ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

DEVELOPMENT OF ENZYME-BASED COATING METHODS FOR THE PREVENTION OF BACTERIAL BIOFILM FORMATION

Ph.D. THESIS

Abdullah SERT

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

BAKTERİYEL BİYOFİLM OLUŞUMUNU ENGELLEMEYE YÖNELİK ENZİM BAZLI KAPLAMA YÖNTEMLERİNİN GELİŞTİRİLMESİ

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ARALIK 2014

Abdullah Sert, **a Ph.D.** student of ITU **Graduate School of Science Engineering and Technology** student ID **521082050**, successfully defended the **thesis** entitled "**DEVELOPMENT OF ENZYME-BASED COATING METHODS FOR THE PREVENTION OF BACTERIAL BIOFILM FORMATION**", which he prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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To LavinyaM,

FOREWORD

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ABBREVIATIONS

AFM **:** Atomic Force Spectroscopy
AHL **:** N-Acyl Homoserine Lactone
APTMOS **:** (3-Aminopropyl)trimethoxysilane
CSLM **:** Confocal Scanning Laser Microscopy
EDC **:** *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride
EPS **:** Extracellular Polymeric Substances
FTIR **:** Fourier Transform Infrared Spectroscopy
Lys **:** Lysozyme
MTMOS **:** Trimethoxymethylsilane
NHS **:** *N*-Hydroxysuccinimide
PAA **:** Poly(acrylic acid)
PAO1 **:** *Pseudomonas aeruginosa* PAO1 strain
QS **:** Quorum Sensing
QSI **:** Quorum Sensing Inhibitor
SiC **:** Silicon Carbide
SS **:** Stainless Steel
SC **:** Sulfochromic Acid

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DEVELOPMENT OF ENZYME-BASED COATING METHODS FOR THE PREVENTION OF BACTERIAL BIOFILM FORMATION

SUMMARY

A biofilm is essentially composed of microbial cells attached to a surface and covered completely with an extracellular polymeric matrix produced by biofilm-forming bacteria. Biofilm formation results in significant changes in gene expression and these changes are related with bacterial cell-to-cell signaling, also known as "quorum sensing". When these bacterial cells approach inert surfaces, they first get bound to these surfaces utilizing their external structures such as flagella, fimbriae or capsular components by weak forces. As the cells remain attached to the surface for some period, they start to secrete extracellular polymeric substances (EPS) in order to compose a biofilm matrix that embeds many layers of bacterial cells when the biofilm develops. Bacterial EPS are generally composed of a wide variety of materials like polysaccharides, proteins, nucleic acids, uronic acid and humic substances. The EPS has several vital functions such as providing an adhesive foundation, structural integrity, bacterial protection and intercellular communication.

Due to highly enhanced resistance to antibiotics and disinfection treatments, uncontrollable and undesirable accumulation of cell aggregates cause serious problems in biomedical applications, infections in humans and corrosion and equipment failure in industrial settings. Studies concerning biofilm effects on human health are generally performed on water systems, prosthesis and implants. Biofilm formation and metabolic activities within may cause serious community health risks. Most of these health risks are nosocomial infections of gastrointestinal, eye and ear etc. Complex laparoscopic devices, which are very sensitive to disinfection and sterilization, used in minimal invasive surgery and other surgical instruments are common places of biofilm formation. Due to these facts, there is a growing demand towards developing strategies to remove and protect the surfaces against biofilm formation.

Several attempts have been made to protect the surfaces of materials, instruments and equipments by addition of antimicrobial, biocidal and non-adhesive substances for coating, addition of diffusible toxic agents and changing surface roughness. In one of these studies, physical and chemical properties of biomaterial surface are modified by coating with a hydrogel. This method was effective in reducing bacterial adhesion but it was difficult to cover the surface uniformly. In another attempt, cuffs on catheters were coated with silver. The drawback of this approach was degradation of the cuff, which results in diffusion of silver ions and loss of antimicrobial activity. Antibiotics have also been coated onto surfaces but with the emergence of microbial resistance, this kind of applications had short-term effect.

One of the solutions to overcome the problem with biofilm formation could be the replacement of biocides with non-toxic alternatives, such as enzymes. Enzymes have been used in several industries ranging from food industry to large scale biocatalysis

and can also be used for the degradation and the removal of the bacterial biofilms. Extracellular Polymeric Substances (EPS) in the biofilm matrix is the essential part of the biofilm development. The complexity and variability of biofilm polymers in the matrix described above could be the utility to use several enzymes like hydrolases and lyases, individually and/or their combinations. This could achieve a sufficient disintegration of the polymeric networks composing the biofilm matrix and detachment of the biofilm from the surface it was attached.

The reasons for using these biocatalysts are that they are environmentally safe, non-toxic to mammalian cells and act on their substrates specifically by retaining their activity for a long period if immobilized. Thus, surface immobilized enzymes could provide surfaces with anti-adhesive and antifouling properties.

The main objective of the present thesis was to apply sol-gel technology and carbodiimide chemistry for immobilization of enzyme molecules to obtain antimicrobial surface. In this project we aim to inhibit formation of bacterial biofilm using covalently attached lysozyme either for use in industrial or medical applications. In order to realize the project, lysozyme molecules were immobilized onto hydrogel-type interlayer, poly(acrylic acid), using azide/nitrene chemistry for covalent attachment of the enzyme molecules. The presence of amine groups in the sol-gel silicate network provides functional sites for covalent bonding of poly(acrylic acid) via the carbodiimide reaction. Spectroscopic characterization of immobilization steps was performed using Atomic Force Microscopy (AFM) and Fourier Transform Infrared Spectroscopy (FTIR). The ability of covalently immobilized enzyme to prevent growth and biofilm formation of *Pseudomonas aeruginosa* was assessed using flow cell and Confocal Laser Scanning Microscopy (CLSM). *Pseudomonas aeruginosa* was used as a model for biofilm forming microorganism. The development of a biofilm in a parallel plate flow cell system containing control and coated test materials was designed to study biofilms growing under a range of conditions (high and low flow rates, incubation times, different temperatures etc.) which facilitates non-destructive imaging of biofilms by using Confocal Laser Scanning Microscopy (CLSM). The activities of enzyme molecules covalently-bound on sol-gel coated surfaces were analyzed during these characterization studies and the anti-biofilm efficiencies of these surfaces were visualized by using confocal microscopy.

BAKTERİYEL BİYOFİLM OLUŞUMUNU ENGELLEMEYE YÖNELİK ENZİM BAZLI KAPLAMA YÖNTEMLERİNİN GELİŞTİRİLMESİ

ÖZET

Biyofilm, canlı yada cansız herhangi bir yüzeye tutunarak mikrobik kökenli polimerik yapıya gömülü kalan mikroorganizma topluluğu olarak tanımlanabilir. Biyofilm oluşumu mikroorganizmaların yaşam alanları içindeki bir yüzeye teması ile başlar ve salgıladıkları çeşitli ekstrasellüler biyopolimerler sayesinde metal, plastik, medikal implant, hücre dokusu gibi çok farklı yüzeylere bağlanabilirler.

Doğada var olan biyofilm yapıları genellikle farklı birçok mikroorganizma türünün oluşturduğu, biyopolimerler ile çevrilmiş heterojen bir yapıdır ve bu yapı biyofilmi oluşturan mikroorganizmalara çevresel koşullardaki değişime adaptasyon kolaylığı, antimikrobiyal kimyasallara karşı dayanıklılık gibi çeşitli avantajlar sağlamaktadır. Aynı zamanda bu katman toksik kimyasalların ve antibiyotiklerin geçişini engelleyerek biyofilm yapısındaki mikroorganizmalara önemli avantajlar da kazandırmaktadır. Hücre katmanları arasında bulunan su kanalları ise biyofilmin gelişmesinde hayati öneme sahiptir. Mikroorganizmalar ihtiyaç duydukları besinlere bu su kanalları sayesinde erişirken, atıkların uzaklaştırılması da yine su kanalları sayesinde kolaylaştırılır ve mikroorganizmaların sinyal moleküller aracılığı ile iletişim kurabilmeleri için de kanal görevi üstlenirler.

Biyofilm oluşumunun istenmeyen bölgelerde gerçekleşmesi insan sağlığını ve endüstriyel verimliliği olumsuz olarak yoğun bir şekilde etkilemektedir. Biyofilm kaynaklı insan sağlığı problemleri, içme suyu kalitesindeki düşüş ve enerji üretim verimliliği gibi birçok konuda biyofilm oluşumunun verdiği hasarların maliyeti milyar dolarları bulmaktadır.

Biyofilm oluşumuna, dental yüzeyler, gıda endüstrisindeki üretim bandındaki kontaminasyonlar ve havalandırma sistemleri gibi birbirinden çok farklı sistemlerde sıklıkla karşılaşılmaktadır. Hem insan ve toplum sağlığı hem de ekonomik açıdan yaratmış olduğu zararlı etkileri nedeniyle, yüzey üzerinde mikrobiyal birikimi engelleyebilecek veya en azından büyümesini ve yayılmasını durdurabilecek yöntemlerin geliştirilmesine yönelik çalışmalar son yıllarda yoğun olarak yürütülmektedir.

Biyofilm sistemlerinin, tehlikeli atıkların işlenmesi ve değerlendirilmesi, endüstriyel atık sularının filtrelenmesi, yeraltı sularının kontaminasyondan arındırılması gibi kullanım alanlarının bulunması ile birlikte, endüstriyel ve biyomedikal uygulamalarda biyofilm oluşumunun, ürün kontaminasyonu, enerji kaybı ve medikal enfeksiyona sebep olmak gibi birçok negatif etkisi vardır. Mikroorganizmaları bertaraf etmek için kullanılan geleneksel antibiyotik ve dezenfektanlar sıklıkla biyofilm yapısı üzerinde yeterli etkinliğe sahip olamamaktadır. Biyofilm yapısını bertaraf etmek için bu kimyasalların yüksek dozda kullanımı, biyomedikal uygulamalarda engel teşkil etmekte, endüstriyel sistemlerde ise çevresel sorunlara yol açmaktadır.

Literatürde farklı endüstriyel ve medikal alanlarda kullanılan malzemelerin, cihazların ve ekipmanın yüzeyleri üzerinde biyofilm oluşumunu önlemeye yönelik çalışmalara rastlanılmaktadır. Bu anlamda en yakın çalışma, kateter manşetlerinin hidrojel ile kaplanarak mikroorganizmaların üremesini engelleyecek şekilde fiziksel ve kimyasal özelliklerinin değiştirilmesine yöneliktir. Bu uygulama mikroorganizmaların yüzeye bağlanma kabiliyetini düşürmesine rağmen, hidrojelin yüzeye homojen bir şekilde tatbik edilmesi oldukça zor bir işlemdir. Bu alanda bir diğer çalışmada ise, antimikrobik etkinliği bilinen gümüş iyonları ile kateter manşetleri kaplanmış, fakat gümüş iyonlarının zamanla ortama difüze olması, kaplamanın antimikrobik etkinliğini kaybetmesine neden olmuştur. Kaplama malzemesi olarak antibiyotiklerin kullanılması ise mikroorganizmaların kullanılan antibiyotiğe karşı direnç kazanmasına ve uygulamanın etkinliğini kaybetmesine neden olmaktadır.

Yüzey üzerinde biyofilm oluşumunu engellemek için kullanılan alternatif yaklaşımlardan bir tanesi de enzimlerin kullanılmasıdır. Biyofilm oluşumunu engellemek için kullanılan toksik kimyasallar ile kıyaslandığında enzimlerin önemli bir üstünlüğü, enzimlerin çevreye zarar vermemesidir. Farklı tür mikroorganizmalar belirli bir yüzeye bağlanmak için farklı polimerler kullanmalarına rağmen yapılan çalışmalarda ticari olarak satılan proteazların biyofilm oluşturan mikroorganizmaların yüzeye bağlanma olasılığını düşürdüğü tespit edilmiştir. Ayrıca lizozim ve Polietilen glikolün kovalent olarak yüzeye bağlandıklarında yüzeyin antibakteriyel ve antiadhezif özellikler gösterdiği tespit edilmiştir.

Çalışmada kullanılan mikroorganizma literatürde biyofilm çalışmalarında sıkça kullanılan mikroorganizmalardandır. Medikal cihazlar da dahil olmak üzere biyotik ve abiyotik birçok farklı yüzeye bağlanabilen ve biyofilm oluşturduğu bilinen gram-negatif bir bakteri olan *Pseudomonas aeruginosa* fırsatçı bir insan patojenidir. Tez çalışmalarında, mikroorganizmaların endüstriyel ve tıbbi alanlarda sıkça kullanılan yüzeylere bağlanarak biyofilm oluşturmalarını engellemek hedeflenmiştir.

Çalışmanın ilk ayağında mevcut kaplama yöntemleri göz önünde bulundurularak, çalışmanın amacına uygun bir kaplama stratejisi belirlenmiştir. Sol-gel yönteminin stabilitesi, oda şartları altında uygulunabilirliği, inert özellikte olması ve ayrıca yüzey üzerinde fonksiyonel grupların oluşmasını sağlayabiliyor olmasından dolayı kaplama aşamasında kullanılması uygun görülmüştür. Sol-gel kaplama sayesinde yüzeyde oluşan fonksiyonel amin grupları doğrudan enzim immobilizasyonu için kullanıldığı gibi alternatif bir yöntemin de geliştirilmesiyle yüzeyde oluşturulan fonksiyonel karboksil grupları da enzim immobilizasyonu için kullanılmıştır. Bu amaçla kullanılan poli-akrilik asit molekülleri yüzeye kovalent olarak bağlanmış ve bunu takiben enzim immobilizasyonu gerçekleştirilmiştir. Uygulanan her iki kaplama tekniğinin de enzimlerin yüzeye aktif biçimde bağlanmasını mümkün kılması karşılaştırma açısından ekstra bir avantaj sağlamaktadır.

Çalışmalar boyunca gerçekleştirilen tüm kaplama aşamaları Fourier Transform Infrared Spektroskopi ve Atomik Kuvvet Mikroskobu yöntemleri kullanılarak karakterize edilmiştir. Sol-jel kaplanan yüzeyler üzerine kovalent olarak bağlanmış enzimlerin aktiviteleri, yapılan spektrofotometrik çalışmalarla test edilmiş ve anti-biyofilm etkinlikleri konfokal taramalı lazer mikroskobu aracılığıyla yerinde görüntülenmiştir. Çalışmalarda kullanılan yüzeyler ise medikal cihazların yapımında ve endüstriyel cihaz ve malzemelerin üretiminde sıklıkla kullanılan paslanmaz çelik yüzeylerdir. Bu yüzeylerin hiçbir muameleye maruz kalmadığı ilk hallerinden enzim

kaplanmış son hallerine kadar tüm adımları karakterizasyon çalışmaları ile incelenmiştir.

1. INTRODUCTION

1.1 Purpose of Thesis

A biofilm is essentially composed of microbial cells attached to a surface and covered completely with an extracellular polymeric matrix produced by biofilm-forming bacteria (Costerton et al., 1995). Biofilm formation results in significant changes in gene expression and these changes are related with bacterial cell-to-cell signaling, also known as "quorum sensing" (Givskov et al., 2008). When these bacterial cells approach inert surfaces, they first get bound to these surfaces utilizing their external structures such as flagella, fimbriae or capsular components by weak forces (Xavier et al., 2005). As the cells remain attached to the surface for some period, they start to secrete extracellular polymeric substances (EPS) in order to compose a biofilm matrix that embeds many layers of bacterial cells when the biofilm develops (Orgaz et al., 2006). Bacterial EPS are generally composed of a wide variety of materials like polysaccharides, proteins, nucleic acids, uronic acid and humic substances (Xavier et al., 2005; Orgaz et al., 2006). The EPS has several vital functions such as providing an adhesive foundation, structural integrity, bacterial protection and intercellular communication (Zhang et al., 2005; Ploux et al., 2007; Leroy et al., 2008).

Due to highly enhanced resistance to antibiotics and disinfection treatments, uncontrollable and undesirable accumulation of cell aggregates cause serious problems in biomedical applications, infections in humans and corrosion and equipment failure in industrial settings. Studies concerning biofilm effects on human health are generally performed on water systems, prosthesis and implants. Biofilm formation and metabolic activities within may cause serious community health risks. Most of these health risks are nosocomial infections of gastrointestinal, eye and ear etc. Complex laparoscopic devices, which are very sensitive to disinfection and sterilization, used in minimal invasive surgery and other surgical instruments are common places of biofilm formation. Due to these facts, there is a growing demand

towards developing strategies to remove and protect the surfaces against biofilm formation (Vijayaraghavan et al., 2006).

Several attempts have been made to protect the surfaces of materials, instruments and equipments by addition of antimicrobial, biocidal and non-adhesive substances for coating, addition of diffusible toxic agents and changing surface roughness. In one of these studies, physical and chemical properties of biomaterial surface are modified by coating with a hydrogel. This method was effective in reducing bacterial adhesion but it was difficult to cover the surface uniformly (Bayston et al., 2005). In another attempt, cuffs on catheters were coated with silver (Bong et al, 2003). The drawback of this approach was degradation of the cuff, which results in diffusion of silver ions and loss of antimicrobial activity (Raad, 1998). Antibiotics have also been coated onto surfaces but with the emergence of microbial resistance, this kind of applications had short-lived effect (Raad et al., 1995; Schierholz et al., 1997; Lelievre et al., 1999).

One of the solutions to overcome the problem with biofilm formation could be the replacement of biocides with non-toxic alternatives, such as enzymes (Kristensen et al., 2008). Enzymes have been used in several industries ranging from food industry to large scale biocatalysis and can also be used for the degradation and the removal of the bacterial biofilms (Orgaz et al., 2006; Kristensen et al., 2008, Leroy et al., 2008). Extracellular Polymeric Substances (EPS) in the biofilm matrix is the essential part of the biofilm development. The complexity and variability of biofilm polymers in the matrix described above could be the utility to use several enzymes like hydrolases and lyases, individually and/or their combinations. This could achieve a sufficient disintegration of the polymeric networks composing the biofilm matrix and detachment of the biofilm from the surface it was attachhed (Kristensen et al., 2008, Leroy et al., 2008).

The reasons for using these biocatalysts are that they are environmentally safe, non-toxic to mammalian cells and act on their substrates specifically by retaining their activity for a long period if immobilized. Thus, surface immobilized enzymes could provide surfaces with anti-adhesive and antifouling properties (Kristensen et al., 2008).

The main objective of the present thesis was to obtain a thin film coating to serve as an "anti-biofilm coating" for the prevention of bacterial colonization. In order to obtain this antimicrobial surface coating, sol-gel technology and carbodiimide chemistry were applied for immobilization of enzymes. Covalent immobilization of the enzymes on stainless steel surfaces which was previously modified by a hydrogel-type layer alone and also with polyacrylic acid by using azide/nitrene chemistry was done. The presence of amine groups in the sol-gel silicate network provided functional sites for covalent attachment of other molecules containing other functional sites. Spectroscopic characterization of immobilization steps and determination of other parameters such as enzyme coating efficiency was performed and determined by using Atomic Force Microscopy (AFM), spectrophotometric assays and Fourier Transform Infrared Spectroscopy (FTIR). Finally, a biofilm study in a parallel plate flow cell system containing control and coated test materials was designed to study biofilms growing under a range of conditions (high and low flow rates, different temperatures etc.) which facilitates non-destructive imaging of biofilms by using Confocal Laser Scanning Microscopy (CLSM). *Pseudomonas aeruginosa* was chosen especially because it is a model organism for biofilm studies.

1.2 Literature Review

1.2.1 Bacterial biofilms

In natural environments, bacteria alternate between planktonic and sessile states in response to environmental factors. The latter form is generally referred to as biofilm mode of growth, which appears to contribute the increased resistance to most antimicrobials and host defence mechanism. Therefore, biofilm can be defined as:

Aggregated bacterial cells surrounded by an adhesive matrix excreted by the cells which are more tolerant to antimicrobials and bacteria are protected from attack by host immune cells.

Over the last decade, the results obtained from various studies on planktonic bacteria have been used to explain the phenomena occurring in micro ecosystems. The observation of planktonic bacteria in such systems has yielded important data, however the study of several environmental habitats has revealed relatively low numbers of planktonic cells. In such environments, total biofilm bacterial count was

estimated to be approximately 1000-fold higher than the planktonic count (Costerton et al, 1995).

There is a realization that microorganisms should not be studied just only as biofilms but also in the context of interactions with their microenvironments. The environment exerts an effect on the metabolism of bacteria and the biofilm research represents the best tool to examinate growth in natural and ecosystems of interest. Planktonic bacteria are highly motile, they have enormous access to nutrients and multiply rapidly, when compared to sessile bacteria.

Biofilms are community of bacterial cells attached to a surface and surrounded by an adhesive matrix excreted (encased in an exopolymeric coat) by the cells. Bacteria can communicate and form biofilm nearly on all surfaces through the quorum sensing pathway, from cellulose to silicone and glass to steel, which are significant materials used for the production of medical instruments. Medical devices have been sterilized by the medical industry with gaseous agents for many years but the majority of the contamination or the corrosion process occurs after the adhesion of the microorganisms and their growth happen inside the human body (de Carvalho, 2007). As it is seen, bacterial biofilm formation on medical instruments causes harsh treatments for human implant surgery. For example, a major number of knee prostheses and catheters had to be changed due to bacterial infections (Schierholz and Beuth, 2001). In food industry, microorganisms can attach and grow on food and biofouling causes important potential hazards. Not only harmless microorganisms but also pathogenic bacteria can form biofilms on food surface. They have the ability to reduce flow and heat transmission, block membrane pores or cause energy losses (Kumar and Anand, 1998).

When bacterial cells contact with inert surfaces, they first attach to the surface by their external structures such as flagella, fimbriae and/or capsular components. When the cells remain attached on the surface they secrete sticky extra cellular substances forming a matrix gel. The matrix consists of mainly polysaccharides, besides of proteins, nucleic acids, lipids, mineral ions and various cellular debris. Several layers of cells embedded in the matrix gel and the layer of cells within the matrix is called biofilm (Costerton et al, 1995).

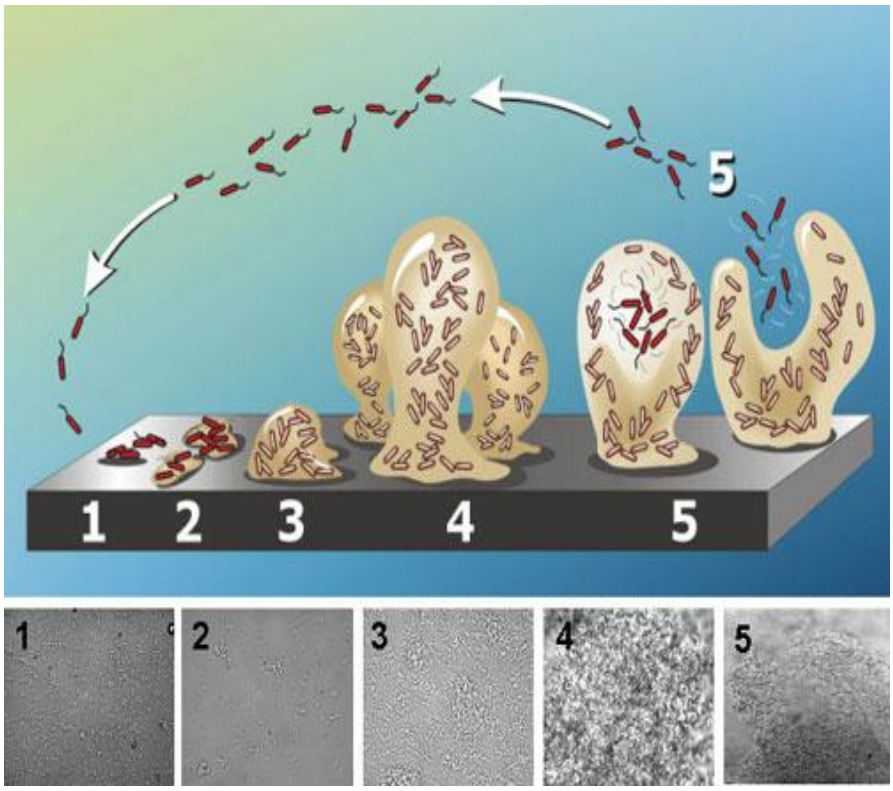


Figure 1.1 : Stages of biofilm formation and development (Url-1).

The accumulation of microorganisms on the surfaces and the formation of biofilm depend on many factors prevailing in the system, such as temperature, humidity and hydraulics of the system, surface material and microbial occurrence in the water. Microorganisms attach on a living or non-living surface, aggregate on their self produced-extracellular polymeric matrix and form biofilm layer. These sessile communities may be any microorganism such as bacteria, fungi, protozoa and any other microorganisms secreting extra cellular polysaccharides. Biofilm layer, formed by bacterial aggregation and attachment on surface, may lead to corrosion causing serious damage. Thick biofilm layers and metabolic activities running inside; make fluid flow more difficult and cause block in water pipes. Biofilm layer also acts like a barrier and affects heat transfer negatively. Even though biofilm layer does not reach a thickness leading corrosion, it may become visible. So, biofilm formed on visible and available surfaces cause visual pollution and also bad odors (Pratt and Kolter, 1999; Hall-Stoodley and Stoodley, 2002).

In Figure 1.1, stages of the biofilm formation and development can be seen. The first stage of the biofilm formation is the initial reversible attachment of free swimming microorganisms to the surface by weak forces. The second stage is the permanent

chemical attachment of these microorganisms in a single layer. In this stage, slimes are produced and irreversible attachment takes place. The third stage is the early vertical development of biofilm in which Extracellular Polymeric Substances (EPS) are also produced. The fourth stage is the production of multiple towers with channels between them. In this stage, maturing of the biofilm occurs by growing, formation of pores and water channels for transfer. The fifth and the last stage of the biofilm life cycle is the detachment of the mature biofilm and the dispersion of the biofilm-forming free swimming microorganisms to any other surface (Url-1, Costerton et al., 1995).

1.2.2 Infections and biofilm

Biofilm formation and metabolic activities within do not only has negative effect on device and system performance, it also cause serious health risks in community. Most of these health risks are nosocomial infections of gastrointestine, eye and ear etc., targeting patients with weaker immune system. It is now well-known that biofilm formation is an important factor in many diseases like native valve endocarditis, osteomyelitis, dental caries, middle ear infections, medical device-related infections, ocular implant infections, and chronic lung infections in cystic fibrosis patients. Bacterial infection in those implants have a potential of serious complications which generally ends up with premature implant removal. This situation is economically harmful and also might be lethal due to the nosocomial infections. (Gupta and Kumar, 2008).

Since microorganisms of biofilm easily and rapidly develop resistance to any condition (disinfectants, antibiotics and other stress factors), such contaminations require continuous, complex and combined treatment methods (Jefferson, 2004).

1.2.3 Quorum sensing

During the evolutional process, bacteria have evolved a wall in order to protect themselves from their enemies. This protection is related with their lifestyle which can be defined as biofilms and can be illustrated as attached to a surface, forming colonies in macro size surrounded with polymers which they produce biologically. This biofilm formation is regarded as a strategy for these bacteria to survive. This confederation provides bacteria some advantages, like increased tolerance and adaptation to several responses. Also, bacteria inside the biofilm structure can

communicate with each other with a special way called quorum sensing. For this communication, bacteria produce small signal molecules, which can diffuse into their environment and provide a concentration-dependent interaction with special receptor proteins. Quorum sensing is related with several vital processes like the control of expression of virulence factors, motility, protection, biofilm formation/maintenance etc. and is evolved to sense and monitor the population density among the bacteria (Bjarnsholt and Givskov, 2008).

QS is discovered in 1970s while studying the bioluminescence in deep-sea fishes (Bjarnsholt and Givskov, 2008). The most intensely studied quorum sensing system is that of the A bioluminescent marine bacterium, *V. fischeri* and its quorum sensing system is one of the most extensively studied system. This bacterium lives in a symbiotic relationship with several eukaryotic hosts. When *V. fischeri* cells are at a very high density, its host produces a special light organ. In this symbiotic relationship, the eukaryotic host provides *V. fischeri* a nutrient-rich environment for a living and *V. fischeri* provides light to its host that can be used for several specific purposes such as an antipredation strategy, or attracting a mate or a prey or warding off predators (Miller and Bassler, 2001).

1.2.3.1 QS systems of gram-negative bacteria

QS systems controlled by N-acyl-L-homoserine lactones (AHL), also known as autoinducers, are widely-studied and well-known examples. These molecules are widely conserved signal molecules that take place in the quorum sensing mechanisms of many gram-negative bacteria. The bacteria first release, then detect and respond to the accumulation of these signal molecules for the coordination of some cellular activities and synchronize the expression of some genes (Dong et al, 2002). These QS systems are involved in several functions and processes of the bacteria like plasmid conjugation, antibiotic production, virulence gene expression and surface motility. The AHL signal molecules are variable among the bacteria and some bacteria can produce more than one type of AHL. Despite these differences, all AHL molecules are composed of an acyl chain with a variable number of carbon atoms. This hydrocarbon backbone might have some replacements at specific positions like a hydroxyl- or oxo-group at the third carbon atom which is the most widely seen one. The acyl backbone is conjugated to a lactonized homoserine by an

amide bond (Bjarnsholt and Givskov, 2008). AHL quorum-sensing molecules are charming and attractive molecules to be used as target for genetic and chemical manipulation. These molecules are highly conserved by having the same homo-serine lactone moiety but differing in terms of the length and the structure of the acyl side chain. The general property of the AHL-mediated gene regulation process is a cell population (density)-dependent regulation named as the quorum sensing (Dong et al, 2002).

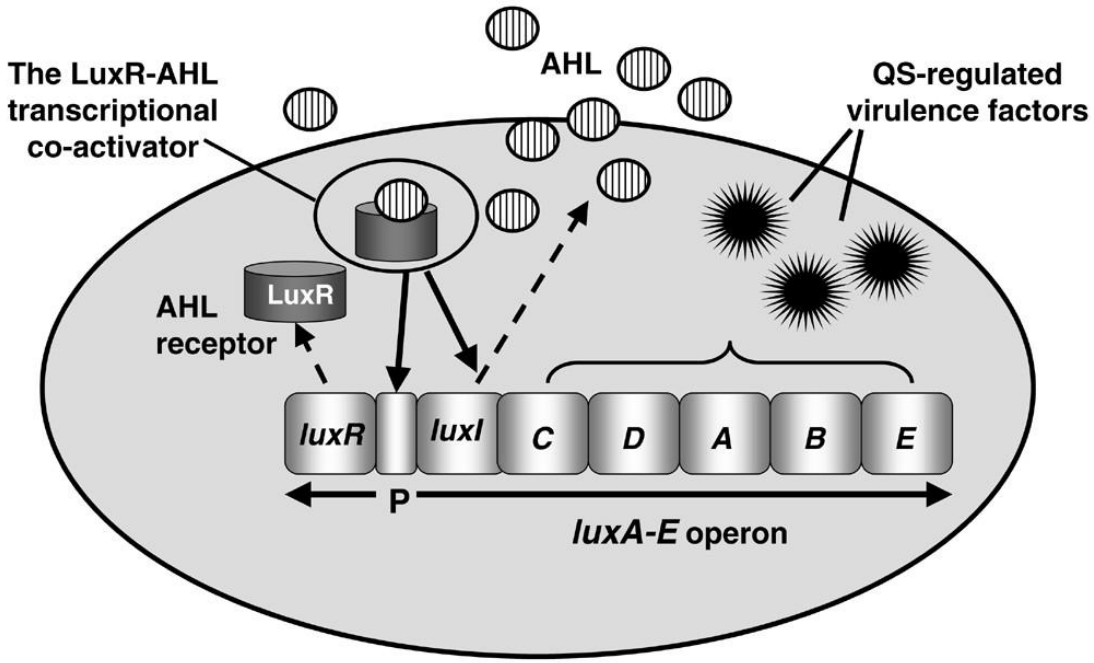


Figure 1.2 : A quorum-sensing model based on acyl homoserine lactone signaling systems (Asad & Opal, 2008).

1.2.3.2 QS systems of gram-positive bacteria

QS systems are also available in Gram (+) bacteria but it is different in terms of signal molecules when compared to the systems of Gram (-) bacteria. Instead of the AHL molecule-based system in Gram (-) bacteria, the QS system in these bacteria work by using small signaling peptides, which are variable in length. These peptides are cleaved and processed inside the cells and then moved out by active transportation. These transported peptides interact with transmembrane receptors of two-component regulatory systems and initiate a response inside the cell. When the density of the bacteria increases, the concentration of the signal peptides also increases and this results with the expression of the QS-regulated genes. For instance the formation of genetic competence and sporulation in *Bacillus subtilis* and the

expression of virulence genes in *Enterococcus faecalis* and *Staphylococcus aureus* are controlled with this QS-based system (Bjarnsholt and Givskov, 2008)

1.2.3.3 QS and biofilm formation

The first evidence of the relatedness with QS system and the biofilm formation was identified for *S. liquefaciens* and *Pseudomonas aeruginosa*. The reason for this relation relies on the advantages of the biofilm structure (Bjarnsholt and Givskov, 2008). The studies of Davies et al showed that a cell-to-cell signal is necessary for the development of a differentiated *Pseudomonas aeruginosa* biofilm which can be defined as the complex multicellular structures composed of individual cells. For this differentiation, the generation of signal molecules are vitally important because if the generation of these signal molecules are blocked, this becomes an obstacle for the differentiation causing an abnormal biofilm formation which is sensitive to outer effects like biocides. So, controlling the biofilm differentiation and its structural integrity by quorum sensing is really important regarding the effects of these biofilms on structures like medical devices or even in wounds or organs (Davies et al, 1998, Bjarnsholt and Givskov, 2008). Biofilm structure is advantageous and ideal for QS regulation of gene expression because the cells in biofilm structure are very close to each other. Bacteria in the biofilm structure have increased levels of tolerance towards antimicrobial agents when compared with planktonic structures. The biofilm structure has natural resistance to antibiotics and other biocides and biofilms in these environments are difficult to eradicate. So the inhibition of these cell-to-cell signals could be helpful in the treatment and removal of biofilm structure. When the QS system and regulation is blocked, biofilms are more susceptible or less tolerant to antibiotics, biocides and detergents. (Bjarnsholt and Givskov, 2008)

1.2.4 Strategies for the prevention of biofilm formation

Several methods exist to prevent biofilm formation on materials. Application of detergents and biocides on substrates are well known procedures and has been widely used. Unfortunately, these methods are not sufficient to prevent biofilm formation and sometimes they have detrimental effects to environment. Due to their hazardous effects, using of biocides in paints or coatings on metallic surfaces is restricted and hence, new strategies have to be considered to protect metallic surfaces from biofilm growth (Dafforn et al, 2011). If a biofilm structure is efficiently formed,

the microorganism in this biofilm structure can tolerate antimicrobial agents at very high concentrations, like 10 to 1000-times that needed to kill genetically equivalent planktonic bacteria. Besides, they are extremely resistant to phagocytosis and degradation, that causes biofilms to be very difficult to eradicate from living hosts (Jefferson, 2004)

In order to protect surfaces against microbial attacks, functional coatings are widely preferred when bulk properties of substrates needed to be conserved. A fine thin film coating should have resistence to corrosion, good adhesion to substrate and precise chemical control in order to meet the required functionality and permanence criteria. Sol-gel technology presents numerous advantages including high biocompatibility, non-toxicity, low-temperature processing and easy application to any kind of substrate. Moreover, sol-gel procedure is carried out in low temperature which gives considerable advantage for preparation of materials in industry (Gupta and Kumar, 2008).

1.2.4.1 Silver ions

Silver is thought to be an ideal candidate for coating devices for the prevention of biofilm formation because free silver ions at defined concentrations may be bactericidal but silver ions are toxic to human cell cultures (Schierholz et al, 2000). Silver ions have a well-known antibacterial mechanism. Metallic silver is inert and does not react with any human or bacterial cells. Besides, silver ions bind to and react with proteins and enzymes, causing structural changes in the bacterial cell wall and membranes and resulting a cellular disintegration and death of the bacterium. The effects of silver on bacteria include binding with cellular DNA, inhibiting enzymes taking place in the respiration and reacting with sensitive thiol groups on bacterial proteins that eventually malfunctions the basal biological activity of the protein (Stobie et al, 2009). Silver ions also bind to bacterial DNA and RNA, which causes an inhibition for the vital processes of the bacterium, however, it has no effect on the human body because there is an absorbtion of the excess silver by the necrotic tissue or the secretion in the urine (Bjarnsholt et al, 2007; Lansdown, 2002). Silver has been used in the past as coatings on many polymeric materials to prevent device-related infections (Kampf et al, 1998; Schierholz et al, 1998; Schierholz et al, 1999). The silver ion (Ag+) binds strongly to electron donor groups such as sulphur,

oxygen, or nitrogen in biological molecules. The silver ions can also act by displacing the other essential ions such as Ca++ or Zn. The antibacterial properties of silver coating were found to be dependent on the activity of silver ion (Ag+) and the rate of silver ions released, which apparently depends on the thickness of the coating. If the amount of silver released is too high, the coating will be cytotoxic. Moreover, Schierholz has reported that the minimum inhibitory concentration of silver towards *Staphylococci* can be up to 10 mg/ml (Schierholz et al, 1998).

Several *in-vitro* and *in-vivo* studies have been performed on several silver-coated catheters and silver-coated vascular prostheses during the last decade and randomised clinical studies have shown equivocal results for such coatings. The variable results for silver-coated surfaces and high concentration of silver ions required for bacterial inhibition and the leaching of silver ions from the surface, which might result in adverse effects to the host environment, such as metal ions accumulation in other body organs, making this coating clinically impractical (Kampf et al, 1998; Schierholz et al, 1998). In a cellular environment containing albumin and halide-ions, the antibacterial activity of silver ions will be decreased due to the specific absorption with albumin and precipitation into insoluble silver chloride. Metallic silver has only slight antibacterial effects because it is chemically stable. Silver-coated medical devices may only be clinically effective when the concentration of free silver ions can be increased and when contact to albumin and chloride ions, and in this case possible cytotoxic effects are also minimized. Due to their controversial clinical efficacy, silver coated medical devices are not well established in clinical use (Schierholz et al, 1998).

1.2.4.2 Enzymes

One of the solutions to overcome the problem with biofilm formation could be the replacement of biocides with non-toxic alternatives, such as enzymes (Kristensen et al., 2008). Enzymes have been used in several industries ranging from food industry to large scale biocatalysis and can also be used for the degradation and the removal of the bacterial biofilms (Orgaz et al., 2006; Kristensen et al., 2008, Leroy et al., 2008). Extracellular Polymeric Substances (EPS) in the biofilm matrix is the essential part of the biofilm development. The complexity and variability of biofilm polymers in the matrix described above could be the utility to use several enzymes

like hydrolases and lyases, individually and/or their combinations. This could achieve a sufficient disintegration of the polymeric networks composing the biofilm matrix and detachment of the biofilm from the surface it was attachhed (Kristensen et al., 2008, Leroy et al., 2008). Besides these enzymes, some enzymes that specifically blocks the bacterial cell-to-cell communications can also disable the bacterial population-density-dependent attack. This is called quorum-quenching mechanism that can be used as a strategy in controlling bacterial pathogens and to build up a proactive defense barrier (Zhang, 2003). Examples to these enzymes are AHL Lactonase and AHL Acylase whose degradation mechanism for AHL can be seen in Figure 1.3. Dong and his colleagues have reported cloning of a gene named as *aiiA*$_{240B1}$, which codes for a novel AHL-inactivating enzyme named as AiiA$_{240B1}$ from the gram-positive bacterium *Bacillus* sp. Strain 240B1. This enzyme inactivates the activity of AHL by hydrolyzing the lactone bond of the molecule. This gene was also transformed to *E. carotovara* strain SCG1 and successfully expressed in this plant pathogen to show that the enzyme produced by this gene significantly reduces the release of AHL signal molecules and the extracellular pectrolytic enzyme activities were also diminished attenuating its pathogenicity to several plants it can act on (Dong et al, 2000). It was also shown that transgenic plants expressing AHL lactonase enzyme show remarkably enhanced resistance to *E. carotovara* infection and attenuated the sypmtoms of the diseases caused by this plant pathogen (Dong et al, 2001). Leadbetter and Greenberg have reported that they have isolated a motile, rod-shaped bacterium from the soil, which was later classified as a strain of *Variovorax paradoxus*, and it had the ability to use N-(3-oxohexanoyl)-L-homoserine lactone molecule as the sole source of energy and nitrogen. Bacteria which synthesize AHL molecules do not degrade them and these molecules are stable at neutral or acidic pH in aqueous solutions. Besides, the homoserine lactone ring is subject to alkaline hydrolysis. Enzymes degrading AHL molecules are therefore important as they could be used commercially for controlling cell-to-cell signaling. As these molecules are stable under acidic conditions, biological degradation of these molecules might be an important tool for maintenance of these signals at low concentrations (Leadbetter and Greenberg, 2000). Lin and his colleagues have reported that they have cloned and expressed a gene which encodes an AHL-acylase from a *Ralstonia* species, strain XJ12B, isolated from a biofilm structure composed of mixed species. The gene responsible for the signal

inactivation in this bacteria, named as *aiiD*, was cloned and expressed in *Escherichia coli*. Their studies have shown that this expressed gene has inactivated three AHL molecules tested by hydrolysing the AHL amide structure, releasing the homoserine lactone and the corresponding fatty acid structure. They have also shown that expression of AiiD in *Pseudomonas aeruginosa* has resulted in quenching of the quorum sensing action of this bacterium and also decreased the swarming ability, elastase and pyocyanin production and the nematode-paralysis action of the bacterium (Lin et al, 2003).

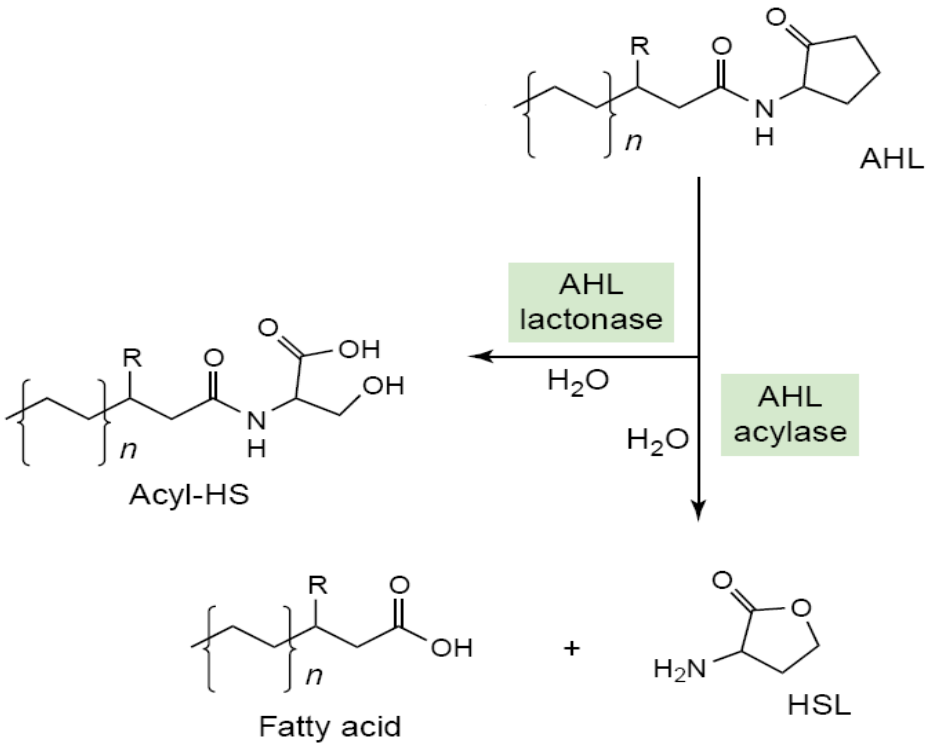


Figure 1.3 : Reactions of AHL Acylase and AHL Lactonase (Zhang, 2003).

1.2.4.3 Furanones

Furanones act as quorum sensing inhibitors (QSI) via intracellular pathways when they are free on the environment or they show their activity when coated on surface. Azide-nitrene chemistry iz utilized for this coating process because furanones do not have reactive chemical substituents and they contain labile lactone ring, which is essential for their antibacterial effectiveness, under both acidic and alkaline conditions that prevent the use of selective and well known immobilization techniques. Azide-nitrene chemistry is compatible with all the furanone compounds

and does not need the existence of reactive groups in the molecular structure of furanone (Al-Bataineh et al, 2006)

Furanone molecules are a part of the chemical defense system, built up by the marine alga, *Delisea pulchra*, which prevents colonization on their surfaces through production of halogenated furanones, reside in vesicles on the surface of the organism. In previous works these compounds and their analogues have been shown to inhibit or prevent growth of both gram negative and gram-positive bacteria (Read and Kumar, 1999; Kjelleberg et al, 1999; Read et al, 2001)

The rationale of the use of these novel molecules are environmentally safe and non-toxic to mammalian cells (Baveja et al., 2004) and act more specifically than the traditional methods.

When the biofilm-forming bacteria is treated with QSI molecules, the concentration of activated receptor proteins is kept at a minimum level, although the density of the bacterial population and signal molecule concentration increase and this prevents the expression of QS-regulated genes. Due to this, bacteria are kept in a harmless and attenuated state. These QSI molecules include synthetic halogenated furanone compounds (Figure 1.4a and 1.4b), penicillic acid (Figure 1.4c) and patulin (Figure 1.4d) which is produced by certain fungi (Bjarnsholt and Givskov, 2008).

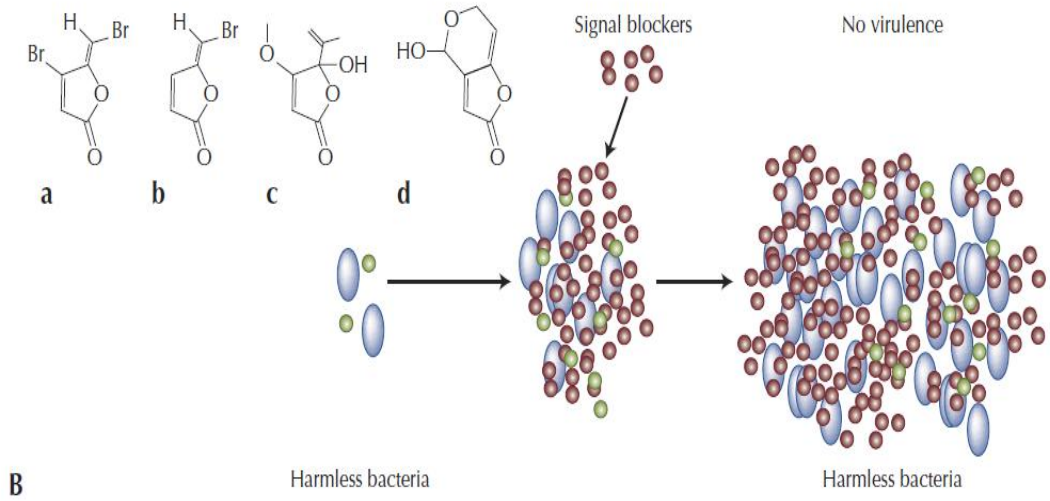


Figure 1.4 : The principle of QS Inhibitors (Bjarnsholt & Givskov, 2008).

1.3 Sol-Gel Technology

Sol-gel technology is a combination of different scientific disciplines and so it is a very useful improvement in science with several applications. Shortly, it can be defined as the process of producing doping scaffolds for several molecules like inorganic, organic and biomolecules by forming a glassy matrix (Gupta and Kumar, 2008). Recently, sol-gel technology has been studied with an increased attention as a new method for the development of modified materials and surfaces as well as the fabrication of optical materials, semiconducting devices, colloidal powders for chromatography applications, hydrogen storage materials, biomedical device coatings, chemical sensors and electrochemical biosensors (Gupta and Kumar, 2008, Yang et al., 2003). This technology has several advantages like simplicity, versatility, inertness, mechanical stability and applicability of chemical or biological modification (Yang et al., 2003). The reasons of sol-gel technology to be used in so many different applications arises from the processing advantages of the technology (Gupta and Kumar, 2008) and also the porosity of the of the matrix (Yang et al., 2003). The application of the technology requires low temperature and its biocompatible and enviromentally-friendly nature makes it ideal for producing bioactive materials. Also, sol-gel technology enables the manipulation and the control of the structure of materials at molecular level which makes it paractical for several applications. Another advantage of the sol-gel derived layers is that they provide a large and active surface area which can be utilized for functionalizing the surface by appropriate biomolecules. Materials obtained via sol-gel technology have become the useful biomaterials for several applications by getting mechanically stronger, biocompatible and bioactive materials like metallic implants with diminished corrosion (Gupta and Kumar, 2008).

As the name implies, sol-gel methodology is the transition of a system from a liquid “sol” phase to a solid “gel” form. This percursor solution is mostly prepared by using silica-based chemicals like tetramethyl-orthosilicate (TMOS) and tetraethyl-orthosilicate (TEOS) (Gupta and Kumar, 2008). Covalent bonding of any molecule to the sol-gel silicate netwotk is accomplished with this suitable functional group (Yang et al., 2003). The initial reaction begins by combining these metal alkoxides with water and a solvent, which is mostly an alcohol, in the presence of a catalyst, which can be either an acid or a base. Once this initial reaction is activated,

simultaneous hydrolysis and condensation reactions take place. Hydrolysis results with the formation of the silanol groups (Si-OH) whereas condensation leads to the production of siloxane bonds (Si-O-Si), releasing alcohol and water as the by-products. These chemical reactions and different processes have major effects on the final gelated product. Thin films can be fabricated on the material by using dip, spin and spray coating techniques. In the transition state from sol to gel phase, the viscosity of the solution increases gradually to form a rigid and porous network structure. Following the gel formation, further processes like heating and drying helps to obtain dense ceramic or glass particles or matrices which can be used for the entrapment or immobilization of different inorganic and organic molecules, including biomolecules (Gupta and Kumar, 2008).

1.4 Pseudomonas aeruginosa

Pseudomonas aeruginosa is a gram-negative opportunistic human pathogen that causes disease which mostly affects the immunocompromised people, having cystic fibrosis or suffering from intense wound caused by burning (Reimmann et al, 2002). The taxonomical classification of *Pseudomonas aeruginosa* is given at Table 1.1. They are proteobacteria under the class of gamma proteobacteria. Some of these opportunistic conditions show up when normal mucosal barriers have been somehow breached or bypassed, when immunologic defense mechanisms have been weakened, when the protective function of the normal bacterial flora has been disrupted by broad-spectrum antibiotic therapy, and/or when the patient has been exposed to reservoirs in a hospital environment (Shirtliff et al, 2002).

The bacterium controls its virulence by cell density via the diffusible signal molecules, also called as autoinducers, named as N-acylhomoserine lactone (AHL) molecules. It utilizes two N-acylhomoserine lactone (AHL)-dependent quorum sensing systems, identified as *las* and *rhl*, that controls the production of population density and growth-phase-dependent virulence factors. This cell-to-cell communication is vital for the virulence of this pathogen by controlling the production of extracellular virulence factors and toxic secondary metabolites through a complex regulatory cascade involving two autoinduction systems which is also known as quorum sensing (Reimmann et al., 2002). *Pseudomonas aeruginosa* produces and responds to two different AHL molecules. One of these molecules are

3-oxododecanoyl-homoserine lactone (3OC12HSL), which is the signal molecule of the *las* quorum-sensing system and also named as PAI. The second molecule is butanoyl-HSL (C4HSL), which is the signal molecule of the *rhl* quorum-sensing system and also named as PAI-2. These two quorum sensing mechanisms take role in the control of several physiological activities and virulence factors which are related with the infection of immunocompromised people and the ones suffering cystic fibrosis (Huang et al., 2003).

Table 1.1 : Taxonomical classification of *Pseudomonas aeruginosa* (Url-2).

Domain	Bacteria
Phylum	Proteobacteria
Class	Gamma proteobacteria
Order	Pseudomonadales
Family	Pseudomonadaceae
Genus	Pseudomonas
Species Group	Pseudomonas aeruginosa group

1.5 Lysozyme

Lysozyme (EC 3.2.1.17) is a hydrolytic enzyme that hydrolyse the β-(1,4) glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine in peptidoglycan, which is the major cell wall polymer of the Bacteria. Due to this hydrolytic activity, lysozymes are involved in bactericidal mechanisms of several organisms like fungi, protozoa, plants, invertebrate and vertebrate animals and even bacteriophages like T4 (Callewaert et al, 2008; Düring et al, 1999).

Lysozyme was discovered by Alexander Fleming in 1922 by accident. The nasal drippings were accidentally occurring in the petri dish with bacterial culture and these cells were lysed. This phenomenon was carefully investigated and the main acting enzyme was identified as lysozyme. Lysozyme belongs to the hydrolases (EC 3.-.-.-) enzymatic class and within the class of hydrolases, lysozyme belongs to the Glycosylases family (EC 3.2.-.-).

Lysozyme reaction is the hydrolysis of the beta (1-4) glycosidic bond between N-acetylglucosamine sugar (NAG) and N-acetylmuramic acid sugar (NAM) and therefore it is possible classify it as Glycosidases; enzymes hydrolyzing O- and S-glycosyl (EC 3.2.1.-) with number 17 (EC 3.2.1.17) in this group (Url-3).

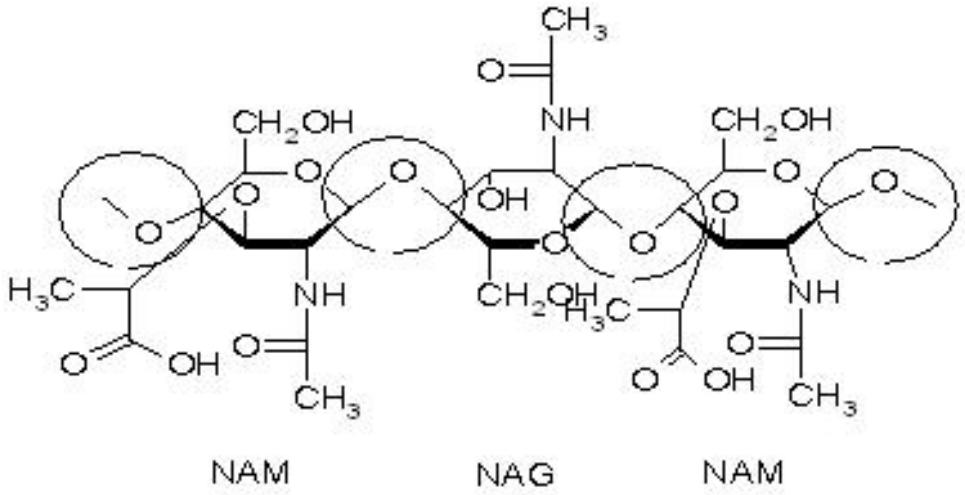


Figure 1.5 : The substrate of lysozyme (The beta (1-4) glycosidic bond between N-acetylglucosamine sugar (NAG) and N-acetylmuramic acid sugar (NAM) to be hydrolysed during the lysozyme reaction are circled) (Url-3).

Most of the Gram-negative bacteria are not affected by the action of lysozyme alone due to the stability of their outer membrane, which avoids the reaching of the enzyme to the peptidoglycan layer. Nevertheless, animals get over this problem by manufacturing additional antibacterial proteins to permeabilize the outer membrane of the bacteria. Lactoferrin is an example of this. Additionally, some of the naturally found lysozymes and the chemically or genetically modified hen egg white lysozyme have been shown to be effective against some of the Gram-negative bacteria without this kind of permeabilizers (Callewaert et al, 2008).Research on heat-denatured hen egg white lysozyme also showed an increased bactericidal effectiveness against Gram-negative bacteria in which the enzyme is partially unfolded, enzymatically inactive and in a hydrophobic dimeric form. This bactericidal activity is based on the insertion of the dimeric form to the membrane of the Gram-negative bacteria and a successive disruption of the membrane (Düring et al, 1999).

2. MATERIALS AND METHOD

2.1 Materials

3-aminopropyltrimethoxysilane (APTMOS, 97%), Methyltrimethoxysilane (MTMOS, 98%), hydrochloric acid %37, methanol, sulfuric acid 95-97% (analytical grade) and potassium bichromate were purchased from Merck. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and poly(acrylic acid) solution (average Mw ~250,000, 35 wt. % in H2O) (PAA) was purchased from Sigma-Aldrich. 316 L stainless steel (SS) coupons (1cm in diameter, 0.2 cm in thickness) were purchased from Gama Metallurgy. Mica sheets (grade V-4, 1 cm in diameter) were purchased from SPI. *Pseudomonas aeruginosa* PAO1 strain (ATCC 15692) was supplied by Arçelik A.Ş. Hygiene Research Center from ATCC, USA. Lysozyme from chicken egg white (specific activity 24700 U/mg) was purchased from Applichem. pSMC21 vector was kindly provided by George A. O' Toole from Darthmouth Medical School.

2.2 Cleaning and Pretreatment of Stainless Steel Coupons

316L stainless steel (SS) coupons (1cm in diameter, 0.2 cm in thickness), purchased from Gama Metallurgy, were cleaned and pre-conditioned according to literature methods (Minier et al., 2005; Chovelon, 1995). The coupons were ground with SiC sandpaper and polished using a 6 μm diamond suspension and rinsed with ethanol, then ultrasonically washed 15 minutes in cyclohexane, 10 minutes in water (three times) then 20 minutes in acetone. They were etched by sulfochromic acid (6 gr of potassium bichromate, Merck, in 100 ml of sulfuric acid 95-97%, Merck) at 60°C for 10 minutes to generate a reactive oxide/hydroxide layer (SS-SC). They were extensively washed with water and dried under a flow of nitrogen.

2.3 Preparation of Stock Sol–gel Solution and Spin Coating

A homogeneous stock sol–gel solution was prepared for the application of spin coating. 20 ul of this solution was applied on the coupon, so the stock solution was prepared according to number of coupons to be coated. For this, 30 volumes of methanol (Merck), 1 volume of 10 mM HCl (Merck), 30 volumes of MTMOS (Merck) and 20 volumes of APTMOS (Merck) (Yang et al., 2003) was mixed in a tube, applied on the middle of the previously polished coupons and spin-coated at 2000 rpm for 30 seconds at room temperature. After coating the coupons, they were dried at 100°C for 1 hour (SS-SC-Sol).

Sol-gel coating studies were also performed by using different polymers for further surface immobilization of enzymes. These polymers were polyethylene, polypropylene, polyvinyl chloride and polyether polyurethane. Before sol-gel coating trials, a surface etching procedure called "flame etching" was performed. The polymer coupons were passed over the flame of a Bunsen burner, however, the surface of some of the coupons have melted already due to the flame. So, flame etching procedure were performed by passing the coupons over the flame very very quickly. For comparison, some of the coupons were used without performing the flame etching procedure. The results of the sol-gel coating was not satisfactory due to instability of the coating on all of the coupons. After the drying step, the thin coating on the polymer surfaces stood up and the coating was not performed on these polymers, so the further studies for enzyme immobilization were performed by using stainless steel coupons.

2.4 Immobilization of Lysozyme Molecules

Following Lysozyme from chicken egg white (specific activity 24700 U/mg) was purchased from Applichem. Covalent immobilized of the enzyme was carried out on the sol–gel coated stainless steel coupons by using the free amine groups exposed on the surface of the sol–gel layer by carbodiimide chemistry. The sol–gel coated coupons were placed in a phosphate buffer solution (0.05 M, pH 6.2) which contains 0.015 M EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, Sigma-Aldrich) and 0.03 M NHS (*N*-Hydroxysuccinimide**,** Merck) for 90 minutes, and immediately transferred to an enzyme solution of 10 mg/ml lysozyme prepared in the same buffer solution for another 90 minutes. Following the coating steps, the

enzyme-coated coupons (SS-SC-Sol-Lys) were rinsed with phosphate buffer solution, pH 6.2, in order to remove the excess unbound and adsorbed enzyme. All the steps for enzyme immobilization experiments were carried out at room temperature (Yang et al., 2003).

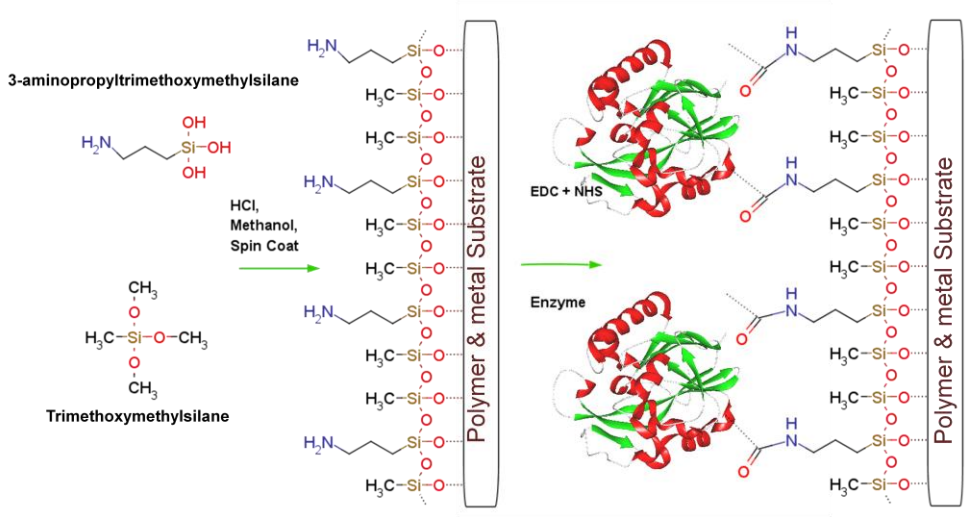


Figure 2.1 : Schematic representation of lysozyme immobilization procedure.

2.5 Surface Characterization

2.5.1 AFM

AFM scanning of the samples were performed in air at room temperature using Ntegra Vita (NT MDT, Zelenograd, Russia). The device was operated in semi-contact mode and images were obtained using silicon cantilever Tap300 Al-G (BudgetSensors) with resonance frequencies of 200-400 KHz, force constant 40N/m. For each sample, several AFM scans (typically 1 x 1 µm) were made to check the surface uniformity. 2-D Fourier transforms of the images were obtained by using FFT analysis software tool and the surface root –mean square roughness which represents the standard deviation of the heights expressed in a three dimensional map were calculated for all images representative of the different coating steps.

2.5.2 FTIR

FT-IR analyses were conducted using Perkin Elmer Spectrum One FT-IR spectrometer equipped with an attenuated total reflectance device. Coated steel surfaces and corresponding compounds for comparison were placed onto a ZnSe

crystal with a 45° mirror angle. All analyses were performed with a resolution of 8 cm-1 using 400 scans.

2.6 Antibacterial Activity of Immobilized Lysozyme Molecules

Lysozyme solutions with different concentrations (0.01 mg/ml, 0.1 mg/ml, 1 mg/ml, 5 mg/ml and 10 mg/ml) were used to obtain the optimum coating solution concentration with different amine-exposing coupons. The enzymatic activity of these lysozyme-coated stainless steel coupons was measured on Micrococcus lysodeikticus cells using a procedure adapted from the classical lysozyme assay (Shugar, 1952). Substrates coated by lysozyme were placed in separate test tubes and covered with 3 ml of a 0.015% (w/v) suspension of M. lysodeikticus diluted from lyophilized bacteria in 66 mM phosphate buffer pH 6.24 and the tubes were placed on a shaker. Monitoring of enzymatic activity was carried out over a period of 6 hours (two more measurements were done at 18th and 24th hours) by taking 1 ml of each suspension every 15 min and the turbidity was measured at 450 nm after which the suspension was put back into the reactor. Two control experiments were carried out to measure non-enzymatic bacterial lysis (= autolysis), whereby the turbidity of a stirred bacterial suspension alone and in the presence of an SS-SC coupon which was not coated with lysozyme was monitored.

2.7 Biofilm Studies on Flow Cell and Their Detection by Using CSLM

2.7.1 Electroporation of *Pseudomonas aeruginosa* PAO1 cells

Pseudomonas aeruginosa PAO1 cells (ATCC 15692) purchased from ATCC was inoculated in Tryptic soy broth medium and were grown at 37 ºC for 16 hours. Following overnight incubation, this culture was centrifuged at 12.000 g for 3 minutes. The cell pellet was washed twice with 300 mM sucrose solution and the pellet was resuspenden in 100 µl of mM sucrose solution carefully. This resuspension of cells was mixed 2 µl (approximately 500 ng) of pSMC21 vector (which was kindly provided by George A. O' Toole from Darthmouth Medical School) and transferred into the electroporation cuvette. 1800 Volts of electricity was applied for the entrance of the vector DNA into the PAO1 cells and the cells were incubated in 1 ml of Tryptic Soy Broth at 37 ºC for 2 hours. Following the

incubation, the cells were diluted with different dilution rates, varying from 10^0 to 10^{-5},spreaded on Tryptic Soy Agar plates including 100 µg / ml ampicillin and 500 µg / ml kanamycin antibiotics and incubated at 37 ºC for 24 hours. Following overnight incubation, colonies were observed on agar plates and some of the single colonies were picked by sterile pipette tips and transferred to 5 ml of Tryptic Soy Broth and incubated at 37 ºC for 24 hours. Growing culture of the cells from single colonies were used for plasmid DNA isolation by using "Roche High Pure Plasmid DNA Isolation Kit". Isolated plasmid DNA replicates were visualized on an agaores gel of %1 concentration.

2.7.2 Biofilm formation and detection studies with CSLM

Single colonies of PAO1 cells containing the pSMC21 vector were used for the first inoculation of the flow cell. 200 µl of the cells were injected inside the flow cell mechanism. For the first hour, no medium flow was given into the flow cells in order to provide the adaptation period and static growth for the cells. When the cells were visualized on the surface of the coupons and composed the initial attached state of the biofilm, they were incubated at 37 ºC for another 24 hours with medium flow over the coupons. The rate of the flow was 3 ml per hour and the cells were observed by using confocal laser scanning microscope.

In order to visualize the PAO1 cells and possible biofilm structures, Leica TCS-SP2 confocal scanning laser microscope was utilized. 10X/1.4 NA dry objective lens was used with 488 nm Argon laser and 515-540 nm emission filters. These filters were appropriate for the visualization of green fluorescent protein (GFP).

Preliminary studies were also performed for the biofilm formation on stainless steel coupons and to visualize it by using Confocal Scanning Laser Microscopy. For the detection of biofilm formation, *Pseudomonas aeruginosa* PAO1 cells were transformed by using pSMC21 vector (kindly provided by George A. O'Toole from Dartmouth Medical School) which contains Green Fluorescent Protein gene with kanamycin and ampicillin resistant genes. Following transformation, these cells were used for the formation of biofilm on the coupons and were visualized by CSLM.

2.8 Alternative Method for the Immobilization of Lysozyme on Sol-gel Coating

As an alternative method for the immobilization of lysozyme, PAA brushes were utilized as a linker between the sol-gel network and lysozyme (Figure 6). Sol-gel coated coupons which were prepared and used for the covalent immobilization of lysozyme by utilizing their free amine groups on their surfaces were reacted with 0.1 w/v% aqueous solution of PAA (average Mw ~250,000, 35 wt. % in H_2O, Sigma-Aldrich) at pH 4 in order to obtain a PAA layer on their surface. Immediately after immersing the coupons, 5 mg/ml of EDC was added. The sample was incubated overnight (16 h) at 4°C with gentle shaking. The coupons were then extensively rinsed with water to remove non-covalently adsorbed PAA (SS-SC-Sol-PAA). Remaining carboxyl groups were then utilized to immobilize lysozyme molecules. These stainless steel coupons with carboxyl groups were placed in a phosphate buffer, pH 6.2 and then 0.1 M EDC and 0.1 M NHS were added. The coupons were kept in this buffer for the activation reaction to take place for 30 minutes. (Cullen, 2008). Following the activation, the coupons were rinsed and reacted in a 10 mg/mL lysozyme solution in phosphate buffer, pH 6.2 for 90 minutes at room temperature. Lastly, the coupons were again rinsed with phosphate buffer to remove excess unbound/adsorbed enzyme and dried under a flow of nitrogen (SS-SC-Sol-PAA-Lys).

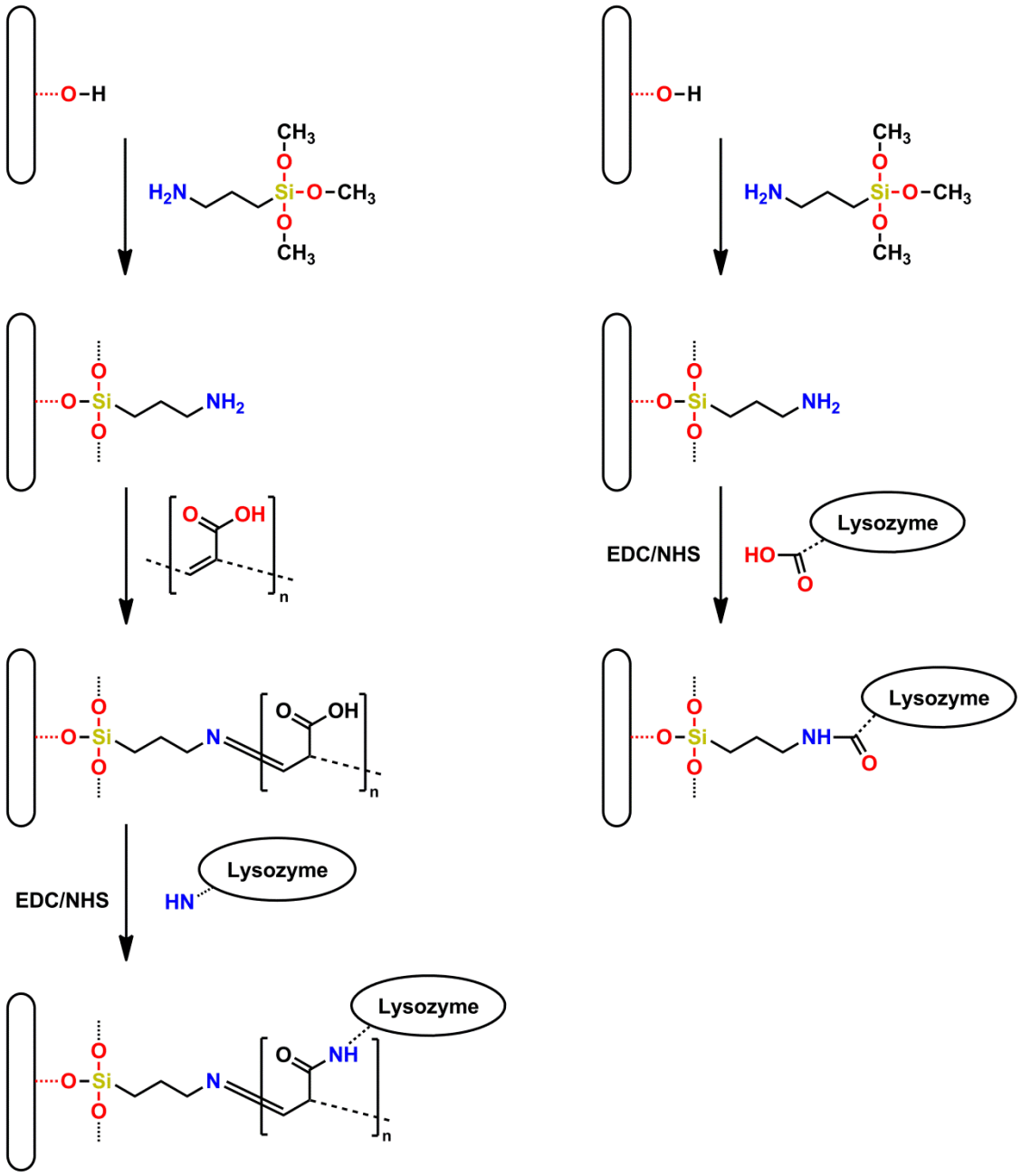


Figure 2.2 : Reaction scheme for the immobilization of lysozyme on steel surface. The amine groups in the sol-gel network are utilized for the addition of carboxyl groups and then the covalent binding of lysozyme from its amine-end (left) or for the direct immobilization of lysozyme from its carboxyl-end (right).

3. RESULTS AND DISCUSSION

3.1 Optimization of Sol-Gel Coating

The first step before the sol-gel coating of the coupons were to clean them and polish them to nanometers-scale. This was performed with SiC sandpaper and polishing diamond suspensions. Following polishing, the coupons were rinsed and washed ultrasonically with different polar and non-polar solvents, including absolute ethanol, cyclohexane, deionized water and acetone. After cleaning the coupons, they were etched by sulfochromic acid solution which is a harsh reactive containing potassium bichromate and sulfuric acid, which initiates the formation of a reactive oxide/hydroxide layer. The coupons were then cleaned with water by rinsing several times dried under a flow of nitrogen. These pretreated coupons were coated with a thin layer of silane film by using sol-gel technology. For this purpose, two silane monomers were used in the studies; 3-(Trimethoxysilyl)-propylamine (3-aminopropyl trimethoxysilane, APTMOS) and Trimethoxymethylsilane (Methyltrimethoxysilane, MTMOS). The amine (NH_2) group in the first monomer provides region for the covalent immobilization of the enzyme (lysozyme). Several different trials were performed (by using different solvents, molar ratios, mixing etc.) for the optimization of sol-gel coating solution. An example of an experimental trial bu using different silane monomer ratios can be seen in Table 3.1. These stock solutions were applied on the previously polished coupons and spin-coated at 2000 rpm for 60 seconds at room temperature. Following the coating, coupons were dried at 100°C for 1 hour. These dried coupons were checked for the stability of the thin films applied on the coupon surfaces. When the ratio of the MTMOS over APTMOS was greater than 2, the stability of the stability of the thin film failed to stay on the coupon surface. Thin film over these coupons were taken off the surface after drying

so further experiments were performed with the ratio of the MTMOS over APTMOS as 1.5.

Table 3.1 : Different APTMOS:MTMOS ratios used in the optimization studies

	1:1	1:2	1:4	1:9	1:99	1:999
Methanol	30 µl	30 µl	30 µl	30 µl	30 µl	30 µl
HCl (10mM)	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl
MTMOS	25 µl	33,3 µl	40 µl	45 µl	49,5 µl	49,5 µl
APTMOS	25 µl	16,7 µl	10 µl	5 µl	0,5 µl	0,5 µl

3.2 AFM Analysis of Stainless Steel Coupons

AFM imaging was utilized in all the steps of the coating procedure. Pretreated (uncoated) coupons, sol-gel coated coupons and sol-gel-coated and then lysozyme-coated coupons were analysed separately to observe the surface differences between these stainless steel coupons. As it can be seen from the figure obviously, uncoated stainless steel coupon has several scratches resulting from the polishing steps (Figure 3.1). However, in Figure 3.2, it can be seen that these scratches were completely covered and filled with sol-gel film. This was an expected result as the polishing steps are harsh steps causing scrathes on the stainless steel surface. These scratches were formed as nanometer-scales and with the aid of AFM-imaging these scratches were obviously visualized. The application of the sol-gel procedure acted as a filling material for these scrathes and also an interface for the immobilization of other chemicals, here the lysozyme molecules. The application of sol-gel coating also changed the surface toplogy of the coupons. The average surface roughness of the coupon decreased from 6.7 nm to 3.2 nm after the sol-gel coating of the stainless steel coupons. In Figure 3.3, the AFM image of the sol-gel coated coupon, which was later used for lysozyme immobilization, can be seen. The AFM imaging shows that the surface of these coupons were totally changed after lysozyme coating step. Possible lysoyme molecules are visualized on the surface of the coupon as a layer of small dots. The physical size of the lysozyme enzyme is defined as 3.36 nm in the

literature (Minier et. al, 2005). Although it is difficult to obtain this datum from the AFM image precisely, the size of the lysozyme enzyme is in a range of 3-4 nm

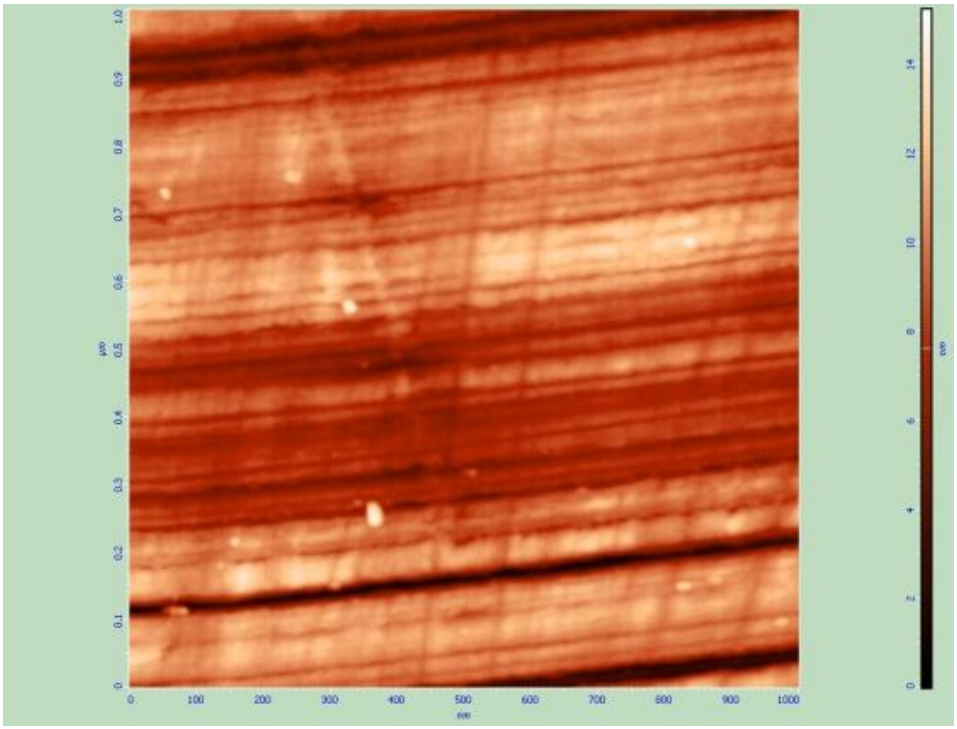


Figure 3.1 : Uncoated (Pre-treated) Stainless Steel Coupon.

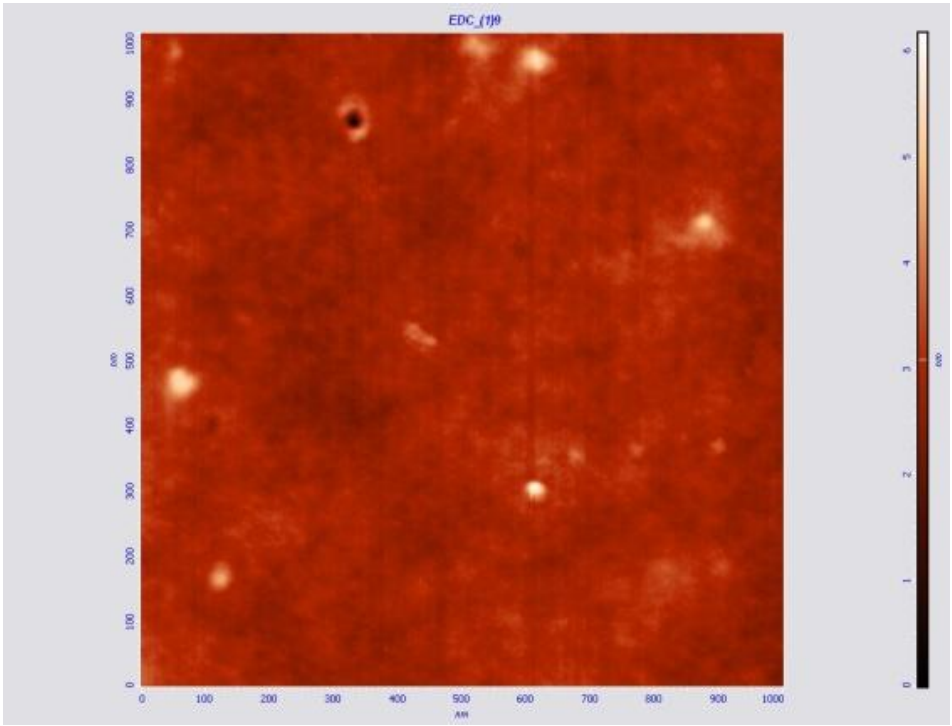


Figure 3.2 : Sol-gel coated, EDC-NHS Treated Coupon.

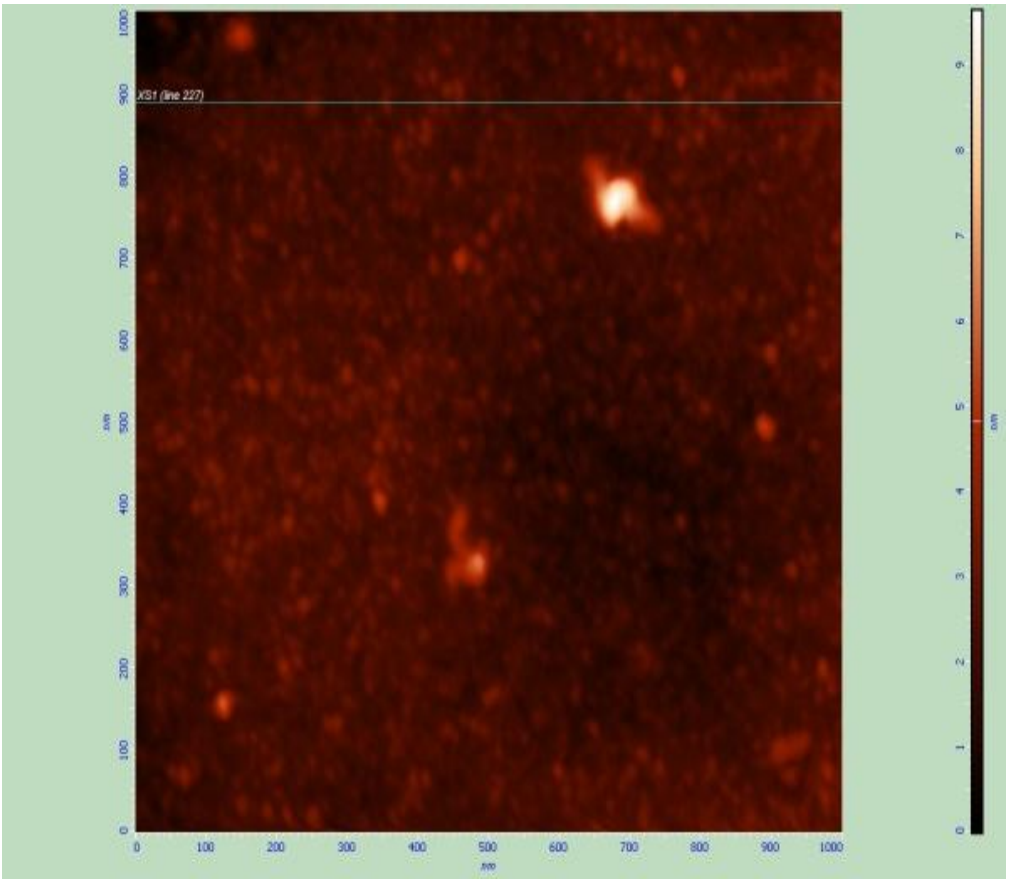


Figure 3.3 : Lysozyme-Coated Coupon.

3.3 FTIR Spectroscopy Analysis

FT-IR spectroscopy analysis was also performed on lysozyme coated coupons. The results of these analysis can be seen in Figures 3.4, 3.5 and 3.6 and 7. The peaks at 1564 and 1486 from sol-gel coated coupon are slightly shifted in sol-gel plus enzyme coated coupons through at 1633 and 1538 and those latter peaks correspond to amide I and amide II bands of the enzyme. The peaks can be seen at 1000 from Si-O bands, and a large peak between 3500 and 2500 is due to C-H and N-H bonds of sol-gel and enzyme.

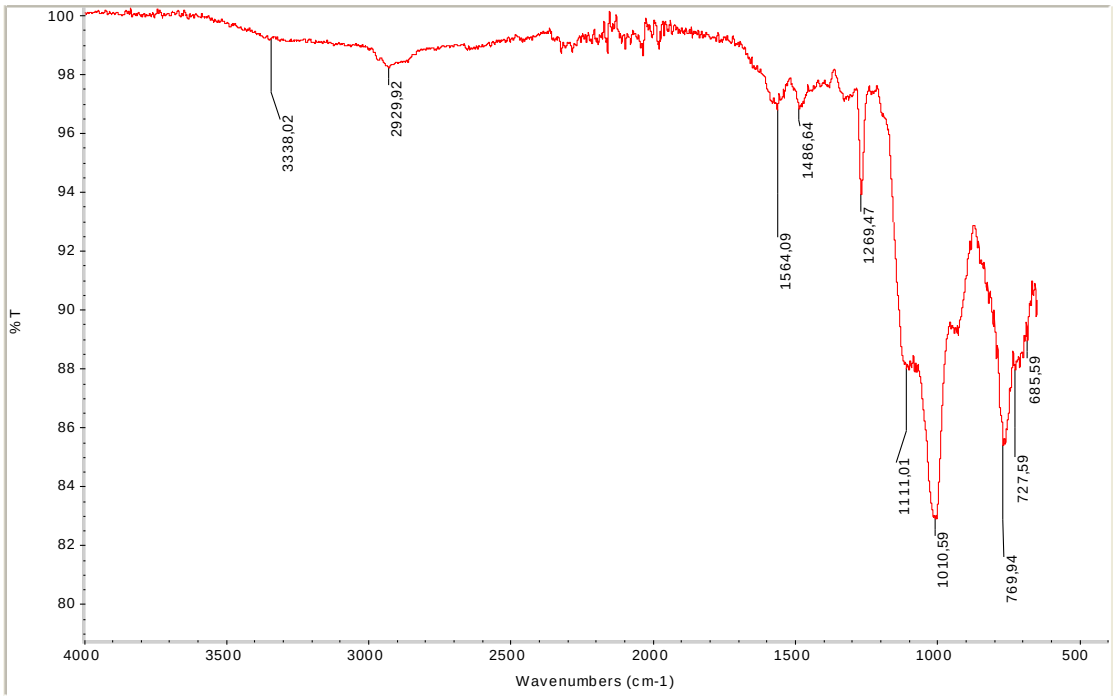


Figure 3.4 : FT-IR analysis result of the sol-gel coated stainless steel coupons.

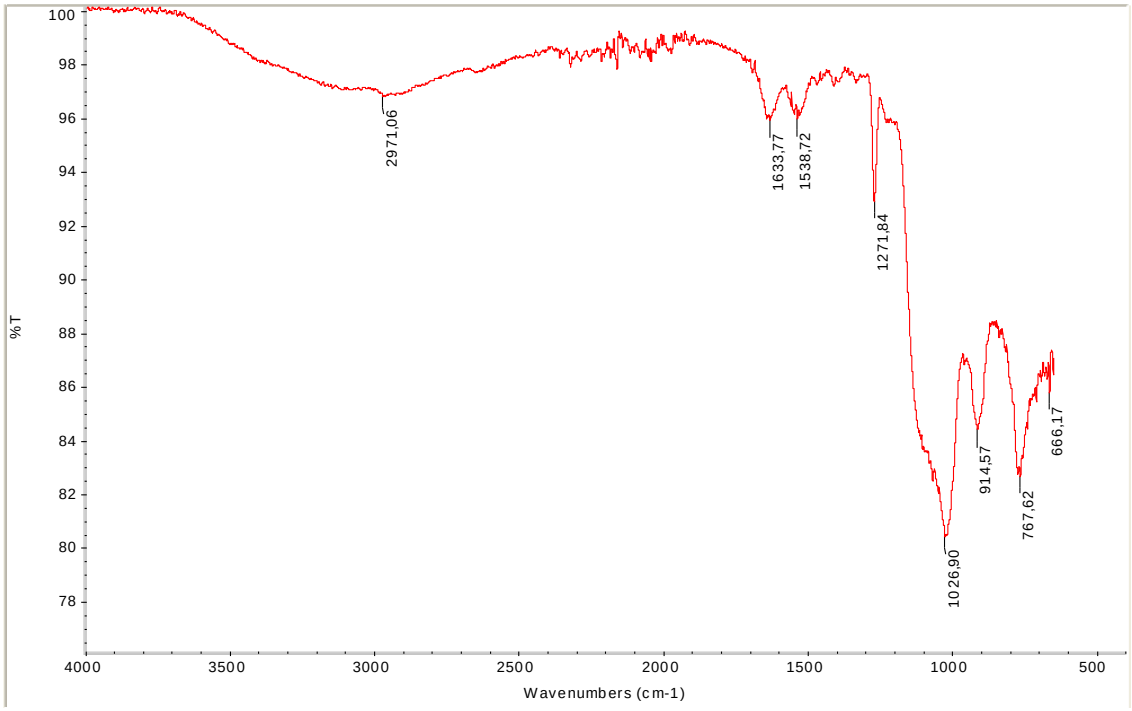


Figure 3.5 : FT-IR analysis result of the sol-gel and lysozyme coated stainless steel coupons.

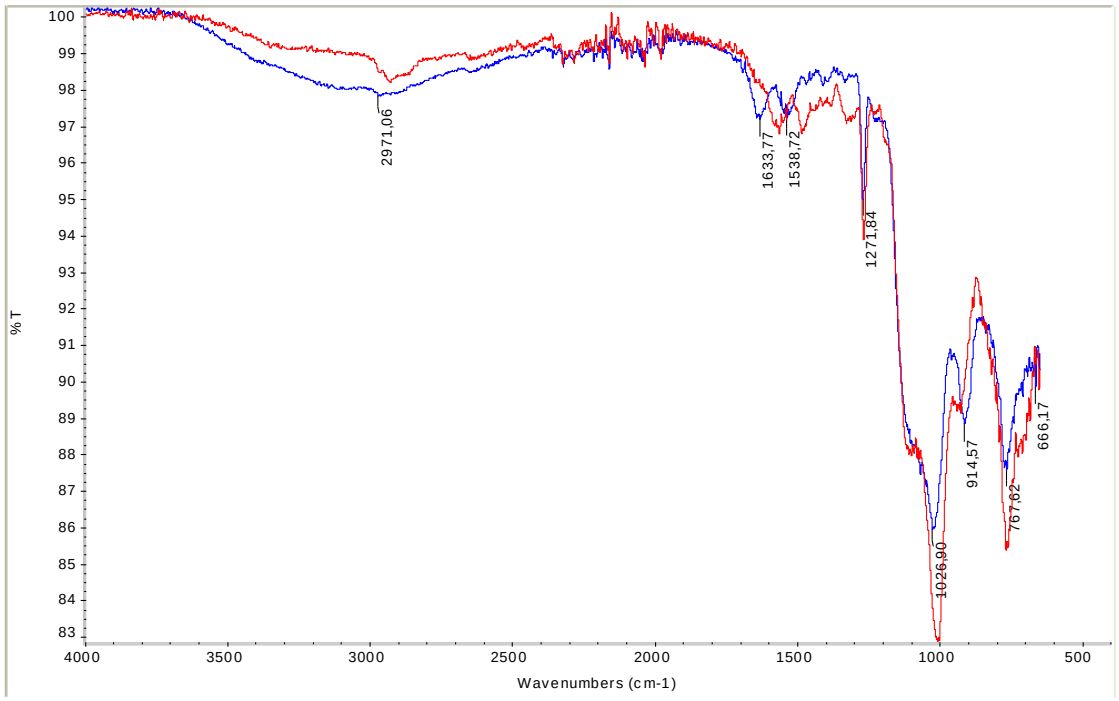


Figure 3.6 : The graph obtained by overlapping the two analysis results. The red lines belong to the sol-gel coated coupon and the blue lines belong to the sol-gel and lysozyme-coated coupon.

3.4 Analysis of the Antibacterial Activity of Immobilized Lysozyme

Lysozyme is a hydrolytic enzyme cleaving the polysaccharidic component of the cell wall of most bacteria and so, inducing the lysis of the cells. The enzymatic activity of lysozyme is generally assayed spectrophotometrically by monitoring the decrease of turbidity of a cell suspension of *Micrococcus lysodeikticus.* To measure the enzymatic activity, six different coupons were immersed in a suspension of *Micrococcus lysodeikticus* and its turbidity was monitored spectrophotometrically during 4.5 h (Figure 3.7). The first trial was performed for the optimum concentration of the lysozyme enzyme solution for efficient coating of the surface. For this purpose, solutions prepared with different enzyme concentrations were used for immobilization procedure.

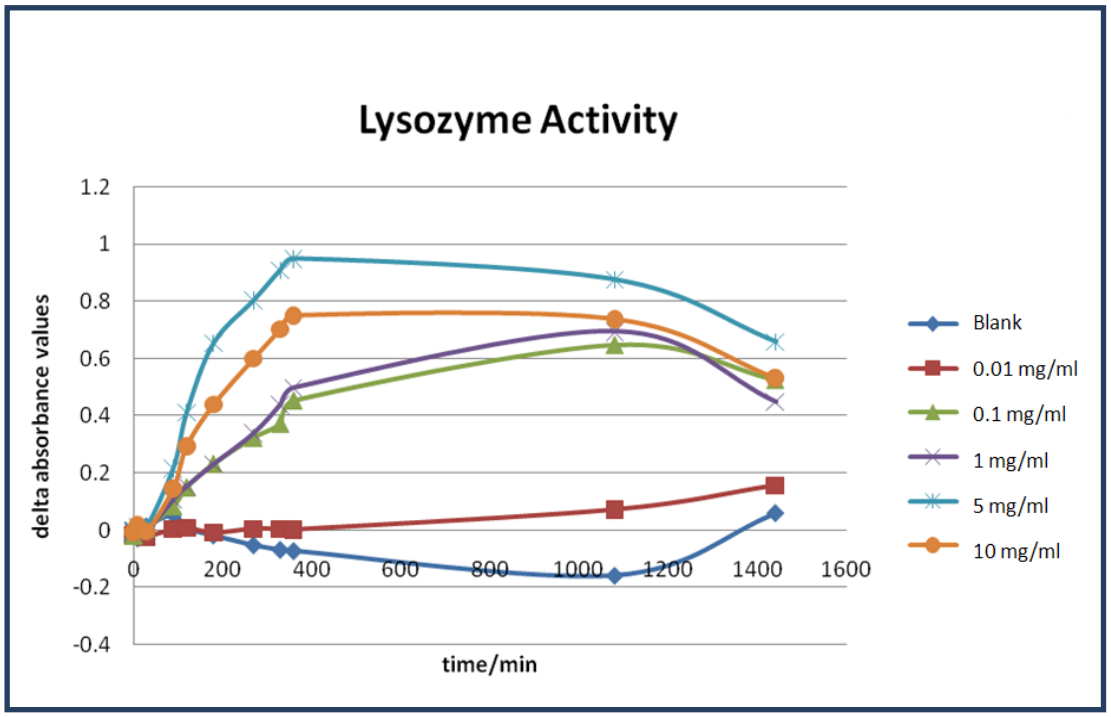


Figure 3.7 : Spectrophotometric results of different enzyme concentrations used for coating and the activities of the coated coupons.

Table 3.2 : Absorbance values obtained by spectrophotometric assay of different enzyme concentrations used for coating.

Time (min)	Pure Culture	Blank	0.01mg/ml	0.1mg/ml	1mg/ml	5mg/ml	10mg/ml
0	0,5116	0,5184	0,5343	0,5351	0,5224	0,5198	0,5255
30	0,5157	0,5182	0,5309	0,5353	0,5201	0,5204	0,5247
60	0,5184	0,5453	0,5349	0,5221	0,5266	0,5313	0,5249
90	0,5414	0,5267	0,5494	0,5174	0,5329	0,515	0,5309
120	0,5883	0,5331	0,5288	0,4475	0,4255	0,3151	0,3861
180	0,6589	0,6497	0,6398	0,4978	0,4953	0,2365	0,3546
270	0,7823	0,7993	0,8069	0,5651	0,5683	0,1448	0,3596
330	0,8694	0,9203	0,9141	0,5952	0,5804	0,1158	0,3195
360	0,9199	0,9868	0,9824	0,6127	0,5465	0,0761	0,2838
1080	0,9461	1,0172	1,0139	0,5626	0,5183	0,0668	0,2656
1440	0,5255	0,6825	0,6095	0,034	-0,0132	-0,1941	-0,056

In Figure 3.7, the graph of the enzyme activity with different enzyme concentrations can be seen. Enzymatic activity towards the bacterial cells is lower when the enzyme concentration is low and higher activity was obtained with higher concentrations, except with 5 mg/ml and 10 mg/ml enzyme concentrations. This was the expected case as the higher lysozyme concentration means more amount of enzymes which can be covalently bound to the free amine groups on the coated stainless steel

surfaces. The only exception was 5 mg/ml and 10 mg/ml enzyme concentrations. This could be explained with the inhibition of the higher amounts of lysozyme and the efficiency of 5 mg/ml lysozyme concentration for the best interaction with the sol-gel coating.

3.5 Biofilm Formation Studies and CSLM Imaging

Pseudomonas aeruginosa PAO1 (ATCC 15692) cells were electroporated with GFP containing vector to be used for the visualization of biofilm formation studies under confocal microscopy. After electroporation and incubation on agar plates, plasmid DNA was isolated from the single colonies and extracted plasmid DNAs were run on agarose gel. The DNA bands observed around the 5148 bp band of the marker in Figure 3.8 belong to the pSMC21 vector.

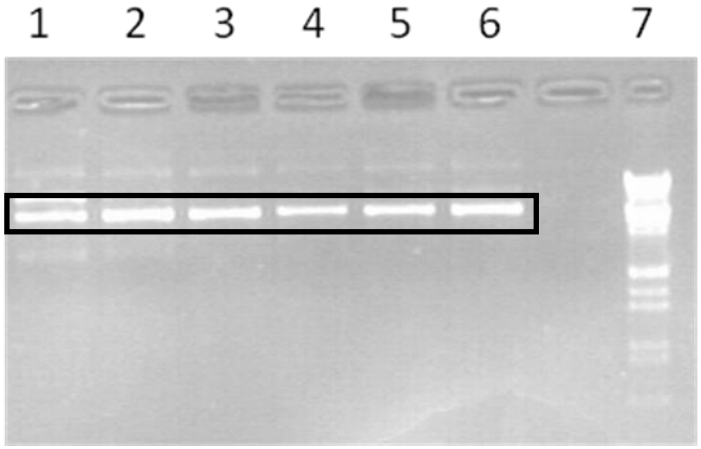


Figure 3.8 : Agarose gel electrophoresis of the plasmid DNA isolated from single colonies of the electroporated PAO1 cells. Tubes 1-2-3: Plasmid DNA of three different colonies grown on kanamycin-containing agar plates after electroporation. Tubes 4-5-6: Plasmid DNA of three different colonies grown on ampicillin and kanamycin-containing agar plates after electroporation. Tube 7: λ DNA standard (bands from up to down, respectively); 21226 bp, 5148 bp, 4973 bp, 4268bp, 3530 bp, 2027 bp, 1904 bp, 1584 bp, 1375 bp, 947 bp, 831 bp, 564 bp.

pSMC21 vector is a 4.8 kb-sized vector constructed by George A. O' Toole and his colleagues from Darthmouth Medical School and was kindly provided by them. The vector contains both the Green Fluorescent Protein (GFP) and two different

antibiotics resistance genes; kanamycin ve ampicillin. This vector is actually the same with pSMC2 vector (Figure 3.9), which was again constructed by O' Toole and his colleagues; just with an addition of the kanamycin-resistance gene. So, the cells containing the pSMC21 vector are able to grow in both ampicillin and kanamycin-containing media with their resistance to both of the antibiotics.

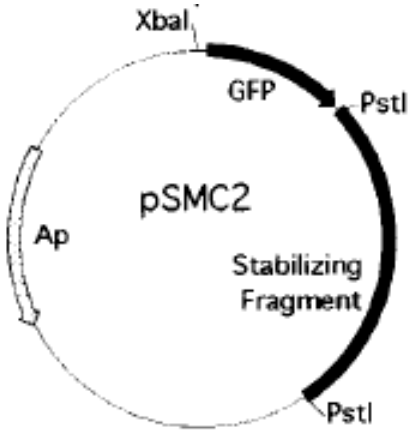
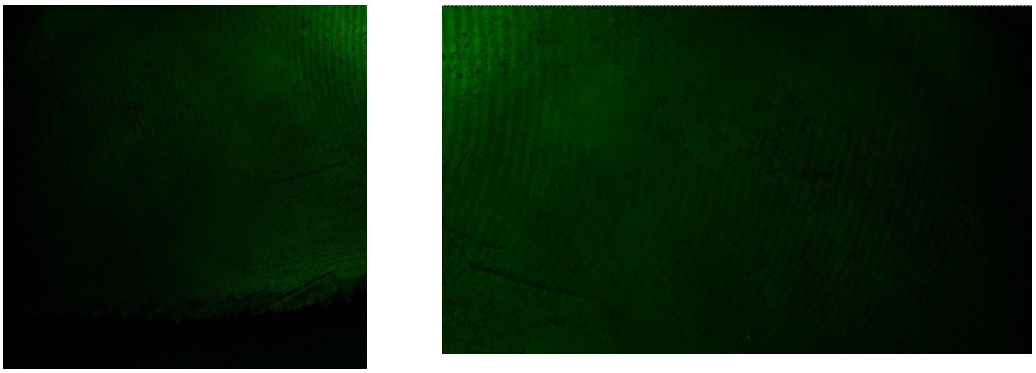


Figure 3.9 : pSMC2 vector map (pSMC21 vector used in the studies also contain the kanamycin-resistance gene).

Following the transformation of the PAO1 cells with the GFP-containing vector pSMC21, all the coating steps of the coupons were analyzed under CSLM for the detection of their anti-biofilm activity. The coupons were placed in the wells of the flow cell and inoculated with the overnight culture of *Pseudomonas aeruginosa* PAO1 cells. The first inoculation culture was left over the coupons for 24 h for the initial attachment and growth of the cells. After the initial attachment, growth medium was flown over the initially-attached cells. After 24 h incubation with PAO1 cells, confocal microscopy images were obtained. Green structures on the whole surface shows the biofilm structure produced by the transformed *Pseudomonas aeruginosa* PAO1 cells (Figure 3.10). The reason of this could be due to the long exposure time of the coupons with the bacterial culture. The total time for the initial attachment and biofilm formation was 48 h and at the end of this time, there might not remain any active enzyme on the surface of the coupons.



Sol-gel coated SS Lysozyme coated SS

Figure 3.10 : SS coupons after 24h incubation with PAO1

3.6 FTIR Spectroscopy Analysis

Surface modification of stainless steel (SS-SC) was analyzed by FT-IR spectroscopy and compared with the FT-IR spectra of bare PAA and Lys. As can be seen from Figure 3.11 and 3.13, SS-SC coupons were successfully coated with sol-gel. In the spectrum of SS-SC-Sol, the bands observed at 1092 cm^{-1} and 1008 cm^{-1} corresponding to stretching of Si-O-C and Si-O-Si indicated that the APTMOS was completely absorbed onto the surface. The weak band at 935 cm^{-1} showed that there were some silanols in the film.

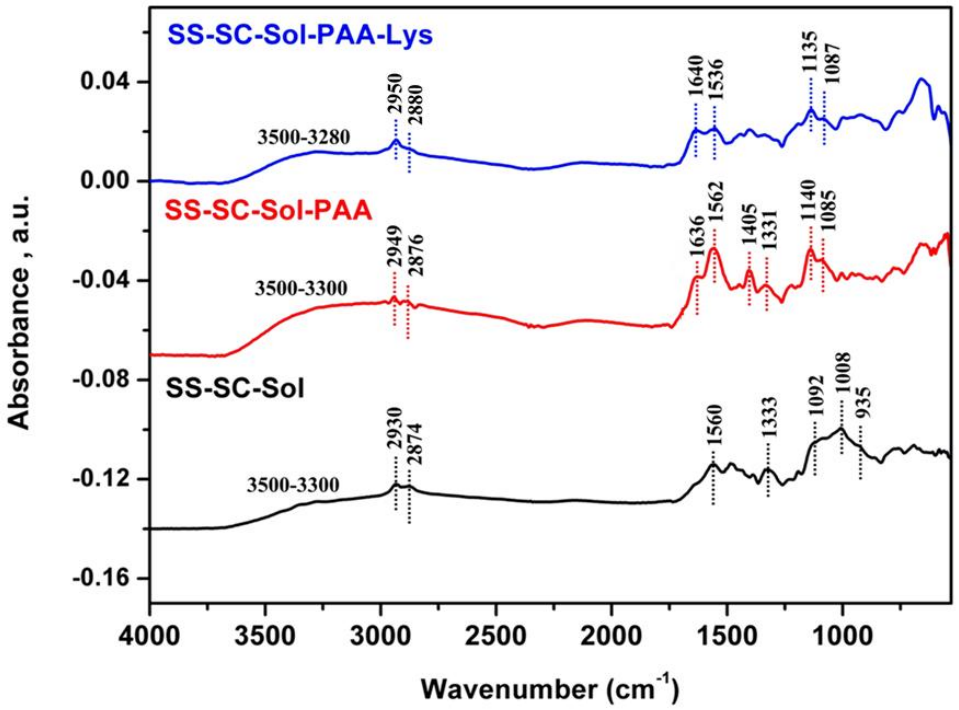


Figure 3.11 : FT-IR spectra of SS-SC-Sol, SS-SC-Sol-PAA and SS-SC-Sol-PAA-Lys.

The absorption of Si-O-C and Si-O-Si stretching decreased a bit after PAA was grafted onto the SS-SC-Sol surface (Figure 3.11). Also the N-H band at 1560 cm^{-1} disappeared while new bands at 1636 (stretching of C=O) and 1562 (bending of N-H) cm^{-1}. For comparison, the FT-IR spectrum of bare PAA was shown in Figure 3.12. As can be seen, bare PAA has C=O stretching band at 1702 cm^{-1} which is the characteristic band of carboxylic acids. When PAA chains were grafted onto the SS-SC-Sol surface an amide was formed and C=O stretching shifted to 1636 cm^{-1}. The O-H stretching of PAA (Figure 3.12) appeared between 3500-330 cm^{-1} together with N-H stretching as a broad band. The immobilization of lysozyme was evidenced by the presence of Amide I (1640 cm^{-1}) and Amide II (1536 cm^{-1}) bands (Figure 3.11). Although these amide bands were overlapped with C=O stretching and N-H bending formed by reaction PAA and SS-SC-Sol, the results were in accordance with FT-IR spectrum of bare Lys (Figure 3.12).

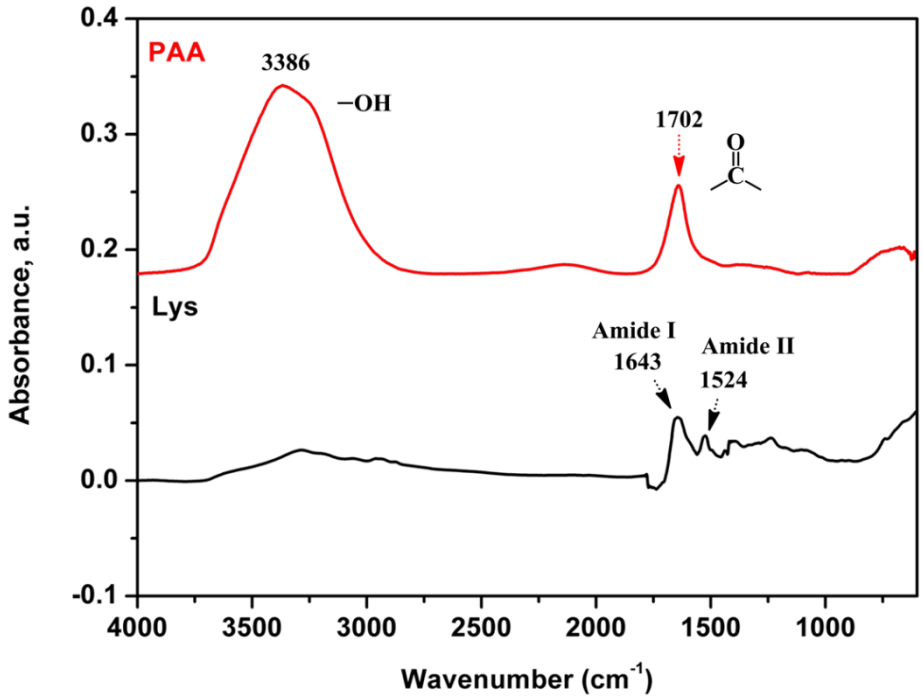


Figure 3.12 : FT-IR spectra of PAA and Lys.

Figure 3.13 shows the FT-IR spectra of SS-SC-Sol and direct immobilization of lysozyme from its carboxyl-end on the SS-SC-Sol surface. Similar results were obtained compared to the covalent binding of lysozyme from its amine-end. As can be seen, Amide I and Amide II bands were observed at 1631 and 1550 cm^{-1} and absorption of Si-O-C and Si-O-Si stretching decreased a bit after lysozyme immobilization.

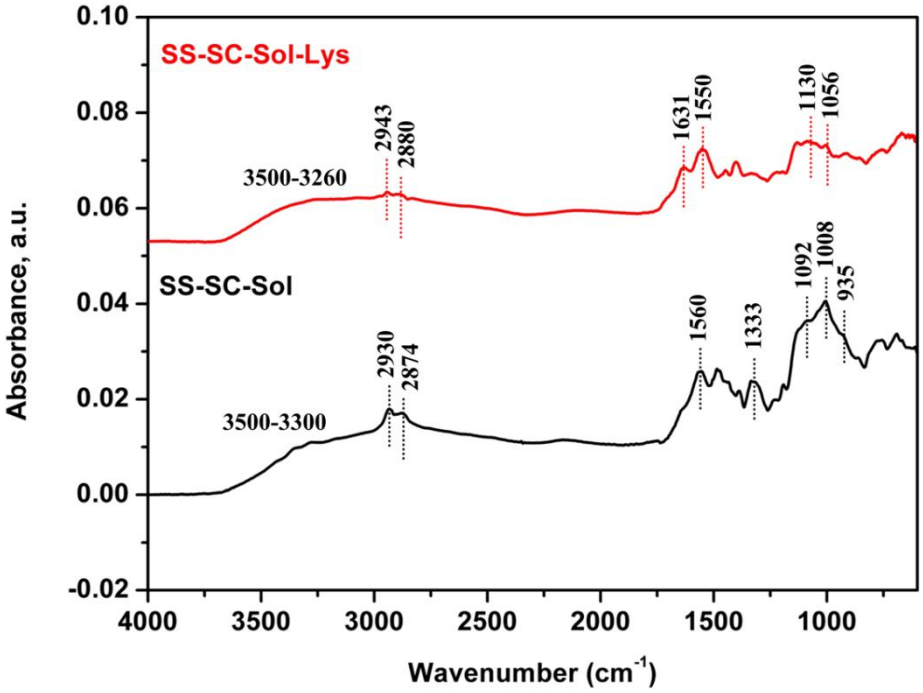


Figure 3.13 : FT-IR spectra of SS-SC-Sol and SS-SC-Sol-Lys.

3.7 AFM Analysis of Coating Steps and Lysozyme-Immobilized Substrates

AFM analysis was performed to study the alteration of the surface topography and the Root Mean Square (RMS) roughness values were calculated for each step of the immobilization process. Figure 3.14 shows the AFM height images of different coating steps.

Mica sheet is an excellent support for AFM, since it has almost atomically flat surface and has an RMS roughness of 0,03 ± 0.01 nm over 1.00 x 1.00 μm^2 scan area (data not shown). RMS roughness of MTMOS/APTMOS treated mica increased to 0,39nm which is consistent with previous reports (Lu, 2011; Libertino et al., 2008). These results are in agreement with FTIR data and it can be argued that AFM results indicates a good sample coverage and silane coating generates a uniform layer on the surface of the discs (Figure 3.13A).

Thin silane film-coated discs were further used for either covalent immobilization of the lysozyme or covalent attachment of PAA layer onto the surface. After grafting the PAA layer, RMS roughness value of the surface increased to 4 ± 0.03 nm, indicating that the surface underwent macroscopic modifications during PAA

grafting step (Al-Bataineh, 2006). The RMS roughness value and obtained AFM image of PAA immobilization step suggest that PAA polymer covers the entire surface, however due to the shrinkage of PAA polymer in dry state results in increase of surface roughness (Tobiesen and Michielsen, 2002).

Surface topography of sol-gel matrix entirely changed due to immobilization of lysozyme while the RMS roughness became to be 0,41nm. The AFM results suggested good sample coverage with a monolayer of lysozyme molecules.

After covalent attachment of lysozyme onto PAA grafted surface, the RMS roughness value decreased to 1.30 nm. Molecular weight of lysozyme is 14,7 kDa and one might compare with PAA (250 kDa) and it might be speculated that relatively small lysozyme molecules not only filled the gaps between polymer chains but also covered the entire surface of PAA chains. These results are coherent with calculated enzyme activity of sol-gel and PAA immobilized lysozyme.

Lysozyme molecules are uniformly immobilized onto both sol-gel and sol-gel-PAA grafted surface with the appearance of peaks with the same height. The size of the lysozyme molecule is given as 3.2 nm by 4.5 nm in the literature (Broutin, 1997). Even it is difficult to get a precise datum from AFM images, it is obvious that the size of the immobilized lysozyme is around 3-4 nm.

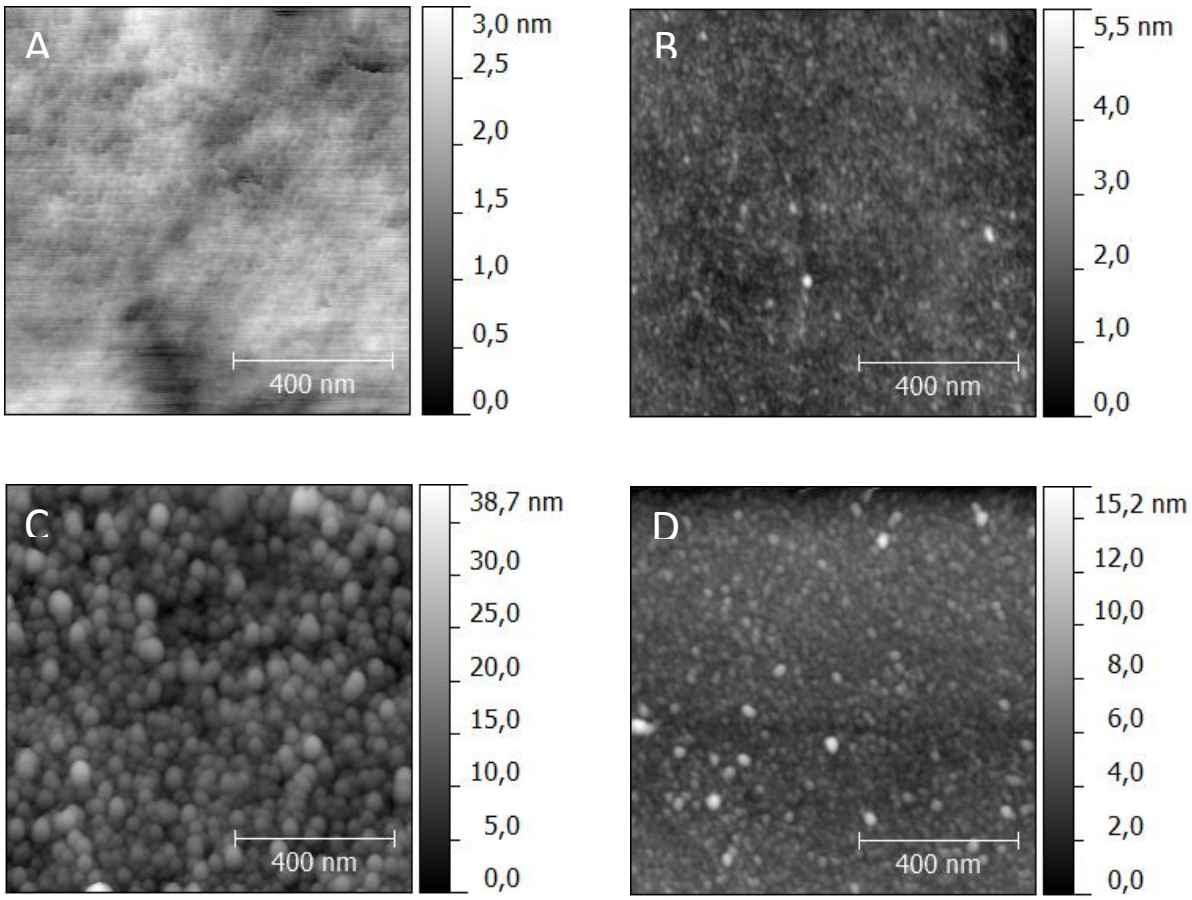


Figure 3.14 : AFM images of stainless steel coupons (A)Mica-Sol, B)Mica-Sol-Lys, C)Mica-Sol-PAA, D) Mica-Sol-PAA-Lys).

3.8 Analysis of the Antibacterial Activity of Immobilized Lysozyme

In Figure 3.15, the enzymatic activity results for six different coated coupons can be seen. According to the results, physical adsorption of lysozyme on stainless steel coupons resulted in poor enzymatic activity (SS-SC-Ads: 0.2 ± 0.01 U, SS-SC-Sol-Ads: 0.14 ± 0.02 U and SS-SC-Sol-PAA-Ads: 0.22 ± 0.44 U) whereas the covalent bonding of lysozyme on the coupons yielded a better activity. When the enzyme is covalently bound on the surface from its amino terminus (SS-SC-Sol-PAA-Lys: 4.2 ± 0.1 U), the activity was 7.5 times higher than that of the enzyme covalently bound on the surface from its carboxyl terminus (SS-SC-Sol-Lys: 0.56 ± 0.37 U). This could arise from the fact that the sol-gel thin film forms a single layer of amine groups and when the enzyme is covalently bound, it becomes very close to the steel surface, where the accessibility of the substrate (*M. lysodeikticus* cells) to the enzyme

becomes limited, causing a hindrance. However, when the enzyme is covalently bound to the carboxyl functional groups of PAA, enzyme molecules might become more relaxed with better accessibility to the substrate. Another reason could be the rigidity of the covalent linkage of lysozyme molecules. In the case of amine containing surfaces, covalently bound lysozyme molecules are directly linked to the surface with higher rigidity. In the case of covalent binding to the polyacrylic acid layer, the enzyme molecules could be covalently bound along the length of the polymer chain (Cullen, 2008). This layer is generally called as the "polyacrylic acid brush" due to its brush-like structure (Cullen, 2008; Dai, 2006). These brushes are advantageous due to their high binding capacity which originates from their high density of reactive functional groups and their ability to swell in appropriate solvents. They do not also have cross-links between polymer chains and this could increase the accessability of proteins to the functional groups within films (Dai, 2006). So, this brush-like structure could supply more regions for binding the enzyme to the carboxylic acid groups resulting in a "multi-layer" enzyme formation.

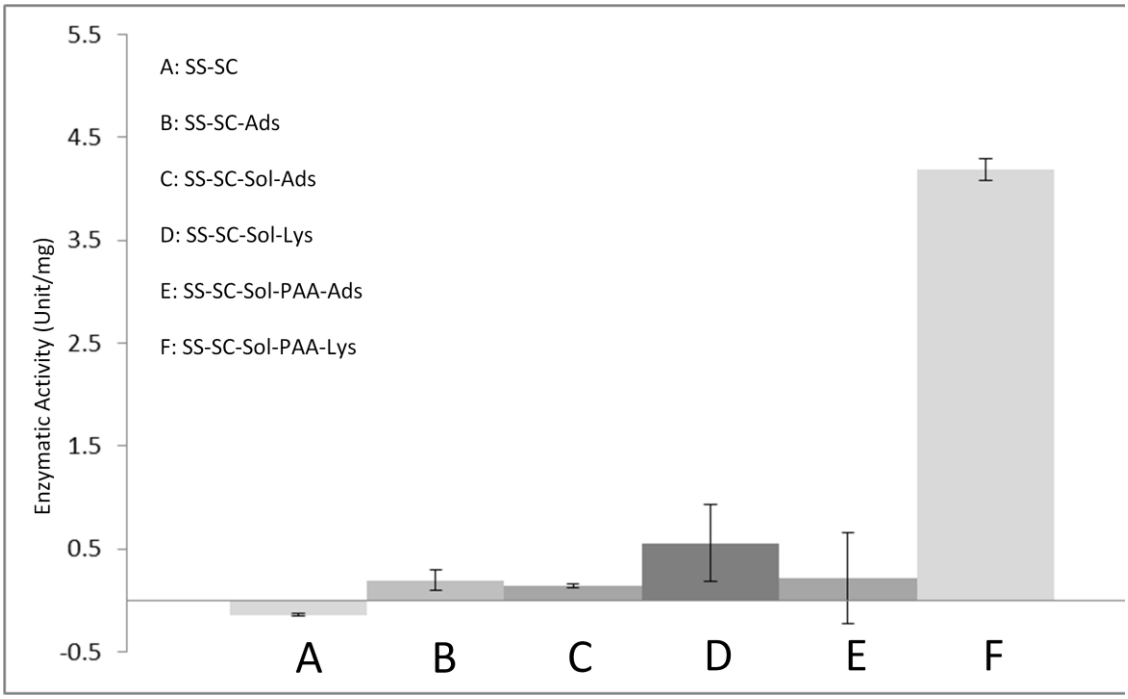


Figure 3.15 : Enzymatic activity of six different stainless steel coupons. (Ads refers to the adsorbed lysozyme on the coupons).

3.9 CSLM Imaging and Analysis of Biofilm Formation

All the coating steps were also analyzed under CSLM for the detection of their anti-biofilm activity. The coupons were placed in the wells of the flow cell and inoculated with the overnight culture of *Pseudomonas aeruginosa* PAO1 cells. The first inoculation culture was left over the coupons for 24 h for the initial attachment and growth of the cells. After the initial attachment, growth medium was flown over the initially-attached cells. After 24 h incubation with PAO1 cells, confocal microscopy images were obtained. There was not an obvious visual difference between the anti-biofilm activities of enzyme-coated coupons and other coupons (Figure 3.16). The reason of this could be due to the long exposure time of the coupons with the bacterial culture. The total time for the initial attachment and biofilm formation was 48 h and at the end of this time, there might not remain any active enzyme on the surface of the coupons. Previous experiments with the enzyme-coated coupons have showed that after 24 h., there was a dramatic change in the activity of the enzyme In order to understand this, the initial incubation time for the initial attachment of the bacteria to the surface could be shorter, like overnight culture (12 h.) but this could affect the initial attachment of the bacteria as this is the first and the most important stage of biofilm formation.

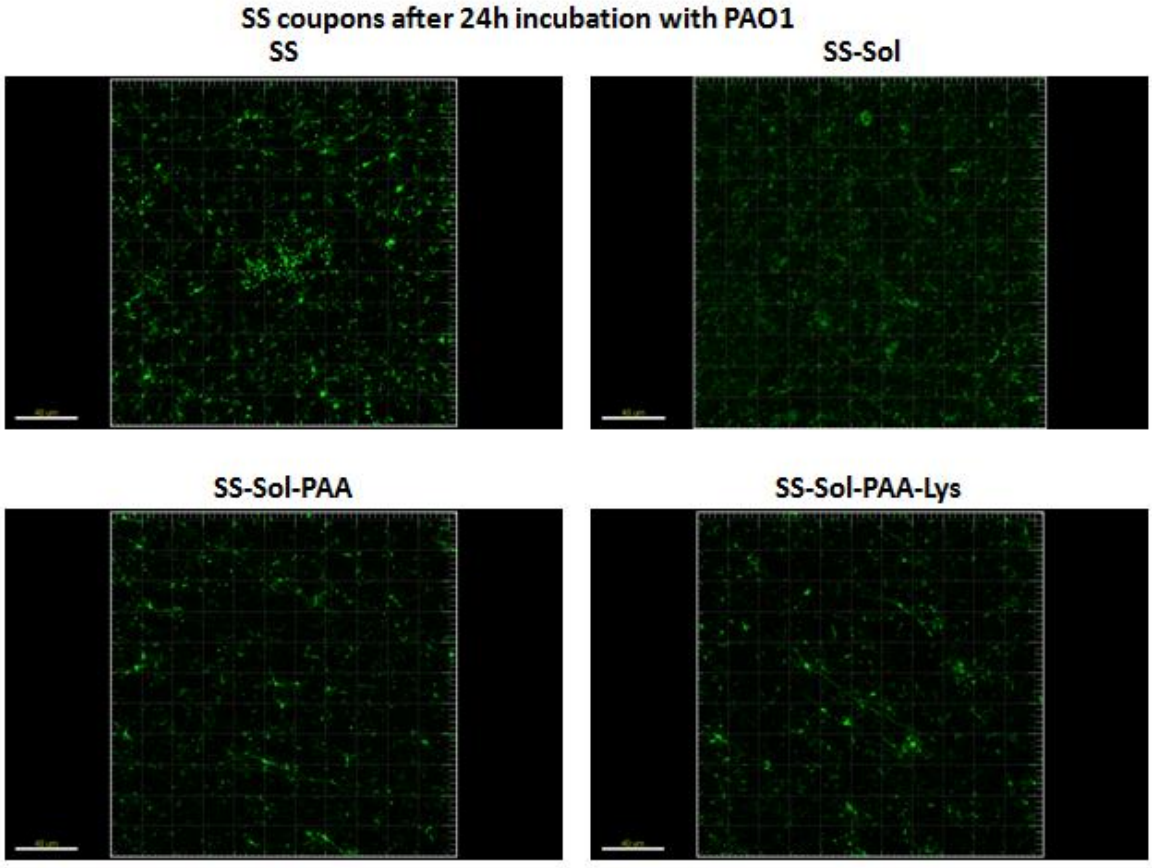


Figure 3.16 : SS coupons after 24h incubation with PAO1.

After confocal microscopy imaging of coated surfaces, serial dilutions were done to obtain the colony forming units on these surfaces. Serial dilutions of the incubated cells on several different stainless steel surfaces were prepared and these dilutions were grown on agar plates. Colony forming units were counted on these plates and were compared with the confocal microscopy images. When consolidating all the data of AFM imaging, confocal microscopy imaging and the colony counting of serial dilutions for different coated and uncoated surfaces showed that sol-gel coating and PAA brush coating increased the surface area when compared to the bare stainless steel coupons. This increase in surface area also increases the possibility of bacterial attachment on these surfaces. That could be the reason stainless steel coupons have the least number of attached cells even in confocal images and also the colony forming units. Sol-gel coating is known to be non-toxic and inert, meaning that it could not have an antibacterial effect on PAO1 cells. However, enzyme coated surfaces have a slightly lower number of colonies when compared to PAA-coated surfaces even the confocal microscopy images seem to be the same. The reason of

this could be that PAA brush coating increased the surface area when compared to the bare stainless steel coupons. This increase in surface area also increases the possibility of bacterial attachment on these surfaces. Lysozyme coated coupons have an antibacterial activity towards the PAO1 cells when compared to PAA brush coating. This could mean that the active enzyme molecules immobilized on the coupons show activity towards the biofilm forming bacterial cells but even it is effective on bacteria, the number of cells attached on the PAA brush is much more higher than the sol-gel coated coupon so the number of colonies on sol-gel coated coupons are lower. This finding shows that the immobilized enzyme shows activity towards the biofilm-forming bacterial cells. It was obvious from the characterization studies that sol-gel coating and PAA brush coating increased the surface area when compared to the bare stainless steel coupons. This increase in surface area also increases the possibility of bacterial attachment on these surfaces as a major drawback. Sol-gel coating is known to be non-toxic and inert, meaning that it could not have an antibacterial effect on PAO1 cells. However, lysozyme coated coupons have an antibacterial activity towards the PAO1 cells when compared to PAA brush coating which can be speculated from the colony counting results. This could mean that the active enzyme molecules immobilized on the coupons show activity towards the biofilm forming bacterial cells but even it is effective on bacteria, the number of cells attached on the PAA brush is much more higher than the sol-gel coated coupon so the number of colonies on sol-gel coated coupons are lower.

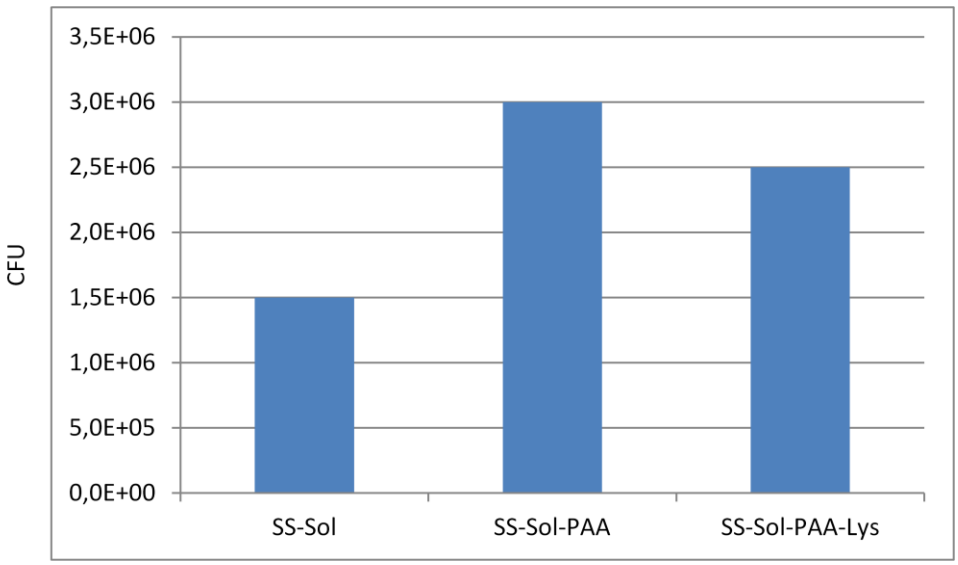


Figure 3.17 : Average CFU values of coated surfaces and control surfaces.

4. CONCLUSION

4.1 Conclusions of the Study

The main objective of the present thesis was to provide a new route towards biofilm-resistant materials by linking enzymes to the surface of these materials with the aid of sol-gel technology and carbodiimide chemistry. To accomplish this, covalent immobilization of the enzymes on stainless steel surfaces which was previously modified by a hydrogel-type layer alone and also with polyacrylic acid by using azide/nitrene chemistry was done. The presence of amine groups in the sol-gel silicate network provided functional sites for covalent attachment of enzymes which also have functional sites. Spectroscopic characterization of immobilization steps and determination of other parameters such as enzyme coating efficiency was performed and determined by using Atomic Force Microscopy (AFM) and Fourier Transform Infrared Spectroscopy (FTIR). Finally, a biofilm study in a parallel plate flow cell system containing control and coated test materials was designed to study biofilms growing under a range of conditions (high and low flow rates, different temperatures etc.) which facilitates non-destructive imaging of biofilms by using Confocal Laser Scanning Microscopy (CLSM). *Pseudomonas aeruginosa* was especially chosen because it is a model organism for biofilm studies. These studies were also combined with the colony counting methods by serial dilutions of these surfaces. It was shown with these studies that lysozyme molecules were successfully immobilized on sol-gel coated coupons with both of the strategies preformed while retaining its activity. AFM and FT-IR analysis have confirmed the immobilization of lyoszyme molecules on stainless steel surfaces and the enzyme activity studies have shown that the enzyme molecules retained their activity after immobilizing on the surface. Biofilm formation studies on these surfaces were important to measure the anti-biolm activities of these surfaces. Sol-gel techique provides a non-toxic and inert surface, meaning that it could not have an antibacterial effect on PAO1 cells. However, enzyme coated surfaces have a slightly lower number of colonies when compared to

PAA-coated surfaces even the confocal microscopy images seem to be the same. The reason of this could be that PAA brush coating increased the surface area when compared to the bare stainless steel coupons. This increase in surface area also increases the possibility of bacterial attachment on these surfaces. Lysozyme coated coupons have an antibacterial activity towards the PAO1 cells when compared to PAA brush coating. This could mean that the active enzyme molecules immobilized on the coupons show activity towards the biofilm forming bacterial cells but even it is effective on bacteria, the number of cells attached on the PAA brush is much more higher than the sol-gel coated coupon so the number of colonies on sol-gel coated coupons are lower. This finding shows that the immobilized enzyme shows activity towards the biofilm-forming bacterial cells. To conclude the established works during these studies, vector containing culture preparation for *Pseudomonas aeruginosa* biofilm imaging was performed. Development of a sol-gel coating platform for enzyme immobilization was achieved and also alternative methods for enzyme coating were established. The different surfaces obtained by using these enzyme coating methods were then characterized with different methods and instruments. As the final work, non-destructive biofilm imaging via flow-cell platform was utilized and the comparison of these alternative methods were accomplished.

REFERENCES

Al-Bataineh, S. A., Britcher, L. G., Griesse, H. J. (2006). XPS characterization of the surface immobilization of bacterial furanones. *Surface Science*, *600*, 952-962.

Asad, S. & Opal, S.M. (2008). Bench-to-bedside review: Quorum sensing and the role of cell-to-cell communication during invasive bacterial infection. *Critical Care*, *12*, 236.

Bayston, R., Path, F.R.C., Bhundia, C., Ashraf, W. (2005). Hydromer-coated catheters to prevent shunt infection. *Journal of Neurosurgery: Pediatrics*, *102*, 207-212.

Bjarnsholt, T., Kirketerp-Møller, K., Kristiansen, S., Phipps, R., Nielsen, A.K., Jensen, P.O., Hoiby, N., Givskov, M. (2007). Silver against Pseudomonas aeruginosa biofilms. *APMIS, 115*, 921–928.

Bjarnsholt, T. & Givskov, M. (2008). Quorum Sensing Inhibitory Drugs as Next Generation Antimicrobials: Worth the Effort? *Current Infectious Disease Reports, 10*, 22–28.

Bong, J.J., Kite, P., Wilco, M. H., McMahon, M.J., (2003). Prevention of catheter related bloodstream infection by silver iontophoretic central venous catheters: a randomised controlled trial. *Journal of Clinical Pathology*, *56*, 731-735.

Broutin, I., Ries-Kautt, M., & Ducruix, A. (1997). Crystallographic analyses of lysozyme and collagenase microgravity grown crystals versus ground controls. *Journal of Crystal Growth*, *181* (1-2), 97-108.

Callewaert, L., Aertsen, A., Deckers, D., Vanoirbeek, K. G. A., Vanderkelen, L. et al. (2008) A New Family of Lysozyme Inhibitors Contributing to Lysozyme Tolerance in Gram-Negative Bacteria. *PLoS Pathog*, *4* (3): e1000019. doi:10.1371/journal.ppat.1000019.

Chovelon, J. M., Aarch, L. E., Charbonnier, M., Romand, M. (1995). Silanization of Stainless Steel Surfaces: Influence of Application Parameters. *J. Adhes*. *50*, 43–58.

Costerton, J. W., Lewandowski, Z., Caldwell, D. E., Korber, D. R. and Lappin-Scott, H. M. (1995). Microbial biofilms, *Annu. Rev. Microbiol. 49*: 711-745.

Cullen, S. P., Liu, X., Mandel, I. C., Himpsel, F. J., Gopalan, P. (2008). Polymeric brushes as functional templates for immobilizing Ribonuclease A: Study of binding kinetics and activity. *Langmuir*, *24*, 913-920.

Dafforn, K. A., Lewis, J. A., & Johnston, E. L. (2011). Antifouling strategies: history and regulation, ecological impacts and mitigation. *Mar Pollut Bull, 62* (3), 453-465. doi: 10.1016/j.marpolbul.2011.01.012.

Dai, J., Bao, Z., Sun, L., Hong, S. U., Baker, G. L., Bruening, M. L. (2006). High-capacity binding of proteins by poly(acrylic acid) brushes and their derivatives. *Langmuir, 22* (9),4274-81.

Davies, D. G, Parsek, M. R., Pearson, J. P, Iglewski B. H., Costerton, J. W., Greenberg, E. P. (1998). The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science, 280,* 295–298.

de Carvalho, C. C. (2007). Biofilms: recent developments on an old battle. *Recent Pat Biotechnol,* 1 (1), 49-57.

Dong, Y. H., Xu, J. L., Li, X. Z., Zhang, L. H. (2000). AiiA, an enzyme that inactivates the acylhomoserine lactone quorum-sensing signal and attenuates the virulence of Erwinia carotovora. *Proc. Natl. Acad. Sci. USA, 97,* 3526-3531.

Dong, Y. H,, Wang, L. H., Xu, J. L., Zhang, H. B., Zhang, X. F., Zhang L. H. (2001). Quenching quorum-sensing-dependent bacterial infection by an N-acyl homoserine lactonase. *Nature, 411,* 813-817.

Dong, Y. H., Gusti, A. R., Zhang, Q., Xu, J. L., Zhang, L. H. (2002). Identification of quorum-Quenching N-acyl homoserine lactonases from Bacillus species. *Applied and Environmental Microbiology, 68* (4),1754-1759.

Düring, K., Porscha, P., Mahn, A., Brinkmann, O., Gieffers. (1999). The non-enzymatic microbicidal activity of lysozymes. *FEBS Letters, 449,* 93-100.

Givskov, M., Rasmussen, T. B., Ren, D. & Balaban, N. (ed.). (2008). *Control of biofilm infections by signal manipulation,* p. 13-38. Springer, Berlin.

Gupta, R. & Kumar, A. (2008). Bioactive materials for biomedical applications using sol-gel technology. *Biomedical Materials, 3* (3), 1-15. doi: 10.1088/1748-6041/3/3/034005.

Hall-Stoodley, L. & Stoodley, P. (2002). Developmental regulation of microbial biofilms. *Current Opinion in Biotechnology, 13,* 228–233.

Huang, J. J., Han, J. I., Zhang, L. H., Leadbetter, J. R. (2003) Utilization of acyl-homoserine lactone quorum signals for growth by a soil Pseudomonad and Pseudomonas aeruginosa PAO1. *Applied and Environmental Microbiology, 69* (10), 5941-5949.

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

Kampf, G,, Dietze, B., Grosse-Siestrup, C., Wendt, C., Martiny, H. (1998) Microbicidal Activity of a New Silver-Containing Polymer, SPI-ARGENT II. *Antimicrobial Agents and Chemotherapy, 42* (9), 2440-2442.

Kjelleberg, S., Steinberg, P.D., Holmstrom, C., Back, A. (1999). Inhibition of gram positive bacteria, PCT International Application PP3034.

Kristensen, J. B., Meyer, R. L., Laursen, B. S., Shipovskov, S., Besenbacher, F., Poulsen, C.H. (2008). Antifouling enzymes and the biochemistry of marine settlement, *Biotechnology Advances*, *26*, 471–481.

Kumar, C. G. & Anand, S. K. (1998). Significance of microbial biofilms in food industry: a review. *International Journal of Food Microbiology, 42*, 9-27.

Lansdown, A. B. (2002). Silver. I: Its antibacterial properties and mechanism of action. *Journal of Wound Care*, *4*, 125–30.

Leadbetter, J. R. & Greenberg, E. P. (2000). Metabolism of acyl-homoserine lactone quorum sensing signals by Variovarax paradoxus. *Journal of Bacteriology*, 182 (24), 6921-6926.

Lelievre, H., Lina, G., Jones, M.E., Olive C., Forey, F., Roussel-Delvallez, M., Nicolas-Chanoine, M., Bebear, C., Jarlier, V., Andremont, A., Vandenesch, F., Etienne, J. (1999). Emergence and Spread in French Hospitals of Methicillin-Resistant Staphylococcus aureus with Increasing Susceptibility to Gentamicin and Other Antibiotics. *Journal of Clinical Microbiology*, 37 (11): 3452-3457.

Leroy, C., Delbarre, C., Ghillebaert, F., Compere, C., Combes, D. (2008). Effects of commercial enzymes on the adhesion of a marine biofilm-forming bacterium. *Biofouling*, *24* (1),11–22.

Libertino, S., Giannazzo, F., Aiello, V., Scandurra, A., Sinatra, F., Renis, M., and Fichera, M. (2008). XPS and AFM characterization of the enzyme glucose oxidase immobilized on SiO(2) surfaces. *Langmuir, 24* (5), 1965-1972. doi: 10.1021/la7029664.

Lin, Y. H., Xu, J. L., Hu, J., Wang, L. H., Ong, S. L., Leadbetter, J. R., Zhang, L. H. (2003). Acyl-homoserine lactone acylase from Ralstonia strain XJ12B represents a novel and potent class of quorum-quenching enzymes. *Molecular Microbiology*, *47* (3), 849-860.

Lu, Q., Wang, J., Faghihnejad, A., Zeng, H., Liu, Y. (2011). Understanding the molecular interactions of lipopolysaccharides during E. coli initial adhesion with a surface forces apparatus. *Soft Matter*, *7* (19), 9366-9379.

Miller, M. B. & Bassler, B. L. (2001). Quorum Sensing in Bacteria. *Annu. Rev. Microbiol*. *55*, 165–99.

Minier, M., Salmain, M., Yacoubi, N., Barbes, L., Méthivier, C., Zanna, S., Pradier, C. M. (2005). Covalent immobilization of lysozyme on stainless steel. Interface spectroscopic characterization and measurement of enzymatic activity. *Langmuir*, *21*, 5957–5965.

Orgaz, B., Kives, J., Pedregosa, A.M., Monistrol, I.F., Laborda, F., SanJose, C. (2006). Bacterial biofilm removal using fungal enzymes. *Enzyme and Microbial Technology, 40*, 51-56.

Ploux, L., Beckendorff, S., Nardin, M., Neunlist, S. (2007). Quantitative and morphological analysis of biofilm formation on self-assembled monolayers. *Colloids and Surfaces B: Biointerfaces*, *57*, 174–181.

Pratt, L. A. & Kolter, R. (1999). Genetic analyses of bacterial biofilm formation. Current Opinion in Microbiology, 2, 598–603.

Raad, I. (1998). Intravascular-catheter-related infections. *Lancet, 351* (9106): 893-898.

Raad, I., Darouiche, R., Hachem, R., Sacilowski, M., Bodey, G. (1995). Antibiotics and Prevention of Microbial Colonization of Catheters. *Antimicrobial Agents and Chemoterapy*, *39* (11), 2397-2400.

Read, R., Kumar, N., Willcox, M. D. P., Zhu, H., Griesser, H., Muir, B., Thissen, H., Hughes, T. (2001). Antimicrobial coatings, PCT International Application, PQ6812.

Read R. & Kumar, N. (1999). Production of furanones, PCT/AU99/00285.

Reimmann, C., Ginet, N., Michel, L., Keel, C., Michaux, P. et al. (2002). Genetically programmed autoinducer destruction reduces virulence gene expression and swarming motility in Pseudomonas aeruginosa PAO1. *Microbiology*, *148*, 923-932.

Schierholz, J. M., Steinhauser, H., Rump, A. F. E., Berkels, R., Pulverer, G. (1997). Controlled release of antibiotics from biomedical polyurethanes: morphological and structural features. *Biomaterials*, *18*, 839-844.

Schierholz J. M., Lucas, L. J., Rump, A., Pulverer G. (1998). Efficacy of silver-coated medical devices. *Journal of Hospital Infection*, *40*, 257-262.

Schierholz, J. M., Beuth, J., Pulverer, G., Konig, D. P., Scharlack, R. S., Kampf, G., Dietze, B., Wendt, C., Martiny, H., Groe-Siestrup, C. (1999). Letters to the Editor: Silver-Containing Polymers. *Antimicrobial Agents and Chemotherapy*, *43*(11), 2819-2821.

Schierholz, J. M., Fleck, C., Beuth, J., Pulverer, G. (2000). The antimicrobial efficacy of a new central venous catheter with long-term broad-spectrum activity. *Journal of Antimicrobial Chemotherapy*, *46*, 45-50.

Schierholz, J. M. & Beuth, J. (2001). Implant Infections: a haven for opportunistic bacteria. *Journal of Hospital Infection*, *49*, 87-93.

Shirtliff, M. E., Mader, J. T., Camper, A. K. (2002). Molecular interactions in biofilms. *Chemistry & Biology*, *9*, 859–871.

Shugar D. (1952). Measurement of lysozyme activity and the ultraviolet inactivation of lysozyme. *Biochem. Biophys. Acta*, *8*: 302-308.

Stobie, N., Duffy, B., Hinder, S. J., McHale, P., McCormack, D. E. (2009) Silver Doped Perfluoropolyether-Urethane Coatings: Antibacterial Activity and Surface Analysis. *Colloids and Surfaces B: Biointerfaces*, *72*, 62–67.

Tobiesen, F. A. & Michielsen, S. (2002). Method for Grafting Poly(acrylic acid) onto Nylon 6,6 Using Amine end groups on Nylon Surface. *J. Polym. Sci. Pol. Chem., 40*, 719-728.

Url-1, <*www.biology.binghamton.edu/davies/images/biofilm.jpg*>, Retrieved on 05.08.2013.

Url-2, <*http://microbewiki.kenyon.edu/index.php/Pseudomnas_aeruginosa*>, Retireved on 12.11.2013.

Url-3, <*http://lysozyme.co.uk*>, Retrieved on 06.09.2013.

Vijayaraghavan, R., Chandrashekhar, R., Sujatha, Y., Belagavi, C.S. (2006). Hospital outbreak of atypical mycobacterial infection of port sites after laparoscopic surgery. *Journal of Hospital Infection, 64,* 344-347.

Xavier, J. B., Picioreanu, C., Rani, S. A., van Loosdrecht, M. C. M., Stewart, P. S. (2005). Biofilm-control strategies based on enzymic disruption of the extracellular polymeric substance matrix - a modelling study. *Microbiology, 151,* 3817–3832.

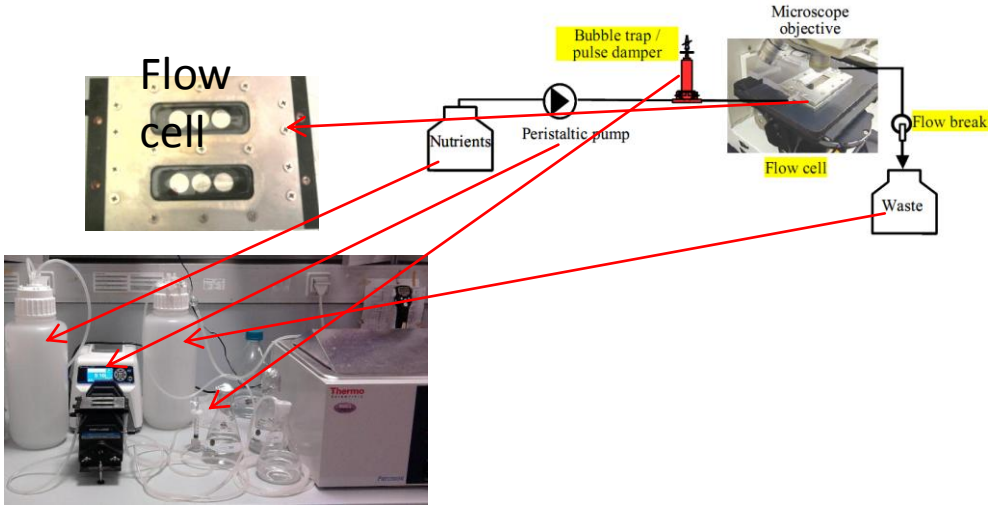
Yang, X., Hua, L., Gong, H., Tan, S. T. (2003). Covalent immobilization of an enzyme (glucose oxidase) onto a carbon sol–gel silicate composite surface as a biosensing platform. *Analytica Chimica Acta, 478,* 67–75.

Zhang, T., Ke, S.Z., Liu, Y., Fang, H.P. (2005). Microbial characteristics of a methanogenic phenol-degrading sludge. *Water Science and Technology, 52* (1-2), 73-78.

Zhang, L. H. (2003). Quorum quenching and proactive host defense. *TRENDS in Plant Science, 8* (5), 238-44.

APPENDIX

APPENDIX A: Experimental set-up for the confocal microscopy imaging of the biofilm formation on coupons placed in the flow cell



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