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Investigating the Antibacterial Activity of Antimicrobial Peptide and Silicate Integrated Flexible Biomaterial

Abstract

Osteomyelitis is a disease in which the process of bone destruction and loss is caused by microorganisms. Osteomyelitis has an increasing incidence rate that could cause chronic and acute infections. Bone infection has caused recurrent and permanent damage in 40% of the patients. 2-5% of infections caused by medical devices used in orthopedic surgical operations are osteomyelitis. More than half of osteomyelitis cases are caused by gram-positive bacteria such as *Staphylococcus aureus* and *Staphylococcus epidermidis*. In order to prevent the occurance of osteomyelitis due to the medical device, there is a dire need of integrating bactericidal and bacteriostatic agents into biomaterials matrix that are used in the design of medical implantation. The flexible biomaterial is currently widely used in the treatment of bone defects such as cancer, bone loss due to skeletal trauma and infection, bone fractures, and congenital deformities of the facial and skull bones. β-tricalcium phosphate (β-TCP) graft material is used in a flexible biomaterial due to its high osteocompatibility and mechanical strength. Flexible biomaterial is a fully synthetic, osteoconductive and flexible bone graft material and act as a temporary bone tissue scaffold for aiding tissue regeneration. In addition, the flexible bone scaffold is biocompatible and radio-opaque and is absorbed in a controlled manner. To the best of our knowledge the available flexible biomaterials in the marked those are in use of bone defect treatments does not possess antibacterial activity. Antibacterial biomaterials are direly needed to prevent microbial complications after implant surgery.

In terms of antibiotic treatments to eradicate an infection at the site of implantation, the formation of biofilms at the implant side reduces treatment efficacy due to increased antibiotic tolerance of the biofilm and decreased penetration efficiency through the extracellular polymeric substances (EPS) of biofilm matrix, degradation of antibiotics by the enzymatic components of EPS, and increased resistance.

Antibiotic resistance and biofilm-associated antibiotic tolerance have addressed the urgent need to investigate novel antimicrobial agents as alternatives to antimicrobial drugs and development of antibacterial agent integration strategies to the biomaterials. Antimicrobial peptides (AMPs), are a class of naturally occurring, peptides that play an important role in the innate immune system of various organisms have been extensively studied and have the potential to serve as small-molecule antibiotics. In this study by using the human LL-37 peptide as a template, a synthetic antimicrobial and anti-biofilm peptide, SAAP-276, has been synthetically synthesized. Antimicrobial peptide-loaded biomaterials have been prepared and the potential of the prepared flexible biomaterial in inhibiting the bacterial cell adherence to material surface, providing bactericidal effect and inhibiting the biofilm formation was investigated.

All in all in this study, we aim to integrate SAAP-276 AMP into the commercially available Powerbone flexible strip biomaterial (TerraMed) used in bone tissue regeneration. The physicochemical characterization of prepared flexible material and its components have been explored. For this purpose; Dynamic light scattering (DLS) was used for size determination and scanning electron microscopy (SEM) was used for morphology analysis of the particulate ingredients in addition the porosity of the Powerbone flexible biomaterials before and after the peptide integration processed to porous structure. Tensile test was performed to compare the mechanical strengths biomaterials before and after the peptide integration. The antibacterial activity of the obtained flexible biomaterial against in vitro planktonic *S. aureus* (Gram-positive) bacteria in order to investigate the bactericidal properties of the design for preventing the biofilm formation of the flexible biomaterial designs. The antibacterial activity of the antimicrobial peptide integrated biomaterial was investigated by using, Resazurin and Crystal Violet assays on *S. aureus*. According to the results of the Resazurin and Crystal Violet test, it was observed that the cell viability of the bacterial strains and the presence of EPS decreased with the increasing SAAP-276 content in the biomaterial by preventing the bacterial cell adhesion on the biomaterial surfaces. All in all the obtained results shows SAAP-276 peptide loading into flexible biomaterials is and promising approach for the prevention of bone infection.

Keywords: Bone Infection, Anti-microbial Peptides, Flexible Biomaterial, Polycaprolactone, Biofilm, *S. aureus*

Antimikrobiyal Peptit ve Silikat Katkılı Fleksible Biyomalzemenin Antibakteriyel Etkinliğinin İncelenmesi

Öz

Osteomiyelit, kemik yıkımı ve kaybı sürecinin mikroorganizmaların neden olduğu bir hastalıktır. Osteomiyelit, kronik ve akut enfeksiyonlara neden olabilecek artan bir insidans oranına sahiptir. Kemik enfeksiyonu hastaların %40'ında tekrarlayan ve kalıcı hasara neden olmuştur. Ortopedik cerrahi operasyonlarda kullanılan tıbbi cihazların neden olduğu enfeksiyonların %2-5'i osteomiyelittir. Osteomiyelit vakalarının yarısından fazlasına *Staphylococcus aureus* ve *Staphylococcus epidermidis* gibi gram pozitif bakteriler neden olur. Tıbbi cihaza bağlı osteomiyelit oluşumunu önlemek için, tıbbi implantasyon tasarımında kullanılan biyomalzeme matrisine bakteriyosidal ve bakteriyostatik ajanların entegre edilmesine ciddi bir ihtiyaç vardır. Esnek biyomateryal şu anda kanser, iskelet travması ve enfeksiyona bağlı kemik kaybı, kemik kırıkları ve yüz ve kafatası kemiklerinin konjenital deformiteleri gibi kemik defektlerinin tedavisinde yaygın olarak kullanılmaktadır. β-trikalsiyum fosfat (β-TCP) greft materyali, yüksek osteouyumluluğu ve mekanik mukavemeti nedeniyle esnek bir biyomateryalde kullanılmaktadır. Esnek biyomateryal tamamen sentetik, osteokondüktif ve esnek bir kemik grefti materyalidir ve doku rejenerasyonuna yardımcı olmak için geçici bir kemik dokusu iskelesi görevi görür. Ayrıca esnek kemik iskelesi biyouyumludur ve radyoopaktır ve kontrollü bir şekilde emilir. Bildiğimiz kadarıyla, kemik defekti tedavilerinde kullanılan işaretli ürünlerdeki mevcut esnek biyomateryaller antibakteriyel aktiviteye sahip değildir. İmplant cerrahisi sonrası

mikrobiyal komplikasyonları önlemek için antibakteriyel biyomalzemelere şiddetle ihtiyaç vardır.

İmplantasyon bölgesindeki bir enfeksiyonu yok etmeye yönelik antibiyotik tedavileri açısından, implant tarafında biyofilm oluşumu, biyofilmin antibiyotik toleransının artması ve hücre dışı polimerik maddeler (EPS) yoluyla penetrasyon etkinliğinin azalması nedeniyle ve EPS'nin enzimatik bileşenleri tarafından antibiyotiklerin bozulması ve artan direnç nedeniyle tedavi etkinliğini azaltmaktadır. Antibiyotik direnci ve biyofilmle ilişkili antibiyotik toleransı, antimikrobiyal ilaçlara alternatif olarak yeni antimikrobiyal ajanların araştırılmasına ve biyomateryallere antibakteriyel ajan entegrasyon stratejilerinin geliştirilmesine yönelik acil ihtiyacı ele alınmıştır. Antimikrobiyal peptitler (AMP'ler), çeşitli organizmaların doğuştan gelen bağışıklık sisteminde önemli bir rol oynayan, doğal olarak oluşan bir peptit sınıfıdır ve kapsamlı bir şekilde incelenmiştir ve küçük moleküllü antibiyotikler olarak hizmet etme potansiyeline sahiptir. Bu çalışmada insan LL-37 peptidi şablon olarak kullanılarak, sentetik bir antimikrobiyal ve anti-biyofilm peptidi olan SAAP-276, sentetik olarak sentezlenmiştir. Antimikrobiyal peptit yüklü biyomalzemeler hazırlanmış ve hazırlanan esnek biyomalzemenin bakteri hücresinin material yüzeyine yapışmasını engelleme, bakterisidal etki sağlama ve biyofilm oluşumunu engelleme potansiyeli araştırılmıştır.

Sonuç oalrak bu çalışmada, SAAP-276 AMP'yi kemik dokusu rejenerasyonunda kullanılan ticari olarak temin edilebilen Powerbone esnek şerit biyomateryaline (TerraMed) entegre etmeyi amaçlıyoruz. Hazırlanan esnek malzeme ve bileşenlerinin fizikokimyasal karakterizasyonu araştırılmıştır. Bu amaç için; boyut belirleme için dinamik ışık saçılımı (DLS) kullanıldı ve bu gözenekli yapıya işlenen peptit entegrasyonundan önce ve sonra Powerbone esnek biyomateryallerinin gözenekliliğine ek olarak partikül bileşenlerin morfoloji analizi için taramalı elektron mikroskobu (SEM) kullanıldı. Peptit entegrasyonundan önce ve sonra biyomalzemelerin mekanik mukavemetlerini karşılaştırmak için çekme testi yapıldı. Esnek biyomalzeme tasarımlarının biyofilm oluşumunu önlemeye yönelik tasarımın bakterisidal özelliklerini araştırmak amacıyla elde edilen esnek biyomalzemenin in vitro planktonik S.aureus (Gram-pozitif) bakterilere karşı antibakteriyel etkinliği araştırılmıştır. Antimikrobiyal peptit entegre edilen biyomalzemenin antibakteriyel aktivitesi, gram pozitif *S. aureus* üzerinde Resazurin ve Kristal Viyole testleri

kullanılarak araştırıldı. Resazurin ve Kristal Viyole test sonuçlarına göre, bakteri suşlarının hücre canlılığının ve EPS varlığının, biyomateryalde artan SAAP-276 içeriği ile bakteri hücresinin biyomateryal yüzeylere yapışmasını önleyerek azaldığı gözlemlendi. Elde edilen tüm sonuçlar, SAAP-276 peptidinin esnek biyomateryallere yüklenmesinin kemik enfeksiyonunun önlenmesi için umut verici ve umut verici bir yaklaşım olduğunu göstermektedir.

Anahtar Kelimeler: Kemik Enfeksiyonu, Fleksible Biyomalzeme, Antimikrobiyal Peptitler, Polikaprolakton, Biyofilm, *S. aureus*

To my lovely family…

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List of Abbreviations

ECM	Extracellular Matrix
AMP	Antimicrobial Peptides
EPS	Extracellular Polymeric Substances
CWA	Cell Wall-Anchored
FnBP	Fibronectin-Binding Proteins
Cna	Collagen Adhesin
MSC	Mesenchymal Stem Cell
Bbp	Binding Protein
DLS	Dynamic Light Scattering
SpA	Protein A
PCL	Polycaprolactone
β-TCP	β-tricalcium Phosphate
SEM	Scanning Electron Microscope
XRD	X Ray Diffractometer
UV	Ultraviolet
OD	Optical Density
CT	Computed Tomography
PDI	Poly Dispersity Index
ASTM	American Society for Testing and Materials

TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
RPM	Revolutions Per Minute
PBS	Phosphate Buffered Saline
DNA	Deoxyribo Nucleic Acid
SAAP	Synthetic Antimicrobial and Anti-Biofilm Peptide
JCPDS	Joint Committee on Powder Diffraction Standards
ROS	Reactive Oxygen Species
RNA	Ribo Nucleic Acid
MDR	Multi Drug Resistance
CFU	Colony Forming Unit
DMF	Dimethylformamide
HBTU	Hexafluorophosphate Benzotriazole Tetramethyl Uronium
DIPEA	Diisopropylethylamine
MSCRAMM	Microbial Surface Components Recognizing Adhesive Matrix Molecules

List of Symbols

mL	Milliliter
µL	Microliter
µm	Micrometer
Nm	Nanometer
mm	Millimeter
ms	Millisecond
min	Minute
°C	Celsius Degree
mg	Milligram
SiO_2	Silicon Dioxide
µg	Microgram
CaP	Calcium Phosphate
kV	KiloVolt
µA	Microampere
N	Newton
M	Molar
MPa	Mega Pascal
Al	Aluminum

Chapter 1

1. Introduction

1.1 Bone Infection

Osteomyelitis, bone infection is a disease in which the process of bone destruction and loss is caused by microorganisms. Osteomyelitis was observed hundreds of millions of years ago from fossilized animal remains. Osteomyelitis can affect several regions, such as the marrow, cortex, periosteum and surrounding soft tissue [1]. Bone infection causes repeated and permanent damage in 40% of the patients [2]. 2-5% of infections caused by medical devices used in orthopedic surgical operations are osteomyelitis. Bone infection has been known for many years as a challenging disease that is difficult to treat and seriously threatens human life. With the development of medical technology and treatment methods, it has become possible to treat bone infections. However, treatment methods such as implant, biomaterial, or surgical methods with known effectiveness might lead serious side effects on patients. Side effects may cause different complications after the treatment, and they may cause risks on the patient's life.

According to literature, osteomyelitis is mostly caused by gram-positive *staphylococci* and variety pathogens [3]. *S.aureus* colonizes asymptomatically and permanently in some healthy people. 7 out of 10 people, *S.aureus* is either temporarily colonized or absent [4]. *S. aureus* might cause life-threatening infections. Variety of factors take place in the colonization of *S. aureus* and *S. epidermidis* and formation of osteomyelitis. Having a thick cell wall and capsule is one of its most important features. Also, it binds to the existence of various cell wall-anchored (CWA) proteins and extracellular factors. These proteins can be attached to host cells and extracellular matrix (ECM) molecules and have been titled microbial surface components

conceding adhesive matrix molecules (MSCRAMM)[5]. Fibronectin-binding proteins (FnBP) and collagen adhesin (Cna) are also cited as examples [6]. *Staphylococci* can secrete toxins which aid in irruption and spreading in the host. The main targets of these toxins are to destroy host tissue and provide aliments for bacterial survival and growth [7,8].

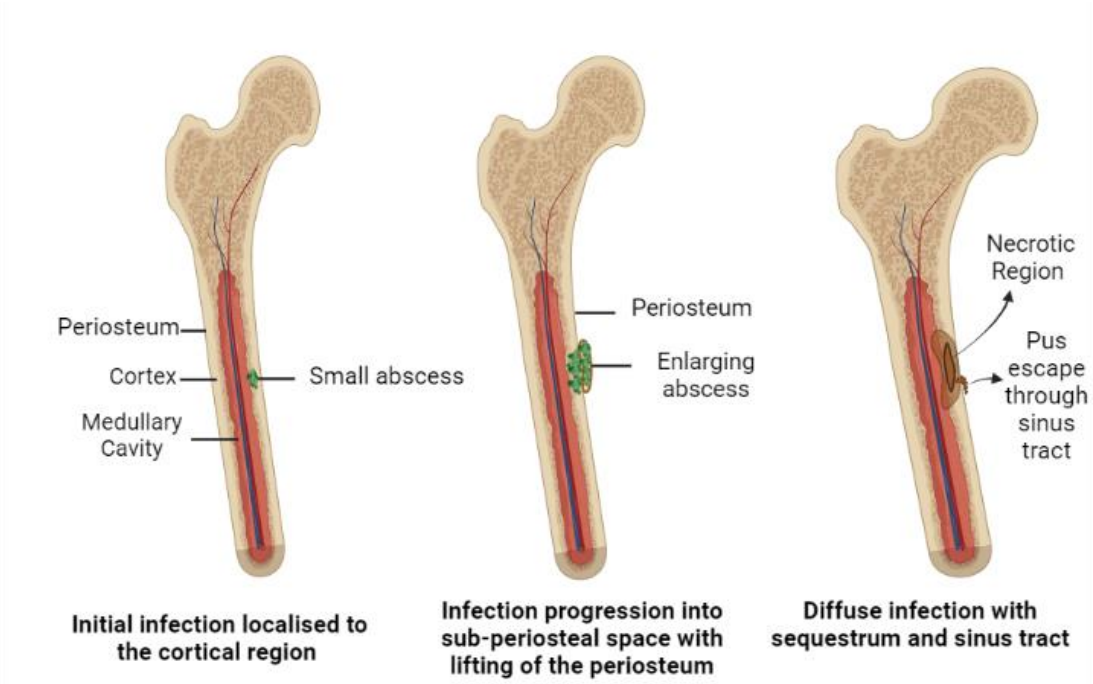

Figure 1: Progression of osteomyelitis.

1.1.1 Classification of Osteomyelitis

Since osteomyelitis is a heterogeneous nature, many classifications have been made. Two major classifications are commonly used for osteomyelitis and these are Waldvogel system and the Cierny-Mader system. These two classifications provide information about the character and source of the disease, while taking into account the physiological state of the patient. Prescribing the treatment of osteomyelitis in the clinical regulation usually related on classification as acute or chronic [9]. In this classification, the degree of tissue damage is often directly related to disease stage.

According to the Waldvogel classification method, an illness can be classed as acute or chronic depending on how persistent it is. It is then further categorized according to the source of the infection in Table 1 [10].

Table 1: Waldvogel classification systems

Waldvogel Classification		
Osteomyelitis Stage	Acute	Initial admission for the same disease
	Chronic	With history of admission for the same disease
Source of Infection	Haematogenous Osteomyelitis	
	Osteomyelitis associated with peripheral vascular disease	
	Osteomyelitis secondary to a contiguous focus of infections	

The four main components of the Cierny-Mader categorization system are the state of the host, the functional impairment brought on by the illness, the place of involvement, and the degree of bone necrosis. [11]. It does not assume it necessary to discrimination between acute and chronic infections. In Table 2, the anatomic type of osteomyelitis is added to the patient's physiologic class in this classification system. The anatomic type of osteomyelitis (I to IV) is combined with the patient's physiologic class (A, B, or C), resulting in one of the 12 clinical staging systems of adult osteomyelitis. For example, Stage IIIA osteomyelitis is defined localized lesion in a good host.

Table 2: Cierny-Mader classification systems

Cierny-Mader Classification		
Anatomic Type	Stage I	Initial admission for the same disease
	Stage II	External osteomyelitis
	Stage III	Localized osteomyelitis
	Stage IV	Diffuse osteomyelitis
Physiologic Class	A- Host	Good or normal immune system and delivery
	B – Host	Compromised locally or systemically
	C - Host	A condition where the treatment of osteomyelitis is worse than the disease itself and does not require treatment or is disability

1.1.2 Pathopysiology of Osteomyelitis

Bone is a connective tissue that is continually rebuilt by the activities of three primary bone cells: osteoblasts, osteocytes, and osteoclasts. Osteoblasts form cells derived from mesenchymal stem cells (MSC) found in the bone marrow and are involved in the production of ECM components of bone. Osteoblasts are mature cells and produce vesicle-shaped osteoid [12]. Osteoid includes collagen and non-collagenous proteins which are collogen type I, proteoglycans and glycoproteins. The glycoproteins found in the ECM are fibronectin, osteonectin, osteopontin, bone sialoprotein and osteocalcin [13,14]. Osteoblasts entering the ECM differentiate to form osteocytes. Osteocytes play a role in detection bone loading and detection of fixation of microcracks and in

the remodeling process of bone. Osteoclasts are the bone-resorbing cells, which operate by decalcifying hydroxyapatite and degrading organic ECM. Osteoclasts work with osteoblasts to protect bone remodeling homeostasis. Bone morphology can be altered by an imbalance in activity between these cells, resulting in pathological bone. [15,16].

1.1.3 *Staphylococcal* Colonization of Bone

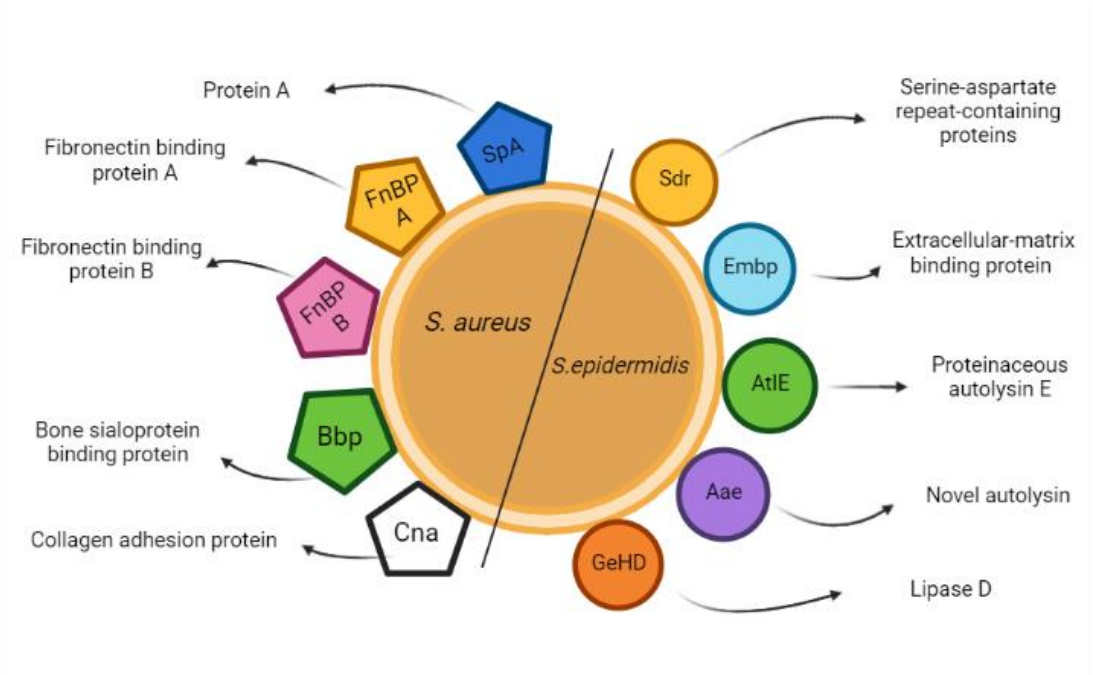

Figure 2: *Staphylococcus aureus* and *S.epidermidis* cell surface proteins

After *staphylococci* bacterium reach the bone, is the attachment takes place which is facilitated by the presence of MSCRAMMs and other cell wall-associated proteins facilitates attachment (Fig.2). *Staphylococcal* colonization on bone can occur through direct interaction with bone cells, plasma proteins, or the ECM. Colonized *staphylococci* may generate a biofilm that promotes infection persistence. Biofilms are populations of microorganisms encased in an extracellular matrix that is adhered to a surface. [17,18]. In addition, *staphylococci* can produce toxins that facilitate spread, causing recolonization and reinfection [19].

Protein A (SpA), FnBP A and B, bone sialoprotein binding protein (Bbp), and Cna are key MSCRAMMS and CWA proteins for the pathogenicity of infection in *S. aureus*.

In addition SpA can be secreted as well as attached to the cell wall of *S.aureus* [2]. SpA has the ability to directly bind to osteoblasts, which therefore allows it to mediate cell death, as well as the suppression of bone production (osteogenesis) and the activation of bone resorption (osteoclastogenesis)[20–23]. Protein A plays an important role in the activation of osteoclasts [23,24].

If *S. aureus* does not contact directly with the host cell, its FnBPs enable binding to host plasma proteins, such as fibronectin and fibrinogen, which can serve as bridge molecules between the bacterium and the host cell receptors. [25,26]. If *S. aureus* persists intracellularly, it will not activate this pathway.

Through Cna and Bbp, *S. aureus* is likewise equipped to interact with the bone ECM. Cna is the only known *S. aureus* cell surface protein that binds to collagen, although Bbp has been shown to bind both bone sialoprotein and fibrinogen. [27–29]. *S.aureus* is well known to form biofilms on medical device implants, allowing for the persistence of infection and the biofilm formation steps are shown in Figure 3.

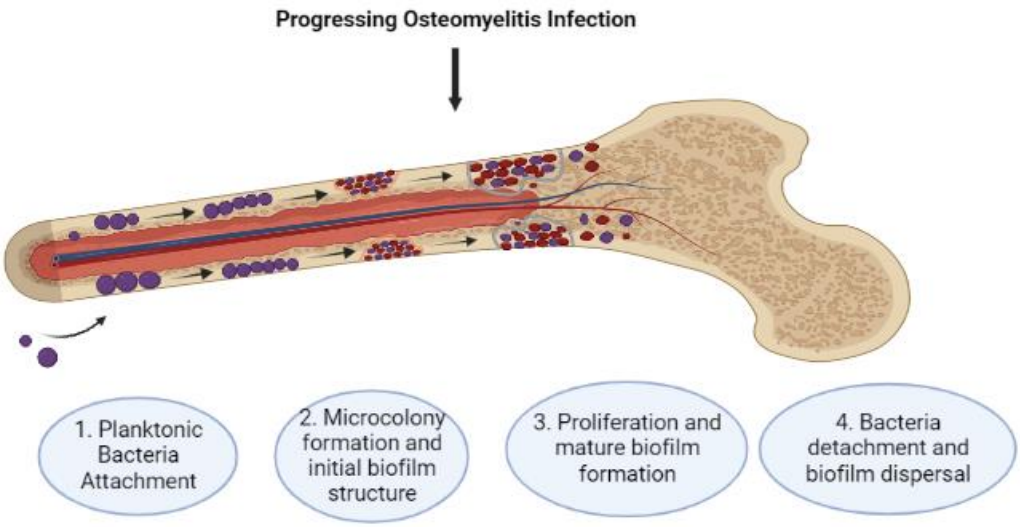

Figure 3: Stages of biofilm development in bone

1.2 Biofilm Matrix and Composition

Biofilms are microbial communities comprised of an organic polymer matrix that enables bacteria to cling to one another or a surface. Biofilm is a network structure composed of polysaccharides and bacterial cells. Bacteria are protected from chemical and physical changes by extracellular polysaccharide matrix.. It enables the storage of nutrients and the elimination of waste. [30]. The structure of the organic polymer matrix inhibits antibiotics from penetrating to the bacterium, leading to a rise in the incidence of diseases caused by antibiotic-resistant pathogens. [31].

The initial phase involves the adhesion of bacterial cells to a surface, which can either be living or non-living. During this stage of the production of a biofilm, the bacterial cells will connect to the surface by making use of their appendages, which may include pili or flagella. The van der Waals force or electrostatic interactions are responsible for the first stage of bonding that takes place between the cell surface and the substrate. [32]. Micro colony formation occur after attachment of bacterial cell to a biotic or an abiotic surface [33]. The second phase of biofilm formation is micro colony formation, which involves the growth and division of surface-attached bacterial cells. [32]. During this accumulation stage, bacterial cells continue to grow and several cell clusters with multilayer structures are produced. Micro colonies that continue to grow become macrocolonies and are surrounded by an extracellular polysaccharide matrix [34]. Water canals around macro colonies facilitate the delivery of nutrients and signal chemicals. Lastly, as seen in Figure 4, biofilm cells can disperse and colonize different habitats when nutrients are scarce. The processes consist of bacterial adhesion to the surface, creation of a monolayer along the surface, maturity of the biofilm through the construction of a three-dimensional structure, and cell dispersion. Typically, biofilm dispersion is caused by environmental changes and is dependent upon the growth circumstances [34].

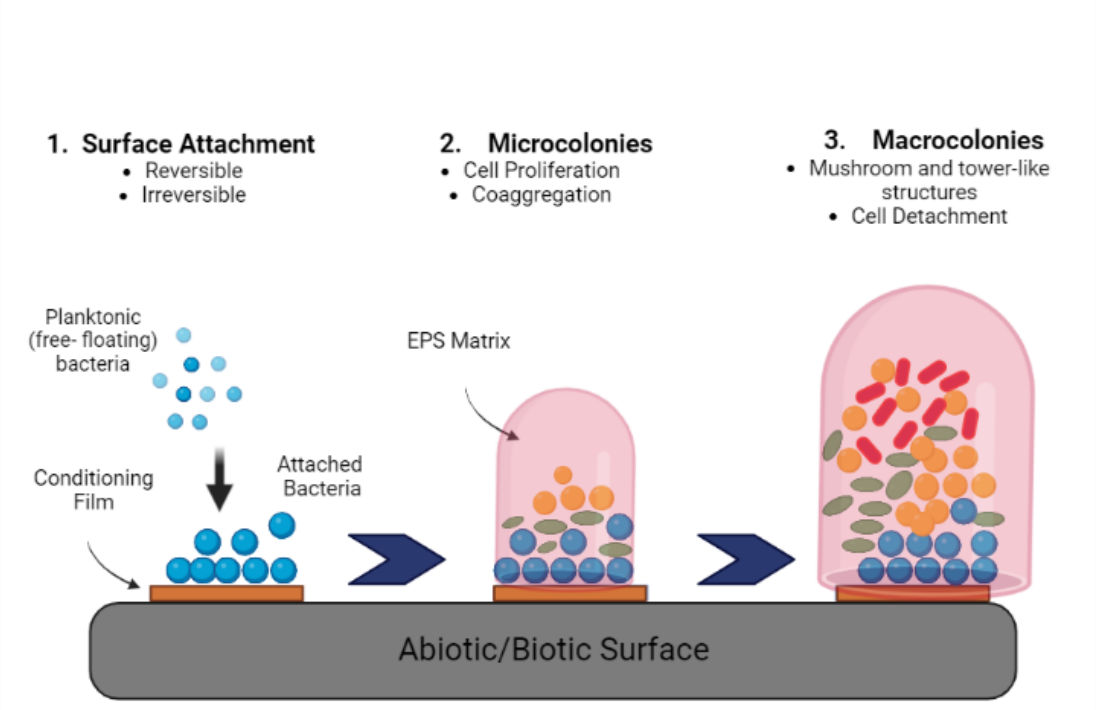

Figure 4: Stages of biofilm formation

Macrocolonies are encased in a matrix of extracellular polymeric substances (EPS). EPS are an insoluble secretion generated by bacterial cells that coats millions of bacterial cells in the form of macrocolonies with a matrix [35]. ECM include polysaccharides, proteins, extracellular DNA, lipids and water [36]. Compared to planktonic bacteria, cells encased in the EPS matrix provide superior protection against external environmental challenges. [37]. Planktonic structures are those in which microorganisms can move freely. The shift of microorganisms from a planktonic structure to a mature and sophisticated biofilm layer is accompanied by profound alterations in bacterial phenotype. The most significant of them is the growth in bacterial resistance to antibiotic drugs. The biofilm growth of bacteria (Figure 5) is far more hazardous than planktonic cell structure [38].

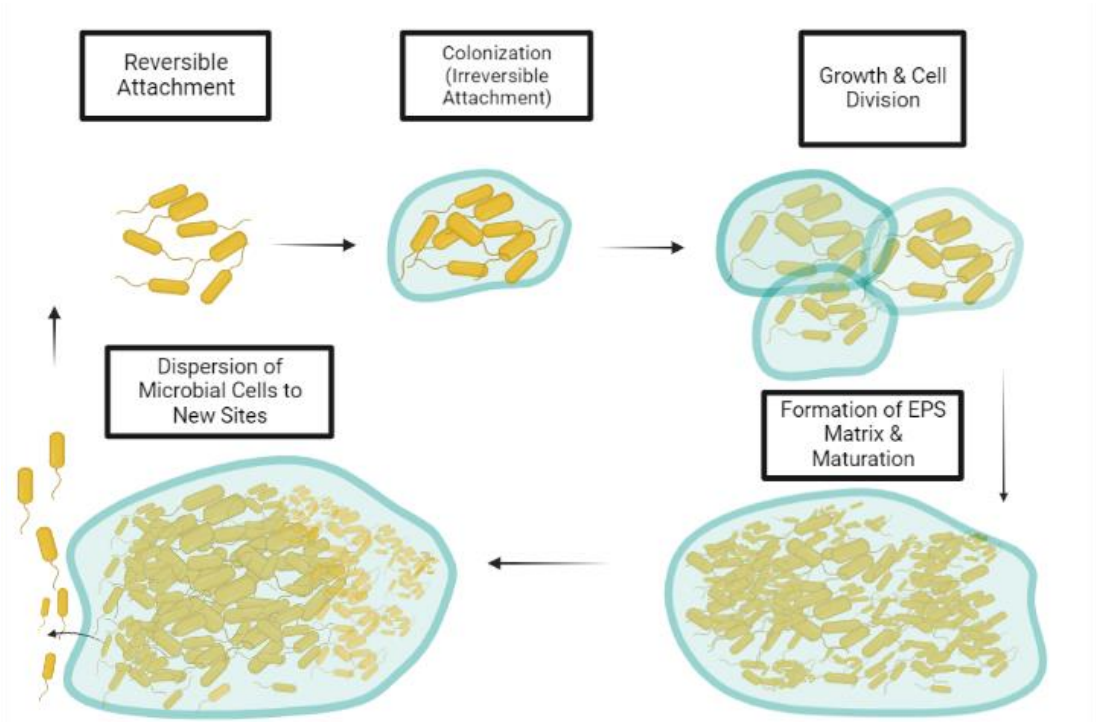

Figure 5: Cycle of biofilm formation

Staphylococcus aureus is one of the gram-positive bacteria present on the flora of the external skin surface and mucosal surfaces, particularly in the respiratory tract. *S. aureus* is assessed as a potent nosocomial pathogen due to the fact that a high nosocomial (hospital-acquired) case-to-infection rate has been reported and it transmits quickly from patient to patient in hospitals [39]. Normally, these bacteria are not detrimental to the body, but when the integrity of the skin is compromised, they can multiply and colonize, leading to infection. [40]. *Staphylococcus aureus* are resistant to all antibiotics nearly [41]. Figure 5 depicts the continuation of the cycle in the absence of the necessary biofilm-dissolving therapy. With cell dispersion, planktonic cells are transported elsewhere, resulting in the formation of a new biofilm at that location.

Overall, untreated infections can lead to severe complications. Consequently, infections should be eradicated using appropriate treatment procedures in order to aid the healing process. In addition to the problems of antibiotic resistance and other traditional treatment techniques, the inactivation problem of bacteria, especially for

the removal of antibiotic-resistant bacteria such as S. aureus, necessitates the development of alternative antibacterial treatment approaches [42]. Several genes control the progression of *S. aureus* biofilms and the conditions that contribute to their growth. The immune responses that are mounted against infections caused by biofilms are weak and ineffectual. Antibiofilm therapies that are effective against *S. aureus* biofilm, which has gained resistance to antibiotics, have been developed. There is a significant connection between infections acquired in hospitals and the production of S. aureus biofilm. The creation of a biofilm by *S. aureus* raises the danger level. *S. aureus* biofilm, which may be found on medical equipment, has the potential to cause patients to develop an infectious condition that is both persistent and recurring. This makes it more difficult to cure disorders. Therefore, this danger is attempted to be reduced by employing effective biofilm eradicating and inhibiting approaches [43]. In the light of this information, we can prevent the formation of biofilm and prevent bone infection disease.

1.3 Antimicrobial Resistance in Bacteria

Antibiotics, such as penicillin or methicillin, which have been routinely used for many years [44] to treat bacterial infections, are another typical therapy technique when the immune system cannot handle the illness. They combat diseases by destroying or blocking bacterial development [45]. Antibiotics cause these effects by interfering with the formation of the bacterial cell wall. In addition, antibiotics alter the cell membrane and impede the creation of nucleic acid and proteins. In rare instances, bacteria modify their reaction to antibiotics, rendering medicines ineffective. The overuse, misuse, and frequent use of antibiotics generates resistance to them [46].

Various biochemical pathways enable bacteria to acquire resistance to antibiotics. By creating an enzyme that deactivates antibiotics, eliminating the antibiotic, offering an alternative target, modifying the antibiotic's target, and decreasing the permeability of the cell membrane, microorganisms prevent antibiotics from entering the cell (Figure 6) [45].

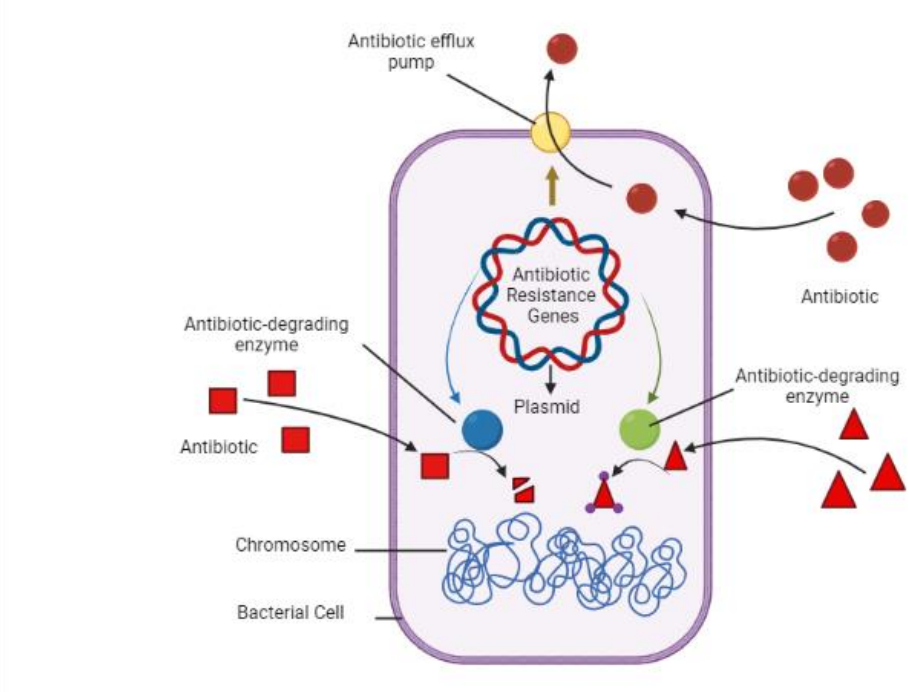

Figure 6: The antibiotic resistance mechanism of bacteria.

It is the creation of biofilms, which is both one of the bacteria's resistance strategies and a virulence mechanism. Biofilm formation is one of the resistance strategies bacteria employ to survive in the presence of antibiotics. Compared to their planktonic form, bacteria in biofilms are 10 to 1,000 times more resistant to conventional antibiotic therapy, which considerably hinders their therapeutic treatment [36]. This infection cannot be treated with antibiotics, the conventional way of treatment. Therefore, antimicrobial peptides are advised for the treatment of biofilm-related illnesses [48]. Antibiotic resistance is a rising concern in both the community and hospital levels. Failure of antibiotic therapy results in a rise in mortality and morbidity, medical costs, and bacterial infectious illnesses. Antibiotic therapy fails due to the prevalence of bacteria with multidrug resistance (MDR) and the development of resistant structures by bacteria to defend themselves from external stimuli [49].

1.4 Antimicrobial Peptides

Peptides are composed of several amino acids connected by peptide bonds. A peptide that has been chemically generated outside of the cell is known as a synthetic peptide.

Peptides offer several benefits, including biocompatibility, chemical modifiability, and simple and inexpensive production. Antimicrobial peptides (AMPs) are sometimes referred to as peptides of host defense. The process by which antimicrobials destroy germs is distinct from that of antibiotics. Therefore, it has the ability to inhibit several antibiotic-resistant microorganisms [50].

AMPs are typically small in size. The length of amino acids is typically between 12 and 50. Due to their limited resistance, antimicrobial peptides are the natural defense molecules of animals, plants, and microbes [51]. Their major function is to eliminate invading and persistent pathogens [52].

According to their amino acid components, architectures, and biological roles, AMPs can be categorized into four groups: α -helical peptides, β -sheet peptides, extended peptides, and loop peptides. α-helical and β-sheet peptides are the most prevalent types and α-helical AMPs disrupt has been identified to disturb bacterial membranes [53]. When they interact with bacterial membranes, α-helical AMPs will transform into amphipathic helical structures, despite the fact that they exist in aqueous solution in a linear state [54]. It is through the disruption of bacterial membranes that β -sheet AMPs exert their antibacterial activity. The peptides' hydrophilic sections interact with the polar headgroups of the membranes through [53].

AMPs have an antimicrobial effect that is broad-spectrum, meaning that they are effective against a wide range of pathogens, including MDR bacteria, Gram-positive and Gram-negative bacteria, fungi, and viruses. AMPs prevent the rapid development of antibiotic resistance unlike the way conventional antibiotics. Electrostatic interactions are the mechanism through which cationic AMPs bind themselves to negatively charged bacterial cell membranes. The adherence is responsible for changes in the electrochemical potential of bacterial cell membranes, as well as damage to cell membranes and a decline in cell morphology. These AMPs are harmless and have no adverse side effects. AMPs prevent microorganisms and bacterial cells from developing drug resistance readily [50,51,53]. Figure 7 demonstrates that antimicrobial peptides inhibit planktonic cells from sticking to surfaces and that biofilm development is not seen.

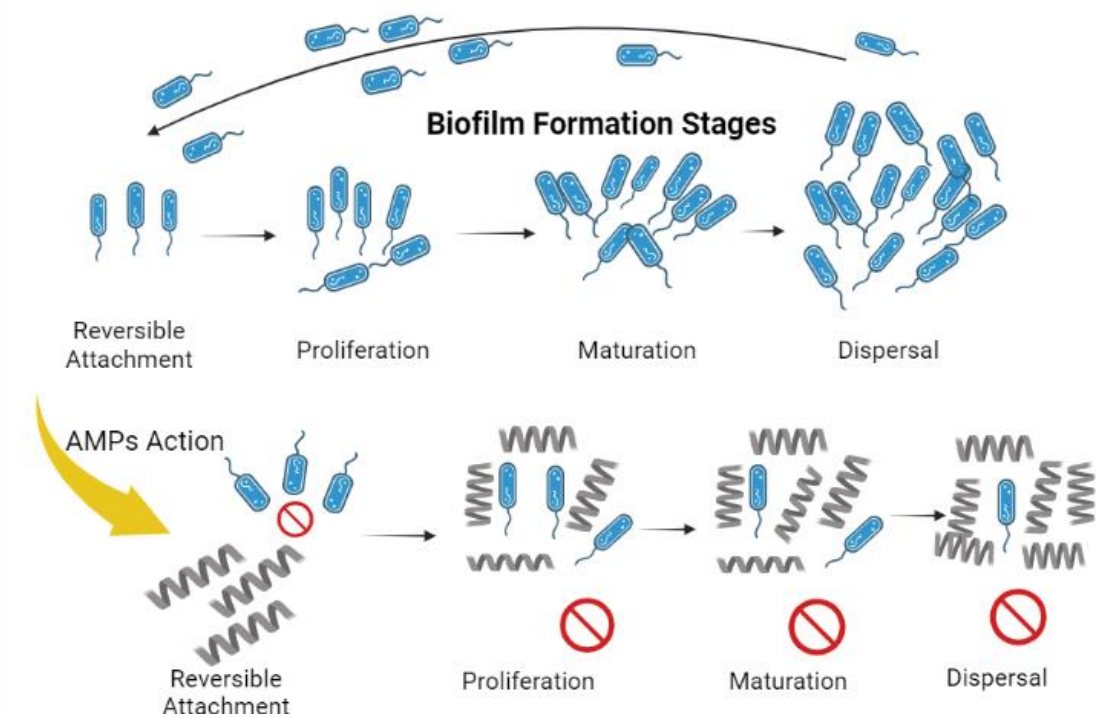

Figure 7: AMPs action on biofilm formation.

Antimicrobial peptides harm bacterial cells' DNA, RNA, and protein production. In this manner, it causes the bacterial cell to die. In general, however, AMPs exert their antibacterial activity by causing bacterial cell membrane damage. Length, charge, secondary structure, and hydrophobicity of AMP peptides influence the AMP-bacterial cell membrane interaction. AMPs should detect bacterial cells preferentially and not damage human cells [55].

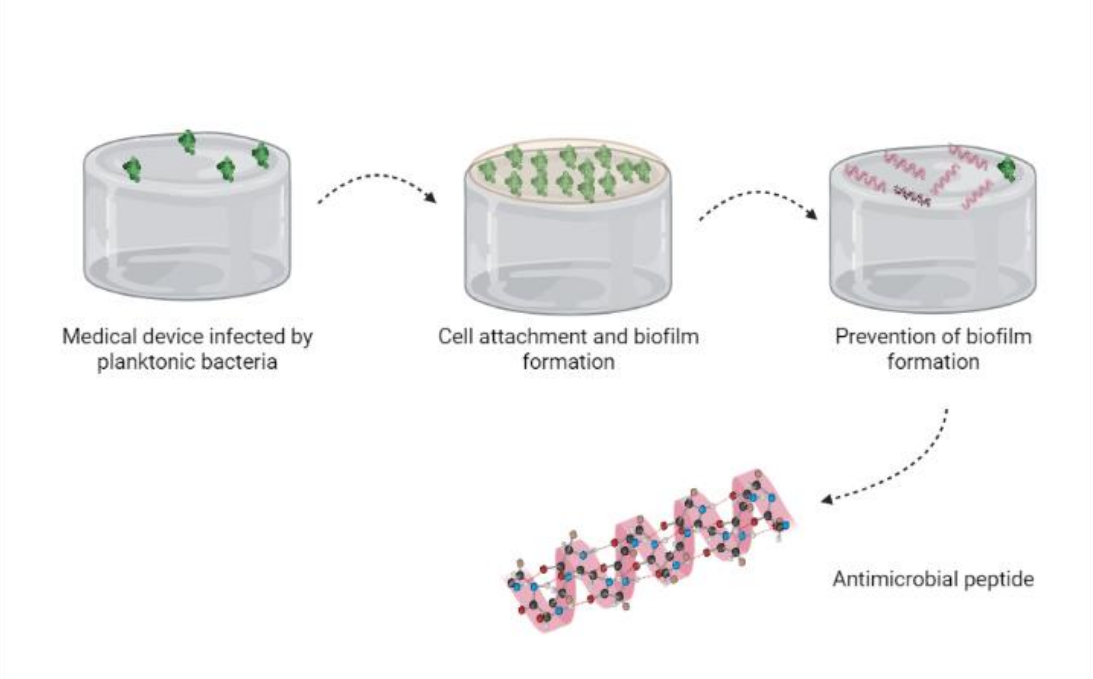

Figure 8: Biofilm inhibition on AMP applied medical device.

In the literature in order to approve the AMP action mode researchers have employed AMP placement on the medical device and explored the adherence of planktonic bacteria on the device. Over time, researchers observed was no biofilm on the surface of the device. This was because the AMPs on the medical device interacted with the planktonic bacterial cells that was reaching to medical device surface and and inhibited biofilm formation as shown in figure 8.

1.4.1 Human Cathelicidin LL-37 Antimicrobial Peptide

LL-37 is the only cathelicidin-derived antimicrobial peptide found in humans. Cathelicidins are tiny, 12-97 amino acid cationic, amphiphilic peptides. The 37 amino acids that make up the LL-37 peptide. Host defense peptides called LL-37 can be immune to infection and help the body detect and get rid of invasive microorganisms [56]. It prevents the production of prefabricated bacterial biofilm at extremely low peptide concentrations [57]. Antimicrobial peptides based on the LL-37 peptide were able to eradicate methicillin-resistant *S. aureus* biofilms from wounded human skin [58]. Antimicrobial peptide LL-37 has a crucial function in preventing local and systemic infections. LL-37 antibiofilm peptide has a good antibacterial impact against *S. aureus* biofilm that is antibiotic resistant [58].

1.4.2 SAAP-276 Antimicrobial Peptide

The antimicrobial activity of LL-37 peptide is susceptible to environmental conditions, hence researchers have generated synthetic peptides that are not as sensitive to environmental factors as LL-37, depending on the amino acid sequence of LL-37 [51]. These synthetic peptides have been designed to exhibit antibiofilm and antibacterial properties under physiological circumstances.

The LL-37 peptide was utilized as a template to generate synthetic peptides. In Figure 9, the synthetic peptides with the highest antibacterial activity are displayed in gray. These peptides' respective amino acid sequences are depicted in the figure [51].

The net charge AMPs is commonly designed as positive, and they are composed of amino acids that are very hydrophobic. Because AMPs are positively charged , they are able to cling to the negatively charged membranes of bacterial cells, which enables them to exert their antimicrobial actions. The hydrophobic portion of AMPs wreaks havoc on bacterial lipid bilayers, resulting in membrane rupture and the eradication of infections. Synthetic antimicrobial peptides share the same characteristic. Synthetic antimicrobial peptides harm bacterial cell membranes and cell walls and limit the generation of excessive reactive oxygen species (ROS). In addition, these synthesized antimicrobial peptides do not affect human red blood cells, even at 10 times greater concentrations [59].

Peptide	Sequence	
LL-37		L L G D F F R K S K E K I G K E F K R I V Q R I K D F L R N L V P R T E S
P139		L K K L W K R V F R I W K R I F R Y L K R P V R
P140		L R R L W K R L V R I I K R I Y R Q L K R P V R
P141		L R R L Y K R V F R L L K R W W R Y L K R P V R
P142		L R R L W K R L V K I L K R W F R Y L R R P V R
P143		L R R L Y K R V V K L W K R L F R Q L R R P V R
P144		L K K L Y K R V A K I W K R W I R Y L K K P V R
P145	(SAAP-145)	L K R L Y K R L A K L I K R L Y R Y L K K P V R
P146		L K K L Y K R L F K I L K R I L R Y L R K P V R
P147		L K K L W K R L A R L L K R F I R Q L R R P V R
P148	(SAAP-148)	L K R V W K R V F K L L K R Y W R Q L K K P V R
P149		L K K V Y K R L A R L L K R Y I R Y L R R P V R
P150		L K K V W K R V A R L I K R W F R Y L R R P V R
P151		L K K L Y K R L F K L W K R L Y R Y L K K P V R
P152		L R R V Y K R L A R L I K R Y L R Q L K K P V R
P153		L R K L W K R V V K I W K R Y L R Q L R R P V R
P154		L R K L W K R L A K I I K R L Y R Y L R R P V R
P155		L K K V Y K R V A R L I K R L F R Y L K R P V R
P156		L R R L W K R L V K L W K R F F R Y L K K P V R
P157		L K K V W K R V F R I L K R F L R Y L K R P V R
P158		L R R V Y K R L F R L W K R I I R Q L R R P V R
P159	(SAAP-159)	L K R L Y K R V F R L L K R Y Y R Q L R R P V R
P160		L K K L W K R L A R L W K R I I R Q L K K P V R
P161		L R R V W K R V A R I I K R L Y R Y L K R P V R
P162		L K R L W K R L F K I L K R Y Y R Y L R R P V R
P163		L R R L W K R V F K I I K R L F R Q L K K P V R
P276	(SAAP-276)	L K R V W K A V F K L L K R Y W R Q L K K P V R

Figure 9: Template of LL-37 peptide [51]

There are a lot of benefits that come along with designing synthetic antimicrobial peptides based on the sequences of other proteins. To begin, these peptides can only be developed to increase their potential in a way that does not have any negative side effects [60]. Second, it is possible to add many functions to the same peptide sequence [61]. Thirdly, the synthesis of synthetic antimicrobial peptides is less costly than the majority of purifying procedures. Lastly, it is possible to construct synthetic peptides with features not seen in peptides used as templates, hence increasing their effectiveness [59]. Antimicrobial activities of synthetic antimicrobial peptides need both electrostatic and hydrophobic interactions with the bacteria. Peptides must possess hydrophobicity in order to permeate the bacterial cell membrane. Multiple physicochemical characteristics, including length, sequence, charge, helicity, hydrophobicity, amphipathicity, and the hydrophobicity/hydrophilicity angle, influence the antibacterial action of AMPs. On the basis of this data, acceptable peptide

sequences must be found [62]. The human cathelicidin antimicrobial peptide LL-37 was utilized as a template for the production of the synthetic peptide SAAP-276. The SAAP-276 peptide is made up of a total of 24 sequences of amino acids. acetyl-LKRVWKAVFKLLKRYWRQLKKPVR-amide is the sequence of amino acids that makes up the antimicrobial synthetic peptide known as SAAP-276 [63]. Studies have demonstrated that the synthetic peptide SAAP-276 is particularly efficient against MDR and *S. aureus* bacteria. *In vitro* suppression of biofilm formation by gram-positive *S. aureus* bacteria is also one of the most important characteristics of the SAAP-276 peptide [51]. In general, synthetic amino acids have a net positive charge and a high hydrophobicity. This characteristic allows synthesized antimicrobial peptides to cling to the bacterial cell membrane. Thus, the proton within the bacterial cell eliminates the proton-motive force and halts the cell's critical processes (Figure 10). However, the literature findings are lacking of integration of SAAP-276 integration into biomedical devices which are in use in the market.

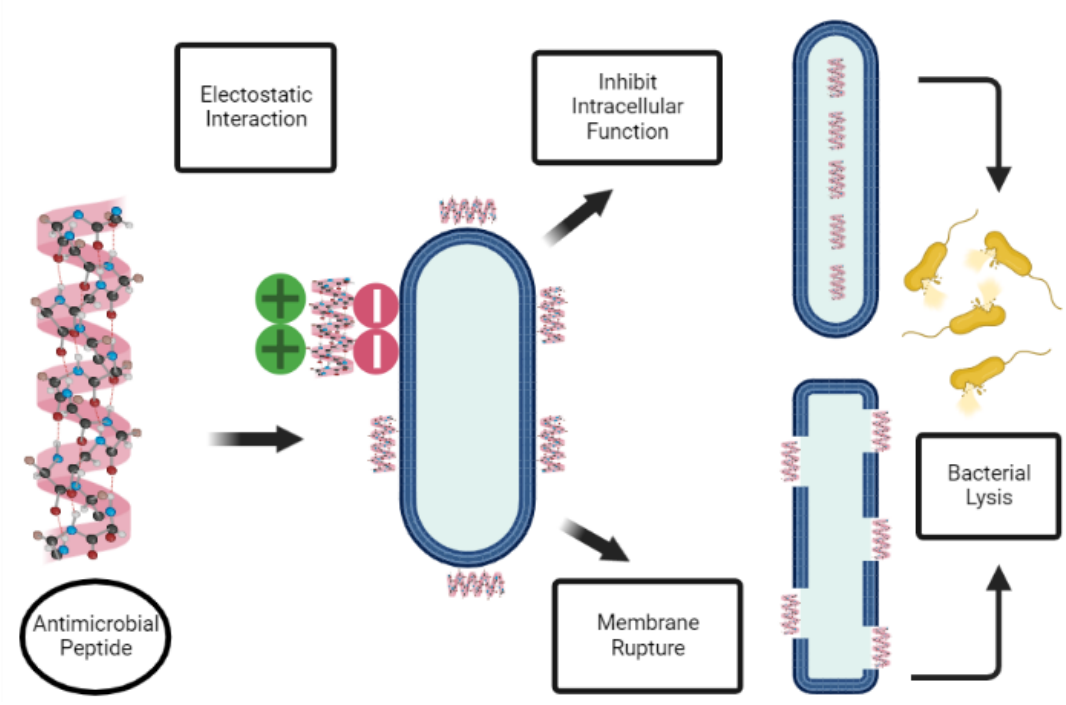

Figure 10: The biological function of antimicrobial peptide.

1.5 Silicate Doped Flexible Biomaterials

Tissue transplantation applications for the repair of damaged hard tissue are widely used in the treatment of bone cancer, bone loss due to skeletal trauma and infection, bone fractures and congenital deformities of the facial and skull bones. Although autografts have appropriate standards, the interest in synthetic bone grafts produced by tissue engineering techniques is increasing day by day due to limiting factors such as damage to the tissue taken area and limited graft availability, and the risk of immune system response in allografts [64]. Many bioceramic materials, including β-TCP and calcium sulfate, are commonly used as bone replacers in both block and granule form.

On the other hand, β-TCP, another biocompatible ceramic, has become the most preferred graft material in recent years due to its high osteo-compatibility and mechanical strength.

Although granule form is the most widely used form of β-TCP- based bone grafts in dental, maxillofacial and orthopedic surgery, filling epiphysis defects in sinus elevation and alveolar crest augmentation and orthopedic surgery, anterior intersomatic fusion and filling of epiphyseal bone spaces during compression of the tibia plate, especially in oral and maxillofacial surgery. Silicate-added flexible strips of different sizes can be used in operations [65,66].

In our current literature analysis evaluations made at the beginning of 2019, there has been an increase in interest in β-TCP-based synthetic grafts in recent years due to the high bioactivity of silicon dioxide (SiO_2) addition. Recent studies have shown that silicate has a critical effect on bone formation relative to its rapid apatite formation ability [66,67]. These studies demonstrated that silicate-reinforced grafts can promote the formation of hydroxyapatite *in vitro*, and also that the small silicate content is better for promoting bone regeneration *in vivo* compared to β-TCP. Silicate-reinforced grafts also showed greater bone fusion in clinical spinal fusion surgery compared to smooth β-TCP. It is aimed to prevent bone infection disease by giving antibacterial effect to the flexible biomaterial, which attracts attention in the sector and is frequently used in operations.

In this study, we aimed to prepare synthetic antibacterial peptide (SAAP-276) loaded flexible biomaterials at different portions and investigate the *in vitro* bactericidal activity by following the *S. aureus* cell viability, bacterial cell adhesion and biofilm formation on the flexible biomaterials. By this way, already existing market product of Powerbone flexible biomaterial could be tuned for providing antibacterial activity in order to prevent bacterial infections after implantation without changing its mechanical and physical properties.

Chapter 2

2. Materials and Methods

2.1 Synthesis of SAAP-276 Peptide

AAPPTEC supplied all of the chemicals utilized in peptide synthesis. The SAAP-276 peptide containing 24 sequenced amino acids (amine-Leu-Lys-Arg-Val-Trp-Lys-Ala-Val-Phe-Lys-Leu-Leu-Leu-Leu-Leu-Lys-Arg-Tyr-Trp-Arg-Gln-Leu-Lys-Lys-Pro-Val-Arg-amide) was produced automatically using a peptide (aappTec LLC-Focus XT). In 5 mL of dimethylformamide (DMF) solution, 200 mg of resin was dissolved. Because 200 mg of Rink Amide-MBHA resin was utilized, amino acids were produced to have a 0.3 M concentration. The de-protection solution was used to remove the 9Hfluorenylmethyloxycarbonyl (Fmoc)-protecting group. This de-protection solution contains 20% (v/v) piperidine in DMF. In addition to preparing the peptide for synthesis, the required ingredients for the synthesis reaction were also produced. For this purpose, 0.5 M 2-[1H-benzotriazol-1-yl]-1,1,3,3-tetramethyluronium hexafluorophosphate, 0.1 M diisopropylethylamine (DIPEA), and HBTU were produced. The reagents HBTU and DIPEA are coupling agents.

Figure 11: Peptide synthesizer (AAPPTEC LLC-Focus X).

The cleavage solution was created to remove the peptide from the resin. Before adding the cleavage solution, the peptide was rinsed three times with 100% ethanol. The cleavage solution was composed of 95 percent Trifluoroacetic acid, 2.5 percent deionized water, and 2.5 percent Triisopropylsilane. This ethanol was extracted using vacuum. The cleavage solution was subsequently added to the peptide-linked resins. After two hours, the cleavage solution was eluted into a cold diethyl ether solution for the precipitation of peptides separated from the resin, as seen in Figure 12. The solution was then centrifuged for 15 minutes at 4500 rpm and 4°C. Following centrifugation, the supernatant was extracted. The SAAP-276 peptide that had been precipitated was left in the fume hood to evaporate diethyl ether. The pellet of SAAP-276 peptide was then dissolved in distilled water and stored at -80°C until lyophilization. The peptide's liquid was extracted using the lyophilizer method by decreasing the pressure The powdered lyophilized SAAP-276 peptide was then kept at -20°C until usage in antibacterial test investigations.

Figure 12: Remove of peptide from resin [68].

2.2 Preparation of AMP and Silicate Doped Synthetic Flexible Biomaterial

β-TCP with the Ca/P: 1.5 ratio was achieved to be employed in the preparation of the flexible biomaterial. For this purpose, appropriate amount of calcium nitrate and ammonium phosphate are placed in deionized water and mixed with a mechanical mixer. After that an appropriate amount of ammonium hydroxide (ammonia) is added to the mixture and mixed. After mixing process was completed, the filtration process was performed. Subsequently, drying was carried out in the oven for two days. As a final process, calcination process is applied and β-TCP form is obtained. The approval of β-TCP formation was carried by XRD analysis.

For the completion of the flexible biomaterial , PCL (Poly L-lactide/Caprolactone Copolymer) is dissolved in chloroform at stirrer and the temperature 20 ℃. It′s called PCL solution. After that, β-TCP, porogen (sucrose) and SiO_2 within determined amount are mix in PCL solution. To create pore structure, removed porogen in distilled water for 3 days and then autoclave process was performed for 15 minutes at 120 °C.

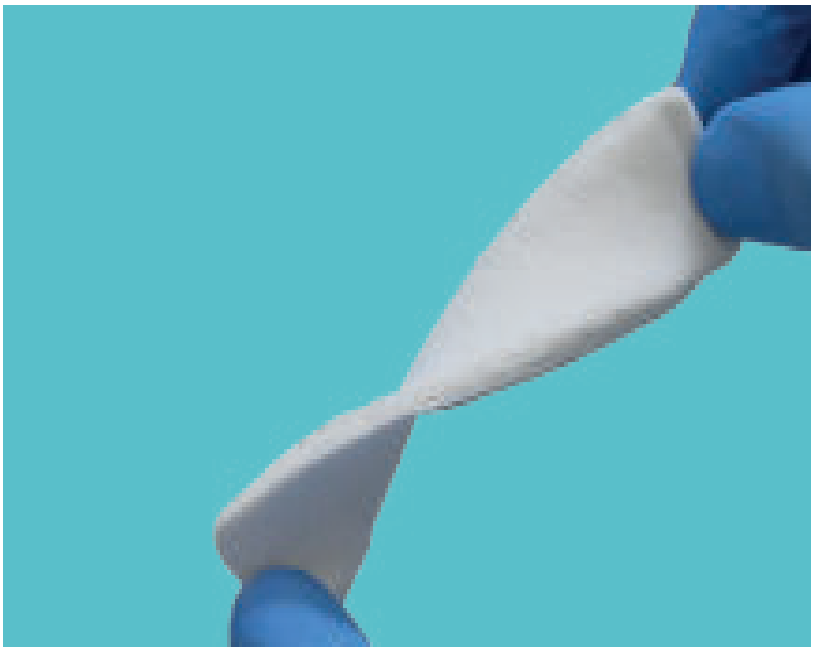

Figure 13: Flexible biomaterial

After the sterilization of flexible biomaterials by autoclave, the material have been taken for pore analysis and mechanical strength investigations. After that, flexible biomaterials were loaded with SAAP-276 solution using impregnation method [69]. In the figure 14, impregnation method, the SAAP-276 are dissolved in a suitable solvent and the porous flexible material is immersed in this solution. As the flexible biomaterial is immersed into the SAAP-276 solution, the peptide molecules are accommodated in the pores of the flexible biomaterials. In order to provide appropriate filing of the pores with SAAP-276 peptides the peptides were dissolved at different concentration and dropped on the biomaterial matrix as SAAP- 276 solution at an exact volume equal to formed the pore volume of the flexible biomaterials [70]. Based on this SAAP-276 solution at different concentrations (1 mg/mL, 2 mg/mL, 4 mg/mL) at the volume equivalent to calculated pore volume of flexible biomaterial has been impregnated as the pore volume of the biomaterial. Drying process was carried out at 36 ℃ in the oven and UV sterilization were carried out for 30 minutes after the impregnation was achieved.

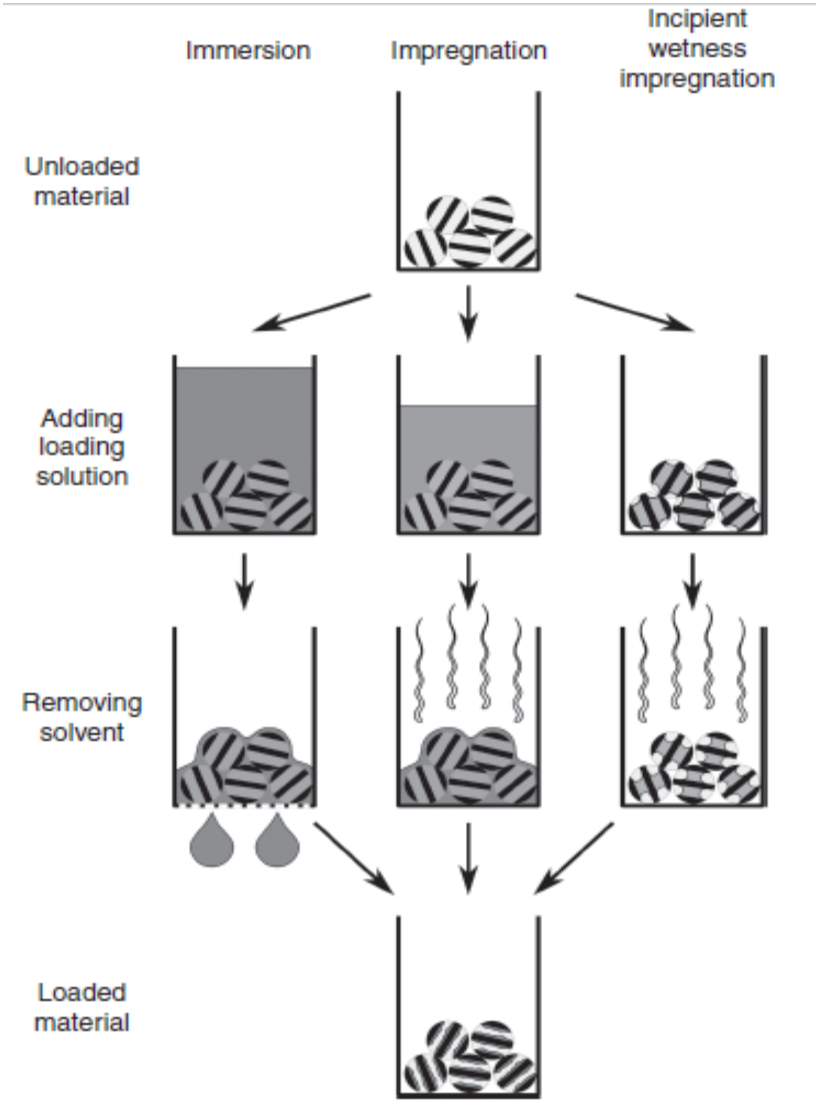

Figure 14: The principles of methods for loading porous materials [70].

2.3 Characterization of Antibacterial Peptide and Silicate Doped Synthetic Flexible Biomaterial

The prepared flexible biomaterials and its components was taken for physicochemical characterization by employing XRD, DLS, SEM, Micro CT analysis and tensile test was applied in order to mechanically characterize the silicate doped synthetic flexible biomaterial with the given details in the below sections.

2.3.1 X Ray Diffractometer (XRD) Analysis

The crystallographic structures of the synthesized β-TCP powders prior to mixing in PCL polymer solution and used in the preparation of flexible biomaterial were determined by X Ray Diffractometer (XRD) (Panalytical Empyrean) analysis. The data obtained were collected and evaluated in the angle range of 10-60 °C and 2Θ.

2.3.2 Dynamic Light Scattering (DLS) Analysis

The hydrodynamic size distribution of purchased SiO_2 powders in water t used for the doping into flexible materials was determined by using a dynamic light scattering (DLS) (Litesizer 500) and fro the analysis the particulate powder was suspended as a 0.025 mg/ml particles dispersion in Milli - Q water.

2.3.3 Scanning Electron Microscope (SEM) Analysis

The morphologies of synthetic silicate doped flexible biomaterial before and after AMP impregnation were observed using a scanning electron microscope (SEM) (Carl Zeiss 300VP, Germany) operated at 5 kV. A thin layer of gold was coated on the surface of the synthetic silicate doped flexible biomaterial by using an automatic sputter coater (Emitech K550X) to reduce the extent of sample arcing during SEM observation. For this analysis, 25*25*2 mm biomaterials were prepared and taken for the SEM analysis.

2.3.4 Micro Computed Tomography (Micro - CT) Analysis

Micro CT test was performed by using a universal testing machine having x-rays. (Scanco Medical μCT 50, Switzerland). The porosity analysis of the silicate doped synthetic flexible biomaterial was carried out according to these scanning parameters: 70 kVp, 114 μA intensity, 20.5 mm FOV/Diameter, 10 μm voxel size, 300 ms integration time, 0,5 mm Al filter. Also, this test was carried out by Ege University Central Research Test and Analysis Laboratory Application and Research Center. For this analysis, 25*25*2 mm biomaterials were prepared and the obtained pore volume has been used to calculate the volume of impregnation solutions for loading of AMPs.

2.3.5 Tensile Test

In the Izmir Katip Çelebi University Biomechanics Laboratory, a universal testing equipment with a 500 N load cell (Shimadzu AGS-X Model, Japan) was utilized for performing tensile tests. The tensile test of the silicate-doped synthetic flexible biomaterial was conducted in accordance with ASTM D638 at a crosshead speed of 50 mm/min. In this test, we have two groups which were before and after steam sterilization and product dimensions are 25*80*2 mm in order to investigate whether the steam sterilization is causing any mechanical destruction compared to flexible biomaterials that is commonly sterilized by gamma irradiation as the market product. At least three tests were conducted to ensure reproducibility.

2.4 Antibacterial Test *In vitro*

Gram-positive *Staphylococcus aureus* (S. aureus ATCC 29213) was utilized in this work. Bacteria from frozen stocks maintained at -80°C were distributed onto agar plates and incubated overnight at 37°C. After one night of incubation on tryptic soy agar (TSA), a single colony was chosen and transferred to 5 mL of tryptic soy broth (TSB). This culture solution was incubated overnight at 37 °C in the orbital shaker (180 rpm).

2.4.1 Antibacterial Activity Determination for SAAP-276

TSB and TSA were used as liquid and solid growth media, respectively. *S. aureus* ATCC 29213 were selected as model bacteria in order to investigate the antibacterial potential of the SAAP-276. Ready to use Resazurin (R&D Systems, USA) was used for Alamar Blue Assay.

The bacteria of each species was adjusted to a concentration of 10^6 CFU/mL in a 96 well-plate containing 50 µL TSB per well. Then, various concentration of SAAP-276 (1, 3, 7, 10, 14 and 17 µg/ mL) were used separately in order to treat 10^6 CFU/mL bacterial culture for the identification of minimal inhibitory concentration (MIC) of SAAP-276 as the AMP. At the same time, 50 µL each concentration of agents and pristine TSB were incubated to be used as blank. After 20 hours of incubation at 37 °C,

10 μL ready to use resazurin (R&D Systems, USA) was added to each well. After 2-4 hours incubation with resazurin, fluorescence intensity was measured at values of 540 excitations and 600 emission using ClarioStar (BMG-LABTECH, Germany) multi-mode plate reader.

2.4.2 Antibacterial Activity Determination for AMP and Silicate Doped Synthetic Flexible Biomaterial

For the investigation, stock SAAP-276 solutions at the concentration of 1 mg/mL (P1), 2 mg/mL (P2), 4 mg/mL (P4) SAAP-276 was prepared in distilled water. These concentration values were employed loaded onto flexible biomaterials via impregnation method. The concentrations were chosen to be above the MIC values of the SAAP-276 AMP. Five major experimental groups which were bare flex in TSB, bare flex in *S.aureus*, P1 flex in *S.aureus*, P2 flex in *S.aureus* and P4 flex in *S.aureus* was used. Bare flex was defined as peptide-free flex. For this test, samples with a diameter of 12.5 mm and a thickness of 1.7 mm were prepared. 90 μL of stock peptide solutions were dropped onto each flexible biomaterial and P1, P2 and P4 flexible biomaterial surfaces' have amount of peptide 0.73 $\mu g/mm^2$, 1.46 $\mu g/mm^2$ and 2.93 $\mu g/mm^2$, respectively. Each group was investigated as at least 3 parallel.

For the investigations the bacterium of each species was adjusted to a concentration of 10^5 CFU/mL in a 24 well-plate containing 500 μL TSB per well. The prepared flexible biomaterials were immersed into 10^5 CFU/mL *S.aureus* bacterial culture in TSB. After 20 hours of incubation at 37 °C the incubated flexible biomaterials was removed and the treated bacterial culture was taken for micro dilution to be spread on the agar plate and taken for colony counting in order to investigate the bactericidal activity of AMP integrated flexible biomaterials.

After the treatment the same incubated flexible biomaterials were washed 3 times with phosphate buffered saline (PBS) and incubated again for 20 hours in fresh TSB in order to investigate the bacteriostatic property of the flexible biomaterial and survival of the adhered bacterial cells on the incubated flexible biomaterials with the bacterial culture in the previous investigation Subsequently, 100 μL of TSB of from each set of sample was taken and transferred to 96 well plate, 10 μL ready to use resazurin (R&D Systems, USA) was added to each well in order to investigate the survival of

the adhered bacterial cells and bacteriostatic activity of SAAP-276 loaded the flexible biomaterials. After 2-4 hours incubation with resazurin, fluorescence intensity was measured at values of 540 excitations and 600 emissions using ClarioStar (BMG-LABTECH, Germany) multi-mode plate reader.

2.4.3 Inhibiting the Formation of *S. aureus* Biofilm on AMP Loaded and Silicate Doped Synthetic Flexible Biomaterial

In the stage of biofilm development, bacteria were cultured aerobically at 37 °C and 180 rpm for 16-18 hours. Using a UV-VIS spectrophotometer with 600 nm wavelength optical density (O.D.), the concentration of a bacterial solution was determined. The bacterial culture solution was diluted in TSB to an optical density (OD) of 0.6, which corresponds to 10^8 colony forming units per milliliter (CFU/mL). As the initial bacterium culture for biofilm development, 10^6 CFU/mL of this diluted bacterial culture was used. In addition, 0.2 percent (w/v) glucose was added to the culture to promote biofilm development. 500 μL of the prepared bacterial solution was put to each well 24 well plate in which the flexible biomaterials' measurements are diameter of 12.5 mm and 1.7 mm thickness were placed at flat bottom. The well plate was placed in an orbital shaker at 37°C and 180 rpm for 18 to 20 hours.

In this study, the prevention *S. aureus* bacterial biofilm formation on antimicrobial peptide and silicate doped synthetic flexible biomaterial was investigated. For this purpose, *S. aureus* biofilms were formed on 4 mg/mL (BF-4 Flex) concentration with flexible biomaterial. The plates which were combined with biofilm and AMP and silicate doped synthetic flexible biomaterials were incubated for 24 hours. After incubation, the biofilms were washed with PBS for elimination of loosely attached bacteria and resazurin assay was employed by adding 10 percent Alamar Blue solution into the total volume of PBS and, fluorescence intensity was measured at values of 540 excitations and 600 emissions using ClarioStar (BMG-LABTECH, Germany) multi-mode plate reader. After resazurin assay, the crystal violet assay was performed. For this purpose, the wells were washed and dried and 200 μL crystal violet solution (2.3% w/v) incubated room temperature for 5 min. The stain was removed and washed twice with distilled water. The biofilms were diluted in 200 μL of 96% ethanol and plates were incubated at room temperature for 1 h [71,72] and the 100 μL from each samples

were taken for spectroscopy investigations The absorbance at 595 nm was measured to evaluate biofilm mass and viability with respect to untreated biofilm control.

Chapter 3

3. Result and Discussion

3.1 Characterization Methods of Antibacterial Peptide and Silicate Doped Synthetic Flexible Biomaterial

3.1.1. X Ray Diffractometer (XRD) Analysis

The XRD plot of the synthesized β-TCP powders is shown in figure 15. When the XRD data of the produced product were examined, it was seen that the maximum peak points were 27.77(214), 31.03(2010), 34.37(220), respectively. When this result is compared with the previous study (Demirci et al.), no difference was observed. In this study, a comparison was made with the JCPDS (Joint Committee on Powder Diffraction Standards) database and it was found that it matched the JCPDS 090169 card number. In addition, it was compared with the β-TCP-based commercially produced product in the study and the XRD graphics were found to be compatible.

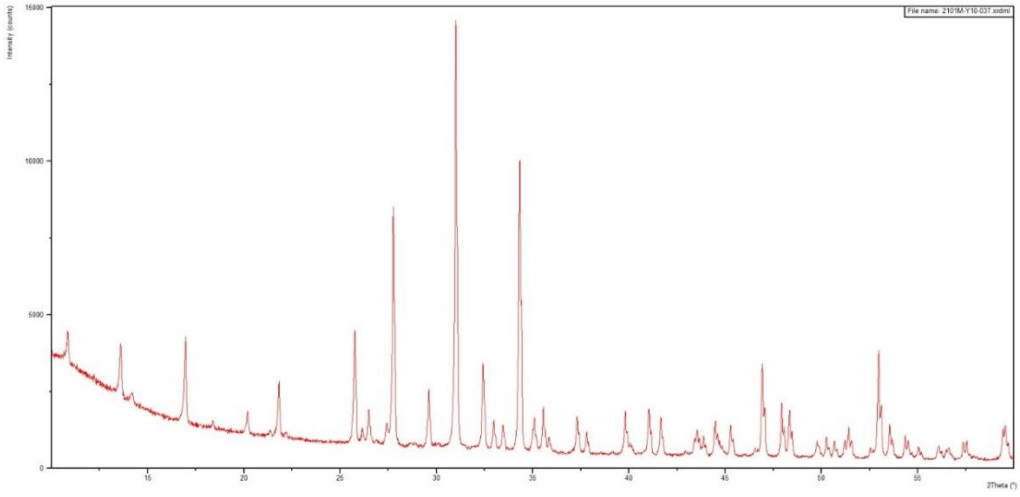

Figure 15: XRD plot for β-TCP powders

3.1.2. Dynamic Light Scattering (DLS) Analysis

The particle size of the SiO_2 was performed in order to investigate the particle size distribution of the sample. In this analysis, 0.025 mg/mL SiO_2 solution was prepared in DIW. The hydrodynamic size of the dispersed SiO_2 from DLS analysis was determined as 434,3 nm ± 47.4 with 23,9 polydispersity indexes (PDI).

Table 3: Hydrodynamic size distribution for SiO_2

Sample	**Measurements Number**	**Polydispersity Index (PDI)**	**Hydrodynamic Radius (nm)**	**Mean Value / Standart Deviation**
SiO_2 (Sigma Aldrich)	1	% 21,46	408,2	434,3 nm ± 47,4 nm
	2	% 24,85	405,7	
	3	% 25,24	489,0	%23,9 ±2,1

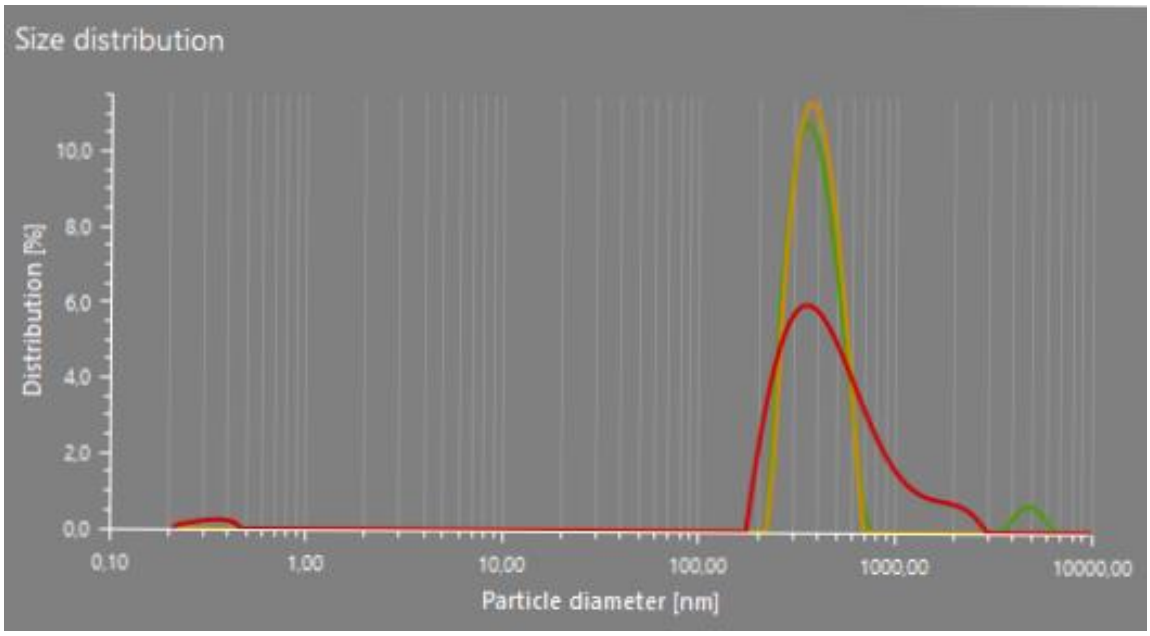

Figure 16: Size distribution plot for SiO_2 powders

3.1.3. Scanning Electron Microscopy (SEM) Analysis

Since biomaterials aim to promote cell adhesion and proliferation, the surface properties of biomaterials are crucial [73]. For instance, the literature describes the sensitivity of osteoblasts to surface roughness [74].

As can be seen from the Figure 17, it has been observed that the obtained products have a porous pore structure. SEM observations showed that the homogeneity and pore size of silicate doped synthetic flexible biomaterial were also similar. The observed porous structure from the figure 17, supports cell adhesion, proliferation, differentiation, and cell-cell interaction. In addition to biocompatibility and biodegradability, a biomaterial must possess a porous structure, high porosity, and surface activity in order to be of high quality and efficacy. The randomized and homogeneous distribution of the porous structure created provides a loaded antimicrobial peptide must be able to inhibition of bacteria growth and prevent biofilm formation in order to be used for aimed [75].

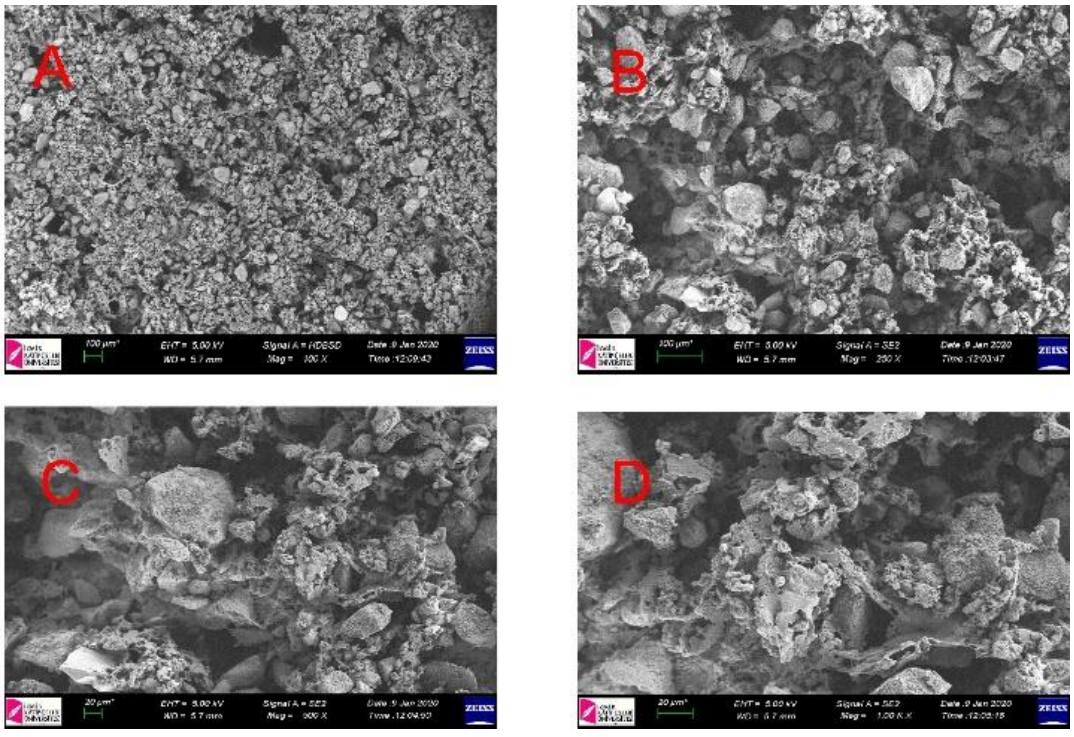

Figure 17: SEM image for flexible biomaterial (A: Magnification 100X, B: Magnification 250X, C: Magnification 500X, D: Magnification 1.0KX)

Figure 18 also includes an image of a peptide-loaded flexible biomaterial. Compared to Figure 17, it is seen that the peptides penetrate into the pore structure of the flexible biomaterial.

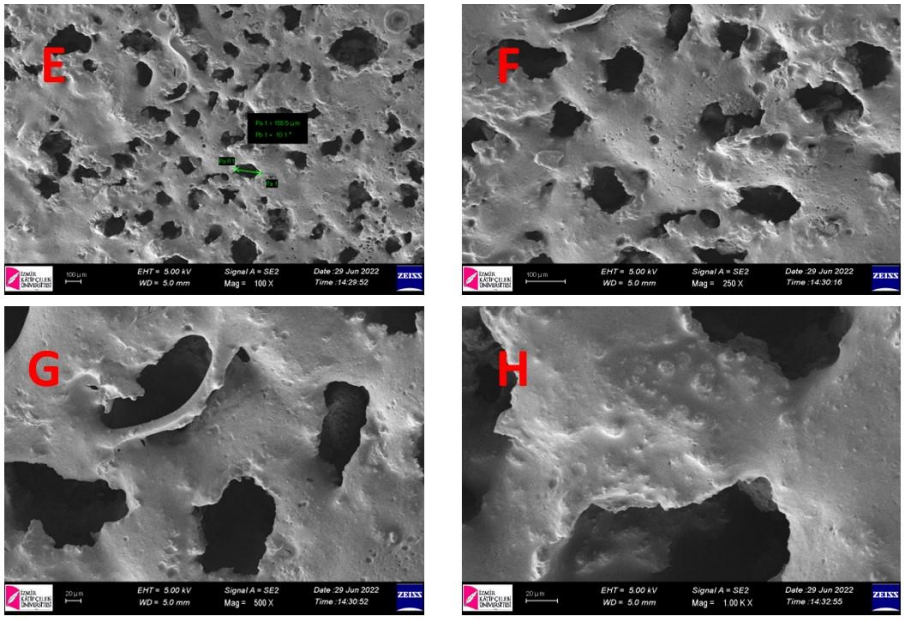

Figure 18: SEM image for peptide impregnated flexible biomaterial (E: Magnification 100X, F: Magnification 250X, G: Magnification 500X, H: Magnification 1.0KX)

3.1.4. Micro Computed Tomography (Micro – CT) Analysis

According to Micro CT test report GIY_MCT_07.08.20_1, our flexible biomaterial has a porosity rate of % 46.23 and mean pore size 76,6 µm. These results used for impregnation of the SAAP-276 as the solution at the exact volume of the pores in the flexible biomaterials. .

3.1.5. Tensile Test

In the AMPs loading technique, the targeted flexible biomaterial application should be biocompatible in a way that does not cause an inflammatory effect in the implanted area, should have decomposition properties suitable for new tissue formation, and should be suitable for mechanical and physical properties. It is expected to have the strength to prevent collapse and act as a scaffold for surgical application and implantation [76].

According to Table 4, the mechanical test results of silicate doped synthetic flexible biomaterials are consistent with each other and are approximately 3.64 MPa. The tensile test of Sample 1,2 and 3 was applied after gamma sterilization. In addition, the mechanical test results of antimicrobial peptide and silicate doped synthetic flexible biomaterials are consistent with each other and are approximately 2,06 MPa in Table 5. The tensile test of Sample 4,5 and 6 was applied after steam sterilization. ISO 5833 standards indicate that bone graft substitutes over 2 MPa mechanical strength are functional throughout bone remodeling and regeneration process [77,78].

Table 4: Mechanical test for silicate doped synthetic flexible biomaterial

Tensile Test	**Test Results**	
	Test Standard	**Tensile Strength (MPa)**
Sample 1	ASTM D638 - ISO 5833 – ISO 13175-3	3.5 MPa
Sample 2	ASTM D638 - ISO 5833 – ISO 13175-3	3.72 MPa
Sample 3	ASTM D638 - ISO 5833 – ISO 13175-3	3.70 MPa

Table 5: Mechanical test for after steam sterilization silicate doped synthetic flexible biomaterial

Tensile Test	**Test Results**	
	Test Standard	**Tensile Strength (MPa)**
Sample 4	ASTM D638 - ISO 5833 – ISO 13175-3	2.01 MPa
Sample 5	ASTM D638 - ISO 5833 – ISO 13175-3	2.20 MPa
Sample 6	ASTM D638 - ISO 5833 – ISO 13175-3	1.98 MPa

When the Table 4 and 5 were compared, a decrease in tensile strength was observed after steam sterilization. PCL has a low melting point of 60°C and therefore weakened its mechanical properties and lowered its tensile strength [79]. However, the mechanical properties are still in the acceptable range as above 2 MPa.

Figure 19: Setup of tensile test

3.2 Antibacterial Test *In vitro*

The antibiotic resistance of bacteria that may form biofilms makes it difficult to treat infections caused by these bacteria. Persistent biofilm infections are caused by gram-positive bacteria called *S. aureus*. Consequently, it poses a serious hazard to human health. By converting into a biofilm mechanism, these bacteria become extremely resistant. The quantities necessary to eliminate bacteria growing on a biofilm-resistant mechanism also destroy the same species' planktonic growth mechanism. It is crucial to prevent biofilm development because biofilm infections are difficult to cure. Due to the insufficiency of antibiotic therapy and post-implantation biofilm development in medical devices, new therapeutic approaches have been developed. In this work, , SAAP-276 synthetic antimicrobial peptide was studied on a synthetic flexible biomaterial. It has been demonstrated that the SAAP-276 peptide is a highly potent in inhibiting the *S.aurues* bacterial cell survival and *S.aureus* biofilm formation. [80].

The action mode of SAAP-276 could be depicted as due to the interaction of negatively charged phospholipids line the surfaces of bacterial membranes and positively (+) charged AMPs adhere to the phospholipid surface of the cell membrane and impair its structural integrity. This destroys the membrane's selective permeability and disrupts the equilibrium between the cell's internal and exterior environments [81]. The obtained results show that , SAAP-276 can immediately manifest its effects. This limits the development of resistance in bacterial cells, as they are able to respond swiftly. Considering the development of antibiotic resistance, the significance of this AMP characteristic increases [81].

In this study, bacterial medium was created in 24-well plates for planktonic study and silica doped flexible biomaterial with different concentrations of AMP was incubated for 24 hours. In addition, biofilm was formed in 24-well -well plates, and AMP impregnated silicate-doped flexible biomaterial were incubated for 24 hours. Resasurin and crystal violet assays were employed Thus, the bactericidal effects of AMP and silicate added flexible biomaterial at different concentrations were determined.

3.2.1 Antibacterial Activity Determination for SAAP-276

Antibacterial activity of SAAP -276 was assessed against *S. aureus ATCC 29213* via Alamar Blue Assay. The results of this assessment was shown in Figure 20 and 21 in terms of cell viability. The effect of various concentration of SAAP-276 antimicrobial peptide (1, 3, 7, 10, 14 and 17 µg/ mL) applications with planktonic *S.aureus* was investigated. In figure 20, Alamar Blue's color changed pink to blue obtained at 17 µg/ mL which is assigned for the minimum inhibitory concentration (MIC) of SAAP-276 against *S.aureus* bacterial culture. According to figure 21, a decrease in cell viability was observed with the spectroscopy investigations with the increased the SAAP-276 peptide concentration compared to the control group. Further antibacterial investigations were performed for the silica doped flexible biomaterials that possess SAAP-276 AMP content above the obtained MIC value.

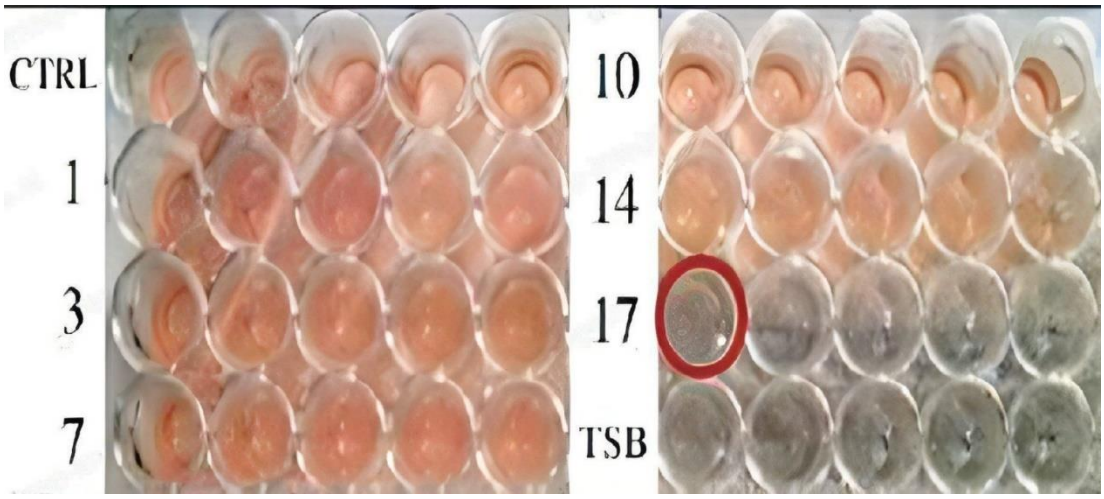

Figure 20: MIC point for SAAP - 276 on *S.aureus*

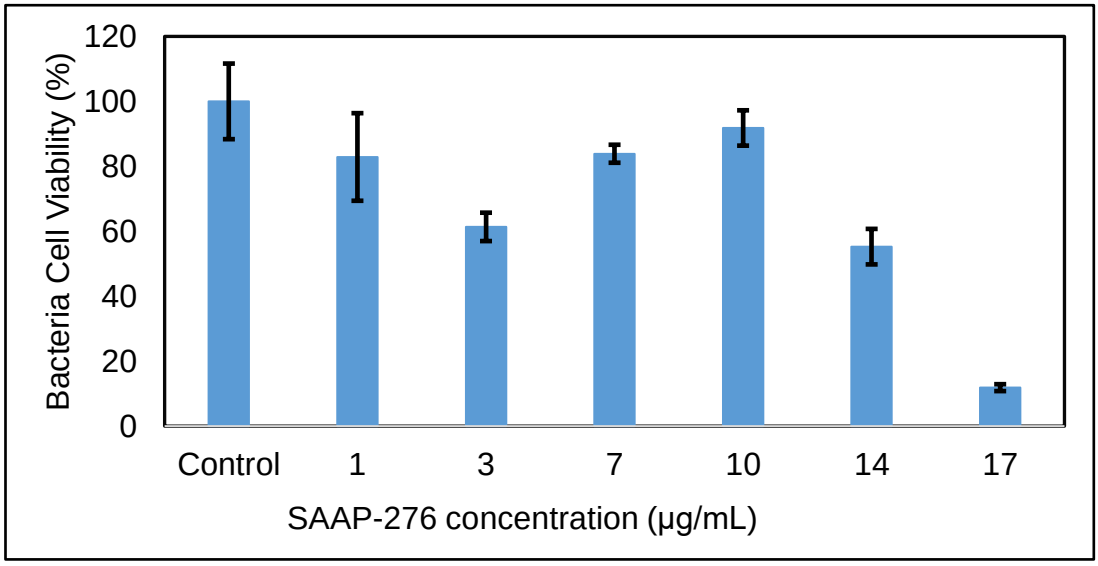

Figure 21: The effect of various concentration of SAAP - 276 Peptide on the *S. aureus*

3.2.2 Antibacterial Activity Determination for AMP and Silicate Doped Synthetic Flexible Biomaterial on *S. aureus*

Antibacterial activity of silicate doped synthetic flexible biomaterial was assessed against *S. aureus ATCC 29213* via colony counting assay. The results of the assessment was shown in Figure 22. The colony counting assay results revealed that the increasing amount of peptides on biomaterials could significantly reduce the viability of *S.aureus* bacterial cell colonies. c. As shown in figure 22, each peptide

groups which are P1 Flex, P2 Flex and P4 Flex was reducing bacterial cell colony by 5 log, 7 log and 9 log *S. aureus ATCC 29213* compared the bare flex respectively. In this results, AMP and silicate doped synthetic flexible biomaterial was efficient enough to provide a bactericidal effect [82].

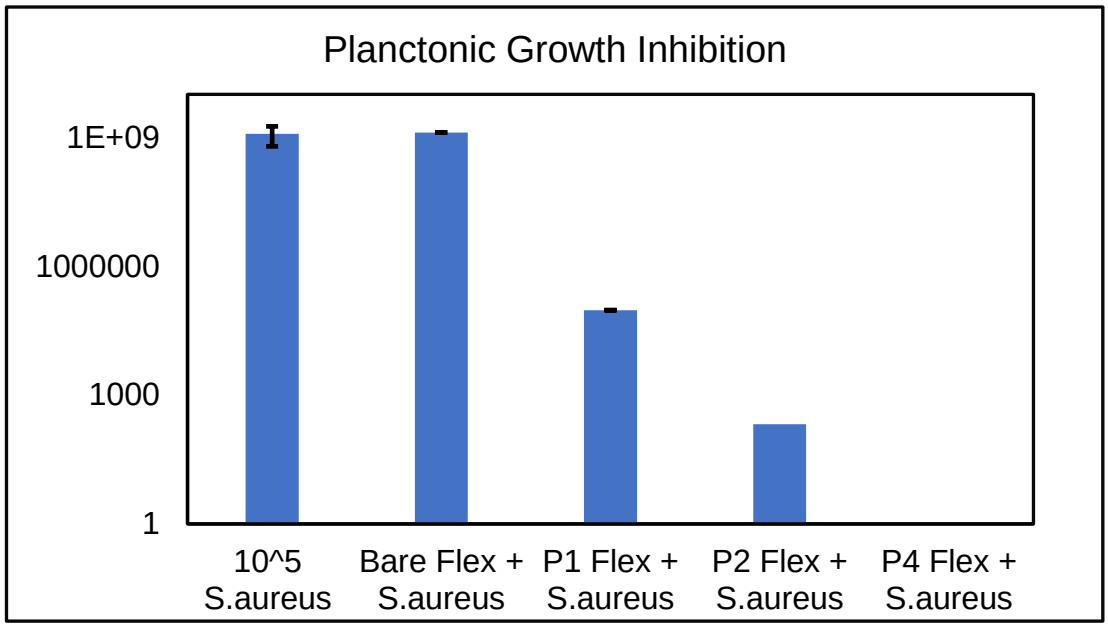

Figure 22: The effect of planktonic growth inhibition AMP and silicate doped synthetic flexible biomaterial on the *S. aureus*

The impact of AMP impregnated silicate doped synthetic flexible biomaterial on preventing the bacterial cell adhesion and eliminating the survival of adhered bacterial cells during the 24h incubation have been presented in Figure 23 and the successful inhibition of bacterial cell adhesion and bacterial cell survival of any adhered bacteria was observed. The bacterial cell viability was observed almost 95% less compared to bare flex. Consequently, Alamar Blue assay established *S. aureus ATCC 29213* is the most susceptible to AMP impregnated and silicate doped synthetic flexible biomaterial and SAAP-276 can prevent the adherence and survival of bacterial cells.

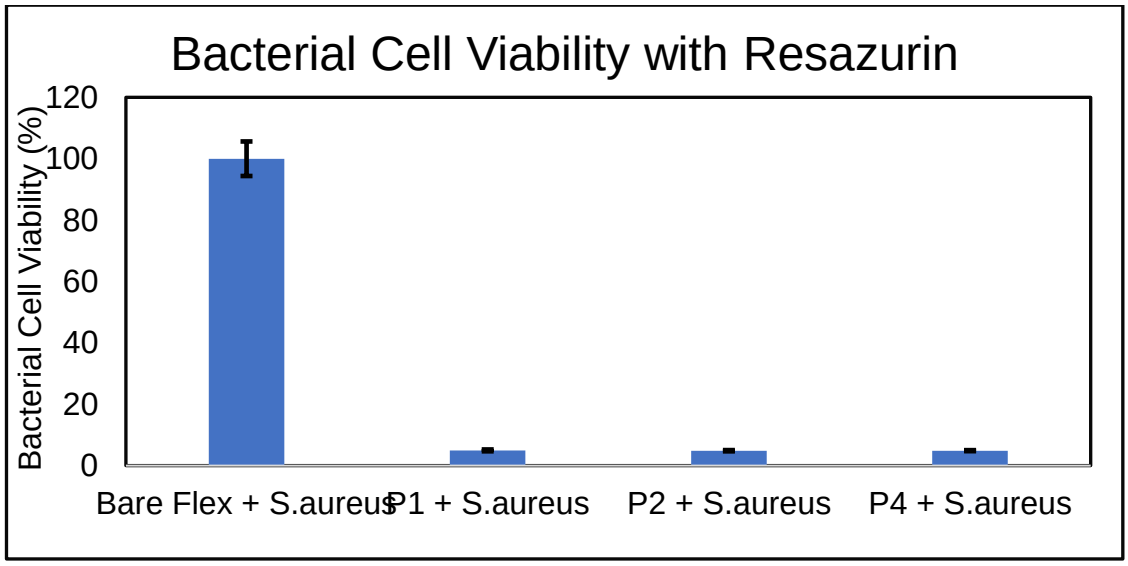

Figure 23: The bacterial cell viability of adhered bacteria on AMP impregnated and silicate doped synthetic flexible biomaterial on the *S. aureus*

3.2.3 Inhibition of *S.aureus* Biofilm Formation by AMP Loaded and Silicate Doped Synthetic Flexible Biomaterial

The resazurin assay (for bacterial cell metabolic activity) and the crystal violet (for biofilm formation inhibition) was used to test biomaterials capacity to inhibit *S. aureus* biofilm formation. Crystal violet was employed due to its known ability to bind to negative charges and therefore target many different molecules of bacteria and EPS. The results of this assessment were shown in Figure 24 and Figure 25 in terms of cell viability and inhibiting the biomass of biofilm. In this study, control group was biofilm formed on bare flexible biomaterials and treatment group was biofilm formed on flexible with the loaded AMP of equivalent to P4 flex biomaterial as given in the above section and named as BF-4 Flex. In figure 24, compared to the biofilm formed on bare flex BP-4 Flex was inhibited cell viability ~% 52 *S. aureus ATCC 29213.*

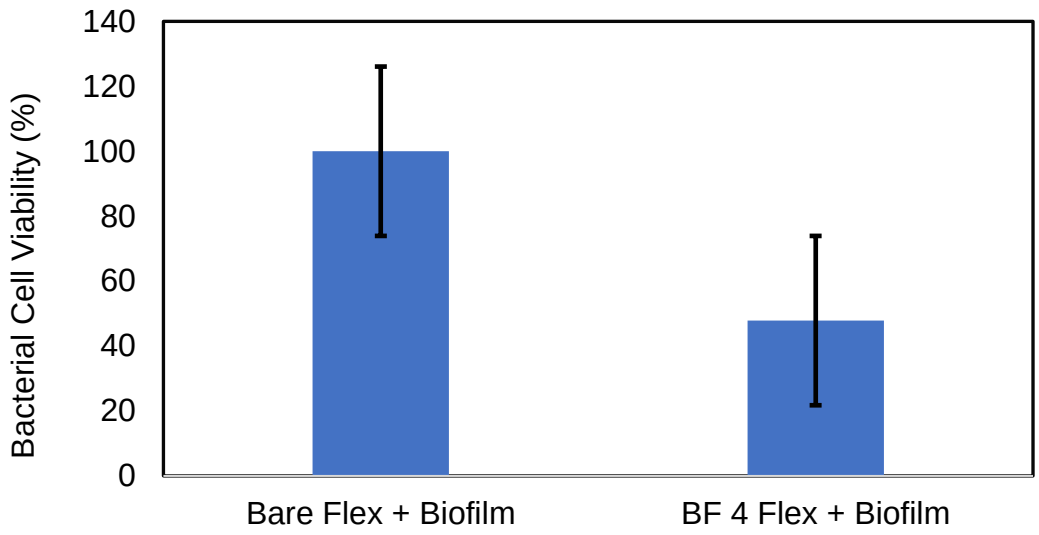

Figure 24: The bacterial cell viability of formed *S. aureus* biofilm on AMP loaded and silicate doped synthetic flexible biomaterial

In Figure 25, BF-4 Flex inhibited biomass of the biofilm ~% 37 *S. aureus ATCC* 29213 compared to bare flex. As the shown in the figures above the highest amount of AMP loaded silicate doped flex biomaterial (P4 or BF-4 Flex) could provide both bactericidal and antibiofilm activity by reducing both the *S. aureus* bacterial cell viability in planktonic form and having a high potential to preventing biofilm formation on the flex material comparing to bare flex. The dominant bactericidal activity of AMP loaded and silicate doped flexible biomaterials compared to antibiofilm activity could be due to the release of peptide form the matrix in time and also the more nutritious environment of biofilm forming conditions that might be overcome the antibiofilm activity of AMP. Even though the viability of the bacterial cell in the formed biofilm is reduced almost 52 % and the biomass was reduced only 37 % this could be related with the potential activity of AMP penetration through the bacterial cell membrane however this might promote the biomass production due to the micro environment stress of the incubation conditions.

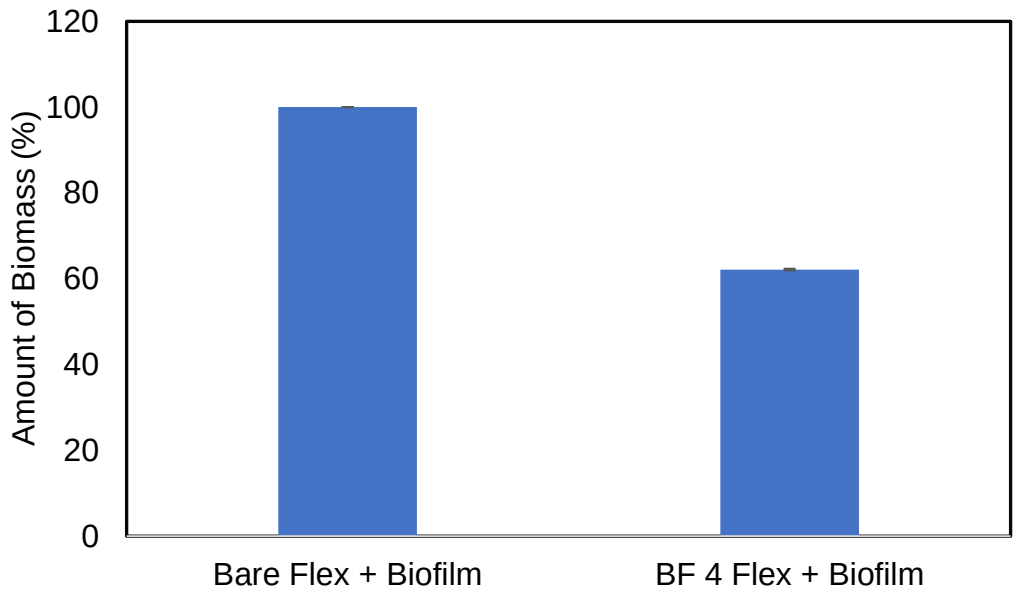

Figure 25: The effect of biomass and EPS with AMP and silicate doped synthetic flexible biomaterial on the *S. aureus* biofilm

Chapter 4

4. Conclusion

In this study, AMP loaded and silicate doped synthetic flexible biomaterials are successfully produced. The characterization of AMP and silicate doped synthetic flexible biomaterials was performed. The obtained results showed that the mechanical properties of the flexible biomaterials do not change significantly compared to commercially availed Powerbone flexible biomaterial product. The antibacterial and antibiofilm activity of AMP loaded and silicate doped synthetic flexible biomaterials against *S.aureus ATCC 29213* was observed successfully. The SAAP-276 peptide utilized in this work inhibited bacterial cells in flexible biomaterials by around 95%. Also, AMP and silicate doped synthetic flexible biomaterials demonstrate inhibited the viable cell in biofilm as (57 %) and reduce the amount of biomass as 37% comparing to bare flex treated control groups. This study we can concluded that silicate-doped synthetic flexible biomaterial and SAAP-276 could be a promising approach to combat antibacterial resistance and osteomyelitis by reducing the planktonic bacterial cell viability, prevention of the bacterial cell adhesion and also inhibiting the biofilm formation. All in all, the study has paved the way for the production of bactericidal biomaterials and will shed light on clinical studies. In future aspects, our aimed which is investigating of AMP loaded flexible biomaterial on gram negative bacteria such as *E.coli, Pseudomonas putida* and studying on cytotoxicity test.

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