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Photodynamic inactivation of *Staphylococcus aureus* using a colloidal system based on clay mineral saponite and photoactive agent phloxine B

Bachelor thesis

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Bratislava – 22/06/2020 (Movilidad)

Resumen:

Inactivación fotodinámica de Staphylococcus aureus usando un sistema coloidal basado en el mineral de arcilla saponita y el agente fotoactivo floxina B.

Bratislava – 22/06/2020

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Las infecciones asociadas a la atención médica (HCAI) se adquieren a los pacientes mientras reciben atención médica en hospitals y clínicas principalmente mediante el uso de dispositivos invasivos. Estas infecciones relacionadas con el dispositivo son causadas por las comunidades de biopelículas de microorganismos unidos a las superficies. La biopelícula vive en una matriz autogenerada de sustancias poliméricas extracelulares debido a su tolerancia inherente y "resistencia" a diversas terapias antimicrobianas. *S. aureus* es uno de los microorganismos más relevantes en infecciones de dispositivos médicos. En esta tesis, se hace brevemente una descripción general de los efectos adversos del HCAI, como la morbilidad, la mortalidad y las pérdidas económicas para los pacientes y su colosal impacto en todo el mundo. Junto con el resumen teórico, este informe también se centró en explorar una estrategia innovadora para erradicar la biopelícula de *Staphylococcus aureus*. El sistema coloidal se basó en el mineral arcilloso saponita (0.1g / L) modificado con TMODAB (0.08 mM) en el sistema coloidal A y TMODAB (0.06 mM) en el sistema coloidal B y con el fotosensibilizador inmovilizado floxina B (0.05 mM). Este sistema fue irradiado por luz halógena verde. Inicialmente, se cultivaron una cepa estándar y dos aisladas procedentes de clínica de *S. aureus*, y se determinaron las curvas de crecimiento. El análisis de PCR se realizó para identificar la presencia del gen *MecA*, ya que determina las cepas resistentes a la meticilina. El sistema coloidal A, B había mostrado una reducción de $4\log_{10}$ en el crecimiento bacteriano usando condiciones de luz en comparación con los grupos de control sin irradiación, lo que infiere la efectividad del sistema coloidal. Estos hallazgos podrían considerarse un análisis preliminar como un contribuyente significativo en el camino de una técnica innovadora para erradicar los HCAI.

Palabras clave: Staphyococcus aureus; minerales de arcilla; sistema coloidal; fotoinactivación; floxina B; saponita; bromuro de trimetiloctadecilamonio.

Abstract:

Photodynamic inactivation of *Staphylococcus aureus* using a colloidal system based on clay mineral saponite and photoactive agent phloxine B.

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Healthcare-associated infections (HCAI's) are acquired to patients while receiving health care in hospitals, clinics mostly by using invasive devices. These device-related infections are caused by the biofilm communities of micro-organisms attached to the surfaces. Biofilm lives in a self-generated matrix of extracellular polymeric substances owing to their inherent tolerance and 'resistance' to various antimicrobial therapies. *S. aureus* is one of the most relevant micro-organisms in medical device infections. In this thesis, an overview of HCAI's adverse effects such as morbidity, mortality and economic losses to patients and its colossal impact around the world been described briefly. Along with the theoretical overview, this report also focused on exploring an innovative strategy to eradicate *Staphylococcus aureus* biofilm. A colloidal system was based on clay mineral saponite (0.1g/L) modified by TMODAB (0.08 mM) in Colloidal system A and TMODAB (0.06 mM) in Colloidal system B and with immobilized photosensitizer phloxine B (0.05 mM). This system was irradiated by green halogen light. Initially, a standard strain and two clinical isolates of *S. aureus* were cultured, and growth curves were determined. PCR analysis was performed for the identification of the presence of the *MecA* gene as it determines methicillin-resistant strains. The colloidal system A, B had shown $4\log_{10}$ reduction in bacterial growth using light condition compared to the control groups without irradiation inferring the effectiveness of the colloidal system. These findings could be considered as preliminary analysis as a significant contributor in the road of an innovative technique to eradicate HCAI's.

Keywords: *Staphyococcus aureus*; clay minerals; colloidal system; photoinactivation; phloxine B; saponite; trimethyloctadecylammoniumbromide.

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Abbreviations:

HCAI's -	Health care-associated infections
MRSA -	Methicillin-resistant *S. aureus*
PDI -	Photodynamic inactivation
CLABSI	Central line-associated bloodstream infection
CAUTI -	Catheter-associated urinary tract infections
VAP -	ventilator-associated pneumonia
SSI -	Surgical site infections
MDR -	Multi drug resistant
CWA	cell wall-anchored proteins
ECM -	Extra cellular matrix
eDNA -	external DNA
PDT -	Photo dynamic therapy
PDI -	Photo dynamic inactivation
ALT -	Antibiotic lock therapy
CVC -	Central venous catheter
PBP -	Penicillin binding protein
EPS -	Extracellular polymeric substances
PIA -	Polysaccharide intercellular adhesin
PS -	Photosensitizer
ROS -	Reactive oxygen species
PCR -	Polymerase chain reaction

Introduction:

Healthcare-associated infections (HCAI's) are acquired by patients while receiving health care treatment in hospital or clinics (Haque *et al*., 2018). These are regarded as one of the major complications of modern medical therapy and contribute morbidity and of patients (Hopmans *et al*., 2020). These HCAI's are seen in lower, middle, as well as the higher income group countries (Rosenthal *et al*., 2011). Still, it is shown that this type of infections is 3 to 5 times more common in limited-resource countries (Rosenthal *et al*., 2010). In European countries, it is estimated that about 5 to 7% of adult in-patients would acquire these infections during their treatment time in hospitals (European Centre for Disease Prevention and Control, 2013) causing mortality about 3.5 % in these patients (Cassini *et al*., 2016). Moreover, annual costs incurred by hospitals average 13'7 million € with > 99% attributed to patient management (Guest *et al*., 2019).

Microbial biofilms are responsible for 65-80 % of all disease caused by bacteria in human-related infections (Hall *et al*., 2014a). Microbial Biofilm forming activity on the medical devices is considered as the vital factor for the prevalence and severity of HCAI'S. Biofilms are owing to their inherent tolerance and 'resistance' to the known antimicrobial therapies (Sklyar *et al*., 2017). Today, numerous techniques are used to combat these structures, which includes the use of antibiotics. Both systemic antibiotics and methods similar to blocking devices with antibiotics have been effective for the prevention and treatment of biofilms (Justo & Bookstaver, 2014). Surface coating by nanoparticles has been used to prevent immature biofilms and to detach the mature from the surface. The use of different peptides proved with promising results against both $Gram^{+}$ and $Gram^{-}$ bacteria and *Candida* species (Thankappan *et al*., 2013). Another technique includes the modification of surface polymers that serves as a strategy to prevent contamination by biofilms formed by bacteria and fungi. Non-covalent immobilization of water-insoluble and organo-soluble cationic polymers onto a surface has been used in some studies to highlight its anti-biofilm ability. These techniques have succeeded to some extent with their limitations, providing scope for new techniques to handle these HCAI'S (Hoque *et al*., 2015).

S. aureus is one of the most relevant micro-organisms in terms of the formation of biofilms. Usually, it has strong biofilm formation ability (Ou *et al*., 2020), in addition to being complex due to the existence of strains resistant to antibiotics. The severity of

infection increases with the formation of multi-layer biofilm, making the infection difficult to treat with antibiotics. In addition, biofilm cells are resistant to phagocytosis by neutrophils and macrophages and resistant to antimicrobial peptides (Foster, 1996). Because of the extensive antibiotic resistance among nosocomial pathogens choices for their eradication have been limited (Talha *et al.*, 2020). Still, numerous investigations have been carried out in order to find techniques and substances with effect on the antimicrobial activity against methicillin-resistant *S. aureus* (MRSA) that must be accompanied by no human cell toxicity and hemolytic activity (Matiadis *et al.*, 2020).

Photodynamic inactivation (PDI) is a method used to fight against bacteria which is based on the use of a photosensitizer, light of a proper wavelength and oxygen, which combined leads to oxidative stress and killing of target cells (Kossakowska-Zwierucho *et al.*, 2020). PDI inactivation is currently considered as a novel and promising method to inactivate bacteria and fungi due to its environmental-friendly characteristic, low-energy consumption and low-cost inputs (Chen *et al.*, 2020). Phloxine B (PhB) is a photosensitizer which is used in PDI and can enhance photocytotoxic effects, including the inhibition of cell proliferation or cell death (Qi *et al.*, 2014).

Saponite (Sap) is a naturally occurring trioctahedral smectite. Sap layered particles carry a negative charge, and the negative charge is compensated by hydrated inorganic cations, which are exchangeable and can be replaced by a variety of other cations. The properties of Sap can be modified, which has been used in the development of organoclays, some of which exhibit antimicrobial properties (Gaálová *et al.*, 2019).

Hybrid systems composed of clay minerals and adsorbed organic dyes represent materials with unique optical, photophysical and photochemical properties (Bujdák, 2018). Known the bactericidal properties of clay minerals and the advances in the photoinactivation technique in the field of bacterial elimination, the point of this investigation was to combine both techniques and the design of colloidal systems formed by Sap and trimethyloctadecylammoniumbromide (TMODAB) incorporated with PhB and irradiation with a green light. The purpose is to show the bactericidal power of this technique in the eradication of *S. aureus*.

1. Overview

1.1 Healthcare-associated infections:

Healthcare-associated infections (HCAI'S) are among the major complications of modern medical therapy, and they can develop either as a direct result of healthcare interventions such as medical or surgical treatment (in secondary care) or from being in contact with a primary healthcare setting (Wilson *et al.,* 2019). To further understand the risk of developing an HCAI's, it is essential to understand the routes of transmission to the host. Micro-organisms can be acquired from several reservoirs, such as human skin, water and food sources (Bulut & Oncul, 2020). These reach the new host either directly by contact with the infected person or indirectly, due to airborne contamination, consumption of contaminated food or contact with contaminated surfaces. The new host can come into contact with these micro-organisms through inhalation, ingestion, breaks in the skin barrier following surgery or insertion of intravenous lines, or through mucous membranes (Percival *et al*., 2015).

Factors associated with higher incidence included low-income household's income inequality index, crowding, low education, and being a medically underserved area. Conversely, factors related to lower Methicillin resistant *staphylococcus aureus* (MRSA) infection incidence were high-income households, expensive homes, rural areas, high education, and health insurance (See *et al*., 2017). The most critical HCAI's are those related to invasive devices: central line-associated bloodstream infection (CLABSI), catheter-associated urinary tract infections (CAUTI), ventilator-associated pneumonia (VAP) as well as surgical site infections (SSI) (Al-Tawfiq & Tambyah, 2014). HCAI's occur as a result of infection by several agents, most commonly bacteria ($Gram^+$ and $Gram^-$) (Zhang *et al*., 2019), but also fungi, parasites and other microbial agents (Vandecandelaere *et al*., 2012). Many of these device-associated infections and SSI are caused by multi-drug resistant organisms (MDRO) such as MDR *Pseudomonas aeruginosa* or MRSA (Fan *et al*., 2019).

1.2 CLABSI

Central venous catheters are utilized for the delivery of fluids, blood products, nutritional solutions or medications, and for access in dialysis treatment (Cavanna *et al*., 2020). The use of CLABSI has been associated with a substantial risk of infectious complications, including access-site cellulitis, septic thrombophlebitis, bloodstream infection or endocarditis (Donelli, 2006). CLABSI is a frequent cause of healthcare-associated infections, increasing healthcare costs and decreasing the quality of life for critically and chronically ill patients (Malek & Raad, 2020). This also has a significant financial impact on patients as the increase in the days in hospital by 6.1 days. As this increase lead to cause an increase of the mean additional cost of antibiotics and burdening the patient on average about 10500 € per patient. These infections in turn increases the attributable additional mortality rate by 20% (Ramirez *et al*., 2007). The dominant micro-organisms causing these infections are bacteria *S. aureus, Staphylococcus epidermidis,* and fungi such as *Acinetobacter*, and *Candida albicans* were the most common colonies in CLABSI (He *et al*., 2019). The treatment of CLABSI relies on catheter removal and systemic antimicrobials. However, antibiotic lock technique can be used as an attempt to eradicate biofilm formed on the inside lumen of the catheter in case of uncomplicated long-term catheter-related BSI (Gominet *et al*., 2017). Critical elements of prevention of these infections are hand hygiene, avoidance of insertion of unnecessary catheters or full sterile barrier precautions at insertion (Buetti & Timsit, 2019).

1.3 CAUTI

Urinary catheters are tubular latex or silicone devices that are used to measure urine output, collect urine during surgery, prevent urine retention or control urinary incontinence (Flores-Mireles *et al*., 2015). The infection may be originated from those organisms that colonize the periurethral skin, which migrate into the bladder via the mucous layer that forms between the epithelial surface of the urethra and the catheter (Stickler, 2008). The current costs for the hospital are estimated to be approximately 820 € per patient, as the cost of hospitalization increase and even causes death to critically ill patients (Kennedy *et al*., 2013). For patients undergoing catheterization, the risk of developing a catheter-associated infection increases by approximately 10% each day (Talsma, 20077/10/2020 3:14:00 PM). The main causative agents of this type of infection are a variety of Gram-, with a predominance of strains belonging to *P. aeruginosa*. Other isolated strains in this catheters include *Acinetobacter*

baumannii, Klebsiella ornithinolytica or *C. albicans* (Djeribi *et al*., 2012). These catheters are to be replaced frequently to avoid infections. Still, immensely increase the costs as well the risk increase during the main removal. Even, some complications can occur due to this procedure. To prevent these infections, it is essential to reduce unnecessary catheters use (Kennedy *et al*., 2013).

1.4 VAP

VAP is a nosocomial infection that is not present at the time of intubation of patients requiring ventilation and develops more than 48 h after the initiation of mechanical ventilatory support (Osman *et al*., 2020). For VAP, hospital lengths of stay and medical care costs are also higher for critically ill patients who develop VAP. Also, it has an estimated attributable mortality of 9% (Hunter, 2012), but the most important is that this type of infection is increasingly attributed to antibiotic-resistant bacteria (Kollef, 2015). The micro-organisms that have been documented to colonize ETTs and grow in the form of a biofilm are numerous, including the multidrug-resistant bacterium MRSA and Gram- such as *K. pneumoniae*, *E. coli*, *P. aeruginosa* and *Acinetobacter* spp. (Ramirez *et al*., 2007).

1.5 SSI

SSI's are those wound infections that occur following a surgical procedure, and they are derived from laparoscopy, infections in stitches or interventions (Nasser *et al*., 2020) and they may be related to dehiscence, infection, and poor surgical technique (Fiani *et al*., 2020). SSI's are associated with severe morbidity and mortality, especially in high-risk patient populations. It is estimated that 750,000 surgical site infections occur in the US each year, using 3.7 million extra hospital days and costing more than $ 1.6 billion in excess hospital charges each year (Edmiston *et al*., 2006). SSI's can occur due to the contamination of a wound by micro-organisms derived from the patient's skin. *S. aureus*, *S. epidermidis*, *Candida* or *E. coli* are some of the micro-organisms that have been found to cause these infections (Kathju *et al*., 2009).

HCAI	MICRO-ORGANISMS
Medical device-related	
CAUTI	*Coagulase-negative staphylococci (CNS), C. albicans, A. baumannii, Proteus mirabilis, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, S. aureus, S. epidermidis*
Central-line-associated septicaemia	*CNS, C. albicans, K. pneumoniae, P. aeruginosa, S. aureus, S. epidermidis*
VAP	*Candida, K. pneumoniae, P. aeruginosa, S. aureus, S. Epidermidis*
Surgical-site infection	*Candida, E. coli, Staphylococcus spp., MRSA, P. aeruginosa, S. aureus, S. epidermidis*
Surgical wound, prosthesis-related infection	

Table 1: An overview of the most commonly isolated micro-organisms found in biofilm-related HCAI's (Percival *et al*., 2015).

2. Characterization of *S. aureus*

2.1 General characterization of *S. aureus*

This taxonomical classification belongs to http://taxonomicon.taxonomy.nl/Support.aspx

Domain	: *Bacteria*
Super phylum	: *Terrabacteria*
Phylum	: *Firmicutes*
Class	: *Bacilli*
Order	: *Bacillales*
Family	: *Staphylococcaceae*
Genus	: *Staphylococcus*
Species	: *aureus*

This bacterium belongs to the group of Gram+ bacteria and metabolism is generally facultative anaerobic, and *S. aureus* is usually in the form of un-encapsulated cocci. Also, it is a usual member of the microbiota of the body, frequently found in the upper respiratory tract and on the skin. It is typically seen as a commensal organism in the human microbiota. Still, it can occasionally become an opportunistic pathogen causing infections such as in the skin, respiratory tract and also food poisoning.

2.2 MRSA

MRSA is refractory to certain antibiotics such as beta-lactams (except 5th generation cephalosporin). These antibiotics include methicillin oxacillin, penicillin, and amoxicillin. MRSA strains are distinct from general *Staphylococcus* strains concerning the composition of the membrane, ability to form a thicker biofilm, and, importantly, ability to modify the target of antibiotics to evade their activity (Song *et al*., 2020). Much of the genetic diversity of MRSA and other pathogens occurs within the accessory genome, where mediators of virulence, immune evasion and antibiotic resistance are commonly found (Turner *et al*., 2019).

Resistance to β-lactam antibiotics is frequently associated with the production of β-lactamase blaZ ((Stapleton & Taylor, 2002). Methicillin-resistance is conferred by the presence of *Mec A* gene predominantly, in which the *MecA* gene resides on a genomic island termed the *Staphylococcal* Cassette Chromosome *mec* (SCC *mec*) (Venkatvasan *et*

al., 2020). This gene codes for penicillin-binding protein 2a (PBP2a) that differs from other PBPs because its active site does not bind methicillin or other beta-lactam antibiotics (Petrovic-Jeremic *et al*., 2008). It is involved in cross-linking of peptidoglycan in the bacterial cell wall. PBP2a has a lower binding affinity for β-lactam antibiotics compared to the general PBP proteins resulting in resistance (Harkins *et al*., 2017). Studies confirm that MRSA illnesses lead to a more extended hospital stay as well as a longer recovery time. These types of infections also carry high costs (Dhand *et al*., 2011(Lasa *et al*., 2005)).

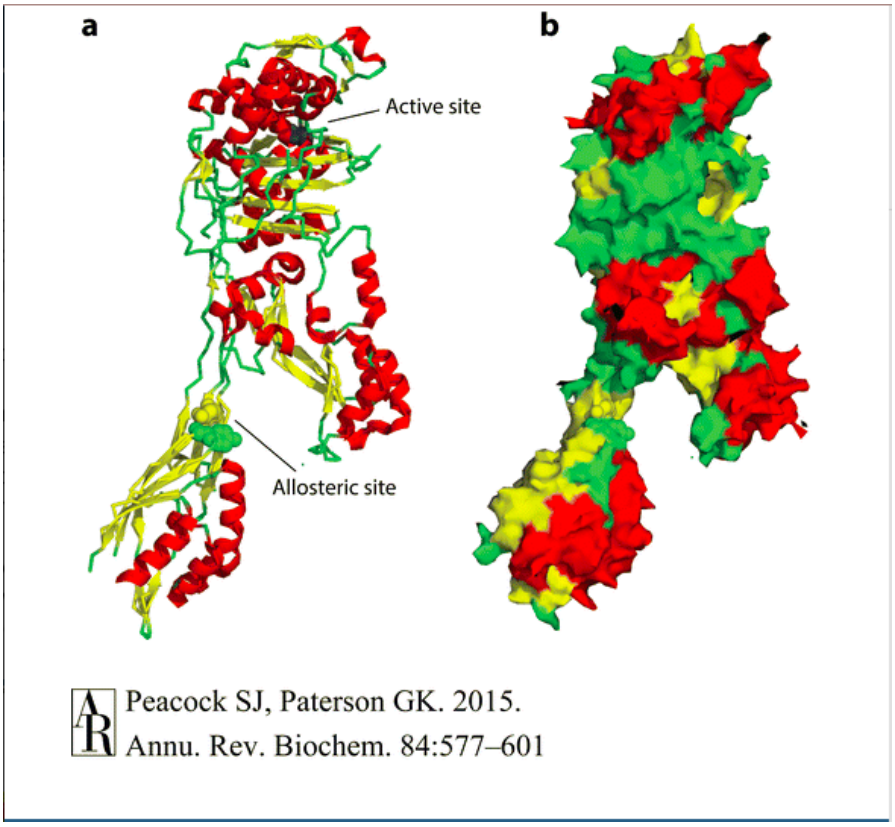


Figure 1: Structure of a PBP2a's monomer of MRSA (Peacock and Paterson, 2015).

3. Biofilm

Biofilm is a community of micro-organisms attached to a surface that live in a self-generated extracellular polymeric substances (EPS). It is a complex community of associated cells on a surface, including in a highly hydrated polymer matrix whose water circulates through open channels of the structure (Teschler *et al*., 2015). The cells forming a biofilm have properties and gene expression patterns that differ from those of planktonic cells. Among them, phenotypic variations in enzymatic activity, cell wall composition and surface structure stand out (Lasa *et al*., 2005). EPS may vary in chemical and physical properties, but it is primarily composed of polysaccharides and proteins (Donlan, 2002). The matrix structure is highly viscoelastic, has rubber properties, is resistant to traction and mechanical breakage. It has the ability to adhere to interphase surfaces, including internal spaces of porous media, through extracellular polysaccharides that act as adherent gums. It has water channels through which oxygen, nutrients and waste substances circulate that can be recycled (Flemming & Wingender, 2010). The biofilm micro-organisms are immobilized, very close to each other and this fact allows intense interactions. The interactions among biofilm micro-organisms include communication through chemical signals in a code called "*quorum sensing*". It is responsible for the ability to sense and respond to the environment, and this cellular communication promotes the expression of particular genes (Srivastava *et al*., 2011). This biofilm matrix also works as a means of protection and survival against adverse environments, barrier against phagocytic invaders and the entry and diffusion of disinfectants and antibiotics (Song *et al*., 2014). Micro-colonies are the primary component of every biofilm, but it is demonstrated that the structure of micro-colonies can vary among the biofilms formed by the different sp. One factor taken into account is the composition of the extracellular matrix, differences of which in components may give rise to different types and structures in micro-colonies (Tolker-Nielsen, 2015). All these properties increase the resistance to antibiotics and other antimicrobial compounds, and it makes a need to find measures against the formation of biofilms as well as their destruction (Lasa *et al*., 2005).

3.1 Phases of formation

Biofilm formation is a process in which microorganisms move from a free-living, unicellular state to a multicellular sedentary state, where subsequent structured

communities with cellular differentiation are formed. Biofilm development occurs in response to environmental extracellular signals and self-generated signals.

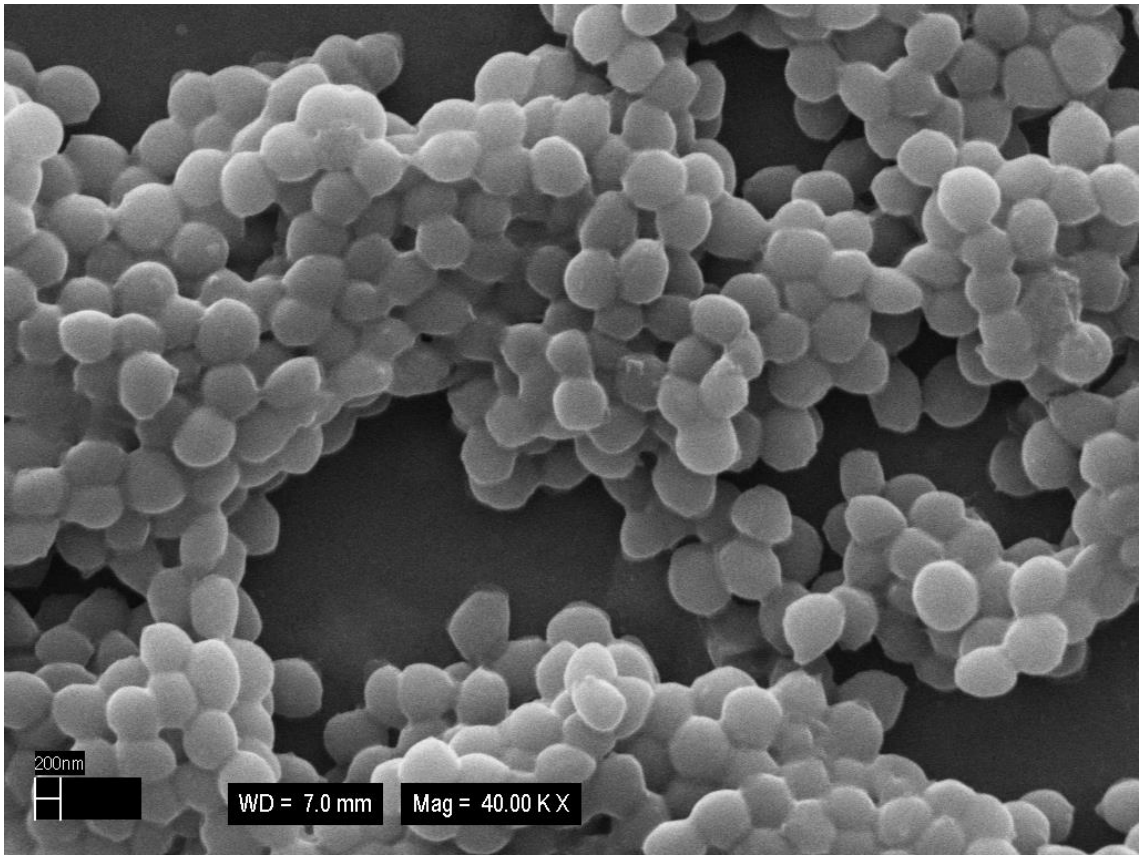


Figure 2: *S. aureus* biofilm SEM photograph obtained from Nitin Chandra Teja Dadi.

3.1.1 Attachment

Cells attach to abiotic surfaces via hydrophobic interactions or to biotic surfaces via microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). They are a specific type of bacterial adhesion proteins that are covalently linked to the peptidoglycans of the cell wall of Gram+ bacteria and interact with ECM molecules (Sridharan & Ponnuraj, 2016). The planktonic cells attach to biotic surfaces using some cell wall-anchored proteins (CWA) specifics for host matrix substances (Moormeier & Bayles, 2017). When the environmental conditions are favorable, the production of adhesives on its external surface increases. In this way, it increases its cell-cell and cell-surface adhesion capacity.

3.1.2 Multiplication

After the attachment, cells will begin to divide and accumulate. Before the production of an ECM in which to embed, the newly formed daughter cells are vulnerable to detachment. To increase the stability and maintain the immature biofilm, some bacteria such as *S. aureus* are known to produce factors that help to stabilize cell-to-cell interactions. This stage is known as the multiplication stage because it is time the cells use to division and accumulation (Moormeier & Bayles, 2017).

3.1.3 Exodus

It is an early dispersal event that coincides with micro-colony formation and results in the biofilm restructuring. It is mediated by nuclease-dependent degradation of eDNA. A period of the mass exodus of cells occurs in which a subpopulation of cells is released from the biofilm via eDNA degradation to allow for the formation of three-dimensional micro-colonies. Only a subpopulation of cells expresses the gene *nuc* which encodes the expression of nuclease. Because of it, the detachment of some cells of the population is avoided (Moormeier & Bayles, 2017).

3.1.4 Maturation

Finally, the biofilm reaches its maturity stage, with a three-dimensional architecture and the presence of channels through which water, nutrients, chemical communication compounds and nucleic acids circulate. The matrix maintains the cells together what increases the interaction between them with intercellular communication and synergistic consortium formation. The grouping and reproduction of cells lead to an improvement of their density in the medium, and it increases the communications through a mechanism called *quorum sensing*. It regulates the genetic expression of a cell group and is produced by releasing molecules called auto-inductors. When bacteria begin to clump and reproduce and improve their density in the medium, that density increases their communications through a mechanism called quorum sensing. This mechanism can regulate the gene expression of a whole group based on their cell density and is produced by releasing molecules called auto-inductors.

3.1.5 Dispersal

In this phase, the number of bacteria is already very high, and the cells are embedded in the ECM. As they are already integrated, and the number of cells increases, an exponential rise in *quorum sensing* will occur, which entails biofilm matrix modulation and dispersal of cells via protease activation and/or phenol soluble modulins (PSM) production (Moormeier & Bayles, 2017).

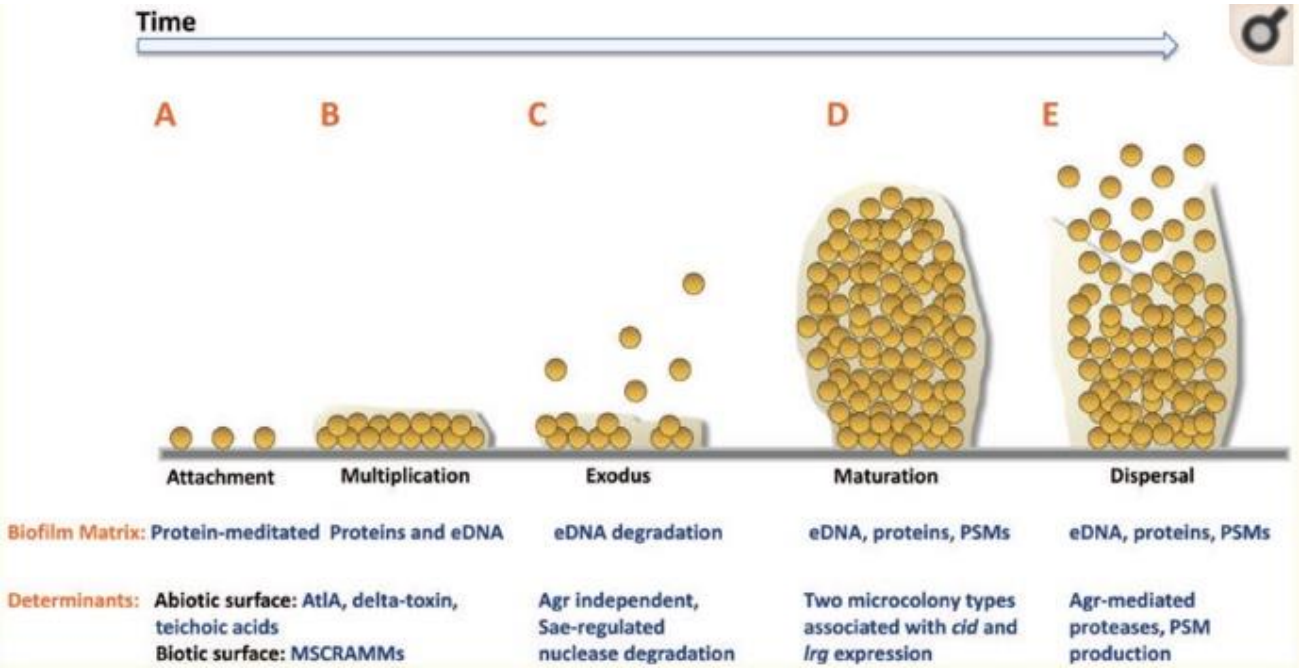


Figure 3: Model of *S. aureus* biofilm development (Moormeier & Bayles, 2017).

4. Mechanisms against HCAI's associated with medical devices

4.1 Measures against biofilms

In the measures against biofilms, we are able to find approaches tested in medical devices as catheters as antibiotics as doripenem and tobramycin (Mataraci Kara & Ozbek Celik, 2018). New anti-bacterial operators to treat diseases brought about by *S. aureus* have as of late been presented, together with the partially synthetic streptogramins quinupristin/dalfopristin, the synthetic tigecycline, oxazolidinone linezolid and daptomycin (Qin *et al*., 2020). However, *S. aureus* biofilms may slow the distribution of antibiotics via charge interaction, size exclusion, the viscosity of the matrix, and possible adsorption to proteins. EPS may also inactivate antibiotic molecules prior to reaching biofilm cells (Jefferson *et al*., 2005).

In addition, lock therapies and techniques changing the properties of materials used on medical devices. Antibiotic lock therapy (ALT) is carried out by introducing antimicrobial agents in drops directly into the catheter itself and blocking it later (Bujdáková, 2016). In catheters, commonly antibiotic lock solutions combine a highly concentrated antibiotic with an anticoagulant to allow for local instillation into the catheter lumen. It is essential that the lock solution has a low risk of toxicity and adverse events (Justo & Bookstaver, 2014). Aldo substances as ethanol or chlorohexidine are used in this technique, but there are drawbacks to the use of ethanol as it may not be compatible with all catheters (Hogan *et al*., 2014). However, ALT failed in some research in the treatment against infections caused by *S. aureus*.

Also, antimicrobial peptides (AMPs) have great potential as anti-biofilm agents. It has been reported that antimicrobial peptides could reach the inner structure of *S. aureus* cells through membrane and bind to bacterial DNA (Cui *et al*., 2020). Also, in vitro studies have been carried out both for the development of anti-adhesive surface coatings and to kill biofilm-forming cells (Sánchez-Gómez & Martínez-de-Tejada, 2016(Ghadiri *et al*., 2015). Moreover, polysaccharides have shown *in vitro* a reliable anti-bacterial power against the *S. aureus* micro-organism affecting the cell wall.

4.2 Clay minerals

Clay minerals have been used from the beginning for numerous purposes, having a large number of applications in biological systems. Throughout history, clay minerals have been used for healing and protective purposes. Due to some of the physicochemical properties, they have such as high surface reactivity, good rheological behavior or high acid absorption capacity (Ghadiri *et al.*, 2015).

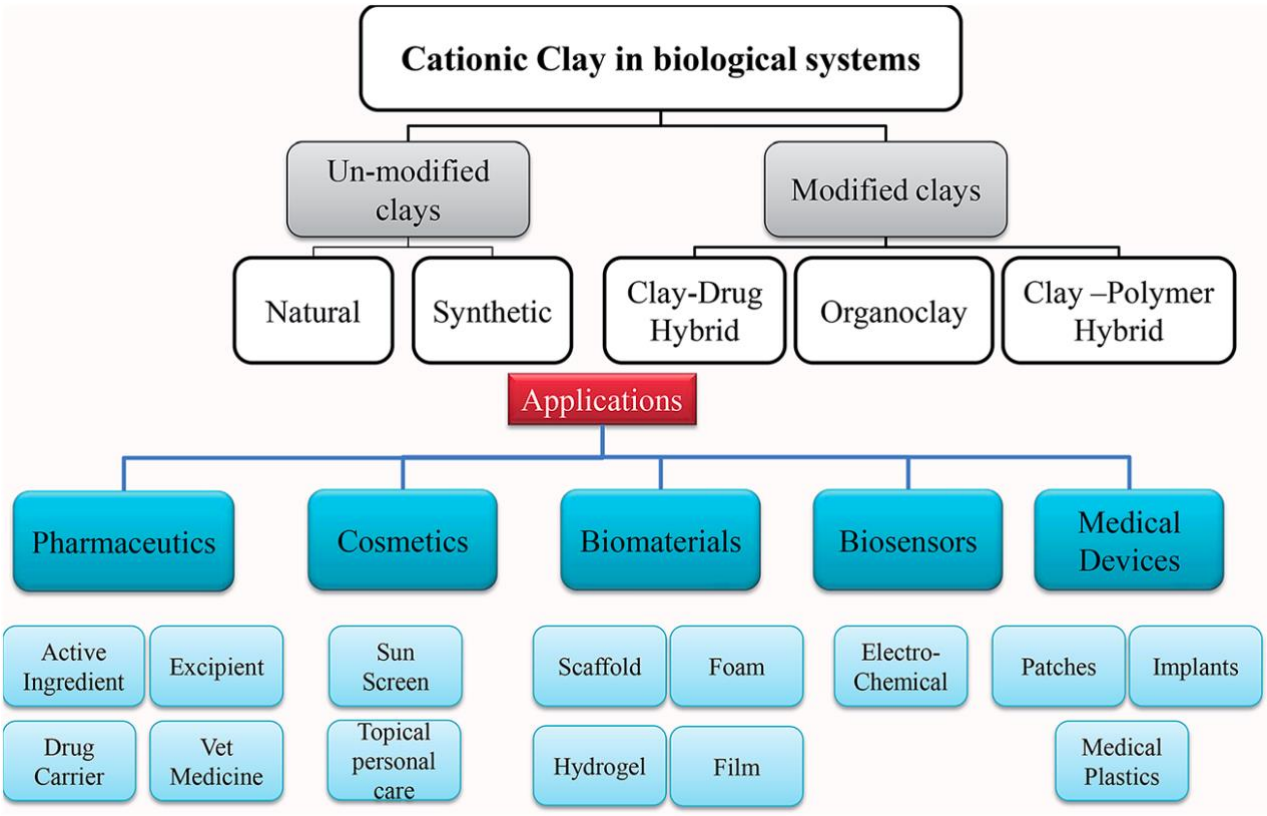


Figure 4: Applications of modified and unmodified cationic clay minerals in various biological systems (Ghadiri *et al.*, 2015).

4.2.1 Physical properties of clay minerals

Clay minerals are members of the phyllosilicate or sheet silicates family. Clay minerals can be positively charged in which case they are known as cationic clay minerals (cationic-CM) or they can manifest negative charge. The latter is known as anionic clay minerals. The charge that these materials have is the main reason for their ion exchange capacity (Ghadiri *et al.*, 2015). Modified clay minerals are well known to have the ability to adsorb natural and anthropogenic toxic compounds. They have bacteriostatic and bactericidal activity, which is due to their abilities to alter the permeability of cellular

membranes allowing intracellular low molecular-weight components to diffuse out. This process results in cellular death (Plachá *et al*., 2014).

4.2.2 Applications of clay minerals

These minerals can act as active ingredients or as excipients. As active ingredients, they have been utilized in pharmaceutical products and to reduce toxic effects of bacteria in the digestive system (Holešová *et al*., 2014). These can also be used as excipients which are substances introduced into specific pharmaceutical formulations to improve its organoleptic characteristics or its physical-chemical properties, and to facilitate the pharmaceutical formulation's preparation (Carretero, 2002). Clay minerals have also been used in the development of protective devices due to the ability to absorb toxic compounds and bactericidal activity (Plachá *et al*., 2014).

4.2.3 Saponite

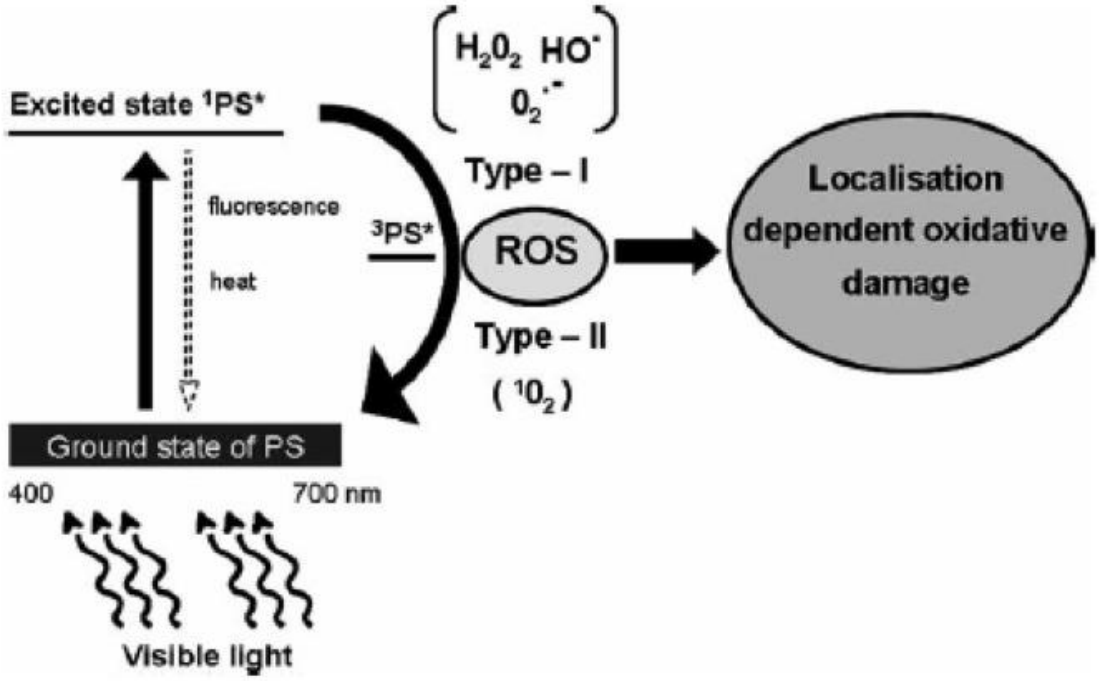
The Sap is a trioctahedral magnesium silicate that belongs to the group of smectite clays (Sprynskyy *et al*., 2019). There are *in vitro* studies that reveal the potential of this clay as anti-bacterial or bacteriostatic against Gram- bacteria (Gaálová *et al*., 2019) Also, solid or colloidal materials with embedded PS are promising agents from the medical or environmental perspective. Colloids based on layered silicates of the Sap including it modified with dodecyl ammonium cations (C12) and a photosensitizers (PS) has also been used to test the antimicrobial effect (Donauerová *et al*., 2015).

4.3 Photodynamic inactivation

In the strategies against micro-organisms, one of the approaches which are being developed and improved is PDI. It is a promising modality based on the combination of a PS that is selectively localized in the target cell and illumination of the target with visible light, resulting in photo-damage and subsequent cause of cell death (Tardivo *et al*., 2005). Utilizes a combination of a PS, visible light, and oxygen leads to formation of cytotoxic reactive oxygen species (ROS) resulting in the oxidation of several cellular components and rapid cell inactivation. It destroys pathogenic micro-organism (de Melo *et al*., 2013). The PS transfer the absorbed light energy to adjacent molecular oxygen leading to the generation of ROS that rapidly reacts with a variety of substrates producing damages in biomolecules. These changes generate a loss of biological functionality leading to cell death (Reynoso *et al*., 2019).

The best way of achieving this goal of antimicrobial PDI is to ensure that the PS has a pronounced cationic charge, as in general, microbes have a more pronounced negative charge compared to mammalian cells. Therefore, cationic PS binds selectively (Alrabiah *et al*., 2019). One of the advantages of PDI as a potential clinical antimicrobial therapy is that it works equally well regardless of the antibiotic resistance status of the microbial cells (Hamblin, 2016). The photodynamic inactivation of antibiotic-resistant bacteria like MRSA was successfully shown on human skin *ex vivo* (Eckl *et al*., 2020).

Figure 5: Mechanism of action of PDI (Maisch, 2009).



4.3.1 Photodynamic inactivation using phloxine B

Halogenated fluoresceins show photoreactivity since they form singlet oxygen when irradiated (Gandin *et al.*, 2008). It is toxic to bacteria through reaction with molecules such as lipids, proteins or nucleic acids, causing cell death (Dahl *et al.*, 1987). Phloxine B (PhB), known as D&C red no. 28 (20,40,50,70-tetrabromo-4,5,6,7-tetrachloro fluorescein disodium salt), is a water-soluble red dye which has an absorption maximum in the visible region at 540 nm (Inbaraj *et al.*, 2005). It is a color additive for food, drugs, and cosmetics (Rasooly, 2005).

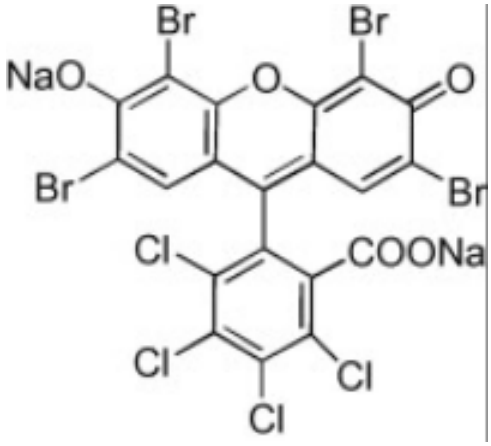


Figure 6: Chemical structure of PhB (Shiotsu-Ogura *et al.*, 2019).

In previous studies, it was shown that PhB leads to a reduction in Gram+ cell number (Brovko *et al.*, 2009). However, the results regarding the toxicity of said dye against Gram- bacteria gave no results, thus confirming the effectiveness of PhB against Gram+ but not Gram- bacteria (Rasooly, 2005). Studies reveal the anti-microbiological capacity of said dye against bacteria of the *S. aureus* type under conditions of ambient room light, without specific light exposure (Rasooly and Weisz, 2002). The cytotoxic activity of PhB is demonstrated higher under light conditions than under dark conditions, but it shows a reduction in both situations (Brovko *et al.*, 2009). In addition, studies have also been carried out using this dye in addition to irradiating it with UV-vis blue light, demonstrating that the CFU/mL (colony forming unit) was suppressed in a way directly proportional to the intensity of the light used to irradiate (Shiotsu-Ogura *et al.*, 2019).

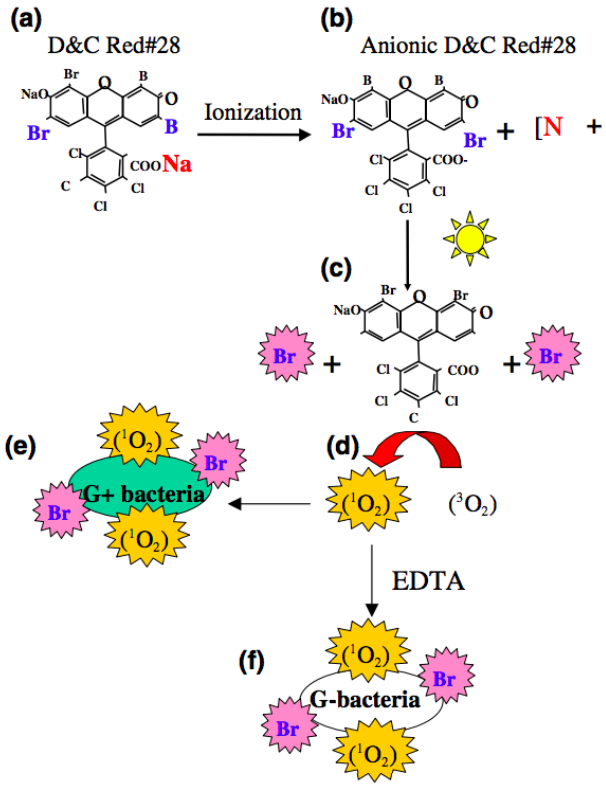


Figure 7: Mechanism of the antimicrobial effects of PhB (Rasooly, 2005).

4.4 Colloidal systems

Although PDI is an efficient technique in many cases, it also has its limitations and chance to improve its activity. Most of the PS is highly hydrophobic and tend to aggregate in the aqueous media. Many PS show low-to-moderate quantum yield in generating ROS and cannot be excited using long-wavelength light, such as red or infrared light, which has immense tissue penetration depth than blue or green light. These factors limit their use in clinical applications (Wijesiri *et al*., 2017). The design of the nanoparticles provides a simple means to incorporate hydrophobic photosensitizing molecules for use in aqueous media. Some investigations with nanoparticles of gold have been done. The hybrid PS display greatly enhanced singlet oxygen generation and outstanding PDI (Wijesiri *et al*., 2017).

5. Goals of thesis

To show light on the adverse impact of HCAI's all over the world and causative micro-organisms responsible for these infections. Explaining the importance of biofilm formation on medical devices and their development of resistance against antibiotics. The necessity for the development of new strategies against these HCAI's

Microbial identification a determination of resistance:

S. aureus standard and clinical isolates will be cultured in MHB and screened for the presence of the *MecA* gene using specific PCR for determining resistant strains.

Antimicrobial effectivity of phloxine B:

The PhB to be tested at different concentrations against standard and clinical strains of *S. aureus* in an antimicrobial assay by PDI to identify the ideal concentration for the antimicrobial hybrid material preparation.

Preparation of hybrid system and antimicrobial testing:

Prepare a hybrid system with Sap and PhB. To identify the appropriate concentrations and ratios of different concentrations of TMODAB and PhB to obtain maximum benefit and acquire the best antimicrobial effectivity.

6. Materials and methods

6.1 Materials:

Muller Hinton broth (MHB)
Mueller-Hinton Broth soil 11g
Distilled water made up to 500 mL
Sterilized by autoclaving (20 min, 120 kPa, 120 °C)
Stored at 4 °C

Muller Hinton agar (MHA)
Mueller-Hinton agar 19 g
Distilled water made up to 500 mL
Sterilized by autoclaving (20 min, 120 kPa, 120 °C)
Subsequently, the soil was poured into a Petri dish in a sterile box.

DEPC water
DEPC 1 mL
Distilled water made up to 999 mL
Stored in a thermostat at 37 °C for 24 h and then sterilized twice by autoclaving (20 min, 120 kPa, 120 °C) and stored at LT.

10x PBS, pH = 7.4
NaCl 80 g
KCl 2 g
Na2HPO4. 2H2O 11.5 g
KH2PO4 2 g
Distilled water 1000 mL
Adjusted with 10 M, followed by 1 M NaOH to pH 7.4
Sterilized by autoclaving (20 min, 120 kPa, 120 °C)
Stored at laboratory temperature (LT)

10x TBE buffer, pH = 8.3
EDTA 9.3 g
Boric acid 55 g
Tris base 108 g
Distilled water made up to 1000 mL
Adjusted to pH 8.3 with 1 M NaOH
Sterilized by autoclaving (20 min, 120 kPa, 120 °C) and stored at 4 °C

1.5 % agarose gel
1.5 g agarose (Serva, Germany)
100 mL of 1xTBE
The mixture was heated until the agarose dissolved.

Then a 5 µL GoodView Nucleic Acid Visualization Dye (Solis Biodyne,Estonia) was added to the contact cooled gel.

1g/L Sap

0.1 g Sap (Kunime Ind., Japan)
100 mL deionized water added to it and stirred until completely dissolved
Sonicated for 5 min (Branson 200 ultrasonic cleaner, USA)
Stored at 4 °C

0.8 mM and 0.6mM TMODAB

0.0314 g and 0.2355g TMODAB (Sigma-Aldrich, Germany)
100 mL Deionized water sterile
The mixture is stirred on heat stirrer and further continued to at 60 ° C in incubator shaker at 150 rpm

10 mM PhB

0.00829 g PhB (Sigma Aldrich Corporation St. Louis, MO, USA)
1 mL of deionized water in 1.5 mL tube.
Vortex well and filtered through 0.22 µm filters (TPP, Trasadingen, Switzerland) and stored at 4 °C

6.2 Microbial cultivation

In this project, three *S. aureus* strains have been tested against the colloidal system. The control strain *S. aureus* 3953 (Czech Collection of Microorganisms, Brno, Czech Republic) and 2 MRSA clinical strains, namely L12 and L18, were tested. Both clinical isolates were acquired from the central venous catheter (Institute of Microbiology, Faculty of Medicine, Comenius University in Bratislava and University Hospital Bratislava). These strains were cultured on Mueller Hinton Agar (MHA, Biolife, Milan, Italy) in an incubator at 37 °C (Multitron S-000115690, Switzerland; 150 rpm) for 24 h (can be used for 1 week).

6.3 Microbial growth curve

A single colony of bacteria seeded into 40 mL of Müller-Hinton broth (MHB, Biolife, Italy) in Erlenmeyer flask. The bacteria were cultured overnight in an incubator at 150 rpm for overnight (o/n) at 37 ºC. The following day the optical density (OD) measured using a spectrophotometer at OD_{600} and adjusted to 0.05 nm in 100 mL MHB and (OD_{600}, Jenway 6300, 1 mL in cuvette), and it also was put into the incubator with shaking. The OD_{600} measurement was taken for every 1 h, the reading was noted for 10 h and graphs plotted basing on the OD_{600}.

6.4 Identification of *MecA* gene

MecA gene was identified for the confirmation of resistance by performing a PCR using a thermocycler in which the single colonies of the bacterial strains were taken as a template. Primers (Metabion International AG, Germany) were used with the sequences shown in Table 2. The PCR cycle (Tables 3 and 4) was performed in a thermocycler (C1000, BIORAD, USA), the reaction mixture for all reaction samples had a final volume of 20 µL. PCR products were then visualized by electrophoresis in a 1,5% agarose gel at 70 V, 90 min (Power Pac, BIO-RAD, USA) with GoodView Nucleic Acid Stain-HGV-II and gel was observed on a UV transilluminator (MUV-21-312-220, Major Science, USA).

Table 2: Sequences of used primers

Primers		**Sequence**	**Amplicon length**
MecA	Forward	GTA GAA ATG ACT GAA CGT CCG ATA A	310 bp (Braoios *et al*. 2009)
	Reverse	CCA ATT CCA CAT TGT TTC GGT CTA A	

Braoios *et al*. 2009 was used for designing primers, but the cycle was designed according to recommendations for hot start mix (5x HOT FIREPol Blend Master Mix) + gradient PCR.

Table 3: Reaction mixture.

Reagents	**Quantity**
5x HOT FIREPol Blend Master Mix	4 µL
DNA template	1 µL
Forward primer	0,5 µL
Reverse primer	0,5 µL
DNA free water	14 µL

Table 4: PCR cycle for the *MecA* gene.

Reagents	**Temperature (°C)**	**Time (min.)**	**Cycles**
Initial denaturation	95	15:00	1x
Deneturation	95	00:20	34x
Annelation	51	01:00	
Extension	72	01:00	
Final extension	72	10:00	1x

6.5 Colloidal system preparation

A colloidal system was prepared with the combination of Sap, PhB. As both Sap and PhB are cationic the surface charge of the Sap was modified by using a quaternary ammonium salt TMODAB. As the Sap is negative charged is covered by the TMODAB with positive charge by this providing PhB a high chance of binding and embedding into Sap. These reagents were prepared with 10 times higher concentrations than the experimental concentrations. Two 250 mL Erlenmeyer flasks were taken, and 100 mL of deionized water was added with 1 g/mL concentration of Sap to each flask and stirred until completely dissolved. Further 2 different concentrations of TMODAB 0.6 mM and 0.8 mM was prepared and combined with Sap. These flasks were sonicated for 10 min (Branson 200 ultrasonic cleaner, USA), and the solutions were transferred to 250 mL cell culture flasks. These flasks were further incubated at 60 ºC with 150 rpm (Thermo shaker PST-60H2-4 Biosan, Latvia) for 24 h allowing Sap and TMODAB binding. After incubation PhB with 0.5 mM was prepared separately in 100 mL deionized water. 50 mL of Sap + TMODAB solution added to 50 mL PhB and continued incubation at 60 ºC for 48 h with shaking. By this preparation, we will receive different solutions such as

i) Sap (1 g/L)

ii) Sap (1 g/L) + TMODAB (0.8 mM) or **A**

iii) Sap (0.5 g/L) + TMODAB (0.4 mM) + PhB (0.25 mM) or **Colloidal A**

iv) Sap (1 g/L) + TMODAB (0.6 mM) or **B**

v) Sap (0.5 g/L) + TMODAB (0.3 mM) + PhB (0.25 mM) or **Colloidal B**

vi) PhB (0.5 mM)

These samples were stored at 4 °C for future antimicrobial assay experiments.

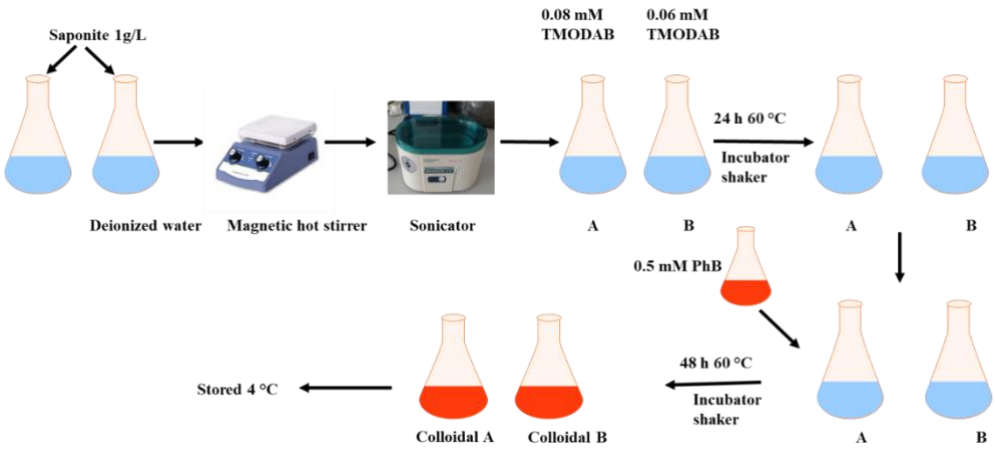


Figure 8: Diagram of the procedure followed during colloidal system preparation.

6.6 Colloidal system antimicrobial assay

An antimicrobial assay was subsequently carried out with the colloidal solutions. In this experiment, the control *S. aureus* 3953 strain was cultivated as mentioned above and used for antimicrobial effectiveness of the colloidal system. Before performing antimicrobial assay growth curve of each strain was determined (Fig. 9, 10 and 11). The overnight culture was prepared with a single colony of bacterial strain in 40 mL MHB at 150 rpm 37 °C. The following day the OD_{600} was measured, and 1 mL was resuspended in 100 mL MHB and OD_{600} were adjusted to 0.05, and it was subcultured again at 150 rpm 37 °C until the OD_{600} reached 0.5 (about 2.5-3 h) to get exponential phase. The cell concentration was approx 2 x 10_7 cells/mL. In antimicrobial assay 24 well polystyrene plates (Sarstedt, Germany) were taken, and wells were added with 1 mL of the bacteria. As a positive control, 1 mL of MHB were pipetted into the wells. The above-prepared samples were higher concentrations; these are to be diluted to the working concentrations. Sap, PhB, A and B of 200 µL combines with 800 µL of MHB. Colloidal A and B of 400 µL added with 600 µL of MHB (according to provider, 22 g per L, it was prepared 22 g per 600 mL). For negative control of infection, 2 mL MHB was added to the wells. All the samples, including control, were performed in 3 parallel wells and performed in dark and irradiated conditions simultaneously.

Final experimental working concentrations:

i) Sap (0.1g/L)
ii) Sap (0.1g/L) + TMODAB (0.08 mM) or **A**
iii) Sap (0.1g/L) + TMODAB (0.08 mM) + PhB (0.05 mM) or **Colloidal A**
iv) Sap (0.1g/L) + TMODAB (0.06 mM) or **B**
v) Sap (0.1g/L) + TMODAB (0.06 mM) + PhB (0.05 mM) or **Colloidal B**
vi) PhB (0.05 mM)

Plates were covered with aluminium foil and preincubated for 1 h at 37 °C. These plates were further transferred to irradiation step in which a green halogen light placed at the top of the plate at 6 cm distance between the light source and 24 well plates. The irradiation step continued for 2.5 hours, and the temperature was monitored every 30 min, the temperature shouldn't exceed over 37 °C. After irradiation, the plates were moved to the incubator and left it to culture for 24 h at 37 °C. The plate in dark condition was also moved to incubation at 37 °C. Next day, wells were resuspended by pipetting tips, and

100 µl of each sample was serially diluted to in 1x PBS MHB and plated (20 µl) on MHA and incubated for 24 h at 37 °C. Next day colonies were counted, and CFU/mL was calculated using the following formula.

$$\frac{CFU}{mL} = \frac{nº\ of\ colonies}{volume\ plated} \times\ dilution\ factor$$

7 Results

7.1 Growth curve

The bacterial growth curve is a graphical representation of the growth of a bacterial population over time. In the growth curves obtained for the control strain (*S. aureus* 3953) could observe that lag phase remains until about two h. We could observe the same in the growth of the MRSA strains (L18 and L12). We were able to observe that the growth during the exponential phase is much faster in the control strain. Fig. 10 shows that the growth curve of the strains L18 reached 0.5 OD_{600} at 4.5 h being the cells around $2x10^7$/mL at this OD_{600}. Similar results were obtained for the L12 strain (Fig. 11). Fig. 9 shows that control-strain needed 3.5 h to reach the OD_{600} 0.5, being 1 h less than the MRSA strains.

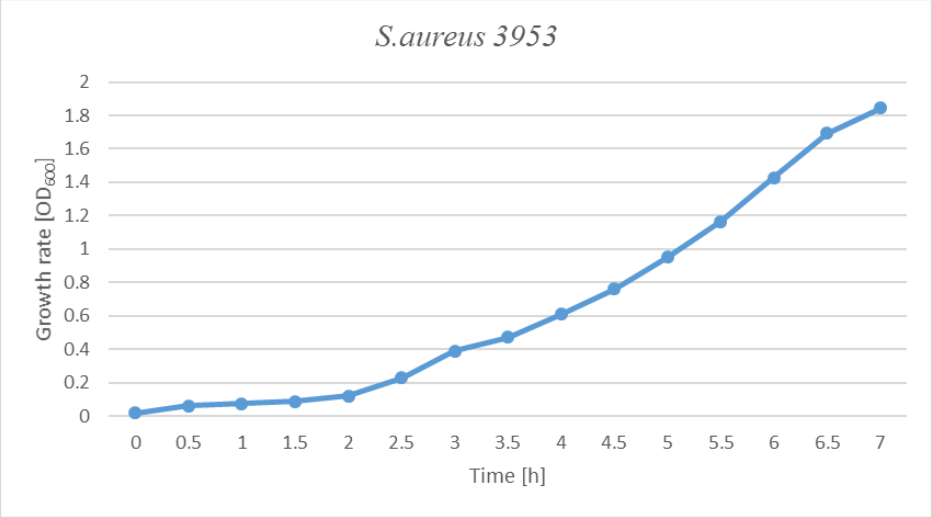


Figure 9: The growth curve of control strain *S. aureus* 3953

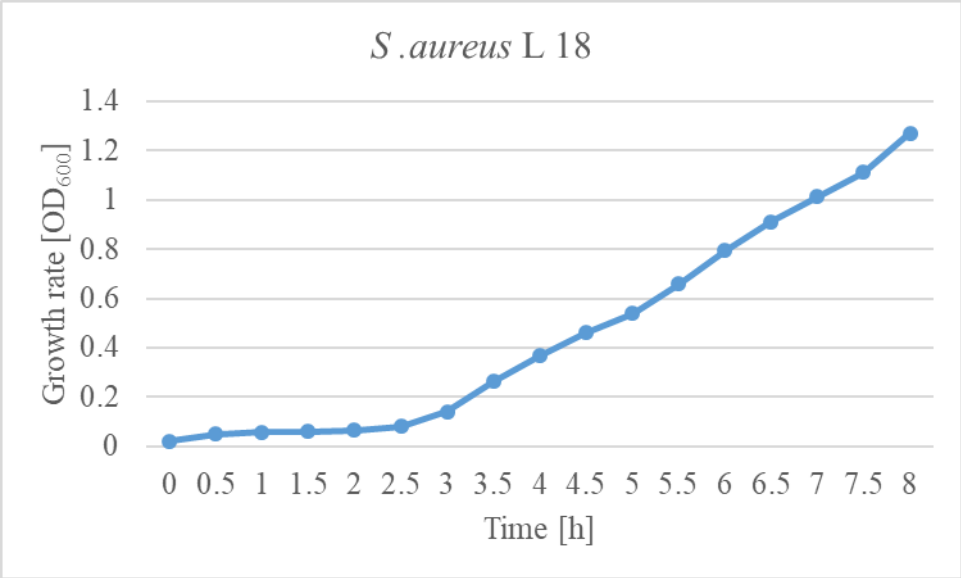


Figure 10: The growth curve of *S. aureus* L18.

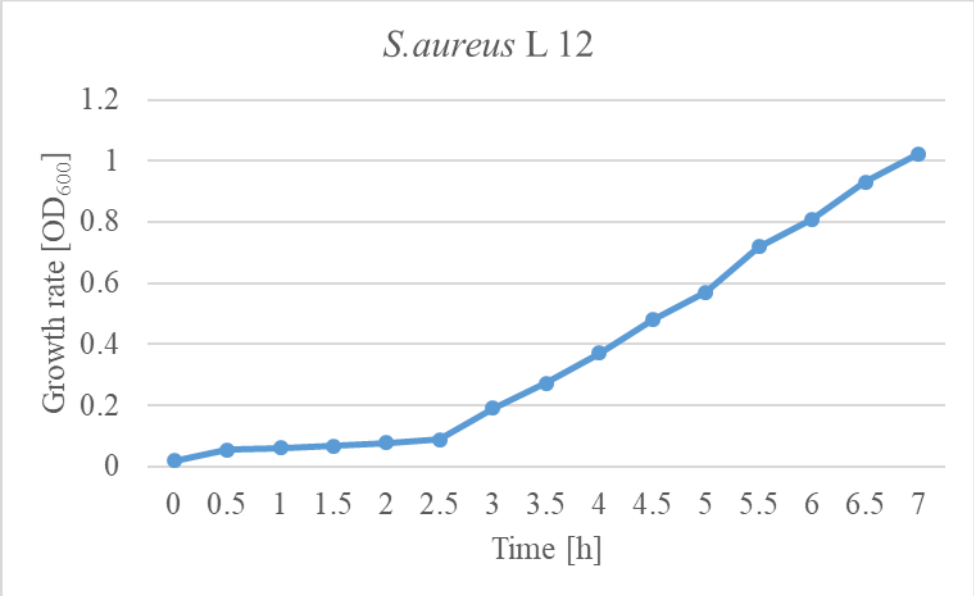


Figure 11: The growth curve of *S. aureus* L12

7.2 Determination of the *MecA* gene

The *MecA* gene identification was performed by PCR followed by agarose gel electrophoresis. Control strain and clinical isolates of *S. aureus* were amplified for the identification of the presence of the *MecA* gene. The results are summarized in Fig. 12. The MRSA strains of L18 and L12 were detected by the presence of a band at 310 bp. It is a specific for the methicillin resistance. In contrast, there was no fragment in the control *S. aureus* 3953 strain that was sensitive to oxacillin.

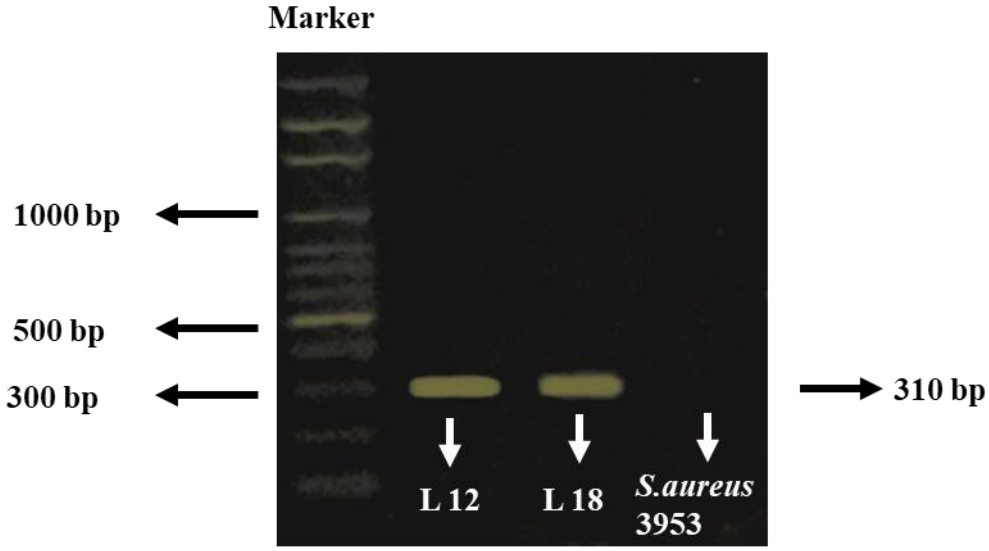


Figure 12: Determination of the *MecA* gene in standard strain *S.aureus* 3953 and clinical isolates L12, L18; Marker 100bp label

7.3 Colloidal system antimicrobial assay

The colloidal solutions were prepared and tested with *S. aureus* 3953 by PDI as the procedure described above (Fig. 8). The control group with MHB and cells and Sap groups have shown a growth rate of 10_{12} CFU/mL in both dark and light conditions whereas A and groups showed a growth rate of 10_{10} CFU/mL, 10_{11} CFU/mL in both dark and light conditions respectively. But in case of Colloidal A and Colloidal B groups, the growth rate was 10_{10} CFU/mL and 10_{11} CFU/mL, respectively in dark condition. However, under the light condition, the growth was shown over $5\log_{10}$ and $4\log_{10}$ reduction, respectively, compared to the control groups. The sample with PhB in dark condition showed the growth of 10_{11} CFU/mL, and $5\log_{10}$ reduction in light condition. This assay shown the complexity of the colloidal system and proved the possible applications as antimicrobial agents. Further research should be focused on the colloidal system preparation for the identification of ideal concentration for better effectiveness.

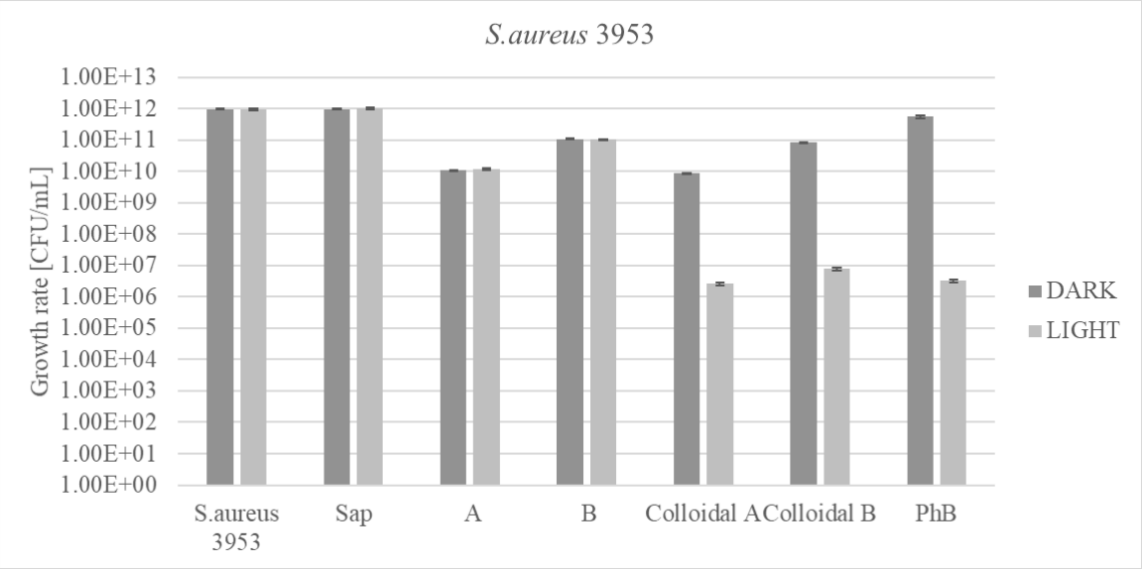


Figure 13: Antimicrobial effectiveness of Sap, two different concentrations of TMODAB in A, B and Colloidal A, Colloidal B and PhB under light and dark conditions.

8. Discussion

In 2019, antibiotic-resistant bacteria and fungi caused more than 2.8 million infections and 35,000 deaths in the United States alone (García *et al*., 2020), the increment in multi-drug resistance has been determined as one of the primary reason for these health problems and also one of the significant focuses of research (Díaz *et al*., 2020). Over 80% of all bacterial infection of animals and humans are related to the biofilm-forming ability of micro-organisms (Hall *et al*., 2014b). *S. aureus* remains one of the most intensively investigated bacterial species as a human and animal pathogen responsible for causing numerous nosocomial and community-acquired infections. In terms of resistance, *S. aureus* infections pose an ever-increasing problem (Kruszewska *et al*., 2004). MRSA has spread worldwide with infection rates <5% in the Scandinavian countries but >40% in Japan, the USA and Southern Europe (Chambers *et al*., 2001) AS higher usage of antibiotics led to the emergence of resistant strains modern strategies been developed to handle MRSA. PDI technique has shown promising results with no known resistance mechanism against PDI (Maisch, 2015) and also demonstrated a significant reduction in the growth rate of MRSA (Wang *et al*., 2019). The inclusion of inorganic materials with antimicrobial properties such as metal oxide nanoparticles or clay minerals in building materials can prevent microbial proliferation or significantly reduce the growth of micro-organisms (Rosendo *et al*., 2020). The antimicrobial properties of clay minerals have made of the materials in studies in which the eradication of bacteria has been proved. One of the clay minerals tested is palygorskite showing good reduction results in the demonstration of the role of the palygorskite clay mineral as an anti-bacterial agent during the incorporation of ZnO nanoparticles onto a clay surface (Hui *et al*., 2019).

In this thesis, a novel hybrid system based on Sap with immobilized photoactive dye PhB was tested in PDI against clinical MRSA strains of L12 and L18. Cationic clay mineral Sap was modified by a quaternary ammonium salt TMODAB which acts as a surface charge modifier and promotes binding of PhB. The concentrations of all 3 components were to be precise as TMODAB is a detergent solution, free moving molecules may be the reason for the toxicity to cells. While A, B have shown growth reduction of bacteria by 2 $\log_{10}$ and 1 $\log_{10}$ compared to their control groups in dark and light conditions, Sap hasn't shown any reduction in the growth rate. This proves the excess unbound TMODAB leakage into the cells that could be the probable reason for the growth reduction in their respective groups. Whereas these A, B were embedded with PhB and prepared solutions Colloidal A, B was in agreement with their A, B group results.

When Colloidal A, B groups were irradiated, the reduction of growth rate is highly significant as colloidal system displayed 4 $\log_{10}$,3$\log_{10}$ reductions respectively. In this antimicrobial assay, the Sap groups haven't shown a reduction in cell growth rate, but in this hybrid system sap role as an antimicrobial agent could be minimum. Still, Sap acts as an excellent carrier (Lackovičová *et al*., 2019) and helps PhB be to bind and adhere and confine in it and increase the effectivity of PDI (Riaz *et al*., 2017). However, the mechanism behind the microbial growth reduction has not been explored, leaving a potential scope for the molecular analysis.

These results are to be considered as preliminary, which have given valuable information and more focus must be given in the combination of the reagents, light source, dyes, and clay minerals as this model could be used for a wide range of applications for medical as well as industrial purposes.

9. Conclusion

HCAI's are one of the most critical problems all over the world, causing adverse effects on human life. *S. aureus* is one of the most prevalent micro-organisms isolated from medical devices associated with HCAI's. These infections are associated with microbial biofilm formation. This micro-organism carries additional problems due to the existence of antibiotic-resistant strains, which, together with the resistance and favorable environment that biofilms give make these organisms challenging to eradicate. Preparation of new hybrid materials by immobilization of PS on clay nanoparticles in combination with PDI could be a viable alternative way. This strategy is widely available, and harmful effects on humans of such materials have not been described yet. Additionally, no resistance has been reported, and its action on resistant or non-resistant strains has shown no difference so far. In the present study, colloidal systems formed by the clay mineral Sap, TMODAB and PDI PhB were tested on *S. aureus* 3953 using the PDI technique. The results were hopeful, demonstrating the microbial effect on the eradication of *S. aureus* of these systems under PDI light conditions. The next steps in such research in the future could be the repetition of this experiment with MRSA to highlight the advantages of this technique, which until now has not shown differences in its performance in resistant and non-resistant strains.

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