ISTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

FUSION PEPTIDES FOR CONTROLLING ANTIMICROBIAL ACTIVITY ON BIOMEDICAL IMPLANTS

M.Sc. Thesis by
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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

BİYOMEDİKAL İMPLANTLARDA ANTİMİKROBİYAL AKTİVİTEYİ KONTROL ETMEYE YÖNELİK FÜZYON PEPTİTLER

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FOREWORD

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ABBREVIATIONS

AMP : Antimicrobial Peptide
BHIB : Brain Heart Infusion Broth
CFU : Colony Forming Unit
FESEM : Field Emission Scanning Electron Microscopy
FITC : Fluorescein 5(6)-isothiocyanate
FTIR : Fourier Transform Infrared Spectroscopy
GEPI : Genetically Engineered Polypeptides for Inorganics
HA : Hydroxyapatite
HABP1 : Hydroxyapatite Bindding Peptide 1
LB- broth : Luria Bertani broth
OD : Optical Density
PBS : Phosphate Buffer Saline
PC Buffer : Potassium Phosphate-Sodium Carbonate Buffer
QCM : Quartz Crystal Microbalance
XRD : X-Ray Diffraction

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LIST OF SYMBOLS

A : Alanine
C : Cysteine
D, Asp : Aspartic Acid
dH_2O : Distilled water
E : Glutamic Acid
F : Phenylalanine
G, Gly : Glycine
H : Histidine
I : Isoleucine
K : Lysine
L : Leucine
M : Methionine
N : Apsparagine
P : Proline
R, Arg : Arginine
S : Serine
T : Threonine
Q : Glutamine
V : Valine
W : Tryptophan
Y : Tyrosine

FUSION PEPTIDES FOR CONTROLLING ANTIMICROBIAL ACTIVITY ON BIOMEDICAL IMPLANTS

SUMMARY

Medical devices such as surgical implants have the potential to become infected with bacteria, leading to many medical problems including degeneration or rejection of the implant. The current treatment of infections is largely dependent on antibiotic therapy; however, traditional antibiotics are facing the increasing challenge of resistant bacterial mutants. A strong need is therefore present to develop effective anti-infectious implants as well as new antimicrobial drugs

The family of antimicrobial peptide (AMP) is one of the promising candidates for infection prophylaxis and treatment. Many of them behave broad-spectrum activity towards Gram-positive and Gram-negative bacteria, viruses, fungi and some parasites. Because of their complex killing mechanisms, the possibility for AMPs to encounter a resistant bacterial strain is much lower than the conventional antibiotics.

Modification of the implant surface with an antimicrobial agent is a potential routine to eliminate infections. Various techniques of immobilizing a biomolecule onto the metal surface have been reported; among them the affinity binding method in which the use of affinity tags to create fusion proteins that can bind to the desired surface has received special attention. In this field inorganic binding pepides offer wide range alternatives as a cross linker between biomolecule and metal suface.

The goal of this research is to investigate if the selected AMP from the literature remain antimicrobial in the case of conjugation with HABP1 and if it is possible to develop antimicrobial implants for further steps of the research.

Genetically engineered hydroxyapatite binding peptide (HABP) and antimicrobial peptide (AMP) that was selected from literature was synthesized conjugately for self immobilization on the model implant material, hydroxyapatite coated titanium surface. The fusion peptide and the AMP-modified titanium was further tested for their antibacterial activity against E.coli and S.mutans strains. According to results, the AMP-HABP1 fusion peptide has remarkable antimicrobial activity against both gram negative and positive bacteria and specific binding affinity to hydroxyapatite surfaces. When bound to hydroxyapatite coated titanium, it can be able to kill bacteria that interact with surface and prevent bacterial adhesion.

BİYOMEDİKAL İMPLANTLARDA ANTİMİKROBİYAL AKTİVİTEYİ KONTROL ETMEYE YÖNELİK FÜZYON PEPTİTLER

ÖZET

Biyoimplant malzemelerinin bakteriler tarafından enfekte edilme riski bulunmaktadır ve bu enfeksiyonlar implantın dejenerasyonu veya vücut tarafından reddedilmesi gibi problemlere neden olabilir. Günümüzde bu tür enfeksiyonların tedavisi büyük ölçüde antibiyotik temellidir; ancak uzun zamandan bu yana kullanılan geleneksel antibiyotikler bakteri mutantlarının geliştirdiği dirençle yüzleşmek zorunda kalmıştır. Bu nedenle yeni antibiyotiklerin ve ayni zamanda etkin antimikrobiyal implantlarin geliştirilmesine şiddetle ihtiyaç vardır.

Antimikrobiyal peptidler enfeksiyonlar karsı savunma ve tedavi için umut verici adaylardan birisidir. Birçoğu gram pozitif ve negatif bakteriler, virusler, fungi ve bazı parazitleri de içine alan geniş bir aktiviteye sahiptir. Kompleks etki mekanizmalarından dolayı antimikrobiyal peptidlerin bakteriyel dirençle karşılaşma olasılıkları klasik antibiyotiklere göre daha düşüktür.

Günümüzde implant yüzeyinin antimikrobiyal maddelerle kaplanması enfeksiyon riskine karşı koymada rutin bir işlem olarak önümüze çıkmaktadır. Biyomoleküllerin metal yüzeylere immobilizasyonu için birçok teknik rapor edilmiştir. Bunlar içerisinde istenen yüzeye bağlanabilen füzyon proteinler oluşturmak için "afinite tag"lerin kullanıldığı afinite ile bağlama yöntemi özellikle dikkat çekmiştir.Bu alanda inorganik yüzeylere afinitesi olan peptidler biyomolekül ve metal yüzey arasında çapraz bağlayıcı olarak geniş bir alternatif listesi sunmaktadır.

Bu çalışmanın amacı literatürden örnek olarak seçilen antimikrobiyal peptidin, hidroksiapatite spesifik bağlanan peptidle konjugasyonu ve ardından implant yüzeyine immobilizasyonundan sonra antimikrobiyal aktivitesini koruyup korumadığını test etmek ve antimikrobiyal implant geliştirme çalışmalarında potansiyel bir aday olup olmadığını göstermektir.

Genetik olarak dizayn edilmiş hidroksiapatite spesifik bağlanan peptid (HABP) ve literatürden seçilmiş bir katyonik antimikrobiyal peptid model implat materyaline kendiliğinden immobilizasyon için konjuge olarak sentezlenmiştir. Daha sonra antimikrobiyal peptid ile kaplanmış titanyumun seçilen E.coli ve S.mutans suşlarına karşı antimikrobiyal aktivitesi test edilmiştir. Sonuçlar, AMP-HABP1 füzyon peptidin gram negatif ve gram pozitif bakterilere karşı kayda değer antimikrobiyal aktivitesi ve hidroksiapatit yüzeylere özel ilgisi olduğunu göstermektedir. Hidrokasiapatit kaplı titanyuma bağlı durumda yüzeyle etkileşen bakterileri öldürebilmekte ve bakteri adhesyonunu engellemektedir.

1. INTRODUCTION AND BACKROUND

1.1 Implant Materials

The use of implant materials and medical devices is an increasingly common and often life-saving procedure[1]. The past half century has seen explosive growth in the use of medical implants. Orthopedic, cardiac, oral, maxillofacial and plastic surgeons are only examples of medical specialists treating millions of patients each year by implanting devices as diverse as pace makers, artificial hip joints, breast implants, to dental implants and implantable hearing aids[1]

Implant materials must be designed to minimise the adverse reactions associated with introducing a foreign material to the body. The immune system will typically attack anything which has originated outside the body, leading to inflammation. It is therefore crucial to choose materials that will have a minimal negative impact on the body.

Regardless of their composition or application, materials used for body repair must meet both biofunctionality and biocompatibility. Biofunctionality concerns the ability of the implant to perform the purpose for which it was designated. These requirements are: (i) mechanical properties such as tensile strength, fracture toughness, elongation at fracture, fatigue strength, Young's modulus; (ii) physical properties such as density in case of orthopedic implants, or thermal expansion in the case of bone cement; and (iii) surface chemistry such as degradation resistance, oxidation, corrosion, or bone bonding ability [2]. Biocompatibility is defined as the ability of a material to perform with an appropriate host response in a specific application [3].

Various types of synthetic substitutes have been developed in order to comply with biofunctionality and biocompatibility. They belong to the following main material classes:

(i) Metals such as titanium, titanium alloys, stainless steel, cobalt–chromium alloys.

(ii) Ceramics such as aluminum oxide, carbon, calcium phosphates, glass–ceramics.

(iii) Polymers such as silicon, poly(methyl methacrylate), poly lactide, poly (urethane), ultra high molecular weight poly ethylene.

(iv) Composites such as ceramic coating on metal implants, or ceramic-reinforced polymers[1].

Titanium is a standard material for medical devices such as hip joints, bone screws, knee joints, bone slides, dental implants, surgical devices, pacemaker cases and centrifuges due to its total resistance to attack by body fluids, high specific strength and low elastic modulus [1,4]. Commercially pure Ti-alloy is widely used in orthopedic and dental implants because of favorable mechanical properties, corrosion resistance[5]. In addition, the body readily accepts titanium since it is more biocompatible than stainless steel or cobalt chrome. However, Ti and its alloy are non-bioactive and it lacks in rapid tissue integration, which results in subsequent development of interfacial fibrous tissue and finally led to the isolation of the implants. Therefore, there is significant interest in the development of technologies that modify Ti surface for improving the interaction between bone cells and metal, a process called osseointegration[5] in order to improve patient outcomes.

Figure 1.1: Titanium Fixer and Screw.

Hydroxyapatite (HA) coatings are applied to orthopedic and dental implants made of titanium (Ti) and its alloys in order to increase their bioactivities[6,7].HA coating on titanium can improve the bonding between the implant and host tissue, leading to uniform bone growth at the implant/bone interface.

Hydroxyapatite is a naturally occurring mineral form of calcium apatite with the formula $Ca_5(PO_4)_3(OH)$, that is similar in composition to the mineral element in human bones. The enamel on teeth is largely composed of a form of this mineral. In nature, hydroxyapatite can appear to have brown, yellow, or green colorations. In its powder form, it is typically white.

Figure 1.2: Hydroxylapatite Crystals**.**

This mineral is often used not only for titanium but also other medical implants. It is bioactive, meaning that it can integrate into bone structures and support growth without breaking down. Initially, the mineral was used mostly for dental implants. Although it is still used for this purpose today, it is also used for other purposes.

Various methods of applying HA coatings have been developed for implants, such as plasma spraying, radio- frequency magnetron sputtering, dip coating, electrochemical deposition, pulsed-laser deposition, and electrophoresis deposition.

Hydroxyapatite can also be used in instances where there are bone voids or defects. This process involves powders, blocks, or beads of the mineral being placed into or on the affected of areas of bone. Since it is bioactive, it induces the bone to grow and correct the problem. This process can be an alternative to bone grafts. It typically results in healing times that are shorter than they would be if hydroxyapatite was not used.

In this study the model implant material, HA coated titanium slides. Surface coating was performed by the method of electro deposition at Material Science of Istanbul Technical University. The HA deposition and cross-sections were characterized using XRD, FTIR, Raman Spectroscopy and FE-SEM at material science at Istanbul Technical University.

1.1.1 Antibacterial Implant Materials

The rapid progress of biomedical technology and an aging population places increasing demands on medical implants to treat serious tissue disorders and replace organ function. In the field of orthopedic implant surgery alone, about 2 million fracture-fixation devices and 600,000 joint prostheses are implanted every year in the United States [8].

Medical devices such as surgical implants, catheters, hip replacements, and joint prostheses have the potential to become infected with bacteria, leading to many medical problems including degeneration or rejection of the implant.

This problem is compounded by the alterations in host defenses associated with the peri-implant region which result in increased susceptibility to infection [9]. Further, it is well recognized that biomaterial surfaces themselves can support the growth of microorganisms which may form biofilms. Those colonies of microbes grow on medical implants and other devices and play a key role in the multi-billion-dollar-per-year problem of antibiotic resistant infections.

Such implant-associated infections are extremely resistant to antibiotics, host defenses [10], and frequently persist until the implant is removed. The risk of infection after surgical implantation ranges from 1% and 7%, but is associated with considerable morbidity, repeated surgeries, and prolonged therapy [11].

As mentioned above, infections are currently a major barrier to the long-term use of medical devices in treating various diseases and abnormalities. While many bacteria are particularly aggressive pathogens in their own right, once bacteria colonize a surface and differentiate into complex communities or biofilms, they become especially difficult to eradicate. Biofilms are considered the leading cause of up to 400,000 cases of catheter-related, bloodstream infections each year In addition, biofilms can arise on virtually any device implanted in the body, including mechanical heart valves, contact lens, artificial hips and knees, and breast implants.

Biofilms are a differentiated, high-density population of bacteria that are embedded in an extracellular polysaccharide matrix that protects the cells from stressful conditions such as desiccation and nutrient limitation. Biofilm formation is a two-step process that requires the adhesion of bacteria to a surface followed by bacteria-bacteria adhesion, forming multiple layers of the bacteria [12]. Once a biofilm has

formed, it can be very difficult to treat clinically because the bacteria on the interior of the biofilm are protected from phagocytosis and antibiotics. Biofilms represent a particular challenge for antibiotic therapy. Cells within a biofilm can be up to 1000 times more resistant to antibiotics than planktonic forms [13]. For these reasons generally the affected device require to be removed surgically.

Common causes of implant-associated infections are *S. aureus* and *S. epidermidis*

[12,14]. *S. aureus* is a common cause of metal- biomaterial, bone-joint, and soft-tissue infections [12,15], while *S. epidermidis* is more common with polymer associated implant infections [16]. *Staphylococci* are Gram-positive, nonmotile, nonspore forming facultative anaerobes that grow by aerobic respiration or fermentation, with diameters of 0.5−1.5 μm. They are characterized by individual cocci, which divide in more than one plane to form grape-like clusters. *Staphylococci* are often found among the normal flora of the human skin and mucous membranes.

The *staphylococci* cell wall is composed of peptidoglycan and teichoic acids [12], and attached to which are adhesins and exotoxins. Many *staphylococci* strains, particularly *S. epidermidis* and some *S. aureus strains*, produce a biofilm[12].

Another one, *S. mutans* is a major cariogenic bacterium in the multispecies bacterial biofilm commonly known as dental plaque [17]. It is a Gram-positive, facultatively anaerobic bacterium commonly found in the human oral cavity and is a significant contributor to tooth decay. *S. mutans* produces copious amounts of extracellular polysaccharide, a key component of plaque and metabolism of sucrose rapidly produces an organic acid which demineralizes tooth enamel[18,19,20]. The *S. mutans* bacteria are facultative, that is, it can live with or without oxygen. When the cells at the bottom of the plaque run out of oxygen, they switch from aerobic respiration to the fermentation of fructose, producing lactic acid which eventually breaks down teeth and causes cavities. These properties make these bacteria responsible for the infections related to dental implants.

Prevention of such infections remains a priority [21].A new strategy for preventing implant-associated infections involves coating the implants with a polymer that contains common antibiotics. Such approaches are currently in clinical trials [21].However, the rising problem of infections caused by multiply antibiotic resistant bacteria, so-called superbugs, limits the value of this approach. In addition, the standard procedure for treating implant-associated infections using high doses of antibiotics over a long period of time, might exacerbate this situation by contributing to selection of antibiotic-resistant bacteria with potential life-threatening complications for patients [21]. The development of an implant coating with broad spectrum antimicrobial activity and one that has no relationship to common antibiotics would be highly advantageous.

Table 1.1: Different types of antibacterial coatings on titanium

	Type of Coating	Fabrication Method	Test Condition	Effect
Antibiotic loaded coatingss	Vancomycin-loaded calcium phosphate coatings	Immersion	In vitro	Effective bacterial inhibition up to 72 h
	Antibiotics incorporated carbonated hydroxyapatite coatings	Biomimetic coprecipitation	In vitro	Inhibit bacterial growth and the release rate of antibiotics is related to the structure of the antibiotics
	Gentamicin-loaded titania nanotubes	The nanotubes were filled via a simplified lyophilization method	In vitro	Reduce bacterial adhesion and enhance Osteoblast differentiation
	Gentamicin-loaded poly(D,L-lactide) coating		In vivo	Reduce implant-related infection
	Vancomycin-loaded sol–gel films	Dipping	In vitro	Zero-order release of vancomycin up to 2 wk
	Vancomycin-bonded surfaces	Covalent Bonding	In vitro	Reduce bacterial colonization even upon repeated challenge
on-antibiotic organic bactericide loaded coatings	Chlorhexidine incorporated hydroxyapatite coating	Surface-induced mineralization	In vitro	Large inhibition zone
	Polylactide coating on anodized surface			
	Polymer and calcium phosphate coatings with chlorhexidine	Impregnation	In vitro	Inhibit the growth and viability of bacteria tested, show cytotoxicity to fibroblasts
	Coatings with the antiseptic combination of chlorhexidine and chloroxylenol	Unclear	In vitro	Produce zones of inhibition against bacteria tested
	Silver coating	Unclear	In vivo	Show no local or systemic side-effect
	Titanium/silver hard coating	Physical vapor deposition	In vitro	Reduce bacterial number on specimen surface and show no cytotoxicity compared with HA surface

1.2 Antimicrobial Peptides

Antimicrobial peptides (AMP) are potential candidates as an alternative to traditional antibiotics. They have emerged as central components of the innate defenses of animals, insects, and plants, and peptides with activities against Gram-negative and Gram-positive bacteria, fungi, enveloped viruses, and eukaryotic parasites have been identified [22]. This group of peptides is generally short (<100 amino acid), form amphipathic structures, often cationic at physiological pH, and can be expressed either constitutively or inducibly by invading pathogens [23].

AMPs are considered to be among the first line in host defense systems, in the sense that they not only can kill microbes directly but also are widely involved in the innate immune response. Many attempts have been made to utilize AMPs as novel antibiotics, because they exhibit a broad spectrum of antimicrobial activity and do not easily induce resistance compared to conventional antibiotics [24,25], although they do eventually evoke resistance [26]. Up to now, hundreds of AMPs have been isolated from natural organisms, while even more have been synthesized in the laboratory.

In this study, AMP was selected from a study performed by Hancock and his colleagues. They created a large library of peptides, and investigated the influence of charged and hydrophobic residues on the antimicrobial activity of tethered peptides, as well as the influence of their positioning within the peptide sequence relative to the tethering surface. From 127 peptides we used one of them, tet127 (KRWWKWWRR) had approximately 90% activity against *Pseudomonas aeruginosa* while tethered on a substrate. These peptide is semi random one exhibiting potent antimicrobial activity in solution [27] and as tethered [22].

1.2.1 Discovery of Antimicrobial Peptides

Antimicrobial peptides were discovered by two independent lines of work: 1) studies on mechanisms by which mammalian phagocytic cells kill bacteria; and 2) studies on the mechanism by which organisms kill bacteria for their survival. In the late 1870s scientists were searching for an agent to kill microbes without causing unacceptable damage to the hosts. Ehrlich, who called this agent a “magic bullet”, in the search for this agent started to work on mammalian granulocytes, and noted the different

staining characteristics of these cell [28].In 1883, Metchnikov described the involvement of granulocytes in the phagocytosis of microbes [29]. Two years later,

Kanthack and Hardy discovered that the degranulation of granulocytes killed phagocytosed bacteria. In the following years Petterson found that aqueous extracts of pus from human emphysema had antimicrobial activity. Petterson and his coworkers desired to identify the compounds responsible for the antimicrobial activity. However, the techniques of the time were insufficient for further investigation of these antimicrobial agents [27]. Approximately two decades later, Fleming's discovery of first lysozyme and then penicilin started a new era for the search of antimicrobial agents [29]. Ten years after the discovery of penicilin Hotchkiss and Dubos isolated tyrocidine and gramicidin antimicrobial peptides from Bacillus brevis, but only gramicidin could be used for very limited applications because of the cytotoxic activity of these antimicrobial peptides on eukaryotic cells. In following decades other antimicrobial peptides were isolated, such as mellitin from bee venom, but they too were toxic and hemolytic[30]. In 1969, Zeya and Spitznagel isolated five cationic antimicrobial proteins from rabbit polymorpho nuclear leukocytes that were not hemolytic, and found that cationic proteins permeate the bacterial cell because of their positive charge [31].In 1978, Weis and Elsbach reported the isolation of a protein, bacterial permeability inducing factor (BPI), from granule proteins of neutrophils of a chronic myelogenous leukemia patient. BPI had additional functions such as the neutralization of endotoxins besides its bactericidal activity [32]. In the early 1980s, cecropins were discovered after a decade of work [30]. Boman and his associates demonstrated that the hymolymph of silk moth pupae had no antimicrobial activity, but the introduction of bacterial debris induced potent antimicrobial activity in the hymolymph. Subsequently, they associated this activity with cecropins and some other antimicrobial peptides such as attacins and lectins. At first, it was thought that these antimicrobial peptides were unique to insects, but later they were isolated from other animals including mammals revealing that these peptides were widely distributed in the animal kingdom and provide enormous survival benefits to the host [33].Because these peptides are very potent against bacteria, but have no toxic or hemolytic effect on host cells, and have a wide taxonomic distribution, their discovery led to the start of a new era in studies of animal antimicrobial peptides [23, 34,35].

1.2.2. Classification of Antimicrobial Peptides

As was mentioned earlier, antimicrobial peptides were discovered as a result of two independent lines of work: first, studies on how mammalian phagocytic cells kill bacteria, and second, on how organisms kill bacteria. Therefore, in the past the origin of antimicrobial peptides was the basis for classification because this type of classification helped to make connections between the function of the antimicrobial peptides was the basis for classification because this type of classification helped to make connections between the function of the antimicrobial peptides originated from a similar group of animals and aspects of the living conditions of these animals. However the later discovery of a large number of peptides from many different animal species and the possession of a group of antimicrobial peptides, such as cecropins, by distantly related animal groups caused this type of classification to become futile. Today, a grouping approach based on the chemical and biochemical characteristics of peptides is preferred.

These antimicrobial peptides can be subdivided by composition and secondary structure into four major groups. One group, including cecropins [36].and magainins [37], exhibit an a-helical structure in lipid membranes. Such peptides are often unordered in solution. A second group includes those, such as the defensins, that adopt an antiparallel b-sheet structure containing one or more disulfide bonds [38].The third group comprises those peptides forming looped structures containing one or more disulfide bonds such as bactenecin [39]. The fourth group involves peptides that contain a high percentage of specific amino acids such as the proline-/arginine-rich bovine peptides, Bac5 and Bac7 [40] and the porcine peptide PR-39 [41].

1.2.3 Mechanism of Action for Antimicrobial Peptides

An essential requirement for any antimicrobial host defense or therapeutic agent is that it has a selective toxicity for the microbial target relative to the host. Ideally, such compounds have affinity for one or more microbial determinants that are easily accessible, common to a broad spectrum of microbes, and relatively immutable. Nature has apparently yielded a class of molecules that meets these constraints in the evolution of antimicrobial peptides. Antimicrobial peptides initially target microbial cells, and thus fulfill criteria outlined above for identifying molecular determinants

of pathogens that are accessible and broadly conserved. As a group, antimicrobial peptides have amphipathic features that mirror phospholipids, thus allowing them to interact with and exploit vulnerabilities inherent in essential microbial structures such as cell membranes[12].

The precise mechanism of action for antimicrobial peptides is yet to be explained. Nevertheless, studies show that prokaryotic membranes are recognized as targets by many antimicrobial peptides. Therefore, a number of models have been proposed to understand the mechanism of action of these peptides. According to one of the models, the mechanism involves the following steps: 1) electrostatic contact between a negatively charged membrane and positively charged antimicrobial peptide, 2) conformation of helical structure and insertion of the peptide into the membrane, and 3) aggregation of several helices to form a pore. It was reported that a micromolar range of antimicrobial peptides sufficient to form a monolayer around a target cell was required for the lysis of bacteria and four or more peptides are required to aggregate and form pores, 5-40 Å in diameter, large enough to kill a target cell. However, it was thought that an organism may be killed in different ways by different peptides, even if they are in the same structural class, or a peptide may operate by different mechanisms on different organisms [42].

The detailed mechanisms are often very specific for a bacterial strain or group. For example, because of the different molecular composition of their cell surface, the alteration of surface charge as a resistance mechanism is accomplished by largely unrelated molecular procedures among Gram-positive and Gram-negative bacteria.

A prominent mechanism of resistance in Gram-negative bacteria is the incorporation of positively charged aminoarabinose in lipid A, which reduces the anionic character of the cell surface and thus the attraction of cationic AMPs. In contrast, Gram-positive bacteria, which do not have lipid A, achieve the same goal by modifying teichoic acids with D-alanyl groups or by including positively charged phospholipids in the cellular membrane [43].

The initial interaction with the target surface significantly influences subsequent peptide dynamics and membrane-disrupting effects. There is widespread acceptance that the initial mechanism by which antimicrobial peptides target microbes occurs via an electrostatic interaction. The facts that electrostatic forces are active over relatively long molecular distances and that lysine and arginine interactions with

phosphate groups in lipid bilayers are particularly strong likely contributes to the initial attraction and membrane-targeting step many antimicrobial peptides.

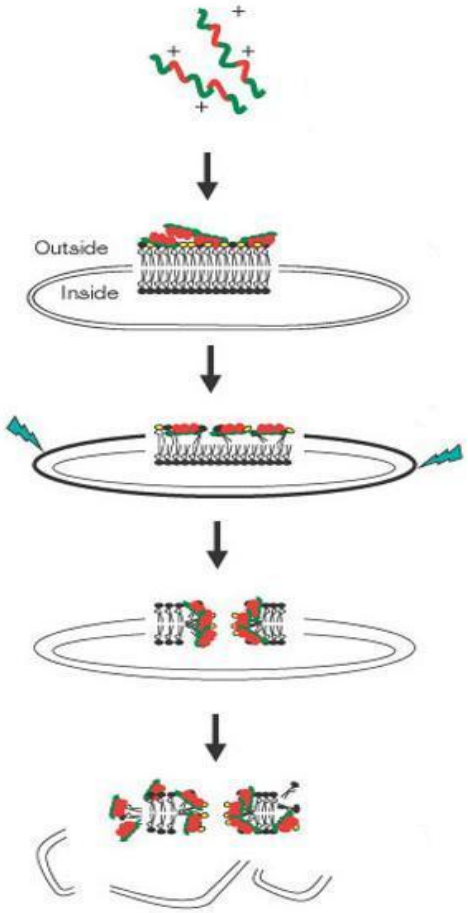

Figure 1.3: Mechanism of Action for Antimicrobial Peptides.

In the case of Gram-negative organisms, there is report suggested a mechanism of peptide interaction with membranes termed self-promoted uptake[44]. This mechanism, similar to that known for aminoglycoside antibiotics, contends that the initial action of the peptide involves a competitive displacement of LPS-associated divalent cations stabilizing the outer membrane. Such LPS displacement is likely to be energetically favorable given that the binding affinity of a typical antimicrobial peptide for LPS is ~3 orders of magnitude greater than that of divalent cations. This hypothesis is supported by studies with polymyxin-resistant pmrA strains of *S. typhimurium*. The LPS phosphate moiety in these strains is highly substituted with 4-amino-4-deoxy-L-arabinose, providing the bacteria a reduced overall negative charge and corresponding increased resistance to cationic antimicrobial peptides[12]. In comparison, Gram-positive organisms lack an outer membrane or LPS; however their cell envelopes are enriched in negatively charged teichoic and teichuronic acids.

The significance of these anionic structures with respect to cationic antimicrobial peptide activity has been demonstrated using a mutant strain of *S. aureus* in which cell wall teichoic acid modification resulted in an increased negative surface charge and was associated with an increased sensitivity to killing by positively charged antimicrobial peptides.

Antimicrobial peptides are preferentially more selective to the procaryotic cell membrane meaning that they selectively kill microorganisms without being significantly toxic to host cells. This might be because prokaryotic cell membranes are more anionic, and prokaryotic cell membranes do not have cholesterol. Studies showed that the presence of cholesterol in the artificial membranes significantly reduced the lytic activity of antimicrobial peptides. Research also demonstrated that besides the antibacterial activity antimicrobial peptides also possesses antitumor, antiviral and antiparasitical activity [45].

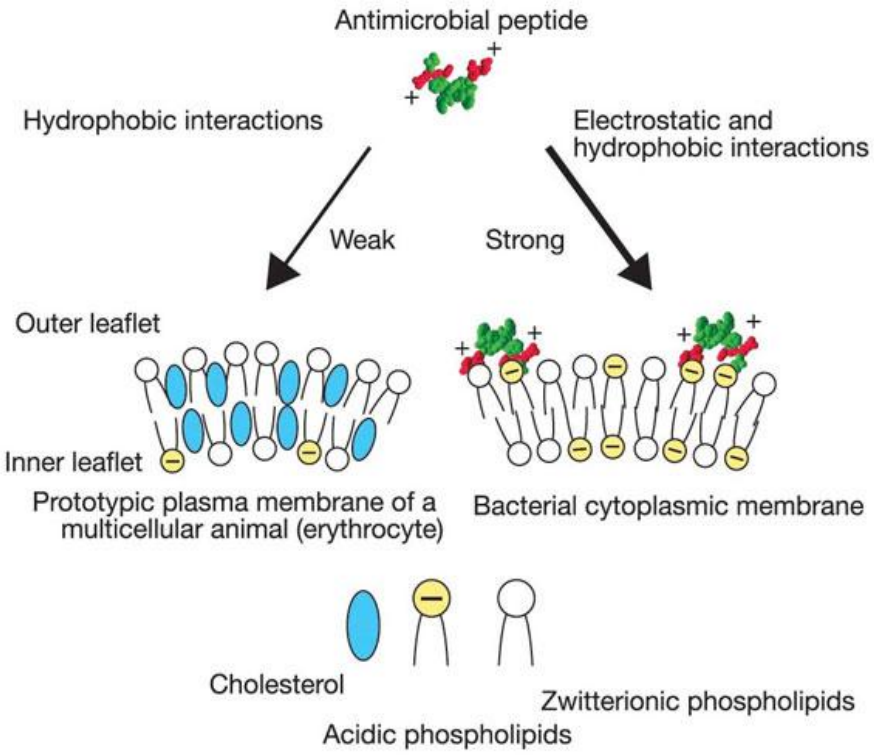

Figure 1.4: The membrane target of antimicrobial peptides of multicellular organisms and the basis of specificity [46].

1.2.4 Activity of Tethered Antimicrobial Peptide

The activity and mechanism of AMPs in solution have been studied for more than 50 years. However, limited attention has been paid to activities of AMPs that are

immobilized on a substrate. One of the earliest experiments by Haynie et al reported the antimicrobial activity of resin-tethered AMPs synthesized using a solid-phase strategy at the concentration of >1,000 μg/ml. The covalently-bonded AMPs were able to significantly reduce the number of viable cells and showed broad spectrum activity against pathogens [47].

A recent publication of Hilpert et al focused on the characterization of a group of highly active AMPs synthesized on a cellulose sheet [22]. The peptides from the most active class were found to show an inhibition rate of almost 100% against *Pseudomonas aeruginosa* (*P. aeruginosa*), even when they were restricted on the cellulose substrate. It was also observed that the activity of the tethered AMPs does not directly correspond with their analogs in free solution. Therefore, attention should be paid to the selection of AMP candidate when the peptide is delivered on a substrate. A higher surface density for most tethered AMPs was required to kill the pathogens than the non-tethered AMPs.

A most possible explanation is that immobilization results in limited mobility of the AMPs, reducing their ability to interact with or penetrate the bacterial membrane. Gabriel et al grafted a less effective AMP LL-37, the human cathelicidin, on a titanium substrate [48]. Antibacterial activity was only observed when the peptide was linked via a flexible poly(ethylene glycol) spacer, which provided improved lateral mobility over direct linking method and short linker coupling. As claimed by Bagheri et al, the most important factors affecting the activity of surface-bound peptide include the length of the spacer and the amount of target-accessible peptide [49]. However, it is speculated that a highly active peptide candidate may be able to compensate the negative parts of a rigid short linker, according to the positive results previously discussed from Hilpert et al.

1.3 Modification of Implant Material With Antimicrobial Agents

There is a number of immobilization strategies to make implant materials antimicrobial. The approaches can be divided into adsorption, covalent coupling, surface coating and affinity binding. These approaches were explained below on the model surface, titanium.

1.3.1 Adsorption

Soaking the implant directly into a solution containing biomolecules is one of the simplest ways to attach the molecules onto titanium surface. In vivo test using the simple adsorption method for alkaline phosphatase delivery showed improved bone formation with the drug-adsorbed titanium implants [50]. Upon contact with air or water, titanium surface is rapidly oxidized with a rigid TiO_2 layer, which is hydrophilic and weakly anionic at physiological pH. Proteins and other biomolecules can react with the oxide layer through van der Waals, hydrophobic or electrostatic forces. These interactions, however, are generally based on reversible phase equilibrium, and the adsorbed quantity and the subsequent release profile are largely dependent on the metal surface treatment,the soaking conditions and the external physiochemical environment.

Figure 1.5: Schematic representation of adsorption immobilization**.**

1.3.2 Surface Coating

Surface coating on titanium implant can serve as a layer of active molecules alone, or can be incorporated with entrapped drugs as a delivery method. Calcium phosphate (CaP) coating is one of the most commonly utilized inorganic coatings. The mineral coating can be deposited onto implant surfaces by plasma spray, electrolytic deposition or biomimetic dip-coating techniques [56-60]. Organic components such as collagen and chitosan are usually co-deposited into the CaP coating to provide a mechanical reinforcement [61-65]. The porous coating can be further incorporated with drugs, proteins or growth factors to achieve different purposes [66].

Collagen and other organic components can be deposited onto titanium surfaces alone, serving as a bioactive layer or a drug delivery vehicle for a controlled release. Collagen is one of the most widely investigated extracellular matrix proteins and has an important role in promoting osteoblast adhesion and differentiation as well as controlling cell progression [67]. Schliephake et al studied the bone formation around a Ti screw coated with collagen to which a cell-adhesive peptide RGD

(Arg-Gly-Asp) was linked. Animal test model with dog mandibles showed significantly improved bone contact and increased volume density of the new bones with the drug-collagen coated screws [68].

Other organic coatings are also investigated utilizing different biomolecules. An animal study on rabbit was performed by Bumgardner et al with chitosan-coated titanium pins. The implants were inserted into the tibia of the rabbits, and the pins with chitosan coatings were proved more supportive for bone formation and osteointegration [69]. Poly(D,L-lactide) and politerefate coatings are reported to be potential candidates as well for controlled slow drug release [70].

1.3.3 Covalent Coupling

Grafting biomolecules on titanium surfaces through covalent coupling provides a stable linkage, which can be retained for several days under physiological conditions [71,72]. This method is expected to retain the surface biomolecules for a longer period than the adsorption and coating delivery routines, and is receiving extensive attention from biomaterial researchers.

Covalent coupling routine starts with the functionalization of the metal surface, usually through silanization. A bifunctional linker is subsequently conjugated onto the surface and links the biomolecules to the surface functional groups. The most commonly used crosslinkers are maleimides, which reacts with the thiol moiety in the cysteine residue more rapidly than with any other groups. This maleimide-involving strategy can be used for cysteine immobilization, and more importantly, the covalent coupling of a bioactive peptide/protein that is linked with a cysteine end.

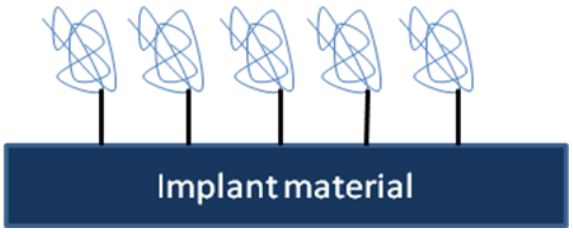

Figure 1.6: Schematic representation of covalent immobilization.

One of the applications for the covalent coupling strategy is the attachment of Arg-Gly-Asp (RGD), a cell-adhesive peptide to titanium surface for modulating the adhesion of extracellular matrix (ECM) proteins. Xiao et al used three different heterobifunctional linkers to immobilize the RGD-cysteine peptide on silanized

titanium surfaces [73]. The silanization step was found to be the key step in controlling the loading reproducibility, and the surface peptide coverage is estimated to be similar regardless of the choice of linker. Ferris et al reported significant increase in new bone thickness and greater pull-out strength in rat femurs with Au-coated titanium grafted with RGD compared with non-RGD implants [74], suggesting that this specific peptide is capable of maintaining its activity when tethered. RGD immobilized on a silicon surface through the same modification routine was also proved to enhance fibroblast adhesion and proliferation [75]. Besides cell-adhesive peptides, attempts have been made to graft antimicrobial molecules on titanium surfaces through covalent bonding as well. Vancomycin covalently bonded to titanium and Ti-6Al-4V alloy is reported to inhibit Staphylococcus aureus colony forming [76,77].

1.3.4 Affinity Binding

In affinity immobilization, biomolecule is immobilized via affinity interactions. A large number of affinity pairs such as lectin-sugar, antigen-antibody, and biotin-avidin are known. Two versions of affinity immobilization are possible. In the first, the surface is precoupled to an affinity ligand and the target biomolecule is added. In the second, the biomolecule is conjugated to another molecule that in turn has affinity toward a surface. The use of affinity tags to create fusion proteins that can bind to the desired surface expands the list further. In this version, inorganic binding peptides conjugated to the target molecule can be used as a new approach.

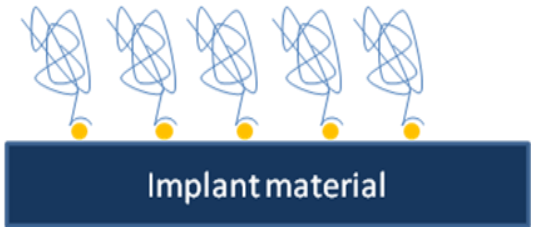

Figure 1.7: Schematic representation of affinity immobilization.

1.4 Inorganic Binding Peptides

In nature, proteins are reported to initiate, catalyze and mediate the fabrication of inorganic nano- and microstructures, which assemble into complex architectures. Therefore, a new emerging research field has been started in nanomaterials design termed molecular biomimetics and nanotechnology [78,79]. The organisms using the

organic-inorganic hybrid systems have evolved to use a part of their proteins in order to produce and bind the inorganic materials in vivo. These organisms synthesize inorganic binding proteins that bind and organize inorganic materials to highly ordered structures to perform excellent functions such as forming protective layers, supportive tissues, transferring ions and developing some optical and mechanical properties in favor of the organism.

Some recent efforts have begun to identify small polypeptides that bind with high affinity to bulk materials using combinatorial biology approaches because of the limited occurrence of naturally inorganic associated proteins, Nowadays, peptide sequences specific to metals, metal oxides and semiconductors and their potential use in material assembly and synthesis[59] have been reported.

The inorganic material commonly include magnetite (Fe_3O_4) particles in magnetotactic bacteria or teeth of chiton [80]; silica (SiO_2) as skeletons of radiolarian [81] or tiny light-gathering lenses and optical wave guides in sponges [82]; hydroxyapatite ($Ca_2C(OH)_3$) in bones [83] and dental tissues of mammals [84] calcium carbonate ($CaCO_3$) in the shells of mollusks [85].

For the selection of material-specific peptides, generally called genetically engineered peptides for inorganics, GEPI [78,79], phage [86] and cell surface display [87,88] have become the major in vivo techniques [89,90,91,87,92,93].

Current approaches for biomolecule immobilization on glass or metal substrates generally require surface functionalization by self-assembled monolayers (SAMs) of bifunctional molecules, such as aminoalkylalkoxysilanes for silica and carboxyl-terminated alkanethiols for gold substrates. Despite their widespread utility, these traditionally available bifunctional molecules have certain limitations, such as causing random orientation of the protein on solid surface and requiring multistep chemical reactions, and the assembled monolayers can be unstable during immobilization. To overcome these limitations, it is preferable to have molecules as direct linkers to the solid substrate of interest that not only have all the desired features of the conventional chemically prepared SAMs but also have specificity to a given solid substrate and assemble onto it efficiently[94].An exciting alternative to chemical coupling may be the use of combinatorially-selected inorganic-binding peptides as molecular linkers and assemblers. In principal, in addition to the specific recognition of inorganic surfaces, combinatorially-selected inorganic-binding

peptides are robust and can be genetically engineered or modified to tailor their functionalities such as synthesizing, binding, erecting and linking of inorganic nanostructures[95].

There has been a surge of research activity utilizing these genetically engineered peptides for inorganics (GEPI), which could be used for synthesis, binding, assembly, and linking of inorganic nanostructures, all under ambient conditions[94]. Recently, peptide sequences specific for platinum, quartz, cuprous oxide and hydroxyapatite, as well as many other materials and minerals, have been identified [78,79, 96-101, 90-100, 92-101]. Those peptides have been also characterized in terms of binding kinetics, affinities, and molecular structure [90, 100,100 and 101].

In terms of immobilization proseduce non-covalent methods has a drawback of leaving the biomolecule from the surface unrestrainedly and also for covalent methods there is need to modify the surface or biomolecule as disadvantage. Briefly, a novel alternative to current chemical coupling may be through the utility of combinatorial inorganic-binding peptides as specific molecular linkers. By this way, it is doable to make desired biomolecule capable of self immobilize onto inorganic surfaces selectively without any chemical treatment for surface activation.

1.5 Model Implant Material: Hydroxyapatite Coated Titanium Slides

Titanium and its alloys with various nanofunctionalized surfaces are used in dental and orthopedic applications. Surface preparation and hydroxyapatite coating is critical in bioactivation of these surfaces in order to enhance osseointegration.

Titanium implant materials are not toxic nevertheless it is necessary to activate the surface. This can be achieved by alkaline treatment. Generated titanium oxide layer increases osseointegration and biocompatibility. Tissue response to titanium implants depends to chemical and physical properties of titanium. While the cavities which are 100-150 diameters act as home for bone cells; narrower than 10μm are necessary for body fluid circulation. For these reasons the surface porosity and smoothness are critical in terms of osseointegration.

HA coating of titanium/titanumoxide surface is required bioactivation by alkali treatment. Sodium titanate and titanium hydroxide groups polymerize on alkali surface and condensed as negatively charged titanium oxide at pH 7.4 [101]. During

the cathodic circulation, those compounds interact with calcium ions and form amorphous calcium titanate (ACT).

In the course of anodic circulation, negatively charged phosphate groups form amorphous calcium phosphate (ACP – $Ca_9HPO_4(PO_4)_5OH$) by reacting with ACT and eventually, with the help of alkali surface conditions occurred during catodic coating , it form apatite crystal [99]. It is indicated that OCP and ACP are pilot of hydroxyapatite (HA) biomineralization and the surface tension of them are lower than HA in aqueous environment [99]. OCP is formed at acidic- neutral conditions, becomes instable and prefers hydroxyapatite formation.

In this study, model implant material is ordered nanotubular titanium oxide slides coated with hydroxyapatite, prepared by Prof. Dr. Mustafa Urgen's laboratory, material science and engineering department, Istanbul Technical University.

By advanced treatments on pure titanium slides, ordered titanium dioxide nanotubular surfaces with 90nm average diameters, 2.5 micrometer deep were prepared [Seçkin and Urgen unpublished data]. Ordered titanium dioxide nanotubular plates were coated with calcium phosphate using a modified simulated body fluid (SBF) solution and pulsed electrodeposition process at 80ºC, with a current density of -10mA/cm^2. Surface coating was characterized using XRD, FTIR and SEM, indicating formation of HA crystal [99].

In nature, hydroxyapatite exists only at the geologic sphere, so what generates the hydroxyapatite is its mineral forms. Hydroxyapatite which is in sclerenchyma may has reduced calcium, hydroxide and phosphate; Ca^{+2} may shuffle across with divalent and trivalent atoms; F^-, OH^- ve CO_3^{-2} may take the place of phosphate groups or HA may not be in the form as its ideal stoichiometry. It is possible to be HA in other carbonated hydroxyapatite phases such as calcium phosphate oxide (CaO), calcium phosphate hydrate, calcium hydrogen phosphate hydroxide or $Ca_{10}(PO_4)_3(CO_3)_3(OH)_2$.

Nanotubular titanium surface coating results demonstrated hydroxyapatite phases was formed. On the FE-SEM images (Fig.1.8), crystals which were in apicular morphology, 15nm diameter, 1,5 micrometer length, high level, spherical ordered dispersed uniformly.

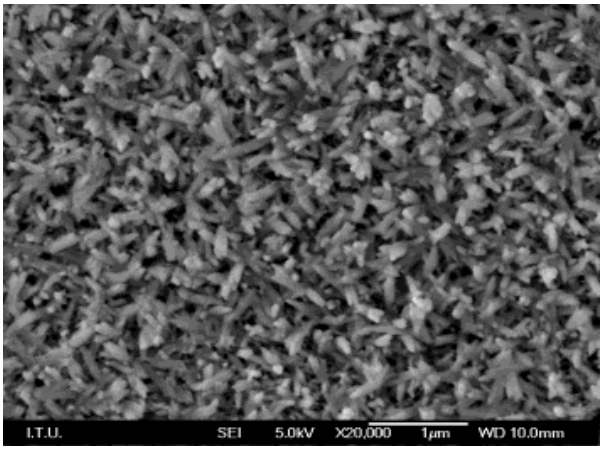

Fig. 1.8: HA deposition on nanotubular titania, characterized by FE-SEM at 80ºC[99].

Peaks that were given at XRD spectrum were compared with HA and CHA peaks given at ICSD 00–89–6437, 01– 89–7834 and 00–019-0272. 20–35θ included peaks typically similar to ones at ICSD datas (Fig 1.9). XRD analysis of the coating indicated that Ha was deposited on the nanotubular titania slides.

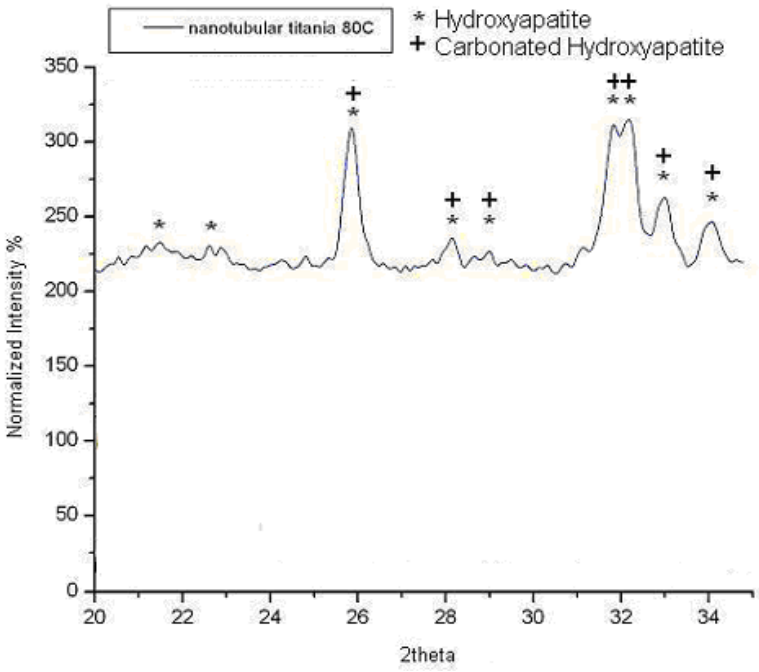

Fig. 1.9: HA deposition on nanotubular titania, characterized by XRD analysis[99].

FTIR spectrums belong to nanotubular titanium oxide had peaks of phosphate at 569, 600, 962 at 1000–1200 cm-1 and OH peaks at 632 and 3568 cm–1 (Fig 1.10).

Surface area of nanotubular titanium oxide slide is 80 times greater than flat titanium. It was reported that hydroxyapatite deposition on ordered titanium dioxide nanotubular slides can provide the alkali environment which is necessary for HA

formation since it has larger surface area that lets hydroxide ion generation and binding on surface.

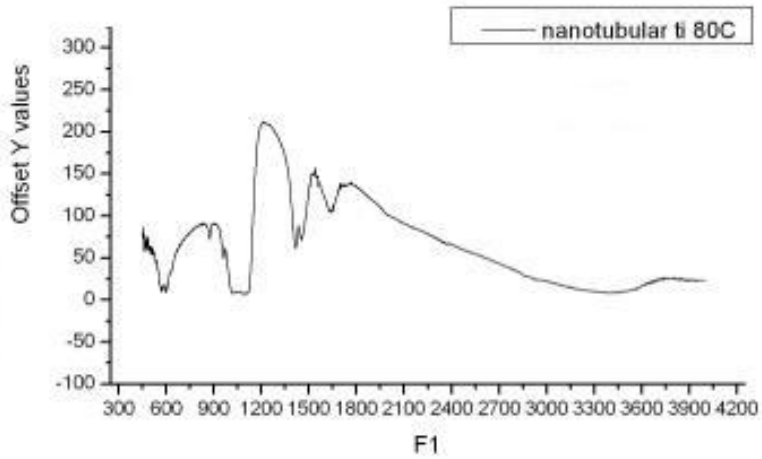

Fig 1.10: HA deposition on nanotubular titania, characterized by FT-IR spectroscopy [99].

1.6 Aim of the Study

The main objective of this study is to investigate if the selected AMP from the literature maintains its antimicrobial property in the case of conjugation with HABP1 and if it is possible to create self-immobilizing antimicrobial fusion peptide on hydroxyapatite surfaces, thus develop antimicrobial implants for further steps of the research. This includes antimicrobial activity assays of this fusion peptide in the free form, QCM experiments and anti-adhesive and antimicrobial effect of the fusion peptide tethered on the model implant material.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Bacterial Strains

2.1.1.1 E. coli ATCC 25922

E. coli ATCC 25922 was used in this study. The strain is not resistance to any antibiotics.

2.1.1.2 S.mutans

S.mutans was used in this study. The strain is not resistance to any antibiotics.

2.1.2 Model Implant Material

HA coated titanium slides (0.5x0.5cm) were prepared by cyclic electrochemical deposition at ITU Chemistry & Metallurgy Faculty Metallurgical & Materials Engineering Department by F. Şermin Utku and Eren Seçkin.

Figure 2.1: HA coated titanium slide.

2.1.3 Peptides

HABP1 is a high affinity sequence, preferentially binds to hydroxyapatite surfaces, one of the genetically engineered peptides characterized by our collaborative group [100].

The peptides were kindly provided as lyophilized form. by Hilal Yazıcı from University of Washington

HABP1 CMLPHHGAC

AMP KRWWKWWRR

AMP-HABP1 KRWWKWWRR GGG CMLPHHGAC

2.1.4 Solutions & Medias

2.1.4.1 Luria Bertani (LB) Medium

10 g tryptone (Acumedia), 5 g yeast extract (Acumedia), 5 g NaCl (Riedel-de-Haen) were dissolved in distilled water and completed up to 1 lt. The pH was adjusted to 7.0-7.5 with 10 M NaOH and sterilized for 15 min. under 1.5 atm at 121 °C. The medium was stored at room temperature.

2.1.4.2 Brain Heart Infusion Medium

37g Brain heart infusion broth (Fluka) was dissolved in distilled water and completed up to 1lt and sterilized for 15 minutes under 1.5 atm at 121°C The medium was stored at room temperature.

2.1.4.3 E.coli ATCC 25922 Overnight Culture

5 ml LB solution was inoculated with E. coli ATCC 25922 stock (from -80°C). The culture was left in the shaker overnight at 37°C, 200 rpm. The overnight culture was prepared freshly for each experiment.

2.1.4.4 S.mutans Overnight Culture

5 ml Brain Heart Infusion Broth solution was inoculated with *S.mutans* stock (from -80°C). The culture was left in the shaker overnight at 37°C, 200 rpm. The overnight culture was prepared freshly for each experiment.

2.1.4.5 Glycerol Stock Solution

80 ml glycerol (Riedel-de-Haen) and 20 ml distilled water were mixed to get 80% (w/v) solution. It was sterilized for 15 minutes under 1.5 atm at 121^{o}C.

2.1.4.6 PC Buffer (Potassium Phosphate-Sodium Carbonate Buffer)

55 mM KH_2PO_4 (Fisher), 45 mM Na2CO3 (Fisher), 200 mM NaCl (Sigma) were prepared in distilled water and the solution was sterilized by 0.2 μm single use syringe filter. The pH was adjusted to 7.2-7.5.

Note: PC buffer can not be sterilized by autoclaving because CO3- ions are converted to CO2 due to high pressure during the autoclave. This conversion causes an increase in pH up to 10.

2.1.4.7 PBS (Phosphate Buffer Saline Buffer)

37mM NaCl(Sigma), 2.7 mM KCl (Sigma), 10mM Na_2HPO_4(Fisher), 1.76mM KH_2PO_4(Fisher) were prepared in distilled water and the solution was sterilized by 0.2 μm single use syringe filter. The pH was adjusted to 7.4.

2.1.4.8 FITC Stock Solution

6mg FITC (BioChemika) was dissolved in 1 ml PBS buffer and stored at +4^{o}C.

2.1.4.9 Ampicillin (Sodium Salt) stock solution

1.28 mg Ampicillin (Sigma) was dissolved in 1 ml deionized water, sterilized by filtration using 0.22μm filter. This is a 1x stock solution and stored at -20^{o}C. It is used as positive control for mibroth micro dilution assay.

2.1.4.10 Cholaramphenicol Stock Solution

1.28 mg Cholaramphenicol (Sigma) was dissolved in 1 ml deionized water, sterilized by filtration using 0.22μm filter. This is a 1x stock solution and stored at -20oC. It is used as positive control for broth micro dilution assay.

2.1.4.11 Peptide Stock Solutions

2.56 mg peptide was dissolved in 1 ml distilled water for each type of peptide. This is a 1x stock solution and stored at -20^{o}C.

2.1.5 Laboratory Equipments

Autoclave: 2540 ML benchtop autoclave, Systec GmbH Labor- Systemtechnik.

Automatic pipettes: Eppendorf.

Centrifuges : Microfuge 18, Beckman Coulter.

Confocal Microscope: Leica TCS SP2 SE Confocal Microscope.

Deep freezes and refrigerators: Heto Polar Bear 4410 ultra freezer, 2021 D deep freezer, Arcelik., 1061 M refrigerator, Arcelik.

Ice machine: AF 10, Scotsman.

Laminar flow cabinet: Airclean 600 PCR Workstation ISC Bioexpress. **Magnetic stirrer:** AGE 10.0164, ARE 10.0162, VELP Scientifica srl.

Orbital shaker: Innova 3100 Water Bath Shaker New Brunswick Scientific

pH meter: MP 220, Mettler Toledo International Inc.: Inolab pH level 1, order#1A10-1113,Wissenschaftlich-Technische Werkstätten GmbH & Co KG.

QCM: KSV Z500 Finland.

Spectrophotometer : DU530 Life Science UV/ Vis, Beckman, UV-1601, Shimadzu Corporation.

Sterilizer : FN 500, Nuve.

Vortexing machine: Reax Top, product# 541-10000, Heidolph2.2. 71.

2.2 Methods

2.2.1 Broth Micro Dilution Antibacterial Assay For Free Peptides

The inhibition effect of the peptides versus their varying concentration was determined according to Broth Micro dilution Antibacterial Assay of Peptides. Briefly, serial dilutions of the peptides (256 μg/ml to 1 μg/ml) were prepared in 96-well microtiter slides and bacterial inoculum in their media was added to each well (~5.0×10^4 CFU/well for *E.coli* and ~1.0×10^4 CFU/well for *S.Mutans*). Total volume was 200μl; 180μl was bacteria and 20μl was peptide solution prepared in dH_2O. Slides were incubated at 37°C 16 to 20 hours and then optical density was monitored at 600 nm. Experiments were repeated three times in duplicate for each bacterial strain for E. *coli* and *S.mutans.*

2.2.2 Modifying the Slides With Peptides

Cleaned and sterilized (UV for 10min.) 0.5x0.5 HA-coated titanium slide was put in 950 μl PC buffer containing 0.1 % detergent. Subsequently, 20 μl of peptide stock was added. The slide in the buffer solution was left overnight at room temperature with constant rotating. The detergent in the buffer provides the peptides to interact with the substrate surface individually.

2.2.3 QCM Experiments

A Quartz Crystal Microbalance (QCM) system (KSV Z500 Finland) was used to prove specific binding of AMP-HABP1 fusion peptide to hydroxyapatite. In a QCM,

quartz crystal is mechanically excited into a resonance by applying an alternating potential across two conducting films deposited on either side of the quartz crystal and the frequency of this oscillation is sensitive to the amount of adsorbed materials on the crystal surface [62]. Also QCM harnesses multiple frequencies which can be defined by overtone numbers n= 3,5,7,9 for determining the properties of the binding process. QCM device was connected to an oscillator circuit that has 5 MHz resonance frequency. In all experiments 7^{th} overtone was taken into account.

In order to demonstrate the affinity of the fusion peptide to hydroxyapatite, an AT-cut quartz crystal surface coated with hydroxyapatite was used. Control experiment was conducted with the same peptide on a bare quartz slide. Slides were cleaned with ultrasonication for 10 min each in isopropanol, ethanol and distilled water respectively. Finally, they rinsed with water again and dried under a stream of pure N_2 before use. To establish a stable baseline, a sufficient amount of PC buffer solution was introduced into the cell before adding the peptide solutions (AMP-HABP1). After this initial treatment/measurement, the solution containing desired amount of peptide was injected into the cell and the frequency change was recorded continuously.

2.2.4 Antimicrobial Activity on Surface

2.2.4.1 Anti-adhesive Effect

Bacterial adhesion characteristics of the functionalized titanium surfaces were assessed via the spread slide method. *S.Mutans* and *E.coli* were cultured in brain heart infusion broth and LB broth respectively. The bacteria were incubated overnight at 37 °C with agitation in the broth. An aliquot of bacterial culture was then added to the broth and incubated for another 2 h at 37 °C. The bacterial culture was centrifuged at 2700 rpm for 10 min. After the removal of the supernatant, the cells were washed twice with PBS and resuspended in PBS at a concentration of 10^7CFU/ml. 1 ml of the bacterial suspension was then added to each substrate in a 24-well slide and incubated for 5 h/12h/24h at 37 °C. The substrates were removed with sterile forceps and gently washed with PBS. The substrates were then placed in broth and the bacteria retained on substrates were dislodged by mild ultrasonication for 2 min in a ultrasonic bath, followed by rapid vortex mixing (10 s). Serial ten-fold dilutions were performed and viable counts were estimated using the spread slide

method. The number of viable bacteria on each substrate surface was counted and expressed relative to the surface area of the substrate (number of bacteria/cm^2).

2.2.4.2 Bactericidal Effect

E.coli and S.mutans were incubated as described above. The bacterial suspension as concentrated by centrifugation and resuspended in PBS at a concentration of 5×10^7 CFU/ml; 1 ml of the bacteria suspension was added to each substrate in 24-well E.coli and S.mutans were incubated as described above. The bacterial suspension was concentrated by centrifugation and resuspended in PBS at a concentration of 5×10^7 CFU/ml; 1 ml of the bacteria suspension was added to each substrate in 24-well slide. Slide chambers were covered and placed in a humidified incubator at 37°C; after 2 h, nonadherent bacteria were removed by with PBS [102].Adherent bacteria were stained with FITC (6 μg/ml) in PBS for 15 min at 37°C; FITC has been observed to only penetrate into cells with compromised membranes [102]. Slides were rinsed with PBS and imaged by confocal microscopy(Leica TCS SP2 SE). Fluorescent (488-nm band-pass filter for excitation of FITC) images were taken at identical locations to determine stained with FITC.

3. RESULTS AND DISCUSSION

3.1 Broth Microdilution Antibacterial Assay For Free Peptides

In this study we used an inorganic binding peptide HABP1 (CMLPHHGAC) which was shown to have great binding affinity toward HA [100], a cationic peptide which was reported to have strong antimicrobial activity (KRWWKWWRR) [22] and a fusion peptide formed by conjugation of them, AMP-HABP1 (KRWWKWWRRGGGCMLPHHGAC).

The inhibition effect of the peptides versus their varying concentration was determined according to Broth Micro dilution Antibacterial Assay of Peptides. As it is shown in Figure 3.1 and 3.2, the fusion AMP-HABP1fusion peptide was not effective as AMP in solution against *E.coli* and *S Mutans* but it is clear that the fusion peptide had antibacterial activity. In addition, HABP1 partially inhibited bacterial reproduction. While an antimicrobial peptide killing the bacteria, it is generally accepted that it uses electrostatic interaction first. Hydroxyapaptite binding peptide 1 also has positively charged amino acid residue. So, it is possible to interact the HABP1 with bacteria membrane end damage it.

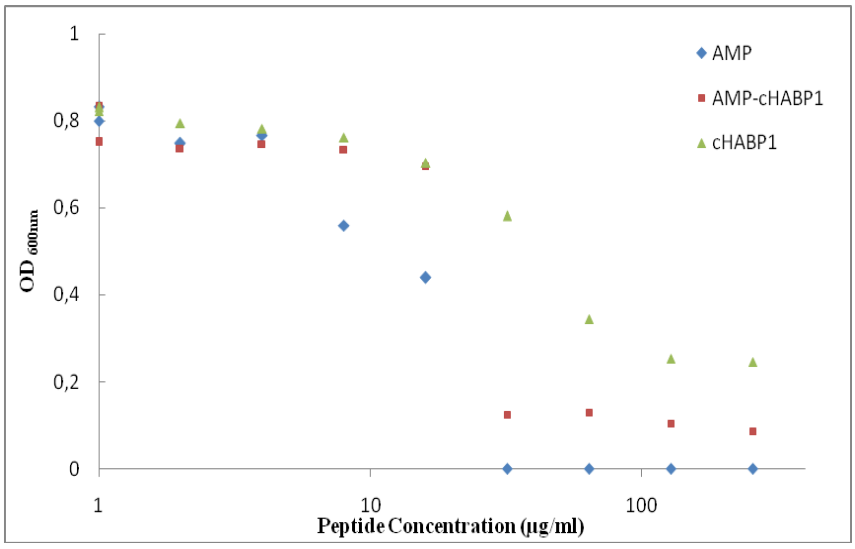

Figure 3.1: Broth microdilution assay results for *E.coli* 5 × 10^7 CFU/ml incubated with peptides at 37°C for 16 hours.

As expected, conjugation with HABP1 decreases the antibacterial activity but it is shown that the new fusion peptide, AMP-HABP1, has remarkable activity against both gram negative and positive bacteria. The lower activity of the fusion peptide may be because positively charge density of antimicrobial peptide was decreased with the addition of HABP1 so it couldn't interact with the negatively charged bacteria membrane as well as the antimicrobial peptide

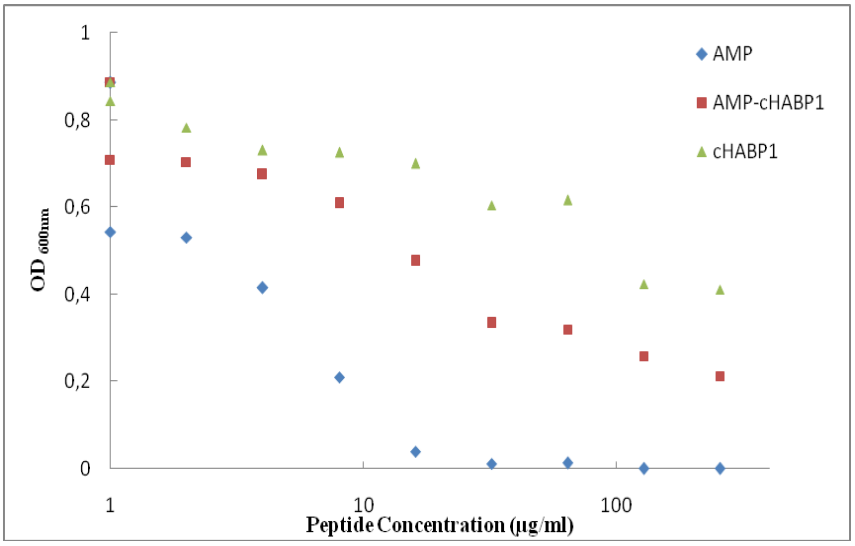

Figure 3.2: Broth microdilution assay results for *S.mutans* 5×10^7 CFU/ml incubated with peptides 37°C for 16 hours.

The figures 3.1 and 3.2 show that while AMP killed *E.coli* at the concentration of 32µg/ml; *S.mutans* was almost killed at the concentration of 16µg/ml AMP (Figure 3.1 and 3.1). Albeit the increasing concentration of HABP1-AMP up to 256 µg/ml there was no completely inhibition of bacteria reproduction compared to AMP.

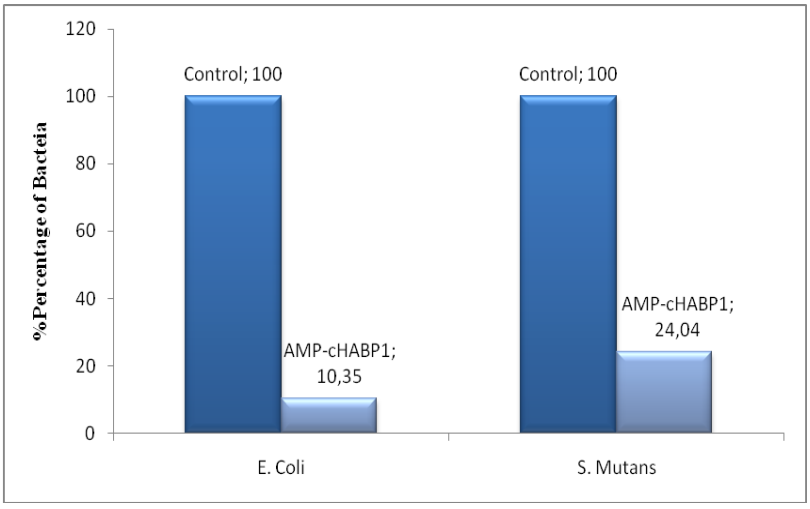

Figure 3.3: *E.coli* and *S.mutans* ratio after the incubation without (control) and with AMP-HABP1 (256µg/ml) for 16 hours.

Under the experiment conditions, AMP-HABP1 killed %89,65 of the E. Coli and %75.96 of S. Mutans maximum (Figure 3.3).

3.2 QCM

Quartz is one member of a family of crystals that experience the piezoelectric effect. Quartz crystal is mechanically excited into a resonance by applying an alternating potential across two conducting films deposited on either side of the quartz crystal. The frequency of this oscillation is sensitive to the amount of adsorbed materials on the crystal surface. During normal operation, all the other influencing variables remain constant; thus mass change on the surfce correlates directly to a change in frequency. As mass is deposited on the surface of the crystal, the thickness increases; consequently the frequency of oscillation decreases from the initial value. By means of the resonant frequency shift, QCM can distinguish microgram of the mass added on the QCM electrode. Therefore it is termed microbalance.

The affinity of AMP-HABP1 fusion peptide to hydroxyapatite surface was evaluated by the QCM experiment. First, the buffer solution was fed into the system for system equilibirum. After the system came into balance, peptide solution prepared in the buffer solution was fed in order to monitor the binding property. Finally, by feeding the buffer again it was observed if the absorbed molecules on the surface dissociate or not.

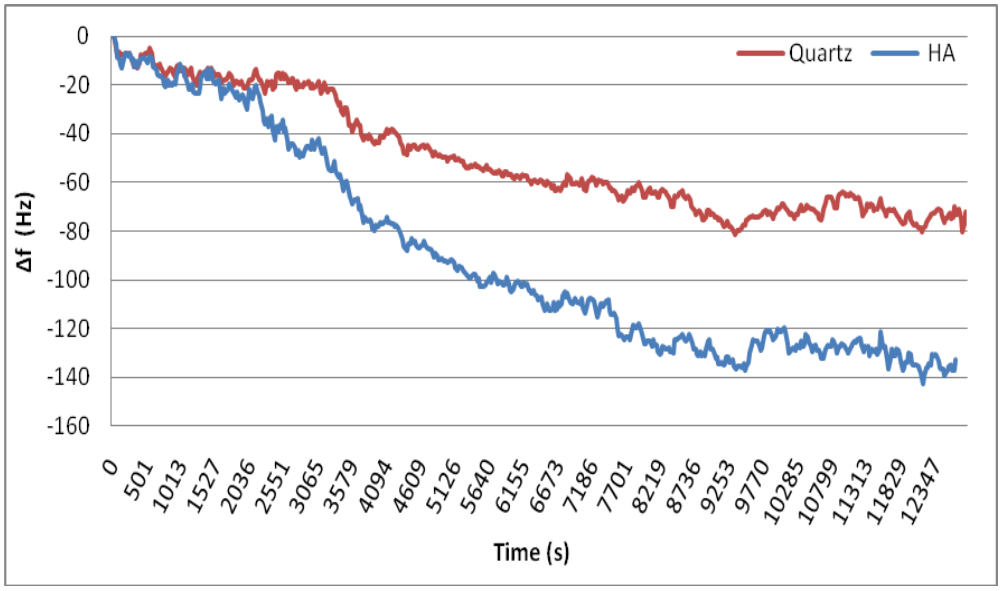

Figure 3.4: Frequency changes observed for 1µM AMP-HABP1 on quartz and hydroxyapatite surface.

While evaluating the results it should be noted that all the biomolecules bind litte amount on any type of surfaces but inorganic binding peptides have specific affinity only certain ones. With monitoring of frequency change which means the adsorbed mass on the surface, it can be said fusion peptide binds to hydroxyapatite specifically compared to bare quartz surface(Figure 3.4). After feeding PC buffer into the system for the monitoring of dissosiation period of the adsorbed peptide, it was observed that there was no remarkable frequency increase demonstrating that these fusion peptides remained on the surfaces.

3.3 Antimicrobial Activity on Surface

The fusion peptide was immobilized on the hydroxyapatite coated titanium surface through the high affinity of HABP1. Also the HABP1 was immobilized on the substrates to see whether it had any antimicrobial activity. Bacterial adhesion characteristics of the functionalized substrates were assessed via the spread slide method. Table 3.1 summaries numbers of bacteria adherent on the substrates.

Table 3.1: Number of adherent of *E.coli* and *S.mutans* cells on surfaces of bare and functionalized substrates.

		Number of bacteria (CFU/cm2)		
		5 Hours	**12 Hours**	**24 Hours**
E.Coli	**HA coated Ti**	$800x10^3$	$564x10^4$	$610x10^4$
	HA coated Ti-HABP1	$380x10^3$	$456x10^4$	$470x10^4$
	HA coated Ti-AMP-HABP1	$8x10^3$	$364x10^4$	$188x10^4$
S.Mutans	**HA coated Ti**	$185x10^1$	$380x10^1$	$392x10^2$
	HA coated Ti-HABP1	$56x10^1$	$240x10^1$	$260x10^2$
	HA coated Ti-AMP-HABP1	-	$160x10^1$	$246x10^2$

For comparable results, relative bacteria numbers were calculated for *E.coli* (Figure 3.5) and *S.mutans* (Figure 3.6). For 5 hours incubation the fusion peptide modified substrates were %100 effective for *S.mutans* while %99 for *E.coli*. However, this

effect decreased with time. When they incubated for 12 hours nearly %32 of *E.coli* and %58 of *S.mutans* were blocked to attach on the surfaces and this percentage was lower when it was 24 hours. Although the decreased activity by the time, it is shown that the fusion peptide modified substrates effective for 24 hours in terms of anti adhesive property.

It is also interesting that, despite it is not very hydrophobic or charged peptide, only the HABP1-modified substrates had anti adhesive activity but less than the fusion peptide modified ones.

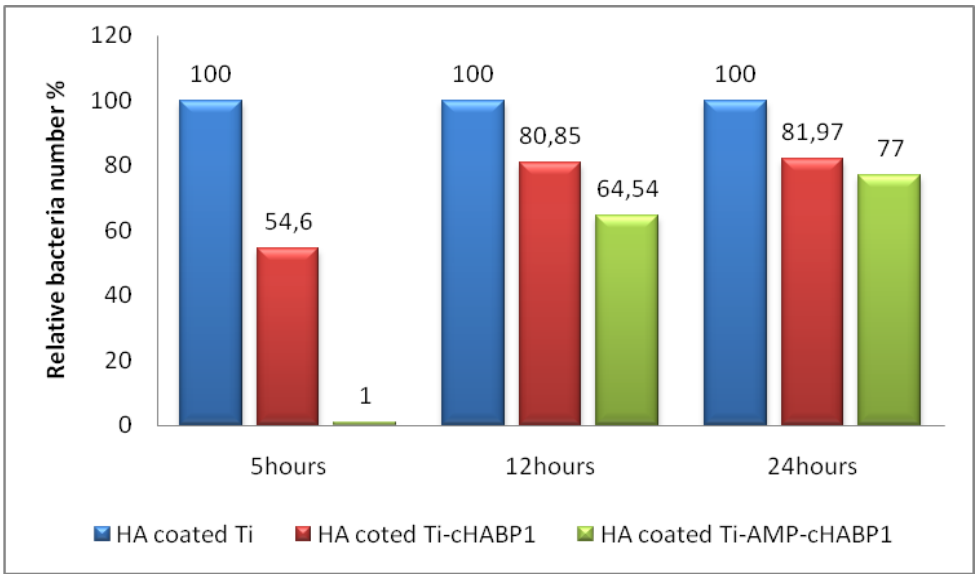

Figure 3.5: Number of adherent of *E.coli* cells on surfaces of bare and functionalized substrates.

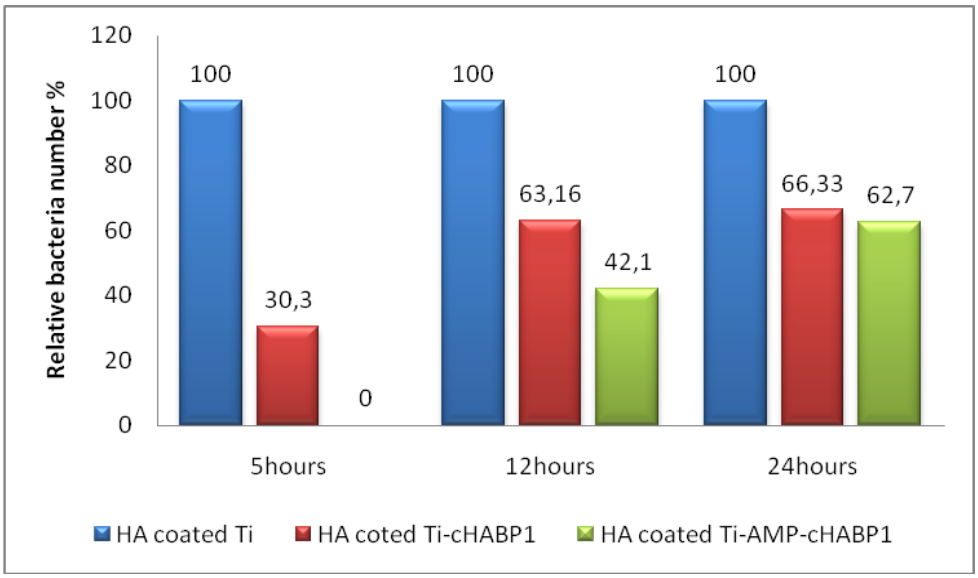

Figure 3.6: Number of adherent of S.mutans cells on surfaces of bare and functionalized substrates.

As there is a growing need for alternative approaches to circumvent the limitations of antibiotic therapies, two approaches have been developed: materials with antimicrobial properties and materials with anti-adhesive properties. The first approach involves materials containing bactericidal substances that are incorporated into or bound to the biomaterial surfaces; the other approach is the modification of substrate materials with the anti-adhesives to prevent the adhesion of bacteria, thus, inhibiting biofilm formation and infection [101].

To determine whether the decrease in bacteria on fusion peptide modified substrates resulted from the bactericidal or anti-adhesive effect of the surfaces, confocal microscope images were taken after staining with FITC. Experiments on modified HA coated titanium slides were performed by exposing the surfaces to an *E. coli* and *S.mutans* suspension for two hours and then rinsing to remove unattached and weakly attached bacteria. The bacteria remaining on the surfaces were imaged in fluorescence after staining with FITC to detect cells with compromised membranes.

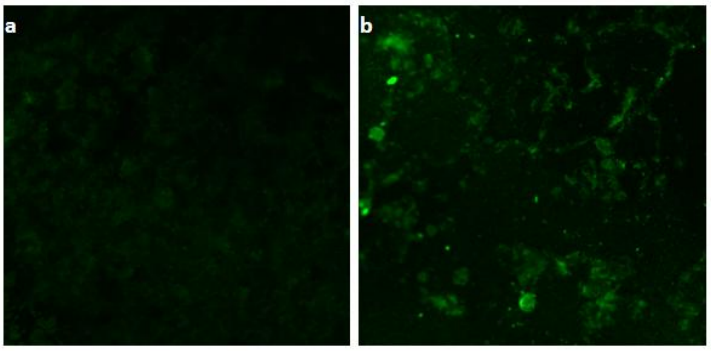

Figure 3.7: Confocal microscopy images for *E.coli* after 2 h incubation on: bare HA coated Ti **(a)** as control, AMP-HABP1 modified HA coated Ti **(b)**.

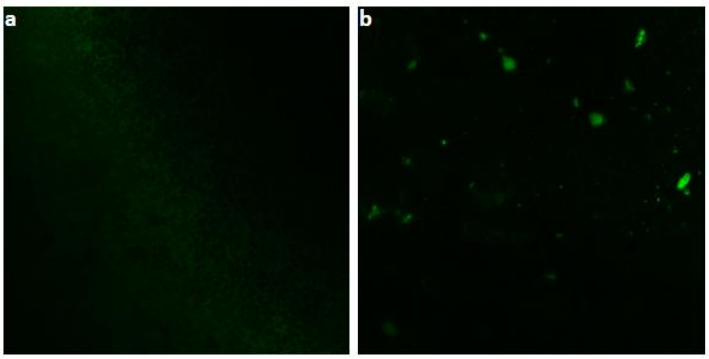

Figure 3.8: Confocal microscopy images for *S.mutans* after 2 h incubation on: bare HA coated Ti **(a)** as control, AMP-HABP1 modified HA coated Ti **(b)**.

FITC (molecular mass of 389.4 Da), a green fluorescent probe, can traverse only through the damaged cytoplasmic membrane of a cell[103].

Images of representative areas of the substrates are shown in Figure 3.7 and 3.8. From these images, attached cells with compromised membranes (from FITC staining) were determined.

We observed that FITC could penetrate only into cells that were treated with the fusion antimicrobial peptides tethered on surfaces that were active on them. The fluorescence images can only provide qualitative information about the effect of the functionalized surfaces. It means that fusion peptide modified hydroxyapatite coated titanium surfaces are able to damage the cell wall and kill the bacteria in first 2 hours, thus provide antimicrobial activity.

4. CONCLUSION

The rapid increase in the use of implantable biomedical devices requires the development of surfaces which can reduce the rate of colonization of the surface by microorganisms. Cationic peptides have previously been reported to meet the criteria for a successful antimicrobial surface coating.

In the present study, the efficacy of binding of AMP-HABP1 was examined via affinity binding strategy applicable to a wide range of biomaterial surfaces and assesed as an antimicrobial surface.

Selected AMP showed antimicrobial activity against to *E. coli* and *S. mutans* strains which is consistent with previous reports. It has been demonstrated that conjugation with HABP1 decreases the antibacterial activity but the new fusion peptide, AMP-HABP1, has remarkable activity against both gram negative and positive bacteria. It may be due to attenuated positive charge density which is important for peptide and bacterial membrane interaction. Broth micro dilution assay shows it is possible to obtain a fusion peptide has remarkable antimicrobial activity and also QCM results indicate that this fusion peptide has specific binding affinity to hydroxyapatite surfaces.

HA coated titanium surface modified with AMP-HABP1 showed reduced bacterial adhesion (total surface coverage by bacteria) and bactericidal effect for both strain of bacteria compared with blank ones. It can be said that the fusion peptide is active when attached to surfaces.

Little is known about the mechanism of antimicrobial peptides tethered on surfaces but it is supposed that when bacteria come into contact with these peptides, the bacteria loses its integrity and destroys itself [104].

This study shows that conjugation the antimicrobial peptides with one of the genetically engineered peptides, HABP1 make them capable of self-immobilization on the hydroxyapatite surfaces and may offer potential for development as a broad-

spectrum antimicrobial coating for biomaterial surfaces, showing good activity against both gram-negative and gram-positive organisms.

Development of antimicrobial surfaces for biomaterials is of great importance in reducing the clinical and economic burden of infection of medical devices and evaluation of methods of attachment for cationic peptides is critical to the development of effective antimicrobial surfaces.

REFERENCES

[1] **Barre`re F., Mahmood T.A., Groot K and Blitterswijk C.A,** 2008. Advanced biomaterials for skeletal tissue regeneration: Instructive and smart functions. *Materials Science and Engineering* R, **59**, 38–71

[2] **Biehl V. and Breme J.,** 1999. *Metallic Biomaterials*. John Wiley & Sons Ltd, Chichester, 37–71.

[3] **Williams D.F.,** 1999.The Williams Dictionnary of Biomaterials, *Liverpool University Press*, Liverpool.

[4] **Hunziker E.B.,** 2001. Articular cartilage repair: basic science and clinical progress. A review of the current status and prospects. *Osteoarthritis and Cartilage*, **10**, 432-463.

[5] **Blackwood D.J. and Seah K.H.W.,** 2010. Galvanostatic pulse deposition of hydroxyapatite or adhesion to titanium for biomedical purposes. *Materials Science and Engineering* C, **30**, 561–565

[6] **Søballe K., Hansen E.S., Brockstedt-Rasmussen H., Hjortdal V.E., Juhl G.I., Pedersen C.M., Hvid I. and Burger C.,** 1991. Gap healing enhanced by hydroxyapatite coating in dogs. *Clin Orthop Relat Res.*, **272**,300–307.

[7] **Lacefield W.R.,** 1993. Hydroxylapatite coatings. In: Hench LL, Wilson J, editors. An Introduction to Bioceramics. *Singapore: World Scientific*., **1,** 223–238

[8] **Darouiche R.O., 2004.** Treatment of infections associated with surgical implants. *N. Engl. J. Med.*, **350**, 1422–1429

[9] **Gristina, A.G.,** 1987. Biomaterial-centered infection: microbial adhesion versus tissue integration. *Science*, **237**, 1588–1595.

[10] **Smith A.W.,** 2005. Biofilms and antibiotic therapy: is there a role for combating bacterial resistance by the use of novel drug delivery systems? *Adv Drug Deliv* Rev **57**,1539–1550.

[11] **Anderson, J. M. and Marchant, R.E.W.,** 2000. Factors favoring colonization and infection. *Biomaterials*, 89–109.

[12] **Harris L. G. and Richards R.G.,** 2006. Staphylococci and implant surfaces: a review. *Injury, Int. J. Care Injured*, **37**, 3-14.

[13] **Willcox M.D.P., 2008.,** A novel cationic-peptide coating for the prevention of microbial colonization on contact lenses. *Journal of Applied Microbiology*, **105**(6), 1364-5072.

[14] **von Eiff C., Proctor R.A. and Peters G.,** 2001. Coagulase-negative staphylococci. Pathogens have major role in nosocomial infections. *Postgrad Med*, **110**(4), 63−76

[15] **Harris L.G., Foster S.J. and Richards R.G.,** 2002. An introduction to staphylococcus aureus, and techniques for identifying and quantifying s. aureus adhesins in relation to adhesion to biomaterials: review. *European Cells and Materials*, **4,** 39-60.

[16] **von Eiff C., Peters G. and Heilmann C.,** 2002. Pathogenesis of infections due to coagulase-negative staphylococci. *Lancet Infect Dis.,* **2**(11), 677−685

[17] **Url-1** < *http://lib.bioinfo.pl/meid:16432* >, accessed at 04.12.2009.

[18] **Url-2** < *http://www.alpharx.com/sm.html* >, accessed at 10.11.2008

[19] **Hamada S. and Slade H.D.**, 1980. Biology, immunology, and cariogenicity of Streptococcus mutans. *Microbiol Rev,*. **44**(2), 331-384.

[20] **Hamada S, Slade H.D. and Loesche WJ.,**1986. Role of Streptococcus mutans in human dental decay. *Microbiol Rev.,* **50**, 352-380.

[21] **Darouiche, R.O.,** 2003. Antimicrobial approaches for preventing infections associated with surgical implants. Clin. Infect. Dis., 36, 1284–1289.

[22] **Hilpert K., Elliott M., Jenssen H., Kindrachuk J., Fjell C.D., Koerner J., Winkler D.F.H., Weaver L.L., Henklein P., Ulrich A.S., Chiang S.H.Y., Farmer S.W., Pante N., Volkmer R. and Hancock R.E.W.,** 2009. Screening and Characterization of Surface-Tethered Cationic Peptides for Antimicrobial Activity. *Chem Biol*., **16,** 58-69.

[23] **Mookherjee N.,** 2007. Cationic host defence peptides: Innate immune regulatory peptides as a novel approach for treating infections. *Cell Mol Life Sci.,* **64**, 922-933.

[24] **Zasloff M.**, 2002. Antimicrobial peptides of multicellular organisms, *Nature,* **415**, 389-395.

[25] **Zelezetsky I. and Tossi A.,** 2006. Alpha-helical antimicrobial peptides—using a sequence template to guide structure–activity relationship studies, *Biochim. Biophys. Acta,* **1758**, 1436–1449.

[26] **Perron G.G. and Zasloff M.,** 2006. Experimental evolution of resistance to an antimicrobial peptide, *Proc. Biol. Sci*., **273,** 251–256.

[27] **Cherkasov, A., Hilper, K., Jenssen, H., Fjell, C.D., Waldbrook, M., Mullaly, S.D., Volkmer, R. and Hancock.,** R.E.W. 2008. Use of artificial intelligence in the design of small peptide antibiotics effective against a broad spectrum of highly antibiotic resistant Superbugs. *ACS Chem. Biol,*. **4** (1), 65–74.

[28**] Spitznagel, J.K.,** 1997. Origins and Development of Peptide Antibiotic Research. In: Antibacterial peptide protocols. Shafer, W.M. (ed.), *Humana press,* Totowa, New Jersey.pp 1-14.

[29] **Carpenter, P.L.,** 1977. Early Development of Microbiology. In 'Microbiology'. W.B. Saunders Company, Philadelphia, London, Toronto. pp. 24-39.

[30] **Boman, H.G.,** 1994. Cecropins: Antibacterial peptides from insects and pigs. In: Phylogenetic Perspectives in Immunity: The Insect Host Defence. *Med. Microbiol. Immunol*., **189**, 169-173.

[31] **Zeya, H.I. and Spitznagel, J.K.,** 1969. Cationic protein-bearing granules of polymorphonuclear leukocytes: separation from enzyme-rich granules. *Science,* **163**, 1069-1071.

[32] **Weis, J., Elsbach, P., Olsson, I. and Odeberg, J.,** 1978. Purification and characterization of a potent bactericidal membrane active protein from the granules of human polymorphonuclear leukocytes. *J. Biol. Chem.,* **253**, 2664-2672.

[33] **Zasloff, M.,** 1992. Antibiotic peptides as mediators of innate immunity. Current *Opinion in Immunology*., **4**, 3-7.

[34] **Boman, H.G., Faye, I., Hofsten, P.V., Kockum, K., Lee, J.Y. and anthopoulos, K.G.,** 1985. On the primary structure of lysozyme, cecropins and attacins from Hyalophora cecropia. *Devel. Comp. Immunol*., **9**:551-558.

[35] **Hancock, R.E. and Lehrer, R.,** 1998. Cationic peptides: A new source of antibiotics. *Tibtech.,* **16**, 82-87.

[36] **Steiner, H.,** 1982. Secondary structure of the cecropins: antibacterial peptides from the moth Hyalophora cecropia. *FEBS Lett*., **137**, 283–287.

[37] **Zasloff, M.,** 1987. Magainins, a class of antimicrobial peptides from Xenopus skin: Isolation, characterization of two active forms, and partial cDNA sequence of a precursor. *Proc. Natl. Acad. Sci. U. S. A.*, **84**, 5449–5453.

[38] **Hill, C. P., Yee, J., Selsted, M. E. and Eisenberg, D.,** 1991. Crystal structure of defensin HNP-3, an amphiphilic dimer: mechanisms of membrane permeabilization. *Science,* **251,** 1481–1485.

[39] **Romeo, D., Skerlavaj, B., Bolognesi, M. and Gennaro. R.,** 1988. Structure and bactericidal activity of an antibiotic dodecapeptide purified from bovine neutrophils. *J. Biol. Chem.,* **263,** 9573–9575.

[40] **Gennaro, R., Skerlavaj, B. and Romeo, D.,** 1989. Purification, composition, and activity of two bactenecins, antibacterial peptides of bovine neutrophils. *Infect. Immun.*, **57**, 3142–3146.

[41] **Agerberth, B., Lee, J.-Y., Bergman, T., Carlquist, M., Boman, H. G., Mutt, V., Jornvall, H. and Zasloff, M.,** 1987. Proc. Natl. Acad. Sci. U. S. A. 84, 5449–5453 1991. Eur. J. *Biochem*., **202**, 849–854

[42] **Merrifield, R.B., Merrifield, E.L., Juvvadi, P., Andreu, D. and Boman, H.G.,** 1994. Design and synthesis of antimicrobial peptides. Antimicrobial peptides. *Wiley Press*, Chichester (Ciba Foundation Symposium 186), pp 5-26.

[43**] Li M., Lai Y., Villaruz A.E., Cha D.J., Sturdevant D.E., and Otto M.** 2007. Gram-positive three-component antimicrobial peptide-sensing system. *PNAS*, **104** (22), 9469–9474

[44] **Hancock R.E.,** 1997. Peptide antibiotics. *Lance,t* **349,**418–422.

[45] **Hultmark, D., Engstrom, A., Anderson, K., Steiner, H., Bennich, H. and Boman, H.G.,** 1983. Insect immunity. Attacins, a family of antibactrial proteins from Hyalophora cecropia. *EMBO*, **2**, 571-576.

[46] **Zasloff M.,** 2002. Antimicrobial peptides of multicellular organisms. *Nature,* **415**, 389-395.

[47] **Haynie S.L., Crum G.A. and Doele B.A.,** 1995. Antimicrobial Activities of Amphiphilic Peptides Covalently Bonded to a Water-Insoluble Resin. *Antimicrobial Agents Chemother,* **39**,301-307.

[48] **Gabriel M., Nazmi K., Veerman E.C., Amerongen A.V.N. and Zentner A.,** 2006. Preparation of LL-37-grafted titanium surfaces with bactericidal activity. *Bioconjug Chem,* **17**,548-550.

[49] **Bagheri M., Beyermann M. and Dathe M.,** 2009. Immobilization Reduces the Activity of Surface-Bound Cationic Antimicrobial Peptides with No Influence upon the Activity Spectrum. *Antimicrobial Agents Chemother,* **53**,1132-1141.

[50] **Piattelli A., Scarano A., Corigliano M. and Piattelli M.,** 1996. Effects of alkaline phosphatase on bone healing around plasma-sprayed titanium implants: A pilot study in rabbits. *Biomaterials,* **17,** 1443-1449.

[51**] Duan K. and Wang R.Z.,** 2006. Surface modifications of bone implants through wet chemistry. *J Mater Chem,* **16,** 2309-2321.

[52] **Tsui Y.C., Doyle C. and Clyne TW.,** 1998. Plasma sprayed hydroxyapatite coatings on titanium substrates Part 1: Mechanical properties and residual stress levels. *Biomateriasl,* **19,** 2015-2029.

[53] **Tsui Y.C., Doyle C. and Clyne T.W.,** 1998. Plasma sprayed hydroxyapatite coatings on titanium substrates Part 2: optimisation of coating properties. *Biomaterials,* **19,** 2031-2043.

[54] **Campbell A.A., Fryxell G.E., Linehan J.C. and Graff G.L..** 1996. Surface-induced mineralization: A new method for producing calcium phosphate coatings. *J Biomed Mater Res,* **32,**111-118.

[55] **Wang J., Layrolle P., Stigter M. and de Groot K.,** 2004. Biomimetic and electrolytic calcium phosphate coatings on titanium alloy: physicochemical characteristics and cell attachment. *Biomaterials,* **25,** 583-592.

[56] **Fan Y.W., Duan K. and Wang R.Z.,** 2005. A composite coating by electrolysis-induced collagen self-assembly and calcium phosphate mineralization. *Biomaterials,* **26,** 1623-1632.

[57] **Suchanek W. and Yoshimura M.,** 1998. Processing and properties of hydroxyapatite-based biomaterials for use as hard tissue replacement implants. *J Mater Res,* **13,** 94-117.

[58**] Khor E. and Lim L.Y.,** 2003. Implantable applications of chitin and chitosan. *Biomaterials,* **24,** 2339-2349.

[59] **Zhang Y. and Zhang MQ.,** 2001. Synthesis and characterization of macroporous chitosan/calcium phosphate composite scaffolds for tissue engineering. *J Biomed Mater Res,* **55,** 304-312.

[60] **Yamaguchi I, Tokuchi K, Fukuzaki H, Koyama Y, Takakuda K, Monma H. and Tanaka T.,** 2001. Preparation and microstructure analysis of chitosan/hydroxyapatite nanocomposites. J *Biomed Mater Res,* **55,** 20-27.

[61] **Garbuz DS., Hu Y., Kim WY., Duan K, Masri BA, OxIand TR, Burt H., Wang R. and Duncan CP.,** 2008. Enhanced gap filling and osteoconduction associated with alendronate-calcium phosphate-coated porous tantalum. *J Bone Joint Surg –Am,* **90A,** 1090-1100.

[62] **Morra M.,** 2007. Biomolecular modification of implant surfaces. *Expert Rev Med Devices,* **4,** 361-372.

[63] **Lebaron R.G. and Athanasiou KA.,** 2000. Extracellular matrix cell adhesion peptides: Functional applications in orthopedic materials. *Tissue Eng,* **6,** 85-103.

[64] **Bumgardner JD., Chesnutt BM., Yuan Y., Yang Y., Appleford M., Oh S., McLaughlin R., Elder S.H. and Ong JL.,** 2007. The integration of chitosan-coated titanium in bone: An in vivo study in rabbits. *Implant Dent,* **16,** 66-79.

[65] **Harris LG., Mead L., Muller-Oberlander E. and Richards R.G.,** 2006. Bacteria and cell cytocompatibility studies on coated medical grade titanium surfaces. *J Biomed Mater Res Part A,* **78,** 50-58.

[66] **Nanci A., Wuest JD. Peru L., Brunet P., Sharma V., Zalzal S. and McKee MD.,** 1998. Chemical modification of titanium surfaces for covalent attachment of biological molecules. *J Biomed Mater Res,* **40,** 324-335.

[67] **Puleo DA.,** 1995. Activity of Enzyme Immobilized on Silanized Co-Cr-Mo. *J Biomed Mater Res,* **29,** 951-957.

[68**] Xiao SJ., Textor M., Spencer N.D. and Sigrist H.,** 1998. Covalent attachment of cell-adhesive, (Arg-Gly-Asp)-containing peptides to titanium surfaces. *Langmuir,* **14,** 5507-5516.

[69] **Ferris DM., Moodie GD., Dimond P.M., Gioranni C.W.D., Ehrlich M.G. and Valentini RF.,** 1999. RGD-coated titanium implants stimulate increased bone formation in vivo. *Biomaterials,* **20,** 2323-2331.

[70] **Davis DH., Giannoulis CS., Johnson R.W. and Desai TA.,** 2002. Immobilization of RGD to silicon surfaces for enhanced cell adhesion and proliferation. *Biomaterials,* **23,** 4019-4027.

[71] **Jose B., Antoci V., Zeiger A.R., Wickstrom E. and Hickok NJ.,** 2005. Vancomycin covalently bonded to titanium beads kills Staphylococcus aureus. *Chem Biol.,* **12,** 1041-1048.

[72] **Edupuganti O.P., Antoci Jr. V, King S.B., Jose B., Adams C.S., Parvizi J., Shapiro I.M., Zeiger AR., Hickok NJ and Wickstrom E.,** 2007. Covalent bonding of vancomycin to Ti6Al4V alloy pins provides long-term inhibition of Staphylococcus aureus colonization. *Bioorganic & Medicinal Chemistry Letters,* **17,** 2692-2696.

[73] **Sarikaya M., Tamerler C., Jen A. K. Y., Schulten K. and Baneyx F.,** 2003. Molecular biomimetics: nanotechnology through biology. *Nature Materials*, **2**(9), 577-585.

[74] **Sarikaya M.,** 1999, Biomimetics: Materials fabrication through biology Proc. *Natl. Am. Sci. U.S.A.,* **25,** 14183

[75] **Frankel, R. B. and Blakemore, R. P.,** 1991. Iron Biominerals. New York: Plenum

[76] **Lowenstam, H.A. and Weiner, S.,** 1989. On Biomineralization (Oxford University Press, Oxford, UK).

[77] **Fong, H., Sarikaya, M., White, S. and Snead, M. L.,** 2000. Nanomechanical properties profiles across DEJ in human incisor teeth. *J. Mater. Sci. Eng.,* **7,** 119–28.

[78] **Glimcher, M and Nimni, M.,** 1992. Collagen cross-linking and biomineralization. *Connect Tissue Res.,* **27,** 73–83

[79] **Paine ML, Zhu DH, LuoW, Bringas P Jr and Goldberg M.,** 2000. Enamel biomineralization defects result from alterations to amelogenin selfassembly. *J. Struct. Biol.,***132,**191–200.

[80] **DeGarmo, E. P., Black, J. T. and Kohner, R. A.,** 1988. Materials and Processes in Manufacturing (McMillan, New York)

[81] **Smith, G. P.,** 1985. Filamentous fusion phage: novel expression vectors that display cloned antigens on the surface of the virion. *Science,* **228,** 1315-17.

[82] **Brown, S.,** 1992. Engineered iron oxide-adhesion mutants of the Escherichia coli phage _ receptor. *Proc. Natl. Acad. Sci. U.S.A.,* **89,** 8651-55.

[83] **Boder, E. T. and Wittrup, K. D.,** 1997. Yeast surface display for screening combinatorial polypeptide libraries. *Nat. Biotechnol.,* **15,** 553-57.

[84] **Thai ,C. K., Dai, H., Sastry, M. S. R., Sarikaya, M., Schwartz, D. T. and Baneyx F.,** 2004. Identification and characterization of Cu2O and ZnO binding polypeptides by Escherichia coli cell surface display: towards an understanding of metal oxide binding. *Biotech. Bioen.,* **87,** 129-137

[85] **Brown, S.,** 1997. Metal-recognition by repeating polypeptides. *Nat. Biotechnol.,* **15,** 269-72

[86] **Whaley, R. S., English, D. S., Hu, L. E., Barbara, F. P. and Belcher, M. A.,** 2002, Selection of peptides with semiconducting binding specificity for directed nanocrystal assembly. *Nature,* **405,** 665-68.

[87] **Seeman N.C. and Belcher, A.M.,** 2002. Emulating biology: Building anostructures from the bottom up. *Proc. Natl. Am. Sci. U.S.A.,* **99,** 6451-55.

[88] **Peelle, B. R., Krauland, E. M., Wittrup, K. D. and Belcher, A. M.,** 2005. Design criteria for engineering inorganic material-specific peptides. *Langmuir,* **21,** 6929-6933.

[89] **Kacar T., Ray j., Gungormus M., Oren E. E., Tamerler C. and Sarikaya M., 2009**. Adsorption Kinetics of an Engineered Gold Binding Peptide by Surface Plasmon Resonance Spectroscopy and a Quartz Crystal Microbalance. *Adv. Mater,* **21,** 295–299.

[90] **Taktak B.,** 2008. Genetic Design and Synthesis of Bi-Functional Protein For Bionanotechnology. *Master Thesis.*

[91] **Naik, R. R., Stringer, S. J., Agarwal, G., Jones, S. E. and Stone, M. O.**, 2002. Biomimetic synthesis and patterning of silver nanoparticles. *Nature Mater.,* **1,** 169-71.

[92] **Nam, K. I., Kim, D. W., Yoo, P. J., Chiang, C. Y., Meethong, N., Hammond, P. T., Chiang, Y. M. and Belcher, A. M.,** 2006. Virus-Enabled Synthesis and Assembly of Nanowires for Lithium Ion Battery Electrodes. *Science,* **312,** 885-89.

[93] **Oren, E. E., Tamerler, C., Sahin, D., Hnilova, M., Seker, U. O. S., Sarikaya, M. and Samudrala, R.,** 2007. A Novel Knowledge-Based Approach to Design Inorganic Binding Peptides. *Bioinformatics,* **23,** 2816-2822.

[95] **Zin, M. T., Ma, H., Sarikaya, M. and Jen A.K.Y.,** 2005. Assembly of Gold Nanoparticles using Genetically Engineered Poypeptides, *Small,* **1**(7) 698-702.

[96] **Sarikaya, M., Tamerler, C., Schwartz, D. T. and Baneyx, F. O.** 2004. Materials assembly and formation using engineered polypeptides. *Annual Review Of Materials Research,* **34,** 373-408.

[97] **Thai, C. K., Dai, H., Sastry, M. S. R., Sarikaya, M., Schwartz, D. T. and Baneyx, F.** 2004. Identification and characterization of Cu2O and ZnO binding polypeptides by Escherichia coli cell surface display: towards an understanding of metal oxide binding. *Biotech. Bioeng.*, **87,** 129-137

[98] **Hnilova, M., Oren, E.E., Seker, U. O. S., Wilson, B., Collino, S., Evans, J. S., Tamerler, C. and Sarikaya, M.** 2008. Effect of Molecular Conformations on the Adsorption Behavior of Gold-Binding Peptides. *Langmuir,* **24** (21), 12440–12445.

[99] **Utku F. S., Seckin E., Goller G., Tamerler C. ve Urgen M.**, 2009. Nanomats2009.

[100] **Gungormus M., Fong,H., Kim W., Evans J.S., Tamerler C. and Sarikaya M.,** 2007. Regulation of in vitro Calcium Phosphate Mineralization by Combinatorially Selected Hydroxyapatite-Binding Peptides. *Biomacromolecules,* **9,** 966–973.

[101] **Zhang F., Zhang Z., Zhu X., Kang E and Neoh K., 2008.** Silk-functionalized titanium surfaces for enhancing osteoblast functions and reducing bacterial adhesion. *Biomaterials,* **29,** 4751–4759.

[102] **Statz A.R., Park J.P., Chongsiriwatana N.P., Barron A.E. and Messersmith P.B.,** 2008. Surface-immobilized antimicrobial peptoids. *Biofouling,* **24,** 439–448.

[103] **Zaslof M.** 2002. Antimicrobial peptides of multicellular organisms. *Nature,* **415,** 389–395

[104] **Url-3** < *www.physorg.com/news152466952.html* >, accessed at 22.04.2010

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