilm formation the splints were exposed intraorally for 48 h (see Figure 2). Whilst the enamel slabs were exposed orally, specimens were avoided from any cleaning procedures; furthermore, the volunteers refrained from using any agents (as toothpastes and mouth rinses) in cleaning measures. As well as during meals, the splints were removed and kept in a wet chamber. After formation of the biofilms, they were further exposed to various treatments ex situ.

The enamel specimens were removed from the splints and washed with sterile water. They were individually placed in wells of a 96 well plate and used in the experiments. For calcium peroxide nanoparticles, as the nanoparticles were dispersed in ethanol, initial experiments included 2 % ethanol in the treatment and 32 µg/mL concentration was examined. Later the method was modified to evaporate the ethanol. 20 µL solution to make 32, 96, 144, and 192 µg/mL was added in wells of 96 well plate. After ethanol evaporation, PBS was added. This was then gently stirred to redisperse calcium peroxide nanoparticles. The enamel specimens with biofilms were subsequently placed in the wells, this was further incubated for 18 h at 37 ˚C.

The effect of Tob with and without the combination of calcium peroxide nanoparticles was examined. Tob was dissolved in PBS and the concentrations of 1000, 500, 250 and 128 µg/mL were exposed on the enamel specimens with the biofilms for 18 h at 37 ˚C. The combination experiments involved the treatment between above mentioned concentrations. Controls included the incubation of biofilm specimens with PBS alone, calcium chloride dissolved in PBS (used in same concentration as calcium peroxide nanoparticles), and 300 µg/mL O_2-PFH loaded liposomes prepared in PBS at 37 ˚C for 18 h.

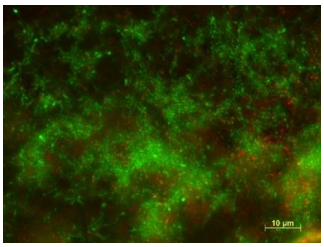
To assess the viability of bacteria within the biofilms on enamel specimen surfaces, staining with LIVE/DEAD® BacLight™ Bacterial Viability kit L7012 was performed and the analysis was done using fluorescence microscopy. SYTO9 dye stained all bacteria and this was observed as green fluorescence under the microscope, and the dead bacteria were stained with propidium iodide and this was observed as red fluorescence. For this the specimens were incubated with Baclight stain for 10 min in dark, mounting oil was used to observe the bacteria under 100X objective and the imaging was performed using fluorescence microscope (Axio Imager 2 Microscope, Zeiss MicroImaging, Göttingen, Germany). At least 4 representative images from different random positions within the specimen were taken with a 1000-fold magnification, and the image was processed with AxioVision 4.8 (Carl Zeiss Microimaging, Göttingen, Germany) software. The biofilm viability was analyzed by counting living bacterial cells (green fluorescence) and dead bacterial cells (red fluorescence) using a custom-made analysis routine written on the MATLAB with its Image Processing Toolbox (MATLAB with Image Processing Toolbox version: 9.10.0 (R2021a), Natick, Massachusetts: The MathWorks Inc.; 2021). The customized routine identifies bright objects, whose diameter lies in a specified range using top-hat-filtering, morphological opening and a watershed-segmentation, separating the bacterial clusters. The

objects appearing only on red channel or on green and red channel were counted as dead, and those appearing only on the green channel were counted as living bacteria.

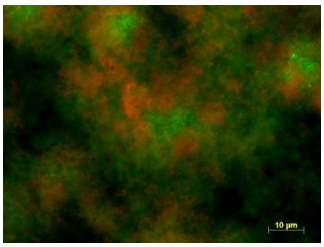
Images were also analyzed for bacterial coverage on the enamel surface specimens considering the complete area observed under the microscope and the bacterial growth over it. This was measured using the ImageJ software (National Institutes of health, Public Domain, BSD-2).

- **Results**

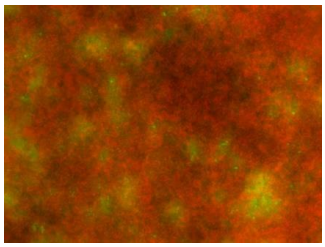
The in situ grown oral biofilms were treated ex situ with testing solutions. The initial treatment protocol involved calcium peroxide nanoparticles used at a concentration of 16 µg/mL and Tob at 1000 µg/mL. However, it was observed that the untreated control in PBS also showed dead bacteria (see Figure 30), which was due to the growth conditions within oral cavity. The calcium peroxide nanoparticles when dispersed in ethanol showed enhanced activity at a lower concentration used in testing (see Figure 30). And the concentration of Tob used was excessively high and lethal for oral bacteria.



Control (in PBS)



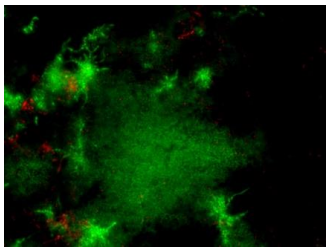
Calcium peroxide nanoparticles (16 µg/mL) dispersed in ethanol (2 %)



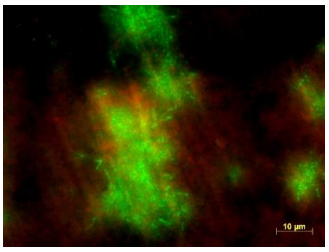
Calcium peroxide nanoparticles (16 µg/mL) dispersed in ethanol (2 %) and Tob (1000 µg/mL)

Figure 30 Fluorescence microscopy images for treated in situ grown oral biofilms

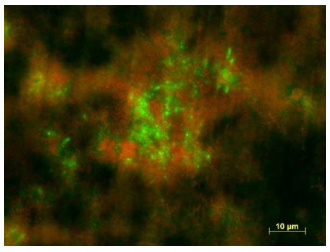
Therefore, in the next experiment the effect from ethanol alone was studied without the calcium peroxide nanoparticles. It was observed that the ethanol had toxic effect on the oral bacteria (see Figure 31).



Control (in PBS)



PBS and ethanol (2 %)



Tob (250 µg/mL) and ethanol (2 %)

Figure 31 Fluorescence microscopy images for treated in situ grown oral biofilms- study of the effect observed from ethanol

Thus, the method was further modified for ethanol evaporation. And the calcium peroxide nanoparticles were used at a lower concentration initially of 32 µg/mL. However, the lower concentration did not show expected bacterial load reduction (see Figure 32).

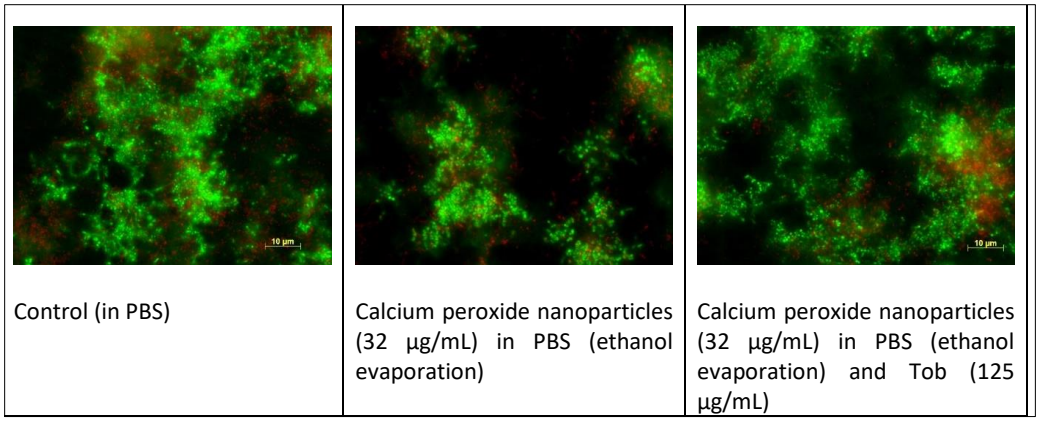


Figure 32 Fluorescence microscopy images for treated in situ grown oral biofilms- effect observed from lower concentration of calcium peroxide nanoparticles

For selection of calcium peroxide concentration, it was used in 1, 3, 4.5, and 6 times the concentration used in in vitro experiments; i.e. 32, 96, 144, 192 µg/mL (see Figure 33). The calcium peroxide nanoparticles showed an intrinsic antimicrobial effect at the concentration of 96 µg/mL. It was observed that the bacterial load at the concentration of 144 and 192 µg/mL decreased. Therefore, 96 µg/mL concentration of calcium peroxide nanoparticles was selected for further experiments.

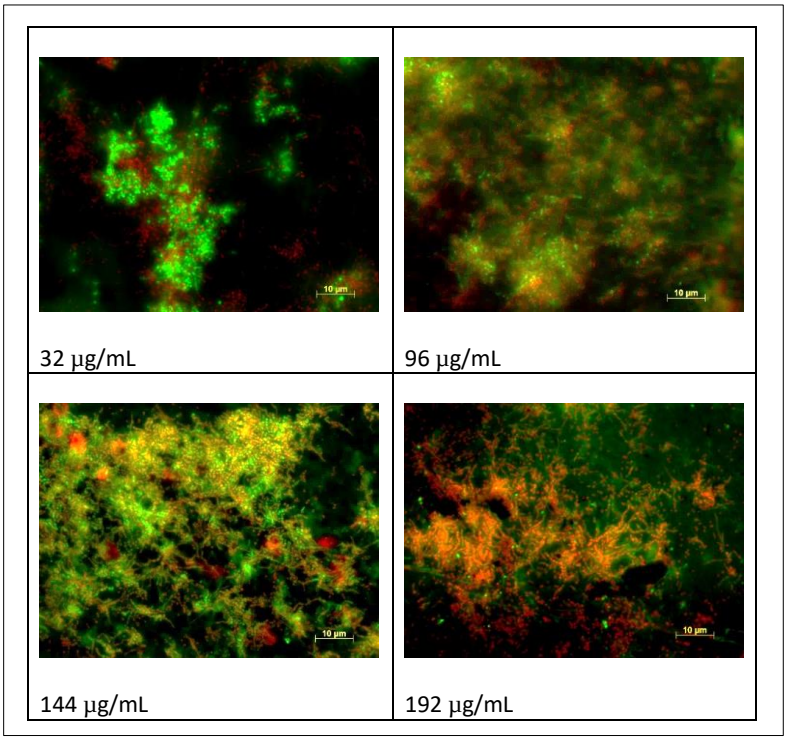


Figure 33 Fluorescence microscopy images for treated in situ grown oral biofilms- study for selection of concentration of calcium peroxide nanoparticles

Further, for selection of the concentration of tobramycin sulphate, concentrations of 128, 250, 500 and 1000 µg/mL were studied (see Figure 34). It was observed that at concentration of 128 µg/mL the bacterial load was least affected when compared to concentrations of 250, 500 and 1000 µg/mL. Thus, a sublethal concentration of 128 µg/mL was selected for further experiments to observe the combination effect from calcium peroxide nanoparticles.

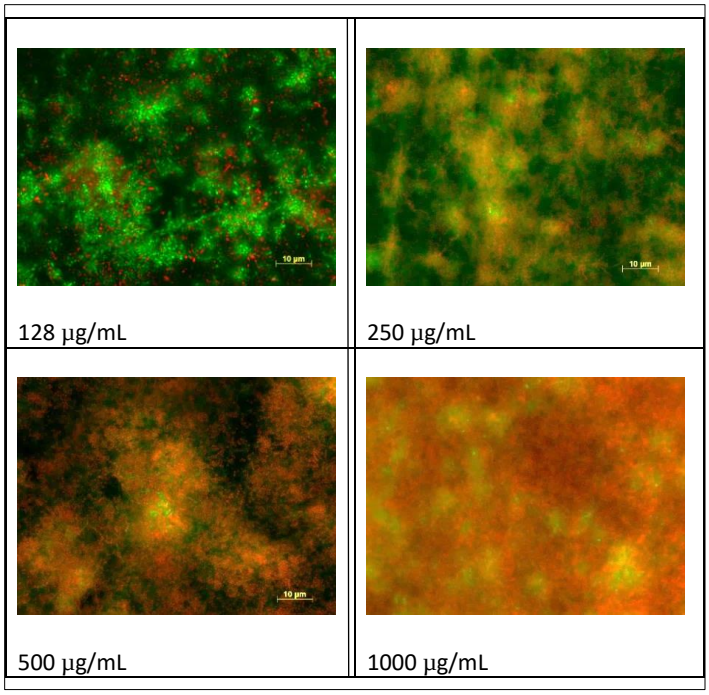


Figure 34 Fluorescence microscopy images for treated in situ grown oral biofilms- study for selection of concentration of tobramycin sulphate

The combination between calcium peroxide nanoparticles at the concentration of 96 µg/mL and tobramycin sulphate at 128 µg/mL was studied. It was observed that the calcium peroxide nanoparticles had an intrinsic antimicrobial effect, but also enhanced the antibiotic activity from tobramycin and reduced the bacterial load furthermore (see Figure 35).

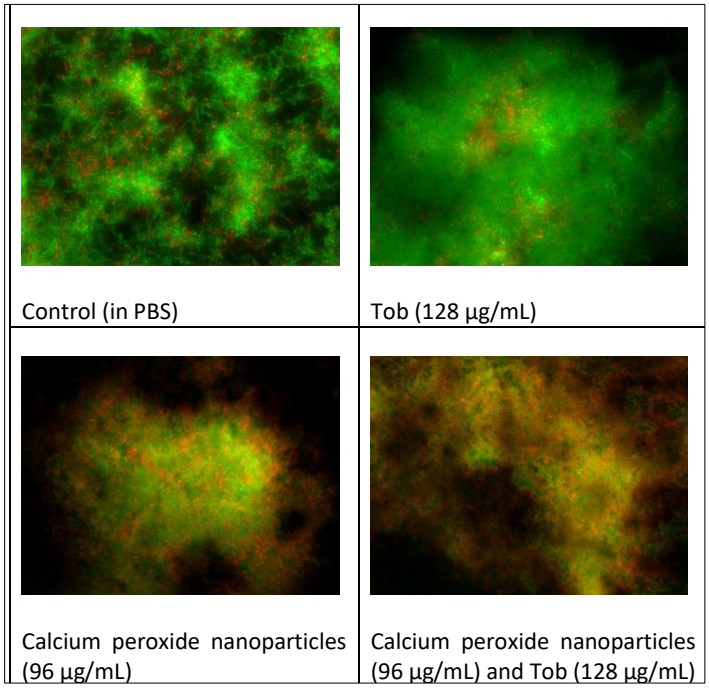


Figure 35 Fluorescence microscopy images for treated in situ grown oral biofilms- combination treatment of calcium peroxide nanoparticles and tobramycin sulphate

For comparison of the activity observed from calcium peroxide nanoparticles, the controls for its counterparts as used for in vitro experiments were studied. For the effect from oxygen the O_2-PFH

liposomes (300 µg/mL) and for the calcium released- calcium chloride (96 µg/mL) was used in control treatments. It was observed that the bacterial load was not affected when compared to the other treated samples (see Figure 36).

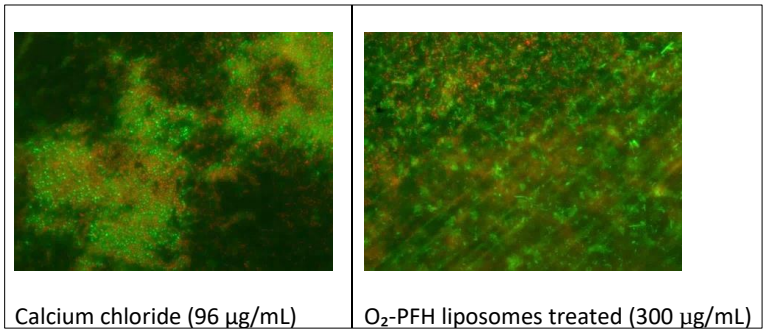


Figure 36 Fluorescence microscopy images for treated in situ grown oral biofilms- study for calcium chloride and O_2-PFH liposomes tested as controls

The bacterial load was then analyzed for % dead bacteria using the fluorescence intensity measurement from image analysis in MATLAB software. It was observed that for the untreated sample the % dead bacteria were nearly 40 %, which was also the case in several other treatments i.e. with only tobramycin sulphate, calcium chloride, and O_2-PFH liposomes. However, for calcium peroxide nanoparticles alone treated samples the % of dead bacteria increased to 60 % and for the combination with tobramycin increased furthermore to 70 % (see Figure 37).

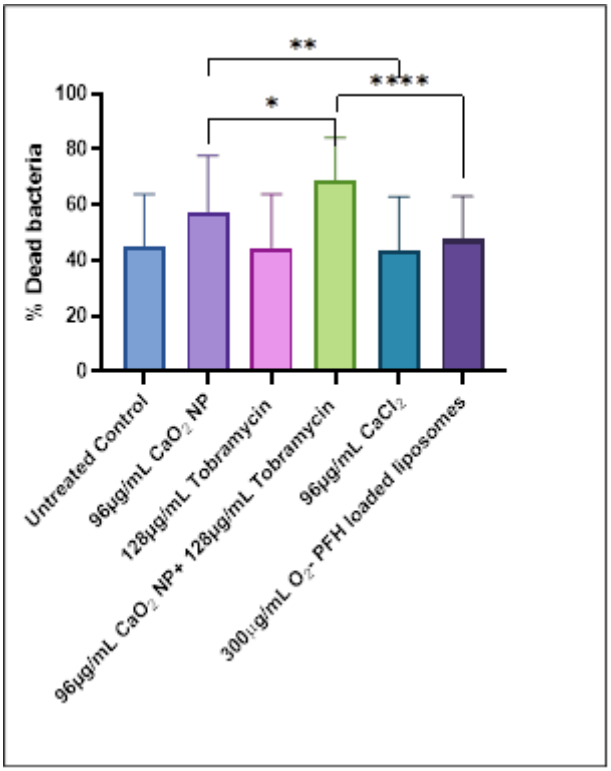


Figure 37 Graphical representation of % dead bacteria calculated using MATLAB software from fluorescence signal image analysis

The bacterial coverage over the surface of the specimen was also calculated using fluorescence intensity in the ImageJ software. It was observed that the bacterial coverage was in the range of 50 ± 30 % fairly distributed over the specimens throughout the treatments (see Figure 38).

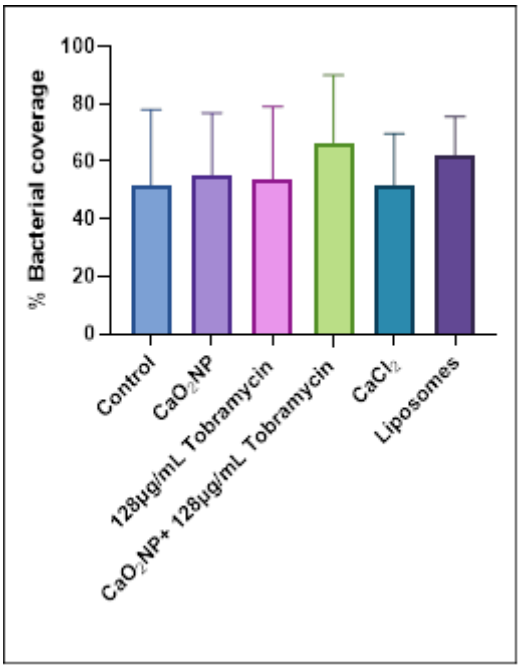


Figure 38 Graphical representation of % bacterial coverage on specimens calculated from fluorescence image analysis using ImageJ software

- **Discussion**

This study aimed at evaluating the antimicrobial effectiveness of calcium peroxide nanoparticles (CaO_2 NPs) and the synergistic interaction between the nanoparticles and tobramycin sulfate (Tob) on in situ formed oral biofilms. The first series of experiments showed that ethanol used as a dispersant had a direct toxic effect on oral bacteria, thus underlining the importance of the control of experimental factors. This effect was reduced by ethanol evaporation, which enabled the identification of the intrinsic antimicrobial activity of the nanoparticles.

CaO_2 NPs were active against a wide range of microorganisms and showed significant antimicrobial effect at 96 µg/mL and at higher concentrations. This is consistent with previous studies that have shown that nanoparticles are capable of inducing ROS and increasing the bacterial killing rate with the minimum toxicity at high concentrations[225]. The Tob was also found to have antibacterial activity in a dose dependent manner and therefore sublethal concentrations of 128 µg/mL were used to look for additive effects.

The combination of CaO_2 NPs and Tob indicated an enhancement of bacterial killing compared to individual treatments. This additive effect was possibly accounted by the disruption of bacterial membranes due to the ROS generation by CaO_2 NPs which facilitated the penetration of Tob. Studies have shown similar combinations to improve efficacy against biofilm-associated bacteria[226]. Control experiments involving oxygen-releasing liposomes and calcium chloride confirmed that the antimicrobial effect of CaO_2 NPs is a combination of both oxygen or calcium ion release.

The results were then analyzed and it was seen that the combination treatment led to a reduction in bacterial viability, as 70% of the bacteria were killed, while 40% killing was observed in the untreated controls. It was also observed that the bacterial coverage on the treated surfaces was not much different between the treatments, indicating that the biofilm architecture was not physically affected but the bacterial viability in the biofilms was greatly reduced. As suggested in ROS treated biofilms, the biofilms retained structural similarities[227].

- **Summary**

From the findings of this study, it has been seen that CaO_2 NPs can be considered as a potential adjuvant to antibiotics in the treatment of oral biofilms. The nanoparticles had a notable in vitro antimicrobial activity which was mainly attributed to ROS production and the nanoparticles were also found to enhance the effect of Tob even at sub lethal concentrations. The combination therapy resulted in increased bacterial kill without affecting the architecture of biofilms implying that it would be useful in preserving tissue structure during the treatment. These findings are in conformity with increasing evidence that supports the use of nanomaterials in combating antimicrobial resistance of biofilms and improving the efficiency of antibiotics. There is a need to continue with research to improve on the formulation of nanoparticles so that they can be delivered to the desired site of action with minimum harm to the surrounding tissues in the clinical practice. There is therefore the need to undertake more investigations in order to analyze the consequences of such therapies on oral microflora and other tissues in the future.

Conclusion

The current research work has also been aimed at investigating the possibility of employing nanoparticle based therapeutic systems for the management of the problem of biofilm related infections. This work involved three different types of nanoparticles namely calcium peroxide nanoparticles (CaO_2 NPs), squalenyl nanoparticles and lipid nanocapsules (LNCs) to determine the efficiency of these nanoparticles in breaking down the biofilms of *Pseudomonas aeruginosa* and *Staphylococcus aureus*. All of the experiments performed in this study are in vitro, ex vivo and mechanistic, which has provided a comprehensive understanding of the potential of these nanoparticles and their mechanisms, formulations, and interactions with antibiotics. These results also highlight the potential of nanoparticles in the treatment of biofilms and thus provide a basis for their potential use in a wider range of clinical situations.

The results demonstrated that CaO_2 NPs disrupt biofilm integrity by alleviating hypoxia through oxygen release and generating reactive oxygen species (ROS). This oxidative stress enhanced the antibiotic's penetration and efficacy, overcoming the antibiotic tolerance characteristic of biofilms. The results demonstrated that CaO_2 NPs can break down biofilm structure since hypoxia is relieved through oxygen release and ROS formation. This oxidative stress increased the antibiotic's permeability and effectiveness where it normally faces tolerance when it comes to biofilms. In addition, the calcium from these nanoparticles was seen to be released into the surroundings and help in compaction of the biofilm structure as well as regulate the calcium related signaling pathways that helped in the uptake of antibiotics by the bacterial cells. The gene expression analyses gave a better understanding of the molecular mechanisms that helped in identifying the interactions between the combined treatment and the quorum sensing genes and virulence factors which were found to be down regulated thereby reducing the biofilm resilience and pathogenicity. The experiments with in situ oral biofilms are supported by ex vivo studies that prove the relevance of the findings in more real-life conditions, revealing that CaO_2 NPs are effective in combination with tobramycin in more complex systems. The failure of calcium chloride or other oxygen releasing controls to produce similar results as the ones described above supported the specificity of CaO_2 NPs in biofilm eradication. The results presented here demonstrate that CaO_2 NPs has the potential to act as an adjunct to conventional antibiotics and may find its use in the management of chronic infections and biofilm related conditions.

Squalenyl hydrogen sulphate nanoparticles was another novel strategy for delivering the drugs across the biofilms. These nanoparticles were prepared with the help of surfactants like poly vinyl alcohol (PVA), Tween 80 and albumin and were found to be very effective in delivering tobramycin with or without quorum sensing inhibitors (QSIs) to the biofilms. The present research also showed that the type of surfactant used for the formulation of nanoparticles had a great effect on the performance of nanoparticles and that formulations stabilized with Tween 80 and albumin were more effective in penetrating biofilm and delivering the drug. Interestingly, squalenyl nanoparticles were able to inhibit the growth of bacteria and had a sustained antimicrobial activity thereby enhancing the potential for biofilm removal. And the addition of QSIs to the formulations did not enhance the bacterial killing to any significant degree indicating that the enhancements seen were mainly as a result of the physical and chemical interactions of the nanoparticles with the biofilm matrix. This shows how effective it is to control the composition of the nanoparticles so as to enhance the biofilm infiltration, drug loading and release while at the same time maintaining the activity of the drug.

In the study of lipid nanocapsules (LNCs), the focus was on the ability of LNCs to penetrate biofilms and the antibacterial effect against Staphylococcus aureus biofilms based on the size of the LNCs. The results showed that smaller LNCs (25 nm) were able to penetrate more deeply into biofilms and to a

larger extent, reaching areas that would otherwise be inaccessible. Although the larger LNCs (100 nm) were less invasive, they proved more effective in destroying bacteria because of their higher drug carrying capacity. The findings of this study also point to a major design challenge in the development of nanoparticles, that is, the trade-off between size and delivery efficiency; it is important to select the optimal size for nanoparticles to ensure that they are able to penetrate well but at the same time, can carry enough payload. This was seen when LNCs containing vancomycin produced better results than the free drug treatments and in the process highlight the advantages of nanoparticle based drug delivery in enhancing biofilm directed therapies.

This work increases the current knowledge on the application of nanoparticle-based solutions for biofilm control and presents new perspectives on the mechanisms, efficiency and potentials for enhancement of the systems here studied. All the nanoparticle systems presented in this study have their specific benefits, which prove that the approaches have to be tailored in order to deal with the biofilm problems. Calcium peroxide nanoparticles are identified as a highly effective and stable delivery system of oxygen as well as potential tool for disruption of biofilm matrix, especially when used in conjunction with antibiotics. The capacity of these nanoparticles to reduce hypoxia and regulate the bacterial stress responses may be of particular use in the treatment of chronic infections. Squalenyl nanoparticles improve the biofilm permeation and control the drug release making it suitable for extended antimicrobial therapy. Lipid nanocapsules provide a conceptual framework for the nanoparticle design highlighting the importance of particle size in the management of penetration and drug loading.

From the above findings, it is evident that nanoparticle systems have the capacity of addressing the challenges that are associated with conventional therapies in biofilm treatment. The flexibility and the versatility of these systems make them appropriate for various applications including; the treatment of chronic wounds, control of dental and medical device associated biofilms. Moreover, the synergistic effects that have been seen with the combination treatments show that it is imperative to incorporate nanoparticles with the conventional treatment methods in order to enhance the therapeutic response and prevent the development of resistance.

Although these findings are quite exciting, more research has to be conducted in order to translate these nanoparticle systems from the research realm to the clinical practice. Some of the major challenges include whether how to increase production, the issue of biocompatibility, and the assessment of safety and efficacy of the devices in vivo. It is also important to explore the possibility of the evolution of resistance as a response to nanoparticle-associated stress. Further research should also look into the development of more complex formulations including multifunctional nanoparticles that can perform oxygen release, immune modulation, tissue regeneration, or targeting of certain microbial pathways for instance.

Thus, this work provides a solid base for the creation of nanoparticle-based therapies for the treatment of biofilm-related infections. In this context, nanoparticles have been explored as a way of overcoming biofilm's resistance, increasing the effectiveness of antibiotics, and regulating bacterial virulence which makes these approaches very perspective for the treatment of biofilm-related diseases. Thus, with the help of further improvements and close cooperation between various fields, nanoparticle based therapies are expected to evolve into a powerful tool for the treatment of biofilm infections.

References

1. Hall-Stoodley, L., Costerton, J.W., & Stoodley, P. (2004). Bacterial biofilms: From the natural environment to infectious diseases. *Nature Reviews Microbiology*, 2(2), 95–108.
2. Costerton, J.W., Stewart, P.S., & Greenberg, E.P. (1999). Bacterial biofilms: A common cause of persistent infections. *Science*, 284(5418), 1318-1322.
3. O'Toole, G., Kaplan, H.B., & Kolter, R. (2000). Biofilm formation as microbial development. Annual Review of Microbiology, 54, 49–79.
4. Donlan, R.M., & Costerton, J.W. (2002). Biofilms: Survival mechanisms of clinically relevant microorganisms. Clinical Microbiology Reviews, 15(2), 167–193.
5. Davies, D.G., et al. (1998). The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science, 280(5361), 295–298.
6. Bjarnsholt, T. (2013). The role of bacterial biofilms in chronic infections. APMIS, 121(s136), 1–58.
7. Otto, M. (2012). Molecular basis of Staphylococcus epidermidis biofilm development and strategies for its treatment. Frontiers in Bioscience, 17, 2464–2477.
8. Marsh, P.D. (2003). Are dental diseases examples of ecological catastrophes? Microbiology, 149(2), 279–294.
9. He, J., et al. (2011). Community structure of oral microbiota in elderly individuals with dentures and without. Oral Microbiology and Immunology, 26(2), 113–120.
10. Ciofu, O., et al. (2015). Antibiotic treatment of biofilm infections. APMIS, 123(4), 322–338.
11. Stewart, P.S., & Costerton, J.W. (2001). Antibiotic resistance of bacteria in biofilms. The Lancet, 358(9276), 135–138.
12. Hajishengallis, G. (2015). Periodontitis: From microbial immune subversion to systemic inflammation. Nature Reviews Immunology, 15(1), 30–44.
13. Han, Y.W., & Wang, X. (2013). Mobile microbiome: Oral bacteria in extra-oral infections and inflammation. Journal of Dental Research, 92(6), 485–491.
14. Lamont, R.J., & Koo, H. (2012). Dental plaque biofilm: Ecological interactions in health and disease. Virulence, 3(3), 1–10.
15. Costerton, J.W., et al. (1995). Microbial biofilms. Annual Review of Microbiology, 49, 711–745.
16. Lewis, K. (2001). Riddle of biofilm resistance. Antimicrobial Agents and Chemotherapy, 45(4), 999–1007.
17. Flemming, H.C., & Wingender, J. (2010). The biofilm matrix. Nature Reviews Microbiology, 8(9), 623–633.
18. Kaplan, J.B. (2010). Biofilm dispersal: Mechanisms, clinical implications, and potential therapeutic uses. Journal of Dental Research, 89(3), 205–218.
19. Koo, H., et al. (2017). Targeting microbial biofilms: Current and prospective therapeutic strategies. Nature Reviews Microbiology, 15(12), 740–755.

20. Rumbaugh, K.P., & Sauer, K. (2020). Biofilm dispersion. Nature Reviews Microbiology, 18(9), 571–586.

21. Costerton, J.W., et al. (1999). Biofilms, the customized microniche. Journal of Bacteriology, 181(22), 7212-7216.

22. Fux, C.A., et al. (2005). Survival strategies of infectious biofilms. Trends in Microbiology, 13(1), 34–40.

23. Beloin, C., Renard, S., Ghigo, J.M., & Lebeaux, D. (2014). Novel approaches to combat bacterial biofilms. Current Opinion in Pharmacology, 18, 61–68.

24. Høiby, N., et al. (2010). Antibiotic resistance of bacterial biofilms. International Journal of Antimicrobial Agents, 35(4), 322–332.

25. Petrova, O.E., & Sauer, K. (2012). Sticky situations: Key components that control bacterial surface attachment. Journal of Bacteriology, 194(10), 2413–2425.

26. Kim, J., & Park, H.D. (2013). Engineered approaches to biofilm control. Bioengineered, 4(2), 49–55.

27. Stoodley, P., et al. (2002). Biofilms as complex differentiated communities. Annual Review of Microbiology, 56, 187-209.

28. Kolter, R., & Greenberg, E.P. (2006). The Super-Duper Drug: The Evolution of Quorum Sensing. Nature, 441(7096), 1235-1236.

29. Miller, M.B., & Bassler, B.L. (2001). Quorum sensing in bacteria. Annual Review of Microbiology, 55(1), 165-199.

30. Sutherland, I.W. (2001). Biofilm exopolysaccharides: a strong and sticky framework. Trends in Microbiology, 9(7), 261-264.

31. Fong, J.N., & Yildiz, F.H. (2015). Biofilm matrix proteins. Microbiology Spectrum, 3(1), 1-23.

32. Giridharan, B., et al. (2019). Microbial Biofilm: Its Mechanism of Formation and Its Applications. Current Research in Microbial Sciences, 3, 29-35.

33. An, S.Q., & Lee, S.J. (2017). Effect of flow on biofilm formation and the properties of biofilm. Environmental Engineering Research, 22(1), 63-70.

34. Lappin-Scott, H.M., & Costerton, J.W. (2000). Biofilm formation in nature and disease. Biofilms in Health and Disease, 1-14.

35. Singh, P.K., et al. (2000). Pseudomonas aeruginosa biofilms in cystic fibrosis. *Cystic Fibrosis*, 19(1), 53-68.

36. Otto, M. (2008). Staphylococcus epidermidis–the 'accidental' pathogen. *Nature Reviews Microbiology*, 6(8), 636-646.

37. Mah, T.F., & O'Toole, G.A. (2001). Mechanisms of biofilm resistance to antimicrobial agents. *Trends in Microbiology*, 9(1), 34-39.

38. Bjarnsholt, T., et al. (2008). Why chronic wounds won’t heal: A novel hypothesis. *Wound Repair and Regeneration*, 16(2), 103-112.

39. Marsh, P.D. (2006). Dental plaque as a biofilm and a microbial community—implications for health and disease. *Cureus*, 14(1), 157-173.

40. Hancock, R.E.W., & Sahl, H.G. (2006). Antimicrobial and host-defense peptides as new classes of therapeutic agents. *Nature Biotechnology*, 24(12), 1551-1557.

41. Donlan, R.M. (2001). Biofilm formation: A clinically relevant microbiological process. Clinical Infectious Diseases, 33(8), 1387-1392.

42. Parsek, M.R., & Singh, P.K. (2003). Bacterial biofilms: An emerging link to disease pathogenesis. Annual Review of Microbiology, 57, 677-701.

43. Greenberg, E.P. (2003). Quorum sensing in bacteria. *Nature*, *424*(6945), 134–136.

44. Lebeaux, D., et al. (2014). Management of infections associated with totally implantable venous-access ports: Challenges and perspectives. The Lancet Infectious Diseases, 14(2), 146-159.

45. Vu, B., et al. (2009). Bacterial extracellular polysaccharides involved in biofilm formation. Molecules, 14(7), 2535-2554.

46. Koo, H., et al. (2017). Biofilm in infectious diseases: Characteristics and therapy. Journal of Pathology, 247(5), 605-614.

47. Leid, J.G. (2009). Bacterial biofilms resist key host defenses. Microbe Magazine, 4(2), 66-70.

48. Sutherland, I.W. (2001). The biofilm matrix – an immobilized but dynamic microbial environment. Trends in Microbiology, 9(5), 222-227.

49. Karatan, E., & Watnick, P. (2009). Signals, regulatory networks, and materials that build and break bacterial biofilms. Microbiology and Molecular Biology Reviews, 73(2), 310-347.

50. Stewart, P.S. (2002). Mechanisms of antibiotic resistance in bacterial biofilms. *International Journal of Medical Microbiology*, *292*(2), 107–113.

51. Jefferson, K.K. (2004). What drives bacteria to produce a biofilm? FEMS Microbiology Letters, 236(2), 163-173.

52. Beloin, C., & Ghigo, J.M. (2005). Finding gene-expression patterns in bacterial biofilms. Trends in Microbiology, 13(1), 16-19.

53. Nguyen, T.K., et al. (2020). Smart hydrogels for biomedical applications in the era of COVID-19 and beyond. Advanced Healthcare Materials, 9(20), 2001023.

54. Cos, P., et al. (2010). Anti-biofilm agents: New perspectives for the treatment of microbial biofilm-related infections. Future Medicinal Chemistry, 2(2), 89-103.

55. Harriott, M.M., & Noverr, M.C. (2009). Candida albicans and Staphylococcus aureus form polymicrobial biofilms: Effects on antimicrobial resistance. Antimicrobial Agents and Chemotherapy, 53(9), 3914-3922.

56. Gristina, A.G. (1987). Biomaterial-centered infection: Microbial adhesion versus tissue integration. Science, 237(4822), 1588-1595.

57. Flemming, H.C., et al. (2016). Biofilms: An emergent form of bacterial life. Nature Reviews Microbiology, 14(9), 563-575.

58. Donlan, R.M. (2002). Biofilms: A potential source of infection in prosthetic heart valves. *Clinical Microbiology Reviews*, 15(2), 123-145.

59. Boles, B.R., & Singh, P.K. (2008). A microbial fortress: biofilm formation by the opportunistic pathogen Pseudomonas aeruginosa. *Nature Reviews Microbiology*, 6(2), 98-106.

60. Hall-Stoodley, L., & Stoodley, P. (2005). Evolving concepts in biofilm infections. *Nature Reviews Microbiology*, 3(4), 277-290.

61. Lewis, K. (2008). The presence of persisters in biofilms. *Nature Reviews Microbiology*, 6(1), 93-100.

62. Parsek, M.R., & Greenberg, E.P. (2000). Quorum sensing in Pseudomonas aeruginosa: a potential target for the treatment of biofilm infections. *Current Opinion in Microbiology*, 3(1), 78-82.

63. Zhang, L., et al. (2016). Biofilm formation by bacterial pathogens: Implications for infection and therapy. *Biofilm*, 1(1), 100005.

64. Bassetti, M., et al. (2018). Biofilm infections: A clinical challenge. *Clinical Microbiology and Infection*, 24(6), 583-589.

65. Melville, S. A., & O'Brien, S. K. (2018). Pseudomonas aeruginosa Biofilms in Cystic Fibrosis: Therapeutic Strategies and Drug Discovery. *Journal of Medicinal Chemistry*, 61(19), 8187-8200.

66. Wang, X., et al. (2020). Role of Extracellular Polymeric Substances in Biofilm Formation by Pseudomonas aeruginosa. *Journal of Bacteriology*, 202(14), e00252-20.

67. Acker, J., & Rumbaugh, K.P. (2015). The Role of Biofilms in Chronic Wound Healing: Implications for the Use of Biofilm-Disrupting Agents. *Advances in Wound Care*, 4(5), 317-324.

68. Zong, Z., et al. (2021). Characteristics of Staphylococcus aureus Biofilm in Chronic Wound Infection. *Infection and Drug Resistance*, 14, 67-76.

69. Arciola, C.R., Campoccia, D., & Montanaro, L. (2018). Epidemiology of biofilm infections in medical devices. *Clinical Microbiology and Infection*, *24*(9), 1096–1106.

70. de Vos, W. M., & van der Meer, J. R. (2015). Staphylococcus aureus biofilms: Resilience against Antibiotics. *Clinical Microbiology Reviews*, 28(4), 952-974.

71. Götz, F. (2002). Staphylococcus and biofilms. *Molecular Microbiology*, 43(6), 1367-1378.

72. Flores-Mireles, A. L., et al. (2015). Urinary tract infections: epidemiology, mechanisms of infection, and treatment options. *Nature Reviews Urology*, 12(12), 773-790.

73. Hufnagel, M., et al. (2012). Biofilm Formation of Enterococcus faecalis in Infections. *Current Microbiology*, 65(4), 470-476.

74. Mook, C. E., et al. (2015). Klebsiella pneumoniae Biofilm Formation and Pathogenicity. *Frontiers in Microbiology*, 6, 578.

75. Hall, C. M., & Mah, T.F. (2017). The importance of biofilm in infection and disease. *Biofilms and Biofilm Control*, 21(2), 183-191.

76. Percival, S.L., et al. (2015). Biofilms and their significance in wound healing. *Wound Repair and Regeneration*, 23(3), 239-247.

77. Lee, W., et al. (2019). Antimicrobial Peptides: A New Strategy to Combat Biofilm. *Frontiers in Microbiology*, 10, 1583.

78. Papenfort, K., & Bassler, B. L. (2016). Quorum sensing signal degradation by a novel enzyme. *Nature*, 530(7591), 586-590.

79. Rumbaugh, K. P., et al. (2015). The Role of Quorum Sensing in the Biofilm Formation and Persistence of Pseudomonas aeruginosa. *Clinical Microbiology Reviews*, 28(3), 713-728.

80. Tetz, G., & Tetz, V. (2015). Biofilm-Disrupting Enzymes: A New Approach for Antimicrobial Therapy. *Current Topics in Medicinal Chemistry*, 15(10), 914-926.

81. Campoccia, D., et al. (2013). A Review of Antibacterial Coatings for Medical Devices. *Bioactive Materials*, 3(3), 259-270.

82. Mittermayr, R., et al. (2013). Antibiotic-Impregnated Biomaterials for the Prevention of Infections. *World Journal of Clinical Cases*, 1(6), 140-148.

83. Salvatore, M. R., & Gupta, A. (2019). Responsive Polymer Coatings for Infection Control on Medical Devices. *Advanced Healthcare Materials*, 8(18), 1900686.

84. Bridier, A., et al. (2011). Physical Disruption of Biofilms with Ultrasound: Implications for Therapy. *Clinical Microbiology Reviews*, 24(1), 149-161.

85. Hamblin, M. R., & Yalcin, F. (2018). Photodynamic Therapy for the Treatment of Biofilm-Associated Infections. *Nature Reviews Microbiology*, 16(7), 495-502.

86. Vasiljeva, A., et al. (2018). Electric Fields to Disrupt Biofilms and Improve Antibiotic Efficacy. *Bioelectrochemistry*, 123, 29-38.

87. Kalia, V. C., & Bansal, P. (2015). Synergistic Effect of Antibiotics in Biofilm Eradication: A Review. *Journal of Applied Microbiology*, 119(4), 1154-1170.

88. Nagaoka, I., et al. (2015). Antimicrobial Peptides and Their Combinations with Antibiotics: A Novel Approach for Biofilm Eradication. *International Journal of Antimicrobial Agents*, 46(6), 715-722.

89. Baptista, P. V., et al. (2018). Nanoparticles as Antimicrobials: A Review. *Nature Reviews Microbiology*, 16(5), 297-313.

90. Bikard, D., et al. (2014). Targeted Editing of Bacterial Genomes Using a CRISPR–Cas System. *Nature Biotechnology*, 32(12), 1241-1245.

91. Hyman, P., & Abedon, S. T. (2010). Bacteriophage: Practical Applications for Biofilm Control. *Biofilms in Human Health and Disease*, 2010, 327-334.

92. Bredenbruch, F., et al. (2006). The Pseudomonas aeruginosa biofilm matrix: an overview of the constituents and their roles in biofilm structure and function. Frontiers in Microbiology, 7, 184.

93. Kottwitz, J., et al. (2021). Virulence factors of Pseudomonas aeruginosa: a focus on biofilm formation and immune evasion. Current Opinion in Microbiology, 57, 67-73.

94. Elkins, M. R., et al. (2017). The role of the airway microbiome in cystic fibrosis lung disease. The Lancet Respiratory Medicine, 5(3), 237-245.

95. Gontijo, J. R. S., & Sato, A. M. (2020). Burn wound infection: a review of the clinical importance of Pseudomonas aeruginosa. The Brazilian Journal of Infectious Diseases, 24(3), 246-253.

96. Rego, A. B., et al. (2019). Bone and joint infections due to Pseudomonas aeruginosa: clinical aspects and treatment. Revista Brasileira de Ortopedia, 54(6), 672-677.

97. Keshavarz, K., et al. (2018). The role of Pseudomonas aeruginosa in sepsis: a review of its pathogenicity and treatment options. European Journal of Clinical Microbiology & Infectious Diseases, 37(3), 439-451.

98. Poole, K. (2011). Pseudomonas aeruginosa: resistance to the meropenem and other β-lactam antibiotics. Journal of Antimicrobial Chemotherapy, 66(4), 949-959.

99. Michalopoulos, A. S., et al. (2020). Pseudomonas aeruginosa: An Emerging Opportunistic Pathogen in Intensive Care Units. International Journal of Antimicrobial Agents, 55(6), 105-116.

100. Olakanmi, O., & Adebayo, S. O. (2020). The economic burden of Pseudomonas aeruginosa infections in healthcare settings: a systematic review. International Journal of Infection Control, 16(3), 1-10.

101. Liu, X., et al. (2017). Oxygen Gradients in Biofilms: Implications for Antimicrobial Resistance. *Frontiers in Microbiology*, 8, 1465.

102. Yang, L., & Liao, K. (2020). The Role of Anaerobic Microorganisms in Biofilms: A Review. *Microbial Biotechnology*, 13(2), 318-328.

103. Zhu, Y., et al. (2019). Metabolic Adaptation in Biofilms: The Role of Oxygen in Shaping Microbial Communities. *Nature Reviews Microbiology*, 17(11), 742-755.

104. McBain, A. J., et al. (2004). Biofilms: A Key Factor in the Survival of Bacteria in the Oral Cavity. *Journal of Applied Microbiology*, 96(4), 749-756.

105. Wozniak, D. J., & Ohman, D. E. (1994). Identification of a Pseudomonas aeruginosa gene involved in biofilm formation and virulence. *Infection and Immunity*, 62(7), 2934-2939.

106. Darveau, R. P. (2010). The Oral Microbiome: A New Frontier in Periodontal Disease Research. *Journal of Periodontology*, 81(12), 1796-1802.

107. Kuehn, M., et al. (2013). The Role of Anaerobic Bacteria in Chronic Wound Infections. *Wound Repair and Regeneration*, 21(3), 318-325.

108. McCarty, T. M., & O'Rourke, K. (2015). Anaerobic Infections: Diagnosis and Management. *Infectious Disease Clinics of North America*, 29(4), 817-829.

109. Wang, Q., et al. (2018). "The Oral Microbiome: A Key Player in Health and Disease." *Nature Reviews Microbiology*, 16(9), 630-644.

110. Garcia, R. I., & Haffajee, A. D. (2017). "The Role of the Oral Microbiome in Oral Health and Disease." *Periodontology 2000*, 74(1), 9-24.

111. Sakamoto, M., & RY, H. (2013). "Bacterial Diversity in the Human Oral Cavity." *Journal of Oral Microbiology*, 5, 19803.

112. Zhang, L., et al. (2020). "Oral Microbiome and Its Impact on Systemic Health." *Frontiers in Microbiology*, 11, 579362.

113. Haffajee, A. D., & Socransky, S. S. (2005). "Dental Biofilms: An Overview." *Periodontology 2000*, 38(1), 19-50.

114. Aas, J. A., et al. (2005). "Defining the Normal Bacterial Flora of the Oral Cavity." *Journal of Clinical Microbiology*, 43(11), 5721-5732.

115. Marsh, P. D. (2018). "Infection and Immunity in the Oral Cavity." *Nature Reviews Microbiology*, 16(7), 380-394.

116. De La Fuente-Núñez, C., et al. (2015). "Antibiotic Resistance in the Context of Oral Biofilms." *Nature Reviews Microbiology*, 13(10), 653-669.

117. Gajardo, K., et al. (2019). "The Role of the Oral Microbiome in the Development of Chronic Diseases." *Journal of Oral Microbiology*, 11(1), 1623304.

118. Kassebaum, N. J., et al. (2014). "Global Burden of Severe Dental Caries." *Journal of Dental Research*, 93(7 Suppl), 8S-18S.

119. Kim, K. W., et al. (2018). "Porphyromonas gingivalis and Systemic Inflammation: The Involvement of Toll-Like Receptors." *Frontiers in Microbiology*, 9, 739.

120. Saadoun, M., et al. (2019). "Halitosis: A Review of the Literature." *Journal of Clinical Medicine*, 8(4), 455.

121. Paster, B. J., et al. (2006). "The Human Oral Microbiome." *Genome Research*, 16(2), 200-206.

122. Pihlstrom, B. L., et al. (2005). "Periodontal Diseases." *Lancet*, 366(9499), 1809-1820.

123. Nakano, K., et al. (2015). "Relationship between Oral Dysbiosis and Dental Caries." *Journal of Oral Science*, 57(1), 115-120.

124. Kuczynski, J., et al. (2010). "Direct Sequencing of the Human Microbiome." *Nature*, 464(7285), 59-65.

125. Page, R. C., & Schroeder, H. E. (1982). "Periodontitis in Man and Other Animals: A Comparative Review." *Journal of Periodontology*, 53(12), 697-709.

126. Papadopoulos, A., et al. (2017). "Diet and Oral Health." *Nature Reviews Gastroenterology & Hepatology*, 14(8), 494-505.

127. Vinderola, G., et al. (2018). "Probiotics in the Prevention of Oral Diseases." *Journal of Clinical Dentistry*, 29(1), 1-9.

128. Goll, R., et al. (2013). "The Role of Probiotics in Oral Health." *BMC Oral Health*, 13(1), 41.

129. Veiga, P., et al. (2019). "The Role of Probiotics in Preventing Oral Disease." *European Journal of Clinical Microbiology & Infectious Diseases*, 38(5), 749-762.

130. Baillie, M., et al. (2016). "Calcium Peroxide as a Source of Oxygen: A Review." *International Journal of Pharmacy and Pharmaceutical Sciences*, 8(7), 13-19.

131. Chen, Y., et al. (2019). "Calcium Peroxide-Loaded Hydrogel for Enhanced Wound Healing." *Biomedical Materials*, 14(3), 035003.

132. Dhiwakar, M., et al. (2016). "Endodontic Disinfection Using Calcium Peroxide." *Journal of Conservative Dentistry*, 19(5), 451-454.

133. Scully, C., et al. (2014). "Oral and Maxillofacial Surgery: Calcium Peroxide and its Role in Oral Hygiene." *Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology*, 118(5), 505-510.

134. Zou, Z., et al. (2016). "Application of Calcium Peroxide in Remediation of Contaminated Soils: A Review." *Journal of Environmental Management*, 183, 837-844.

135. Ghosh, D., et al. (2020). "Sustainable Remediation Strategies Utilizing Calcium Peroxide." *Environmental Science and Pollution Research*, 27(35), 44285-44298.

136. Zhang, L., et al. (2021). "Nanostructured Calcium Peroxide for Enhanced Oxygen Release: Synthesis and Applications." *Journal of Nanomaterials*, 2021, 1-10.

137. Alhajj, M., et al. (2022). "Enhancing Wound Healing through Calcium Peroxide and Antimicrobial Combinations." *Wound Repair and Regeneration*, 30(1), 35-45.

138. Kuo, T., et al. (2020). "Controlled Release of Oxygen from Calcium Peroxide-Based Systems for Wound Care." *Materials Science and Engineering C*, 116, 111115.

139. Bassetti, M., et al. (2018). Current Treatment Options for Pseudomonas aeruginosa Infections. Expert Review of Anti-infective Therapy, 16(4), 277-288.

140. Elam, N., et al. (2017). Squalenyl Hydrogen Sulfate: A New Amphiphilic Polymer for Targeted Drug Delivery. Polymers, 9(6), 224.

141. Wacker, M., et al. (2019). Nanoparticles in Drug Delivery: A Review of Self-assembled Systems. International Journal of Nanomedicine, 14, 319-332.

142. Schütz, C., Ho, D.-K. (2021). A New PqsR Inverse Agonist Potentiates Tobramycin Efficacy to Eradicate Pseudomonas aeruginosa Biofilms. Nature Communications, 12(1), 1-12.

143. Ho, D.-K., et al. (2020). Squalenyl Hydrogen Sulfate Nanoparticles for Simultaneous Delivery of Tobramycin and an Alkylquinolone Quorum Sensing Inhibitor Enable the Eradication of P. aeruginosa Biofilm Infections. Journal of Controlled Release, 322, 264-276.

144. Duy-Khiet, H., et al. (2019). Synthesis and Biopharmaceutical Characterization of Amphiphilic Squalenyl Derivative Based Versatile Drug Delivery Platform. Journal of Drug Delivery Science and Technology, 55, 101210.

145. Connelly, C. M., et al. (2022). Effects of Nanoparticle-Encapsulated Antibiotics on Biofilm Dispersal. ACS Nano, 16(1), 189-197.

146. Kahl, B. C., et al. (2014). Activity of Tobramycin in the Presence of Squalenyl Hydrogen Sulfate Nanoparticles. Journal of Antimicrobial Chemotherapy, 69(5), 1330-1337.

147. Liu, Y., et al. (2018). Combination Antibiotic Therapy for Pseudomonas aeruginosa Infections. Infection and Drug Resistance, 11, 1395-1405.

148. Marquette, C., et al. (2020). Quorum Sensing Inhibitors as Enhancers of Tobramycin-Loaded Nanoparticles. Frontiers in Chemistry, 8, 584242.

149. Kuhl, M., et al. (2021). Lower Doses of Antibiotics in Combination with Nanoparticles: Reducing Resistance. Nature Reviews Drug Discovery, 20(10), 819-820.

150. Zhang, X., et al. (2018). Biofilm Disruption by Amphiphilic Polymers. Nature Materials, 17(9), 882-889.

151. Ho, D.-K., et al. (2020). Squalenyl Hydrogen Sulfate Nanoparticles for Simultaneous Delivery of Tobramycin and an Alkylquinolone Quorum Sensing Inhibitor Enable the Eradication of P. aeruginosa Biofilm Infections. Journal of Controlled Release, 322, 264-276.

152. Bloemberg, G. V., et al. (2017). Inhibition of Biofilm Formation and Antimicrobial Synergy Using SHS Nanoparticles. Antimicrobial Agents and Chemotherapy, 61(10), e01774-17.

153. Schütz, C., Ho, D.-K. (2021). A New PqsR Inverse Agonist Potentiates Tobramycin Efficacy to Eradicate Pseudomonas aeruginosa Biofilms. Adv Sci (Weinh), 12(1), 1-12.

154. Hennig, G., et al. (2020). Quorum Sensing Inhibitors as Part of Synergistic Therapies for Multi-drug Resistant Infections. Future Medicinal Chemistry, 12(8), 691-710.

155. Stoodley, P., et al. (2002). Biofilm Formation and Growth: The Role of Material Surface in Infection. Nature Reviews Microbiology, 1(1), 23-30.

156. Kuhl, M., et al. (2021). Nanoparticle-Encapsulated Antibiotics: Enhancing Efficacy in Biofilm Models. Journal of Drug Delivery Science and Technology, 60, 102017.

157. Zhang, X., et al. (2018). Biofilm Disruption by Amphiphilic Polymers: Advances in the Application of SHS Nanoparticles. Advanced Drug Delivery Reviews, 131, 98-113.

158. Ho, D.-K., et al. (2018). Nanoparticles for Antibiotic Delivery in the Treatment of Biofilm-Related Infections: Challenges and Opportunities. Journal of Nanobiotechnology, 16(1), 72-85.

159. Haversack, J., & Nehrbass, U. (2017). "Antibiotic Resistance in Gram-Positive Bacteria: Mechanisms and Management." *Journal of Antimicrobial Chemotherapy*, 72(3), 675-686.

160. Pereira, M.C., et al. (2019). "Lipid Nanocapsules for the Treatment of Infectious Diseases." *Current Medicinal Chemistry*, 26(7), 1247-1262.

161. Parikh, R.H., et al. (2020). "Surface Functionalization of Lipid Nanocarriers: Improving Drug Delivery in Infections." *Pharmaceutical Research*, 37(10), 200-212.

162. Rosenberg, J., et al. (2020). "Lipid Nanocapsules in Infectious Disease Therapy: Mechanisms and Applications." *Drug Delivery and Translational Research*, 10(1), 239-254.

163. Jiao, Y., et al. (2021). "Targeted Nanocarrier Systems for the Delivery of Antibacterial Agents: Recent Advances." *Nanomedicine*, 16(2), 155-169.

164. Tiwari, S.K., et al. (2018). "Enhanced Antibacterial Activity of Lipid Nanocapsules Loaded with Vancomycin." *European Journal of Pharmaceutics and Biopharmaceutics*, 129, 40-50.

165. Wang, Z., et al. (2019). "Efficacy of Lipid Nanocapsules in Delivering Antibiotics to Staphylococcus aureus Biofilms." *BMC Microbiology*, 19(1), 1-10.

166. Choudhary, A., et al. (2020). "In Vivo Efficacy of Lipid Nanocarriers Against Gram-Positive Bacterial Infections." *Frontiers in Microbiology*, 11, 1565.

167. Zhuang, H., et al. (2021). "The Role of Lipid Nanocapsules in Combatting Antibiotic Resistance." *Nature Reviews Drug Discovery*, 20(6), 427-428.

168. Li, Z., et al. (2020). "Formulation and Characterization of Lipid Nanocapsules for Antibiotic Delivery." *International Journal of Nanomedicine*, 15, 3897-3908.

169. Qiu, H., et al. (2019). "Combination Antibiotic Therapy Using Lipid Nanocapsules." *Molecular Pharmacology*, 96(5), 606-617.

170. Montanari, J., et al. (2020). "Lipid Nanocarriers in Clinical Settings: Potential and Challenges." *Clinical Pharmacokinetics*, 59(10), 1213-1226.

171. Sheng, Y., Wang, X., Song, L., Wang, Z., Han, Y., Cui, H., & Sun, H. (2017). Oxygen generating nanoparticles for improved photodynamic therapy of hypoxic tumours. Journal of Controlled Release, 264, 333–340.

172. Rastinfard, A., Nazarpak, M. H., & Moztarzadeh, F. (2018). Controlled chemical synthesis of CaO_2 particles coated with polyethylene glycol: characterization of crystallite size and oxygen release kinetics. RSC Advances, 8, 91–101.

173. Khorshidi, S., Karkhaneh, A., & Bonakdar, S. (2019). Fabrication of amine-decorated nonspherical microparticles with calcium peroxide cargo for controlled release of oxygen. Journal of Biomedical Materials Research Part A, 108, 136–147.

174. Mollajavadi, M. Y., Saadatmand, M., & Ghobadi, F. (2023). Effect of calcium peroxide particles as oxygen-releasing materials on cell growth and mechanical properties of scaffolds for tissue engineering. *Iranian Polymer Journal, 32*, 599–608.

175. Zare, Y. (2016). Study of nanoparticles aggregation/agglomeration in polymer particulate nanocomposites by mechanical properties. *Composites Part A: Applied Science and Manufacturing, 84*, 158–164.

176. Bankar, N., Latta, L., Loretz, B., et al. (2024). Antimicrobial and antibiotic-potentiating effect of calcium peroxide nanoparticles on oral bacterial biofilms. *npj Biofilms and Microbiomes, 10*(106).

177. Albanese, A., Tang, P. S., & Chan, W. C. (2012). The effect of nanoparticle size, shape, and surface chemistry on biological systems. *Annual Review of Biomedical Engineering, 14*, 1–16.

178. Zhang, L., Wang, H., & Zhou, Q. (2018). Surfactant-assisted synthesis of stable calcium-based nanoparticles for biomedical applications. *Colloids and Surfaces B: Biointerfaces, 169*, 278–286.

179. Guo, S., He, H., & Zhou, L. (2017). Stabilization of calcium-based nanoparticles using surfactants and heating techniques. *Materials Science & Engineering C, 75*, 659–666.

180. Song, C., Yuan, H., & Wu, W. (2021). Sustained oxygen release from calcium peroxide nanoparticles for hypoxic tumor therapy. *ACS Applied Bio Materials, 4*, 651–660.

181. Liu, C., Wang, J., & Huang, L. (2020). Dual-action oxygen and peroxide-releasing nanoparticles for combating bacterial biofilms. *Journal of Nanobiotechnology, 18*, 78.

182. Rao, K. P., Patel, A., & Singh, A. (2019). Evaluating the cytotoxicity of calcium-based nanoparticles for biomedical applications. *Toxicology Reports, 6*, 376–385.

183. Kim, J. H., Choi, S., & Park, H. (2016). FTIR analysis of functional groups in calcium peroxide nanoparticles during synthesis. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, 157*, 142–148.

184. Zhao, Q., et al. (2017). Nanoparticle formulation strategies for drug delivery systems. Materials Science & Engineering C, 75, 100–107.

185. Chai, W. L., et al. (2020). Stability and characterization of surfactant-stabilized nanoparticles for drug delivery applications. Journal of Nanoparticle Research, 22(3), 82–96.

186. Liu, Y., et al. (2021). Zeta potential and stability of nanoparticles for pharmaceutical applications. Drug Development and Industrial Pharmacy, 47(9), 1427–1436.

187. Matuszewski, M., et al. (2018). Stabilization of nanoparticles: The role of surfactants in nanoparticle-based formulations. Colloids and Surfaces B: Biointerfaces, 171, 75–83.

188. Jiang, H., et al. (2019). Quorum sensing inhibitors: A promising approach for biofilm-related infections. Journal of Applied Microbiology, 127(1), 1–13.

189. Zhou, Y., et al. (2020). Dual-loading nanoparticles for the treatment of bacterial biofilms. Advanced Drug Delivery Reviews, 170, 71–84.

190. El-Shazly, M., et al. (2020). Dual drug-loaded nanoparticles for the treatment of bacterial biofilms. International Journal of Pharmaceutics, 579(1), 119–129.

191. Zhao, Z., et al. (2019). Stability of drug-loaded nanoparticles: Challenges and strategies. European Journal of Pharmaceutics and Biopharmaceutics, 142, 28–41.

192. Badran, H. M., Zaki, N. M., & Elsheikh, M. M. (2019). Advances in nanoparticle-based approaches for biofilm disruption. Microbial Biotechnology, 12(2), 357–374.

193. Lian, X., Duan, Y., & Li, J. (2018). Surfactant-assisted nanoparticles for drug delivery systems. International Journal of Nanomedicine, 13, 2067–2079.

194. Chen, H., et al. (2020). Nanoparticle-based drug delivery systems for the treatment of biofilm-related infections. Journal of Nanoscience and Nanotechnology, 20(8), 4872–4883.

195. Raghunandanan, D., et al. (2018). Size-dependent behavior of lipid nanoparticles for drug delivery. Drug Delivery, 25(1), 131–141.

196. Singh, S., et al. (2019). Visualization of biofilm penetration by nanoparticles using confocal laser scanning microscopy. Journal of Biomedical Nanotechnology, 15(5), 935–944.

197. Dunne, W. M. (2002). Bacterial biofilms: Clinical implications. Lancet, 360(9337), 529–535.

198. Hu, D., et al. (2020). Relief of biofilm hypoxia using an oxygen nanocarrier: a new paradigm for enhanced antibiotic therapy. *Advanced Science*, 7, 2000398.

199. Létoffé, S., Wu, Y., Darch, S. E., Beloin, C., Whiteley, M., Touqui, L., & Ghigo, J. M. (2022). Pseudomonas aeruginosa production of hydrogen cyanide leads to airborne control of Staphylococcus aureus growth in biofilm and in vivo lung environments. mBio, 13(5), e0215422.

200. Parsons, J. F., Greenhagen, B. T., Shi, K., Calabrese, K., Robinson, H., & Ladner, J. E. (2007). Structural and functional analysis of the pyocyanin biosynthetic protein PhzM from Pseudomonas aeruginosa. Biochemistry, 46(7), 1821–1828.

201. Lépine, F., Dekimpe, V., Lesic, B., Milot, S., Lesimple, A., Mamer, O. A., Rahme, L. G., & Déziel, E. (2007). PqsA is required for the biosynthesis of 2,4-dihydroxyquinoline (DHQ), a

newly identified metabolite produced by Pseudomonas aeruginosa and Burkholderia thailandensis. Biological Chemistry, 388(8), 839–845.

202. Déziel, E., Gopalan, S., Tampakaki, A. P., Lépine, F., Padfield, K. E., Saucier, M., Xiao, G., & Rahme, L. G. (2005). The contribution of MvfR to Pseudomonas aeruginosa pathogenesis and quorum sensing circuitry regulation: Multiple quorum sensing-regulated genes are modulated without affecting lasRI, rhlRI, or the production of N-acyl-L-homoserine lactones. Molecular Microbiology, 55(4), 998–1014.

203. Mukherjee, S., Moustafa, D., Smith, C. D., Goldberg, J. B., & Bassler, B. L. (2017). The RhlR quorum-sensing receptor controls Pseudomonas aeruginosa pathogenesis and biofilm development independently of its canonical homoserine lactone autoinducer. PLoS Pathogens, 13(7), e1006504.

204. Potvin, E., Sanschagrin, F., & Levesque, R. C. (2008). Sigma factors in Pseudomonas aeruginosa. FEMS Microbiology Reviews, 32(1), 38–55.

205. Hodgkinson, J. T., Gross, J., Baker, Y. R., Spring, D. R., & Welch, M. (2016). A new Pseudomonas quinolone signal (PQS) binding partner: MexG. Chemical Science, 7(4), 2553–2562.

206. Ajunwa, O. M., Odeniyi, O. A., Garuba, E. O., et al. (2022). Evaluation of extracellular electron transfer in Pseudomonas aeruginosa by co-expression of intermediate genes in NAD synthetase production pathway. World Journal of Microbiology and Biotechnology, 38, 90.

207. Moradali, M. F., Donati, I., Sims, I. M., Ghods, S., & Rehm, B. H. A. (2015). Alginate polymerization and modification are linked in Pseudomonas aeruginosa. mBio, 6, e00453-15.

208. Meng, L., Cao, X., Li, C., Li, J., Xie, H., Shi, J., Han, M., Shen, H., & Liu, C. (2023). Housekeeping gene stability in Pseudomonas aeruginosa PAO1 under the pressure of commonly used antibiotics in molecular microbiology assays. Frontiers in Microbiology, 14, 1140515.

209. Ding, X., et al. (2018). ROS generation as a mechanism for nanoparticles' biofilm inhibition. *Advanced Drug Delivery Reviews, 139*(1), 70–84.

210. Gao, C., et al. (2020). Synergistic effects of ROS-generating nanoparticles and antibiotics for biofilm eradication. *Nanomedicine, 24*(3), 102127.

211. Yuan, S., et al. (2015). Oxidative stress impact on biofilm formation and antibiotic resistance. *Journal of Biomedical Science, 22*(1), 16.

212. Folkesson, A., et al. (2012). Adaptation of *Pseudomonas aeruginosa* to the cystic fibrosis lung environment. *Nature Reviews Microbiology, 10*(12), 841–851.

213. Rumbaugh, K. P., et al. (2009). Quorum sensing and the social evolution of bacterial virulence. *Current Biology, 19*(9), 341–345.

214. Wong, C. T., et al. (2021). Squalene-based nanocarriers for drug delivery: Advances and therapeutic potential. Frontiers in Pharmacology, 12(1), 622498.

215. Kawashima, Y., et al. (2000). Stabilization of nanoparticles using PVA: Implications for drug delivery. Journal of Controlled Release, 64(1-3), 41–53.

216. Lobato-Calleros, C., et al. (2006). Surfactants and biofilm disruption: Tween 80 and biofilm matrix penetration. Journal of Applied Microbiology, 101(4), 1074–1082.

217. Gulzar, A., et al. (2020). Albumin-stabilized nanoparticles for targeted biofilm therapy. International Journal of Nanomedicine, 15(1), 3077–3091.

218. Hentzer, M., et al. (2003). Attenuation of Pseudomonas aeruginosa virulence by quorum sensing inhibitors. Proceedings of the National Academy of Sciences, 100(18), 11651–11656.

219. Ramasamy, M., et al. (2014). Nanoparticle penetration and diffusion in biofilms: Challenges and opportunities. Biofilm Journal, 1(1), 1–12.

220. Peulen, T.-O., & Wilkinson, K. J. (2011). Diffusion of nanoparticles in biofilms: A study of size and surface properties. Environmental Science & Technology, 45(8), 3367–3373.

221. Gupta, D., et al. (2021). Nanocarriers for overcoming biofilm barriers in chronic infections. Advanced Drug Delivery Reviews, 172(1), 142–164.

222. Wang, L., et al. (2017). Nanoparticle-mediated delivery of antibiotics to biofilm-associated infections. Therapeutic Delivery, 8(2), 79–90.

223. Nobre, C.M.G., König, B., Pütz, N., & Hannig, M. (2021). Hydroxyapatite-based solution as adjunct treatment for biofilm management: An in situ study. Nanomaterials, 11(9), 2452.

224. Schestakow, A., Pütz, N., Guth, M.S., Eisenmenger, T.A., Dudek, J., & Hannig, M. (2022). Influence of a hydroxyapatite suspension on 48-h dental biofilm formation in situ. Archives of Oral Biology, 136, 105388.

225. Wang, L., et al. (2017). Nanoparticle-mediated delivery of antibiotics to biofilm-associated infections. Therapeutic Delivery, 8(2), 79–90.

226. Hu, D., et al. (2020). Smart ROS-generating nanomedicine for synergistic bacterial eradication and anti-inflammatory therapy. Biomaterials, 252, 120078.

227. Deng, H., et al. (2021). Calcium peroxide nanoparticles as a biofilm-disrupting agent for enhanced antibiotic penetration. ACS Applied Materials & Interfaces, 13(12), 13736–13747.

CV and Scientific Output

Neha Bankar

Contact information : Email: nehabankar096@gmail.com, Phone: +4915165787811
Nationality : Indian
LinkedIn : www.linkedin.com/in/neha-bankar-427009168

Professional Profile

Pharmaceutical scientist experienced in formulation development. Enthusiastic about contributing to innovative research in healthcare.

Professional Experience

Doctoral Researcher
Helmholtz Institute for Pharmaceutical Research Saarland, Saarbrücken, Germany
October 2020 – present

Thesis: "Development of nanoparticle formulations (inorganic, polymer, lipid) for controlling pathogenic bacterial biofilms"

- Led the preparation, optimization, and characterization of nanoparticle formulations.
- Conducted PCR, aseptic cell culture, and S2 biosafety grade bacterial culture experiments.
- Managed *in-vitro* and *ex-vivo* testing.
- Coordinated cross-functional teams, including laboratories and hospital clinic.
- Authored scientific reports and papers, presented research findings at international conferences.

Assistant Manager Formulation R&D
Cipla Ltd., Mumbai, India
July 2019 – July 2020

- Development of generic formulations (dry powder inhalers) with GMP standards
- Supported the QA team by compiling and submitting documentation required for regulatory submissions.
- Collaborated with quality control, scale-up engineering, and manufacturing departments to ensure seamless product development and compliance with GMP standards.
- Provided on-site support during product launches.

Intern
Serum Institute of India Pvt. Ltd., Pune, India
July 2017

- Supported the quality control department in documentation processes and stability analysis of viral vaccines.

Education

Doctor of Natural Sciences (Dr. rer. nat.)
Saarland University, Saarbrücken, Germany
October 2020 – present

Master of Pharmacy (Pharmaceutics)
Birla Institute of Technology and Science, Pilani, Hyderabad, India
July 2017 – June 2019, Final Grade: 1.15 (Very good)

Bachelor of Pharmacy
Savitribai Phule Pune University, Pune, India
July 2013 – April 2017, Final Grade: 2.04 (Good)

Skills

- **Soft Skills:** Strategic planning, coordinator, flexible, cooperative
- **Course:** Clinical Research, Regulatory affairs
- **Techniques:** FTIR, HPLC, SEM, CLSM, sterile cell culture
- **Analytical Skills:** Statistical data analysis, stability data evaluation
- **Languages:** English (C2), German (A2), Marathi, Hindi (Native)

Conferences and Seminars

- **Grad School Training** Helmholtz Institute for Pharmaceutical Research Saarland and Saarland University, 2020–2024
- **Controlled Release Society (CRS) Meetings** Local and International Chapters, 2017–2024
- **Lean Six Sigma - Green Belt Certification** Pebble Sierra Academy Pvt Ltd, Hyderabad, India, 2018

Scientific Output

1) "**Dual effect of Calcium Peroxide nanoparticles effective in reducing dose of Tobramycin Sulphate in treatment of *Pseudomonas aeruginosa* biofilm**". Neha Bankar et al. Podium pitch presentation at CRS EU Local Chapters (Germany, Belgium, Netherlands, France and Luxembourg), Annual meeting, March 2022, Aachen.
2) "**Dual effect of Calcium Peroxide nanoparticles effectively reduces dose for Tobramycin Sulphate treatment on Pseudomonas aeruginosa biofilm**". Neha Bankar et al. Poster presentation at HIPS Symposium May, 2022.
3) "**The effect of calcium peroxide nanoparticles on bacterial biofilm barrier**". Neha Bankar, et al. Poster presentation at CRS Germany Local Chapter, Annual meeting, March 2023, Würzburg.
4) "**Adjuvant effect of Calcium Peroxide nanoparticles in controlling oral biofilms**". Neha Bankar, et al. Poster presentation at CRS 2024 Annual Meeting and Expo in Bologna (Italy), July, 2024.

Scientific Article:

Bankar, N., Latta, L., Loretz, B. *et al.* Antimicrobial and antibiotic-potentiating effect of calcium peroxide nanoparticles on oral bacterial biofilms. *npj Biofilms Microbiomes* 10, 106 (2024). https://doi.org/10.1038/s41522-024-00569-7