ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

COVALENT IMMOBILIZATION OF FURANONE DERIVATIVES FOR THE INHIBITION OF BIOFILM FORMATION ON IMPLANT SURFACES

Ph.D. THESIS

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ISTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

İMPLANT YÜZEYLERİNDE BİYOFİLM OLUŞUMUNUN ENGELLENMESİNE YÖNELİK FURANON TÜREVLERİNİN KOVALENT İMMOBİLİZASYONU

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EYLÜL 2018

Emrah Yelboğa, a Ph.D. student of ITU Graduate School of Science Engineering and Technology student ID 521082051, successfully defended the thesis/dissertation entitled "COVALENT IMMOBILIZATION OF FURANONE DERIVATIVES FOR THE INHIBITION OF BIOFILM FORMATION ON IMPLANT SURFACES", which he prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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Date of Submission : 10 July 2018
Date of Defense : 26 September 2018

To my dear love Ayşe,

FOREWORD

I would like to express my deepest gratitude to my supervisors Dr. Nevin Gül Karagüler and Dr. Melek Tüter for their endless understanding and patience; their tremendous support throughout graduate research.

Grateful acknowledgment is made to Dr. Thomas Bjarnsholt and his colleague Dr. Morten Alhede From University of Copenhagen and Dr. Francesco Secundo from Institute of Chemistry of Molecular Recognition, Italian National Research Council (ICRM-CNR) for giving me the opportunity to spent three months in their lab during my EMBO short-term fellowships.

I would like to specially thank to my companion in study; Abdullah Sert; for his knowledge, patience and infinite support. My experiments and study would never end without his help. I would like to thank all friends from Protein Engineering Research Group at Istanbul Technical University.

I would like to thank to my family; my mother Ayhan Yelboğa, my father Zeki Yelboğa and my brother Zekeriya Yelboğa for their complete support and trust in any case during my whole life.

I would like to thank the funding agencies for their financial support throughout my PhD research. This thesis was partly supported by ITU Institute of Science and Technology (Project No: 34066) and TUBİTAK as a part of Joint Research Project from CNR of Italy (Project No: 209T076).

Finally yet importantly, I am extremely grateful to European Molecular Biology Organization (EMBO) that enabled my three-months research visits to University of Copenhagen, Denmark and ICRM-CNR, Milan, Italy respectively.

September 2018

Emrah YELBOĞA
Entrepreneur

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ABBREVIATIONS

2(5H) **:** 5-(Bromomethylene)-2(5H)-furanone

4Z **:** (Z-)-4-Bromo-5-(bromomethylene)-2(5H)-furanone

AFM **:** Atomic Force Spectroscopy

AHL **:** N-Acyl Homoserine Lactone

APTES **:** (3-Aminopropyl)trimethoxysilane

AZA **:** Azidoaniline hydrochloride

CFU **:** Colony Forming Unit

CLSM **:** Confocal Scanning Laser Microscopy

App **:** Appendix

DMEM : Dulbecco's Modified Eagle's Medium

EDC **:** *N*-(3-dimethylaminopropyl)-*N′*-ethylcabodiimide hydrochloride

EPS **:** Extracellular Polymeric Matrix

FBI **:** Foreign Body Infection

FU1 **:** 3-butyl-4-bromo-5(bromomethylene)-2(5H)-furanone

FU2 **:** 5-(dibromomethylene)-3-methylfuran-2(5H)-one

IMD **:** Indwelling Medical Device

MDR **:** Multi Drug Resistant Bacteria

MTMOS **:** Trimethoxymethylsilane

MTS **:** 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfofenyl)-2H\--tetrazolium

OD **:** Optical Density

PAA **:** Poly(acrylic acid)

QS **:** Quorum Sensing

SEM **:** Scanning Electron Microscopy

SiC **:** Silicon Carbide

SS **:** Stainless Steel

SS-SC **:** Sulfochromic Acid Treated Stainless Steel

TCPS **:** Tissue Culture Polystyrene

Trypsin EDTA: Trypsin - Ethylenediaminetetraacetic acid

3-oxo-C12-HSL : N-(3-oxododecanoyl)-L-homo-serine lactone

C4-HSL **:**N-butanoyl-L-homo-serine lactone (C4-HSL)

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COVALENT IMMOBILIZATION OF FURANONE DERIVATIVES FOR THE INHIBITION OF BIOFILM FORMATION ON IMPLANT SURFACES

SUMMARY

A biofilm is essentially composed of microbial cells attached to a surface and completely covered with an extracellular polymeric matrix (EPS) produced by biofilm-forming bacteria. Uncontrollable and undesirable accumulation of cell aggregates constitute serious problems in industrial processes and biomedical applications. Studies concerning biofilm effects on human health are generally performed on water/systems (dentistry 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.Because of these reasons, there is a growing demand for developing strategies to remove and protect the surfaces against biofilm formation .

The benefits of biofilm formation to microorganism include increased protection against antimicrobials and defence mechanisms. A biofilm cannot be considered as a homogeneous distribution of cells but it can be thought as a cluster of cells enclosed within a matrix with channels and pores. These channels facilitate the transport of oxygen and nutrients to the biofilm forming units. Removal of waste products and secondary metabolites also occur through these channels.

Medical devices and implants are highly susceptible to microbial contamination and infection of biomedical devices is a considerable and increasing problem. The aim of our study is to prevent these infections from the application of a thin coating that prevents bacterial colonization on the surfaces of biomedical devices.

The rationale of the study depends on the prevention of microorganism colonization on different surfaces by blocking the communication of these microorganisms through a non-biocidal method. The key molecule of this method is Acyl Homoserine Lactone (AHL), which is a signal molecule used for the communication of microorganisms. These AHL molecules are produced by the microorganisms and secreted out of their cells. These molecules are sensed by other microorganisms and when its density reaches the threshold level, it is taken back to the microorganisms and activates the expression of several genetic elements (virulence factors, antibiotic resistance genes etc.). This communication system is called as “Quorum Sensing”. The blocking of this communication (“Quorum quenching”) is vital to prevent the colonization of microorganisms and infection. In order to achieve this blocking, AHL molecule is the target in our study. Here, analogues of this AHL molecule has been used to block the biofilm formation. Inhibitory effect of AHL analogues 5-(dibromomethylene)-3-methylfuran-2(5H)-one (named as FU1), 3-(bromomethyl)-5-(dibromomethylene) furan-2(5H)-one (named as FU2), (Z-)-4-Bromo-5-(bromomethylene)-2(5H)-furanone (named as 4Z), 5-(Bromomethylene)-2(5H)-furanone (named as 2(5H)) have been examined. Synergistic antimicrobial agent and immobilized AHL analogues composition as inhibitors for adhesion of microorganism have also been investigated.

Furanone molecules are a part of the chemical defense system produced by marine algae named as *Delisea pulchra*, which prevents the colonization on their surfaces [1]. Furanones have been found to interfere with quorum sensing mechanism of many human pathogens and in previous works, these compounds and their analogues have been shown to inhibit or prevent the growth of both Gram-negative and Gram-positive bacteria [2-4]. These molecules act on intercellular communication system both as free molecules and also as surface immobilized molecules [5].

Natural and synthetic furanone compounds have been found to interfere with Quorum Sensing (QS) mechanism of many human pathogens and block this specific pathway. Thus, the strategy for the present study is covalently linking furanone derivatives on surfaces to assess the effect of the immobilized furanone molecules on the adhesion strength of *Pseudomonas aeruginosa* biofilms.

Our goal is to inhibit formation of bacterial biofilm especially for medical applications. In the scope of the literature work, the strategy is to coat the surface with halogenated furanone derivatives.

To obtain surface-immobilized furanones through the sol-gel processes, carbodiimide chemistry and azide-nitrene chemistry have been carried out. In the literature, the immobilization procedure of furanone molecules starts with amine plasma, following polyacrylic acid and 4-azidoaniline treatment to obtain nitrene groups on to the surface, which will be the linker of furanone under UV light.

In this study, furanone molecules have successfully covalently bound to the substrate. Morphological differentiation at each step has been visualised by using AFM and SEM. FTIR results are also coherent with AFM and SEM. PAA attachment has radically increased surface roughness (from 2.059 ± 0.05nm SS-SC to 6.821 ± 0.75) and created niche areas for bacterial attachments. Change in surface morphology also effected CFU counts.

CFU counts and CSLM images showed that FU1 and FU2 are highly effective against biofilm formation and FU2 has superior activity compared to FU1. FU2 has inhibited 78.9% of bacterial adhesion onto the surface while FU1 reduced 65.4% when compared the surface without furanone. 4-Z and 2-5(H) have no activity against biofilm formation. The total bacterial reduction is 4 times higher than AZA samples and 5 times higher when compared with uncoated SS-SC samples.

Tobramycin asserts antibacterial activity when applied with furanone derivatives and it further reduced attachment of bacteria (96.3% reduction for FU2 and 79.4% reduction for FU1) when compared with tobramycin untreated samples (76% reduction for FU2 and 60% reduction for FU1).

Furanone coated substrates were tested in vivo using foreign-body infection model. 68 % mice died before 24th hour of the experiment and furanone coated substrates had higher CFU counts compared to control samples. The relationship between eDNA concentration and CFU also assessed with this experiment and it has been found that eDNA concentration is proportional to CFU count.

This project was performed as a collaboration between Istanbul Technical University in Turkey and the research group from Instituto di Chimica del Riconoscimento Molecolare at CNR (National Research Council) in Italy. Italian group worked on chemical grafting of protease and lysozyme on to the stainless steel coupons and carried out the ATR-FTIR imaging of both the surface coatings and the biofilm samples. Chemically grafted stainless steel surfaces were provided by CNR group.

Arcelik A.S. (partner company from industry) also partly contributed to the project by providing scholarships to researchers. CSLM experiments and animal studies were carried at the University of Copenhagen, Denmark.

Keywords: Halogenated furanone, furanone, carbodiimide chemistry, antibacterial coating, biofilm, quorum sensing, quorum quenching, sol-gel

IMPLANT YÜZEYLERİNDE BİYOFİLM OLUŞUMUNUN ENGELLENMESİNE YÖNELİK FURANON TÜREVLERİNİN KOVALENT İMMOBİLİZASYONU

ÖZET

Biyofilm, yüzeye bağlı mikroorganizmalar tarafından oluşturulan, Ekstraselüler Polimer Matriks (EPS) ile çevrelenmiş bir yapıdır. Endüstriyel ve biyomedikal uygulamalarda kontrolsüz ve istenmeyen bölgelerde oluşan biyofilme bağlı hücre agregatları bu uygulamalarda ciddi ekonomik sorunlara neden olmaktadır. Endüstriyel sistemlerde biyofilm oluşumuna, dental yüzeyler, gıda endüstrisindeki üretim bandındaki kontaminasyonlar ve havalandırma sistemleri gibi birbirinden çok farklı sistemlerde sıklıkla karşılaşılmaktadır. Biyofilm oluşumunun insan sağlığı ile ilgili alanlarda yapılan çalışmalar genel olarak su sistemleri, dişçilik, protez ve implantlar üzerine yoğunlaşmaktadır. 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 bağlı genel sorunlar toplumlarda ciddi sağlık riski teşkil etmektedir. Hastane ortamına bağlı enfeksiyonlar, mide bağırsak enfeksiyonları, kulak, göz hastalıkları, biyofilme bağlı enfeksiyon risklerinin başında gelmektedir. 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.

Doğada biyofilmi oluşturan mikroorganizmalar, salgıladıkları ekstrasellüler polimer matriks ile sarılıdırlar ve bu matriks, çevresel koşullardaki değişimlere karşı biyofilm yapısı içindeki mikroorganizmaları koruyucu bir katman görevi üstlenmektedir. Aynı zamanda bu katman toksik kimyasalların ve antibiyotiklerin geçişini engelleyerek biyofilm yapısındaki mikroorganizmalara koruyucu bir katman görevi görür. Hücre katmanları arasında bulunan kanallar, biyofilmin gelişmesinde hayati öneme sahiptir. Mikroorganizmalar ihtiyaç duydukları besinlere ve oksijene bu kanallar sayesinde erişirken, atıkların ve ikincil metabolitlerin uzaklaştırılması da yine kanallar ile gerçekleşir. Ayrıca mikroorganizmaların iletişim amaçlı kullandığı sinyal moleküllerinin iletimi bu kanallar üzerinden gerçekleşir.

Enfeksiyon riskine karşı biyomedikal alanda biyosidal etkiye sahip antibiyotiklerin uzun süreli kullanımı, zaman içinde patojen mikroorganizmaların bu kimyasallara karşı direnç kazanmasına ve antibiyotiklerin biyofilm oluşumunu engellemede etkisiz kalmasına neden olmaktadır. Biyofilm oluşumunda mikroorganizmalar arasında iletişimi sağlamak amacıyla Açil Homoserin Lakton (AHL), sinyal molekülü olarak kullanılmaktadır. Bu molekül, biyofilmi oluşturan mikroorganizmalarca sentezlenerek hücre dışına salınmaktadır.AHL, diğer mikroorganizmaların hücre duvarında bulunan sinyal reseptörlerince algılanır ve antibiyotik direnç genleri, virülans faktörleri gibi çeşitli genetik elementlerin

ekspresyonunu aktive eder. Bu iletişim sistemi literatürde "Quorum sensing" olarak adlandırılmaktadır. İletişim yolağının bloke edilmesine ise "Quorum quenching" adı verilmektedir ve mikroorganizmaların enfeksiyon ve koloni oluşumunun engellenmesinde hayati öneme sahiptir. Bu çalışmada antibiyotiklerin etki mekanizmasından farklı olarak AHL sinyal yolizinin bloke edilerek, mikroorganizmaların yüzey üzerinde koloni oluşumunun engellenmesi hedeflenmiştir. Sinyal yolizinin ve biyofilm oluşumunun engellenmesi maksadıyla, AHL molekülünün analogları olan halojenlenmiş furanonlar kullanılmıştır. AHL analogları 5-(dibromomethylene)-3-methylfuran-2(5H)-one (FU1 olarak isimlendirildi), 3-(bromomethyl)-5-(dibromomethylene) furan-2(5H)-one (FU2 olarak isimlendirildi), (Z-)-4-Bromo-5-(bromomethylene)-2(5H)-furanone (4Z olarak isimlendirildi), 5-(Bromomethylene)-2(5H)-furanone (2(5H) olarak isimlendirildi) halojenlenmiş furanonlar solüsyon içinde, herhangi bir yüzeye kovalent veya non-kovalent bağ ile bağlı olmadan yapılan çalışmalarda biyofilm oluşumunu engellemede etkin olduklarını göstermişlerdir. Bu çalışmada yüzeye kovalent olarak bağlanan moleküllerin biyofilm oluşumunu engellemedeki etkisi incelenmiştir. Aynı zamanda yüzeye kovalent bağlı furanonun varlığında antibiyotiklerin biyofilm oluşumunu engellemedeki etkileri de incelenmiştir.

Bu çalışmanın amacı, mikroorganizmaların endüstriyel ve özellikle tıbbi alanlarda sıkça kullanılan yüzeylerde biyofilm oluşumunun engellenmesi için furanon türevleri ile kaplı yüzeyler geliştirmektir. Medikal cihaz ve implant yüzeyleri mikrobiyal kontaminasyona ve buna bağlı enfeksiyon oluşum riski barındırmaktadırlar. Biyofilm engellemesine yönelik bugüne kadar yapılan çalışmalar da dikkate alınarak, yüzey üzerindeki oluşumu engellemek üzere, biyofilm oluşumunda büyük öneme sahip "quorum sensing" sinyal yolizini bloke ettiği bilinen halojenlenmiş furanonların farklı türevleri yüzeye kovalent olarak bağlanmıştır. Solüsyon içinde çözünmüş halde furanon türevlerinin patojen mikroorganizmaların biyofilm oluşumunu engellediği daha önce yapılan çalışmalarda kanıtlanmıştır. Bu çalışmada halojenlenmiş furanon türevlerinin kaplama malzemesi olarak kullanımının, *Pseudomonas aeruginosa'*nın yüzeye bağlanması üzerine etkisi incelenecektir. *Pseudomonas aeruginosa* fırsatçı patojeni biyofilm oluşturduğu bilinen ve biyofilm çalışmalarında model olarak kullanılan gram-negatif bir mikroorganizmadır. Geliştirilen yüzeyin, kateter, implant gibi medikal yüzeylerde ve endüstriyel sistemlerde uygulanarak uzun ömürlü koruma sağlanması hedeflenmektedir.

Furanon moleküllerinin yüzeye bağlanmasında sol-jel yöntemi kullanılmıştır. Sol-Jel'in serbest hidroksit grubuna karbodiimid reaksiyonu ile poliakrilik asit bağlanmıştır. Azid-nitren reaksiyonu ile yüzey üzerinde furanonun bağlanabileceği serbest nitren grupları oluşturulmuştur. Nitren gruplarına furanon, UV ışık altında fotokimyasal yöntemler kullanılarak bağlanmıştır. Yüzey kimyasında değişim, moleküllerin yüzeye bağlanışı ve yüzey morfolojisi AFM, FTIR ve SEM ile analiz edilmiştir.

Bu çalışmada furanon molekülleri başarılı bir şekilde yüzeye kovalent olarak bağlanmıştır. AFM ve SEM çalışmaları ile kaplama çalışmalarındaki her aşamada morfolojik değişim görüntülenmiştir. FTIR çalışmaları ile elde edilen bulgular AFM ve SEM ile elde edilen görüntüleri doğrular niteliktedir. Yüzeye PAA bağlanmasından sonra yüzey pürüzlülüğü 2,059 ± 0,05nm'den 6,821 ± 0,5nm değerine çıkmıştır. Yüzey pürüzlülüğündeki değişim CFU sayımlarını da etkilemiştir.

CFU sayımları ve CSLM görüntülemeleri FU1 ve FU2'nin biyofilm oluşumunu engellemedeki etkinliğini doğrular niteliktedir. FU2 molekülünün, FU1'e göre daha etkin olduğu gözlemlenmiştir. AZA bağlı yüzeyle karşılaştırıldığında FU2 bakteriyel bağlanmayı % 76 oranında azaltırken FU1 bağlanmayı % 60 oranında azaltmıştır. 4-Z and 2-5(H) bağlı yüzeylerin biofilm oluşumunu engellemeye yönelik bir etkinliği olmadığı tespit edilmiştir. FU2'nin toplam bakteriyel yükü AZA örneğinden 4 kat SS-SC örneğinden 5 kat daha az olduğu tespit edilmiştir.

Tobramisin furanon türevleri ile birlikte kullanıldığında yüzeye bağlı bakteri miktarını tobramisin kullanılmayan örneklerle (FU2 için % 78,9 oranında, FU1 için %65.4 oranında) karşılaştırıldığında FU2 için % 96,7 oranında FU1 için %79,4 oranında azalttığı tespit edilmiştir.

Furanon bağlı yüzeyler yabancı cisim enfeksiyon modeli kullanılarak in vivo test edilmiştir. Deney farelerinin % 68'inin 24. saatten önce öldüğü ve furanon kaplı yüzeyler CFU sayımının kontrol yüzeylere göre daha yüksek olduğu belirlenmiştir. eDNA konsantrasyonu ve CFU sayımı arasındaki ilişkiye de bu çalışma ile bakılmıştır ve eDNA miktarının CFU sayımı ile orantılı olarak değiştiği saptanmıştır.

Bu proje ITU-Moleküler Biyoloji ve Genetik Bölümü ve İtalya'daki Chimica del Riconoscimento Molecolare, CNR, araştırma grubunun işbirliği ile gerçekleştirilmiştir. İtalya'da görevli grup yüzey kaplamalarının ve biofilm örneklerinin ATR-FTIR görüntülenmesinde görev almıştır. Çelik yüzey üzerine kovalent bağlama çalışmaları CNR grubu tarafından yapılmıştır. Arçelik A.Ş. araştırmacılara burs sağlayarak kısmi olarak katkıda bulunmuştur. CSLM deneyleri ve hayvanlarla gerçekleştirilen deneyler Kopenhag Üniversitesi Danimarka'da gerçekleştirilmiştir.

1. INTRODUCTION

1.1 Literature Review

1.1.1 Bacterial biofilms

In natural environments, bacteria alternate between planktonic and sessile states in response to environmental factors. While planktonic form, passively floating as single cells in aquatic environment, 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. So the community of bacteria is protected from attack by host immune cells.

Over the last decade, there has been a trend toward extrapolating results from studies of planktonic bacteria into environmental systems. These 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 [1-4].

Antonie van Leeuwenhoek made the first investigation of microbial aggregates and it was published in 1684. He scraped the plaque from the teeth and observed what he described as the “animalculi” or “tiny animals” under his primitive microscope.

Microorganisms should not be studied just only as biofilms. They should also be considered in the context of interactions with their micro environment. The environment exerts an effect on the metabolism of bacteria and the biofilm research represents the best tool to examine 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.

In most environments, bacteria are not found in planktonic form but exist as surface-attached communities called biofilms [5]. The benefits of immobilization on a surface to microorganism include increased protection against antimicrobials and increased capacity to withstand environmental changes. Biofilms are the 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. However, the majority of the contamination or the corrosion process occurs after the adhesion of the microorganisms and their growth continue inside the human body [1, 6]. 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 [7]. In the 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 the food surface. They have the ability to reduce flow and heat transmission, block membrane pores or cause energy losses [8].

1.1.2 Biofilm formation

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 extracellular substances forming a matrix gel. 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 extracellular polysaccharides. Synthesis of extracellular matrix molecules occurs after colonization of adhered bacteria. When the cells remain attached on the surface they secrete sticky extracellular substances forming a matrix gel. The matrix consists of main polysaccharides, besides proteins, nucleic acids, lipids, mineral ions and various cellular debris. Matrix produced by bacteria allows co-adhesion of other microorganisms present at the micro-environment. 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. Next stage is the formation of dense bacterial aggregates characteristics of mature biofilms. Several layers of cells embedded in the matrix gel and the layer of cells within the matrix form biofilm [9]. Biofilm formation and maturation stages are given in Figure 1.1.

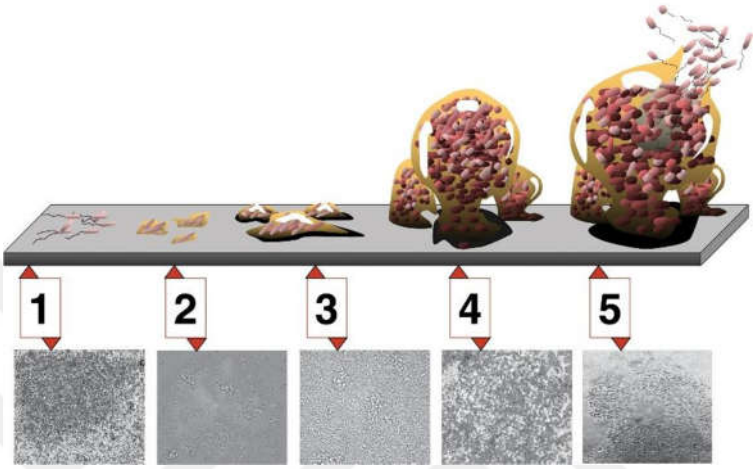


Figure 1.1 : Biofilm formation and maturation in five stages [10].

1st stage, initial attachment; bacteria attaches irreversibly. 2nd stage, irreversible attachment and EPS formation; bacteria adhere to the surface, microcolony forms. 3rd stage, maturation; microcolony grows, EPS covers the entire surface of biofilm. 4th stage maturation and budding formation; Mushroom shaped structure begins to form, biofilm expands. 5th stage, dispersion. Bacteria reenters planktonic state at this step. Each step of development is paired with *P. aeruginosa* biofilm microscopic images [10].

Bacteria present in the biofilm can communicate with each other by means of signal molecules so-called quorum sensing. Signal molecules promote the growth of biofilm, and more biofilm growth causes the production of more quorum sensing signal molecules. Thus, quorum sensing creates a positive feedback loop.

Biofilm layer, formed by bacterial aggregation and attachment on the surface, may lead to corrosion causing serious damage [11]. Thick biofilm layers and the metabolic activities that running inside them make fluid flow more difficult and cause the blocking the water pipes. Biofilm layer also acts as a barrier and affects heat transfer negatively [3, 6]. 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 odours [12].

1.1.3 Infections and biofilm

Biofilm formation and the metabolic activities within do not only have a negative effect on device and system performance, but it also causes serious health risks on the community. Most of these health risks generally target patients with the weaker immune system and mostly cause nosocomial infections of gastrointestinal, eye and ear etc.

There is no definitive studies exist to meet the criteria of Koch's postulates regarding the necessity of biofilms as etiological agents of disease [13] but recently, a minimal set of criteria was proposed for adjudging biofilm etiology of an infection. These criteria include the cluster of bacteria, covered with EPS that exhibit physical and chemical resistance to antibiotics effective on the planktonic cells [14]. However, the most persuasive evidence comes from microscopic observations at infection sites in situ or devices recovered from patients.

1.1.4 Implant associated infections

Bacteria growing in biofilms cause most of the implant-associated infections. Biofilm formation depends on several steps; starts with attachment to an abiotic surface, continues by rapid proliferation and production of EPS by biofilm-forming bacteria to enable intercellular adhesion [11, 15, 16]. One of the most prevalent biofilm former which is widely recognized to cause infections on medically implanted materials is *Pseudomonas aeruginosa* [12]**.** *P. aeruginosa* had been classified under serious threat level and it was declared as one of the six 'top-priority dangerous, drug resistant microbes by Infections Diseases Society of America [17].

Since biofilm-forming microorganisms such as *Pseudomonas aeruginosa* easily and rapidly develop resistance to any condition (disinfectants, antibiotics and other stress factors), such contaminations require continuous, complex and combined treatment methods.

1.1.5 Virulence factors and quorum sensing

QS system of *P. aeruginosa* regulates production of virulence factors which enables microbial cells to evade host defense [18, 19]. QS system controls the production and secretion of small N-acyl-L-homoserine lactone (AHL) diffusible signal molecules. AHL signal molecule concentration depends on cell density and when bacterial density reaches a threshold, the signal molecules bind to a transcriptional regulator which induces transcription of several target genes [18]. *P. aeruginosa* possesses two AHL-mediated QS system: the las and the rhl QS, which, are regulated by LasR and RhlR transcriptional regulators and synthesizes LasI and RhlI. Two AHL signal molecules N-(3-oxododecanoyl)-L-homo-serine lactone (3-C12-HSL) and N-butanoyl-L-homo-serine lactone (C4-HSL) are synthesized by LasI and RhlI respectively [20, 21]. In addition to the AHL signal molecules, a third signalling compound, Pseudomonas Quinolone Signal (PQS) is regulated by LasR and expression of rhlI and rhlR are enhanced by PQS [22, 23]. Therefore it is claimed that PQS has a regulatory function for las and rhl QS systems [16]. The signal molecule N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) has immunomodulatory effects which cause neutrophil apoptosis and thus facilitate infection [24]. Furthermore, it is shown that T-cell proliferation has been inhibited and cytokine IL-12 production and tumor necrosis factor alpha (TNF-α) has been suppressed in the presence of 3-C12-HSL. T-cells, TNF-α and cytokine IL-12 are all involved in the TH1 response, which activates bactericidal activities of macrophages [25].

1.1.6 Antibiotic tolerance and resistance in biofilm

One of the most considerable properties of biofilms is their resistance to antibiotic treatments. Many life-threatening infections are related to biofilm formation, and current treatment of biofilm-related infections with antibiotics is still a major medical problem. Some antibiotic treatments can decrease the number of bacteria present in biofilm but they can not completely terminate transmission of infection in vivo [26].

1.1.7 Antibiotic tolerance in biofilm

If bacteria susceptible to antibiotics during the planktonic mode of growth but stubbornly resist treatment during biofilm mode of growth, the recalcitrance of the biofilm is referred to as tolerance. Antibiotic tolerance of a biofilm allows bacteria to

survive but growth may not occur. Antibiotic concentration might be higher than their planktonic minimal inhibitory concentration. The underlying mechanism of antibiotic tolerance depends on many factors including restricted penetration of antimicrobials, differential physiological activity within the biofilm, the formation of persister cells, specific tolerance mechanisms related to biofilm mode of growth.

Biofilm bacteria are covered within a matrix composed of exopolysaccharides, extracellular DNA and protein. When antibiotics are used in the attempt to treat biofilm-related infections, antibiotics must diffuse into the matrix and reach to bacteria [27]. Laboratory studies showed that the biofilm matrix does not prevent diffusion of antibiotics in general but in some cases, antibiotics can bind to matrix components. It is shown that ciproflaxin and tobramycin penetrate into *P. aeruginosa* biofilm matrix but these antibiotics are effective only against metabolically active bacteria [28]. This finding suggests that the antibiotic tolerance of bacteria is more relevant to the physiological state of microorganism than restricted antibiotic penetration [29].

Subpopulations of microorganisms located different part of the biofilm may have different physiological activity. *P. aeruginosa* biofilms which grown in flow-cells showed, that tobramycin, ciprofloxacin, tetracycline antibiotics kill metabolically more active bacteria found in the upper part of the biofilm, rather than the bacteria found in the lower part of the biofilm. On the other hand, antibiotic colistin, detergent SDS and chelator EDTA are effective against physiologically inactive bacteria located in the lower part of the biofilm [30, 31].

Persister cell formation is thought to be another factor that contribute to antimicrobial tolerance of biofilms. Persister cells are formed by differentiation of subpopulation to dormant state. Unlike to bulk population of biofilm, their lower metabolic activities made them less vulnerable to antibiotics which target them several fundamental metabolic processes such as replication of DNA, cell wall synthesis [32-34].

P. aeruginosa PA14 ndvB gene encodes an enzyme that binds tobramycin and inactivates it by sequestering the antibiotic [35]. The ndvB gene product is specifically produced in biofilms and ndvB mutant cells are much more sensitive to

tobramycin than wild-type biofilms, whereas ndvB mutant and wild-type cells showed no specific tolerance mechanism when grown in planktonic culture.

1.1.8 Antibiotic resistance in the biofilm

Described specific tolerance mechanisms can contribute to the persistence of biofilms and therefore provide the necessary platform for the formation of antibiotic-resistant mutants. It is suggested that 25 to 45% of adult cystic fibrosis (CF) patients are infected with MultiDrug Resistant (MDR) bacteria arise from the several simultaneous mutations in a single bacterial cell of several genes [36]. Advanced studies have demonstrated that in several cases there are hypermutable *P. aeruginosa* isolates associated with MDR [27–30]. 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.

DNA repair systems of either mismatch repair system genes mutS, mutL, uvrD or DNA oxidative lesions repair system genes mutT, mutY, mutM have been found to be responsible for hypermutable phenotype of CF *P. aeruginosa* isolates [37-39]. It has been proposed that hypermutations in those two systems promote the emergence of antibiotic-resistant isolates under selective pressure of antibiotics [37, 40-42].

1.1.9 Prevention of tolerance and resistance in biofilms

Persistent infections result from antibiotic-resistant and tolerant to subpopulations in biofilms. In clinical settings, it has been demonstrated that in vitro biofilm susceptibility testing is more reliable than planktonically determined susceptibility testing [43]. From biofilm susceptibility test of 100 CF isolates, it has been shown that standard antibiotic dosage results in sub-optimal antibiotic concentration at the site of infections in biofilm-related infections [44]. Subinhibitory concentrations of antibiotics make biofilms more tolerant and resistant against antibiotics[45]. It has been shown that subinhibitory concentrations of beta-lactam antibiotics [46], aminoglycosides [47] and quinolones [48] promote biofilm formation.

Combinatorial treatment is a possible way to eliminate biofilm formation in vitro and in vivo [42, 49]. Antioxidants have a combinatorial effect when used with antibiotics by decreasing mutability and resistance of biofilms [50]. Furthermore, prevention of

resistant biofilm formation can be achieved by applying pairs of antibiotics which belong to different classes with synergistic activities starting at the early stage of infection as suggested in the European Consensus Document on Early Intervention and Prevention of Lung Disease in Cystic Fibrosis [51].

1.1.10 Current biofilm removal strategies

In order to protect surfaces against microbial attacks, functional coatings are widely preferred when bulk properties of substrates need to be conserved [52]. A fine thin film coating should have resistance to corrosion, good adhesion to the substrate and precise chemical control in order to meet the required functionality and permanence criteria.

Sol-gel technology possesses numerous advantages including high biocompatibility, non-toxicity, low-temperature processing and easy application to any kind of substrate [53]. Moreover, the sol-gel procedure is carried out at the low temperature which gives a considerable advantage for preparation of materials in industry [54].

1.1.11 Immobilization of antimicrobials

Several immobilized antibiotics such as cefazolin [55], minocycline-rifampin [56, 57], teicoplanin [58, 59], vancomycin [60, 61] and antimicrobials like silver [62], chlorhexidine [63], salicylic acid [64], quaternary ammonium compounds [65, 66], caspofungin [61] have been tested on biomaterials including catheters, prostheses and other implants. Clinically tested chlorhexidine implanted catheters have been shown to have no effect against preventing the disease [67, 68]. Another study has been reported that chlorhexidine coated catheters resulted in anaphylaxis response [69]. Quaternary ammonium compounds and silver have displayed toxic effect on human cells [70, 71]. Antibiotic usage as implant coatings have the disadvantage of development of resistance by the biofilm forming microorganisms and makes this kind of therapy has short life-time and ineffective [72-75].

Silver ions have been used as the antibacterial coating on many materials to inhibit bacterial attachment on biomedical devices. Silver ions can either bind to electron donor groups of sulphur, oxygen and nitrogen in biological molecules or they can substitute with some essential ions such as Ca^{2+} or Zn^{+}. Silver ions can demonstrate cytotoxic effect when the concentration is too high [76]. Therefore the concentration

of silver ion embedded in coating and diffusion rate of silver ions into the medium is crucial for reliable antibiofilm effect [77].

The most important drawbacks of silver ion coating are that high concentration of silver ions needed for the inhibition of bacterial attacment, leaching of silver ions from the surface and accumulation of silver ions in other body organs make this strategy clinically impractical.

1.1.12 Proteins and peptides

Within this decade many peptides and proteins have been reported to be utilized as implant coatings, scaffolds for artificial organs and grafts. Collagen is one of the promising protein that has been widely investigated in the literature. Bovine collagen has been chemically modified to improve its adhesion properties as a biomaterial [78]. Collagen creates a biomaterial surface which supports the adhesion body cells for tissue integration and inhibit growth of bacteria. In one study, covalently bound RGD peptide and collagen activity had been conducted for the adhesion strength of *E. coli* and *S. aureus.* RGD had no effect on the attachment of the strains. On the other hand, collagen reduces *E. coli* adhesion and increases adhesion of *S. aureus.* In one study RGD peptide was incorporated into chitosan coated substrate. Adherence of ostebolast cells was stimulated and bacterial adhesion to surface decreased [79]. Although peptides possess a number of advantages, such as applicability, facilitating adhesion of body cells, having no cytotoxic effect, they have some drawbacks like susceptibility to proteolytic degradation, pH and/or salinity-dependent activity and loss of activity due to binding to serum proteins [80].

1.1.13 Quorum sensing inhibitors

In nature, organisms protect their surfaces from microbial colonization by synthesizing chemical compounds. Halogenated furanones are produced by marine red algae species *Delisea pulchra* to protect itself against microbial colonisation. Furanones have been found to interfere with (QS) mechanism of many human pathogens and biologically active against many gram negative and gram positive microorganisms [81, 82].

The rationale of the study depends on the prevention of microorganism colonization on different surfaces by blocking the communication of these microorganisms

through a non-biocidal method. The key molecule of this method is Acyl Homoserine Lactone (AHL), which is a signal molecule used for the communication of microorganisms. These AHL molecules are produced by the microorganisms and secreted out of their cells. These molecules are sensed by other microorganisms and when its density reaches the threshold level, it is taken back to the microorganisms and activates the expression of several genetic elements (virulence factors, antibiotic resistance genes etc.). This communication system is called as “Quorum Sensing”. The blocking of this communication (“Quorum quenching”) is vital to prevent the colonization of microorganisms and infection. In order to achieve this blocking, AHL molecule is the target in our study. Thefirst way is to use an analogue of this AHL molecule to block the system or the breakdown of this AHL molecule enzymatically by using two specific enzymes. Furanone compounds lack any reactive chemical groups and their lactone ring is labile under both acidic and alkaline conditions.

1.1.14 Furanone molecules as QSIs

Among many synthetic and natural QSI molecules, natural halogenated furanones also has shown antibiofilm properties. In the literature, many natural and synthetic brominated furanones have been identified as the effective QSIs in both gram-negative and gram-positive bacteria [83-89]. In one study, brominated furanones have been found to interfere production of polysaccharide matrix, repression of the eps operon and therefore prevent biofilm formation by gram-positive bacteria *B. Subtilis [85]*. In another study a halogenated furanone compound (Z-)-4-bromo-5-(bromomethylene)-2(5H)-furanone incorporated into nafion polymer inhibited *P. aeruginosa, E. coli* and *B. subtilis* surface colonization [90]. Covalently bound furanones on reduced bacterial adhesion onto stainless steel substrate with this study [91]

The project aims to obtain surface-immobilized furanones through the sol-gel processes, carbodiimide chemistry and azide-nitrene chemistry. In the literature, the immobilization procedure of furanone molecules starts with amine plasma, and following with polyacrylic acid and 4-azidoaniline treatment to obtain nitrene groups on to the surface, which will be the linker of furanone under UV light (Figure 1-2).

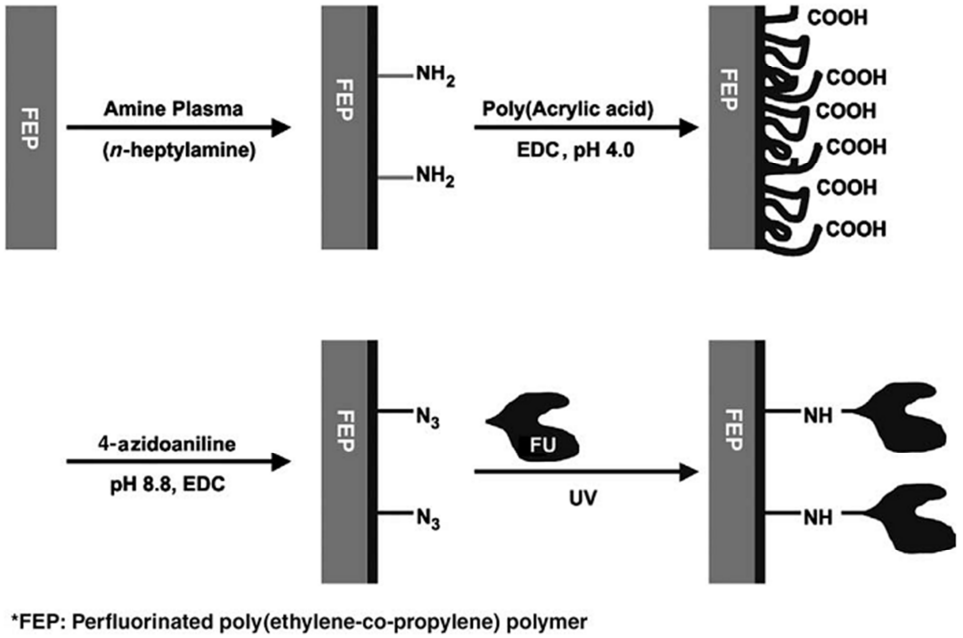


Figure 1.2 : Schematic diagram of the furanone coating structure [103].

As an alternative way can be using a silane monomer having an amino group (3-(Trimethoxysilyl)-propylamine, CAS: 13822-56-5, $C_6H_{17}NO_3Si$) to obtain a thin film with amino groups on the surface, which can be then utilized for the immobilization of furanone with an extra step.

The alternative strategy of furanone coating could be the entrapment of "Quorum Quenching Enzymes" in the sol-gel matrix to obtain a scaffold coating structure. Organic molecules added to the sol become entangled in the developing network and consequently entrapped in the final xerogel [92].

1.2 Purpose of Thesis

A biofilm is essentially composed of microbial cells embedded in an extracellular polymeric matrix produced by biofilm-forming bacteria. Uncontrollable and undesirable accumulation of cell aggregates constitutes serious problems in industrial processes and biomedical applications. Studies concerning biofilm effects on human

health are generally performed on water/systems (dentistry systems), prosthesis and implants. Biofilm formation and metabolic activities in biofilm can lead to serious health risks. Most of these health risks are nosocomial infections of gastrointestinal, eye, ear etc. Because of these reasons, there is a growing demand for developing strategies to remove and protect the surfaces against biofilm formation.

The benefits of biofilm formation to microorganism include increased protection against antimicrobials and defence mechanisms. A biofilm cannot be considered as a homogeneous distribution of cells but as a cluster of cells enclosed within a matrix with channels and pores. These channels facilitate the transport of oxygen and nutrients to the biofilm forming units. Removal of waste products and secondary metabolites also occur through these channels.

The main objective of the present thesis was to apply sol-gel technology and carbodiimide chemistry for immobilization of furanone compounds to obtain antimicrobial surface (Figure 1.3). Here we aimed to inhibit formation of bacterial biofilm using covalently attached furanone derivatives either for use in industrial or medical applications. Furanone compounds have been found to interfere with quorum sensing mechanism of many human pathogens and block this specific pathway. In order to realize the project, furanone compounds were immobilized onto hydrogel-type interlayer, poly(acrylic acid), using azide/nitrene chemistry for covalent attachment of the 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 (Figure 1.4 and Figure 1.5). Spectroscopic characterization of immobilization steps has been performed using Atomic Force Microscopy (AFM), Fourier Transform Infrared Spectroscopy (FTIR), Scanning Electron Microscopy. Cell viability of furanone compounds has been tested using MTS assay. The ability of covalently attached furanone compounds to prevent growth and biofilm formation of *Pseudomonas aeruginosa* has been evaluated using flow cell and Confocal Laser Scanning Microscopy (CLSM). *Pseudomonas aeruginosa* was used as a model biofilm forming microorganism. Within the scope of this project, there is a possibility of establishing a research base at the cutting edge of a new field of biomedical equipment expanding the possibility to produce materials with a higher level of safety.

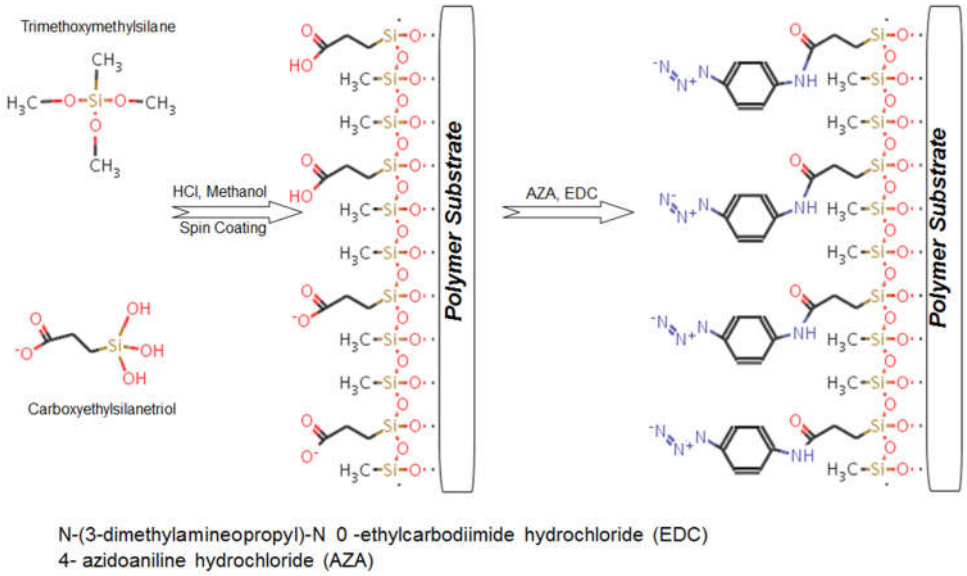
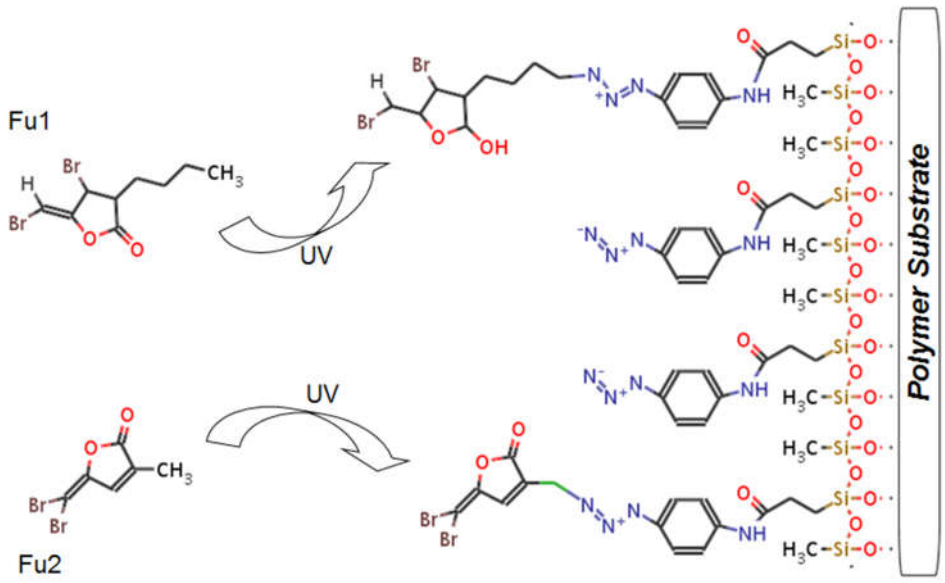


Figure 1.3 : Sol-gel coating onto polymer substrate.

In order to have a long-lasting anti-biofilm effect, covalent attachment of furanone compounds onto the surface is a method of choice. It is aimed for inhibition of biofilm formation on materials that are commonly used for medical devices, equipment and industrial systems. In this thesis, uncoated, furanone coating substeps and furanone coated substrates have been analyzed. Characterized surfaces are then used as substrate to test the biofilm formation of *Pseudomonas aeruginosa* PAO1 which is the well known model as biofilm forming microorganism. Gram-negative bacterium *P. aeruginosa* is an opportunistic human pathogen and it is attached to many abiotic and biotic surfaces including medical devices. It is the most common bacterium found in life threatening infections of the immunocompromised patient [93]. The extracellular polymeric substance produced by biofilm-forming bacteria protects colonized bacteria and ensures that they are resistant to [2, 94, 95] antibiotics. Combinatorial effect of antimicrobials and furanone molecules has also been investigated. In vivo effectiveness of furanone coating has been tested using foreign body infection model.



Fu1: 3-butyl-4-bromo-5(bromomethylene)-2(5H)-furanone
Fu2: 5-(dibromomethylene)-3-methylfuran-2(5H)-one

Figure 1.4 : Novel method for furanone immobilization onto sol-gel layer.

Our strategy is to coat medically relevant surface with halogenated furanone derivatives. The stainless steel material which is commonly used for medical devices, types of equipment and industrial systems was coated for the inhibition of biofilm formation.These halogenated furanone compounds have been found to interfere with quorum sensing (QS) mechanism of many human pathogens and block this specific pathway. We have aimed to assess the potential of furanone coatings for inhibition of biofilm formation by real-time monitoring of sessile bacteria under a range of environmental conditions. QS inhibition experiments will be performed in situ as previously reported [96-99]. In the literature, there are findings of the anti-biofilm activity of furanone molecules in solution [100]. This project aims to find a solution to biofouling by covering the surface with furanone-based compounds which are thought to be highly active against both gram-negative and gram-positive

microorganisms without having any cytotoxic effect on cells [101, 102].

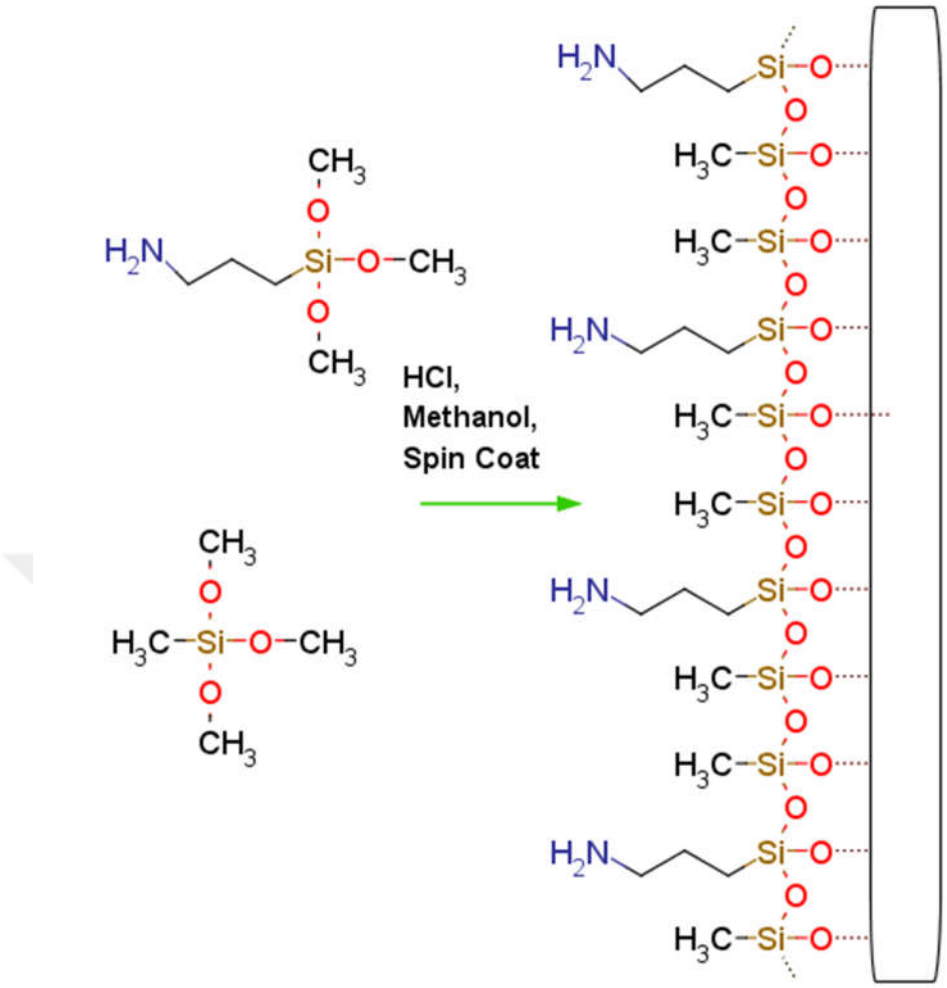


Figure 1.5 : Amino groups can be utilized for furanone immobilization.

Particularly, the achievement of the study will be articulated with the described objectives as explained below:

i) Developing a biofilm in a parallel plate flow cell system containing control and coated test materials, which facilitates non-destructive imaging of biofilms.

ii) To estimate furanone coating efficiency on the test materials by using AFM.

iii) Quantitative assessment of synthetic furanone compounds; to prevent growth and in-vitro biofilm formation of *P. aeruginosa* PAO1 which covalently immobilized on test materials to determine the fluorescent intensity of the samples using CLSM).

iv) Understand the mechanism of action of furanone-based coatings by real-time monitoring of *P. aeruginosa* biofilm in a flow cell system. The furanone compounds were covalently immobilized on to the test coupons by using the sol-gel method and azide/nitrene chemistry [103, 104]. Furanone molecules Fu1: 3-butyl-4-bromo-5(bromomethylene)-2(5H)-furanone (2-5(H)) and Fu2: 5-(dibromomethylene)-3-methylfuran-2(5H)-one, coated on stainless steel, have been tested for anti-biofilm activity by comparing with negative controls (Figure 1.6).

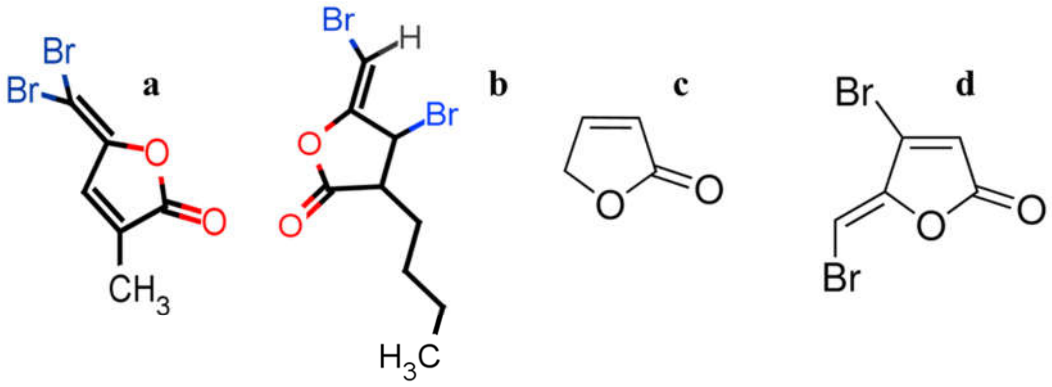


Figure 1.6 : Brominated furanone molecules used in this study: a.FU1, b. FU2, c. 2(5H), d. 4-Z

AFM imaging has been utilized to assess surface topography, coating continuity of polymers and thin films deposited by sol-gel. AFM has a powerful imaging capability at nanometer or sub-nanometer resolution. AFM was also utilized in determining film thickness with high accuracy. In the proposed work, surface roughness, surface topography, coating continuity and the film thickness of the coated test materials, after grafting with furanones have been characterized by AFM FU1 and FU2 used as model furanones for AFM studies.

Continuous-culture once-through flow chambers [30] was used in conjunction with CLSM. The flow cell have been designed for insertion of removable discs, which were coated with furanone derivatives, and control samples to evaluate biofilm formation and testing anti-biofilm activity on different surfaces. The study of attachment of microorganisms on control and furanone coated surfaces had been monitored in the presence of fluorescent protein GFP in plasmid-based mini-Tn7 transposon system (pBK-miniTn7-gfp3) [105]. The GFP provides the visualization of spatial growth patterns within the biofilm. The labelling technique had been used successfully in combination with flow cell to investigate initial attachment and

organization of microorganisms in the biofilm [106]. Factors including such as cluster size, activity, uniformity and distribution of cells within biofilm had been obtained by combining GFP and CSLM labelling and monitoring techniques. These techniques have given additional information about biofilm architecture and their response to surface-coated furanones. Initial attachment of microorganisms to the surfaces, structural homogeneity and physiologic profile of microorganisms within the biofilm was analyzed using CSLM. Biomass, mean thickness, substratum coverage and surface to volume ratio has been calculated using COMSTAT, a computer program developed for quantifying biofilm structures [107].

Although there is a huge number of reports available dealing with furanone compounds in the solution, there are very few reports dealing with the immobilization of furanone compounds on surfaces and there is no report available which attempts to inhibit *P. aeruginosa* biofilm formation on furanone coated surfaces. Other discriminative features of the research from other works in the literature include real-time and non-destructive monitoring of biofilm formation from time zero. Flow cell system provides a platform in which experiments can be carried out under the same environmental conditions and allows simultaneous monitoring of both surface coated and control test materials.

For this study furanone molecules have been selected according to the following criteria: Furanone molecules effective against biofilm formation (I), long lasting antibiofilm effect (II), doesn't have any significant cytotoxicity (III), molecules tested in vivo (IV), if there is any antibiofilm activity at subinhibitory concentration (V).

In this thesis, it is planned to determine the effects of surface coated furanone derivatives on opaque surfaces of biomedical importance to reveal their antibacterial potential as the surface coating material.

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 H_2O) (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.

Flow Cell System Components are composed of silicone glue (3M Super Silicone Sealant Clear), 6 mm and 35 mm thick polycarbonate sheet plastic, 50x24 mm glass coverslips, 2 ml syringe (Terumo), 3mm outer diameter, 1mm inner diameter silicone tubing 3 mm outer diameter 1mm inner diameter Marprene® (Watson-Marlow) tubing, peristaltic pump (Watson-Marlow, 205S), Schott medium bottles, clear polypropylene plastic connectors and T-connectors (Cole Parmer EW-06365-xx), 3,175 mm and 1,588 mm reduction connectors, bubble traps were required for assembly of flow cell system.

2.1.1 Components for bubble trap construction

35x80x45 mm polycarbonate blocks, 5 ml syringes with inner diameter of 12,5 mm, 9x2 mm rubber gaskets (M-seals, 221355), silicone glue, stoppers (Nordson EFD part no. 7017976) were used for assembly of bubble traps.

2.1.2 Materials for inoculation and running

Fresh overnight culture of *P. aeruginosa* as inoculum, flow-cell system, syringes with needles (Terumo LU-100, 27G), clamps were used after assembly of the flow-cell system.

2.1.3 Equipment for CSLM of flow cell grown biofilms

CLSM microscope (Zeiss LSM710), scalpels, Imaris (Bitplane), ImageJ, COMSTAT version-2, Java runtime environment were used during confocal imaging and data editing. The fresh overnight culture of *P. aeruginosa* as inoculum, flow-cell system, syringes with needles (Terumo LU-100, 27G), clamps were used after assembly of the flow-cell system.

2.1.4 Cell viability materials

Dulbecco's Modified Eagle Medium (DMEM; low glucose) (Gibco - Invitrogen, USA), Dulbecco's Modified Eagle Medium (DMEM; high glucose) (Gibco - Invitrogen, USA) cat no: 12800-017, Penicillin/Streptomycin Solution (Biochrom Ac, Germany), CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS)(Promega, USA), 96-well plates (Orange Scientific, Belgium), Elisa Plate Reader (Bio-Tek, Elx800, USA), Trypsin-EDTA (0,25 % w/v trypsin/0,02 % EDTA, Sigma)

2.2 Immobilization of Furanone Compounds

The following immobilization strategy is based on azide/nitrene chemistry. Other immobilization strategies such as diimide mediated amide formation are chemically more selective and well established but most furanone compounds lack any reactive chemical groups and lactone ring is sensitive to both acidic and alkaline conditions. It is criticized that lactone ring structure and $-CBr^2$ or -CHBr groups are important elements for antibacterial effectiveness [82, 101].

Azide/nitrene chemistry based immobilization is compatible with all furanone compounds synthesized. There is no need to adapt or change immobilization steps for given furanone compound. Immobilization steps were performed using hydrogel-type interlayer poly(acrylic acid) (PAAC).

Summary of the immobilization protocol is given as shown in the scheme in Figure 2.1, followed by a detailed description of each immobilization step.

2.2.1 Substrate activation and cleaning

Coupons were cleaned and preconditioned according to literature methods [79, 80]. Briefly, cold-rolled stainless steel plates (AISI 316L) 2 mm in thickness were cut and

turned into discs of 1 cm in diameter. The specimens were ground with SiC sandpaper and polished using a 6 μm diamond suspension and rinsed with ethanol, then ultrasonically washed 15 min in cyclohexane, 10 min in water (three times) then 20 min in acetone. They were etched by sulfochromic acid at 60 °C for 10 min to generate a reactive oxide/hydroxide layer (SS-SC). They were extensively washed with water and dried under a flow of nitrogen.

2.2.2 Preparation of stock Sol-Gel solution and spin coating

A homogeneous stock sol–gel solution was prepared by mixing 0,3 ml of methanol, 0,01 ml of 10 mM HCl, 0,3 ml of MTMOS and 0,2 ml of APTMOS [104] in a small test tube at room temperature. Stock solutions were freshly prepared daily prior to the application of the spin coating. The stock solution was applied on the previously polished coupons and spin-coated at 2000 rpm for 30 seconds at room temperature. Following the coating, coupons were dried at 100 °C for 1 hour (SS-SC-Sol).

In order to by-pass the amine plasma step, we will apply a sol-gel step. This step includes a thin bioactive sol-gel coating by using two silane monomers; Trimethoxymethylsilane (Methyltrimethoxysilane, CAS: 1185-55-3, $CH_3Si(OCH_3)_3$) and Carboxyethylsilanetriol (CAS: 18191-40-7, $C_3H_7NaO_5Si$) in an acid-catalyzed process (HCl, Methanol) [104]. At the end of this process, a sol-gel coating having carboxyl groups on the surface will be obtained, which will be further utilized for the immobilization of furanones via azide-nitrene chemistry.

2.2.3 Poly(acrylic acid) attachment onto sol-gel layer

Stainless steel surfaces were functionalized with amino groups of sol-gel matrix. In brief, poly(acrylic acid) solution was deposited on the hydroxylated surface, which enabled attachment of azidoaniline molecules via carbodiimide chemistry. Covalent binding of furanone molecules was carried on dark room conditions due to the light sensitivity of azide groups.

As shown in Figure 2.1, immediately after sol-gel coating, the interfacial layer was applied by soaking the SS-SC-Sol samples into 0,1 % aqueous solution of PAA at pH 4. EDC (5 mg/ml) was added to the solution after immersing samples into PAA solution. The samples were kept in a shaker at 4 °C overnight. Samples were

immersed and then washed twice with Milli-Q water to remove excess and non-covalently bound PAA from the surface (PAA).

2.2.4 Azidoaniline attachment onto PAA layer

SS-SC-Sol-PAA samples were immersed into the aqueous solution of 4-azidoaniline hydrochloride (2 mg/ml) under dark room conditions. Immediately after soaking samples into the AZA, 5 mg/ml EDC was added into the solution and then left overnight at 4 ºC in the dark with gentle shaking.

2.2.5 Furanone immobilization onto AZA layer

10 mg/ml furanone compounds in acetone was applied evenly to the SS-SC-PAA-AZA samples under dark room conditions. Samples were incubated until complete evaporation of acetone at room temperature. The samples are then illuminated with UV light for 3 minutes and washed twice with ethanol and water then dried with nitrogen.

In order to verify existence of covalently attached furanones on the surface, same experiments were carried on except UV illumination to the SS-SC-PAA-AZA layer was omitted. Under this conditions furanone compound was expected to be washed off from the surface and confirmation of this study was performed with CSLM.

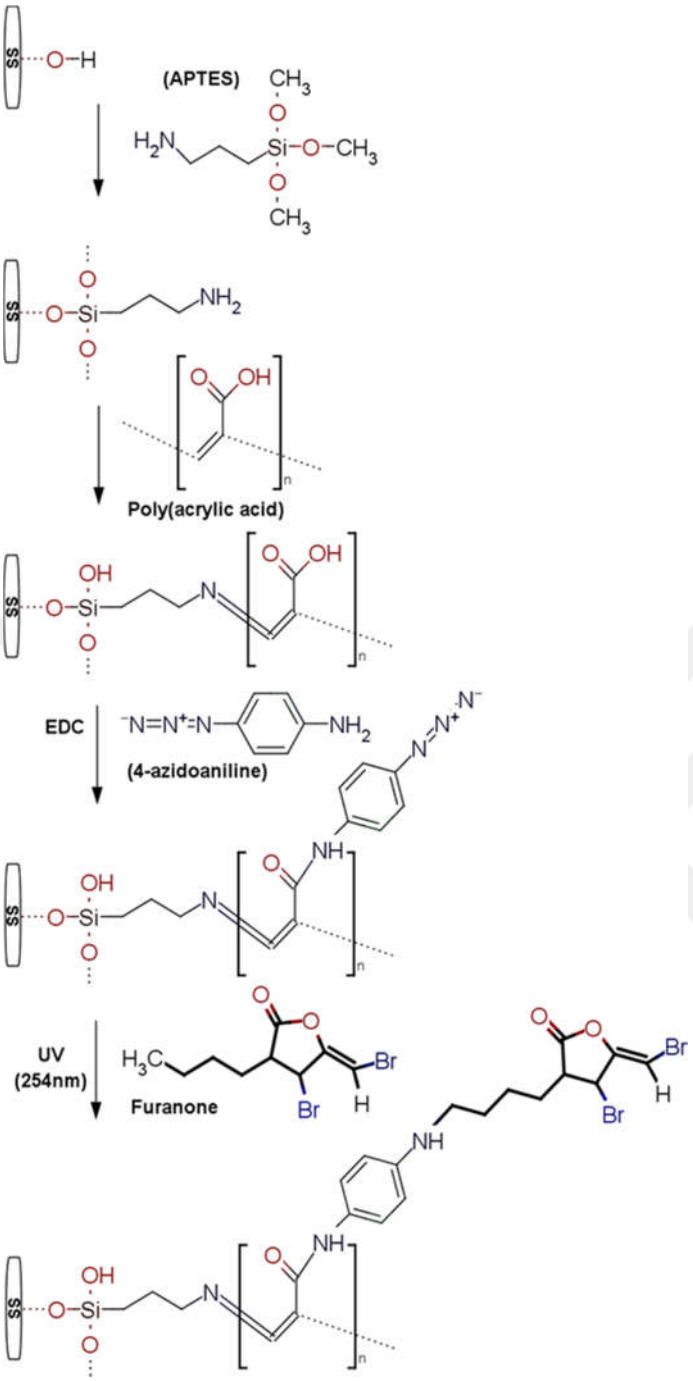


Figure 2.1 : Furanone coating steps for SS-SC substrate

As shown in Figure 2.1, immediately after sol-gel coating, the interfacial layer was applied by soaking the SS-SC-Sol samples into 0,1 % aqueous solution of PAA at pH 4. EDC (5 mg/ml) was added to the solution after immersing samples into PAA solution. The samples were kept in a shaker at 4 °C overnight. Samples were immersed and then washed twice with Milli-Q water to remove excess and non-covalently bound PAA from the surface (SS-SC-Sol-PAA).

2.3 Surface Characterization

2.3.1 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 40 N/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.

2.3.2 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 ºC mirror angle. All analyses were performed using 400 scans, with a resolution of 8 cm^{-1}.

2.3.3 SEM

Glass coverslips were used as substratum instead of SS-SC substrate in Scanning Electron Microscope analysis (SEM). Coating steps were imaged by SEM as previously described [108]. Briefly, intermediate coating steps and furanone coated substrate were fixed in 2 % glutaraldehyde, postfixed in 1 % OsO_4, point-dried using CO_2 and sputter-coated with gold as a standard procedure. All samples were investigated with a Philips XL Feg30 SEM operated at 2-5 kV accelerating tension. The magnifications used were 1.500 X and 5000 X.

2.4 Cell Viability Test of Immobilized Furanone Compounds

2.4.1 Data processing and evaluation of cell viability

Mean test absorptions were calculated and expressed as a percentage of control cells. Each value represents the mean of 3 experiments for materials non-cured and held

for 24 hours, and 4 experiments for freshly-cured materials, using at least 6 replicates of each extract per experiment. Extracts were rated as severely, moderately, or slightly cytotoxic, where activity relative to controls was less than 30 %, between 30 % and 60 %, or greater than 60 %, respectively. Statistical analysis using a one-way variance (ANOVA) was carried out. Differences were considered to be significant at a P-value of less than 0,05. The difference among individual groups was evaluated by the Student's t-test, with a p-value less than 0,05 being considered significantly different.

2.4.2 Cell viability assay

Mouse L929 fibroblastic cells which are the well known model for the investigation of cell viability were used in this study. The viable cell amounts were determined by using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfofenyl)-2H\--tetrazolium (MTS) method after 12 h and 24 h of proliferation.

L929 cells were maintained in Dulbecco's Modified Eagle Medium (Gibco, high glucose) supplemented with 10 % fetal bovine serum, 100 U/mL penicilin and 10 µg/mL streptomycin in a 37 ºC, humidified, 5 % CO_2 balanced air incubator. Medium was changed every 3 days. Trypsin-EDTA (0,25 % w/v tyripsin 0,02 % EDTA, Sigma) was used in order to detach the cells.

Two different furanone compound coated and uncoated steel coupons were used for the assay. $5x10^3$ cells were seeded onto each sample in 24-well plates and waited for two hours for cell attachment. Then 1 mL medium added to each wells. Cells on tissue culture polystyrene (TCPS) surface of 24-well plates used as negative control, which considered to be an ideal condition of cell growth. One sample without cells was used as internal control for each sample.

CellTiter 96® One Solution Cell Proliferation Assay kit, (Promega, WI) was used to determine the cell density on the samples. The experiment was performed in triplicate for each sample type at 12 h and 48 h. DMEM low glucose medium was mixed with MTS solution with a ratio 5:1. Cell seeded samples were transferred into a new, sterile 24-well plate. MTS - medium mixture (400 µL) was added onto each sample in 24-well plate and incubated in dark for 2,5 h at 37 ºC in a CO_2 incubator. After incubation, 200 µL of solution from each well was transferred into a new 96-well plate. Absorbance was measured at 490 nm using an Elisa Plate Reader (Bio-

Tek, Elx800, USA). The percentage of cell viability was calculated by multiplying the ratio absorbance of the sample versus control by 100. Cytotoxicity was rated based on cell viability were rated as severely for 30 %, moderately for 30-60 %, slightly for 60-90 %, not cytotoxic for 90 % cell viability.

Cytotoxicity was rated based on cell viability relative to controls; extracts were rated as severely, moderately, slightly, or not cytotoxic based on the activity relative to the values obtained for controls: 30 %, 30–60 %, 60–90 % or 90 % respectively.

Percent cell viability was calculated by dividing the absorbance values of experimental wells by those of control wells and multiplying by 100. Cytotoxicity was also rated as severely cytotoxic for <30 % cell viability, moderately for 30–59 %, slightly for 60–90 % or not cytotoxic for >90 %.

2.5 Antibacterial Activity of Furanone Compounds

2.5.1 Media for biofilm studies

For all biofilm studies A-10 and FB media were used.

Table 2.1 : ABtrace media for biofilm studies

A-10 Media	Final Concentration in media
$(NH_4)_2SO_4$	15,1 mM
$Na_2HPO_4•2H_2O$	33,7 mM
KH_2PO_4	22,0 mM
NaCl	0,051 mM
FB Media	Final Concentration in media
$MgCl_2$	1 mM
$CaCl_2$	0,1 mM
Trace Metals	1 ml

ABtrace minimal media was composed of 1 Liter of A-10 was mixed with 9-liter FB and glucose (1 mM final concentration) added into solution.

2.5.2 Flow cell system construction

Flow-cells and bubble traps were constructed from Poly carbonate (PC) autoclavable material. Media flasks, a non-pulsating peristaltic pump, bubble traps, flow chambers, silicone tubing, Marprene® tubing, waste containers and connectors were components of the flow cell system. Each flow cells contain three independent channels and constructed from plastic base. The substratum is glued on top of the flow cells using silicone glue. The substratum was glass coverslips either uncoated or coated with furanone. Each step of coating steps was also used as substratum. Bubble traps were made from the single-use syringe mounted on top of a plastic base. Media passing through bubble trap will float to the top of the syringe and cannot pass downstream of the trap. After assembly of components media will pass from media flask through the pump to the bubble tramp and finally through the flow cell. The effluent from the flow-cell was directed toward waste container.

The assembled system was autoclaved and sterilized with 0,5 % sodium hypochloride and followed by several flushes with sterile water, just to be sure of a system is free of contamination.

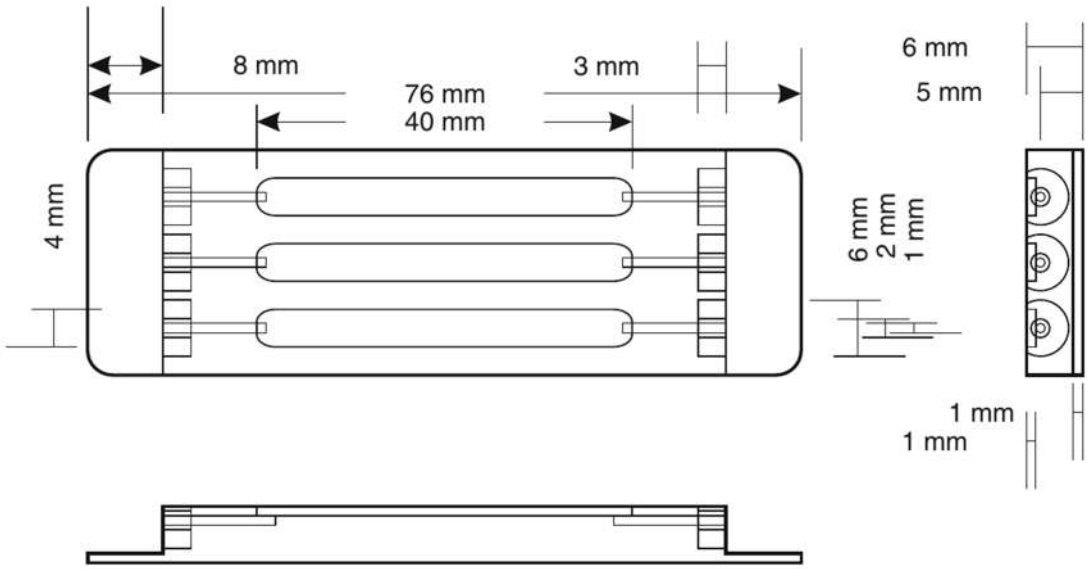


Figure 2.2 : Drawing of the flow cell [109]

1 ml of silicone glue was filled into the syringe and thin threads of silicone were placed between flow channels and at each end. Glass substratum is attached to flow-cell using silicon glue. 50x24 coverslips are used as substratum. Coverslip was firmly pressed toward the flow-cell.

2.5.3 Assembly of the flow-cell system

Assembled system is shown in Figure 2.3. The system is composed of a medium container, lead-in tubing, Marprene® pump tubing, a peristaltic pump, lead-out tubing, bubble traps, connecting tubing, three channel flow cells, waste tubing and a waste collection container. All components except the pump were autoclaved prior to final assembly.

The system was filled with sterile water by taking off the stoppers on top of the bubble traps. Stoppers were soaked in 96 % Ethanol. Stoppers were put again when the bubble traps were filled and the rest of the system was filled with sterile water. The peristaltic pump was run for 10 minutes to remove all air bubbles out of the system.

Sterilization of the flow system was done by pumping 0,5 % hypochlorite solution into the system for 4 hours. The system was washed two times again with sterile water to remove hypochlorite solution. Flow-cell system was filled with ABt media.

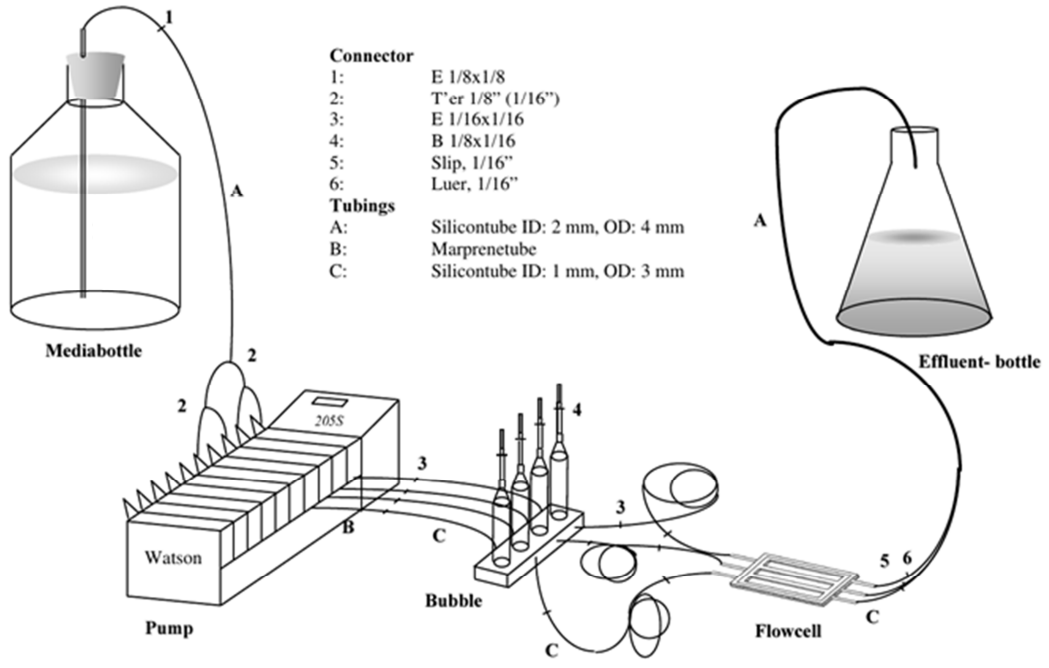


Figure 2.3 : Schematic of assembled flow-cell system

2.5.4 Biofilm formation assay in flow cell

P. aeruginosa strains were streaked on LB plates with the appropriate antibiotics and incubated for 24h at 37 ºC. From each plate a single colony was used for inoculation of 10 ml ABt with 10 % LB. The cultures were grown at 30 ºC for 18 h before they were diluted to an OD of 0,1 in sterile 0,9% NaCl and used for inoculation of the flow channels. The medium flow was kept at a constant rate of 3 ml/h , equivalent to a mean flow velocity of 0,7 mm/s , using a peristaltic pump. Biofilms were grown for 24 h before being shifted to media containing AHL and furanone.

Flow cells are made of plexiglas, covered with the cover slip. A thin layer of silicon was used to assembly flow cell. Assembled system was waited for overnight to dry before use.

Biofilm formation on furanone coated and control material (uncoated, each step of coating test coupons and glass coverslips) surfaces were monitored simultaneously by confocal microscopy for each experiment. Bacteria grown in ABtrace minimal medium was used as a starting culture for initial attachment and placed on and incubated with each biomaterial.

Flow channel will be left at 30 °C for 1 hour. After inoculation, the medium flow will be started and the rate will be set to 3 ml/h. A peristaltic pump will be used to draw media through the system at a constant rate.

The concentration of furanone compounds on the surface of biomaterials will be chosen as 40 µg/cm2, since according to literature data, furanone had no effects on the growth of microorganisms at this concentration [5]. The biofilm consisting of GFP-express-labeled *P. aeruginosa* will be formed on biomaterials at least for 24 h at 30 C. Statistical analysis will be performed in order to compare the effects of each pair of furanone coated surfaces with control surfaces on *P. aeruginosa* biofilm formation. All flow cell experiments were performed in triplicate for each sample type and images obtained after 24^{th} 48^{th} and 72^{nd} hours.

2.5.5 Combinatorial effect of furanone and tobramycin treatment

To study whether the antimicrobial application could improve the antimicrobial efficacy of QSI bioactives against *P. aeruginosa* PAO1, tobramycin was tested for its

MIC value in combination to surface coated furanone. The MIC of tobramycin was selected as 0,75 mg/L according to literature [110].

Uncoated and coated stainless steel coupons were used to evaluate the combinatorial effect of tobramycin and immobilised furanones on the biomass of pre-established 24h biofilms. 6 well plates were inoculated with 200 µl of a $3x10^6$ CFU/ml inoculum of *P. aeruginosa PAO1* strain prepared as previously stated. LB broth was inoculated in each assay as a sterility control. Assay plates were transferred to the incubator (37 °C, 95 % relative humidity) for 48h to allow growth of test biofilms. Following 48h of incubation, coupons were rinsed 3 times by immersing coupons in 6-well plates containing 2 ml of isotonic solution. Rinsed coupons analyzed for cell viability by using CFU counting method. All experiments were performed in triplicate.

2.5.6 Bacterial count

Bacteria grown SS-SC substrate were analyzed for cell viability by standard CFU counting method. Freshly grown *P. aeruginosa* culture diluted to O.D. 0,1 with 0,9 % NaCl. All coupons were cleaned, sterilized and placed in 6 well plates. 10 ml of ABtrace medium was added into each well. Diluted 100 µl of culture was added to each well. After 24 hours of incubation, coupons were taken off and rinsed with isotonic NaCl solution to remove unbound bacteria from the surface of the coupon. Coupons were further soaked in 5 ml of NaCl solution for 5 minutes. Biofilm forming bacteria were scraped off by sonicating in degassing mode for 5 minutes and further 5 minutes in sonication mode. 10 fold of dilutions were prepared for each coupon. Each dilution has incubated in LB plates for 24 hours at 37 ºC.

2.6 CSLM, Image Acquisition and Analysis

Microscopic inspection and image acquisition of biofilm growth were performed with a Zeiss LSM510 confocal laser scanning microscope (CLSM) equipped with detectors and filter sets for monitoring of GFP. Microscopic monitoring was performed with motorized and programmable xy-stage and all images were obtained with a 63x/1.3 oil objective. At the beginning of each experiment, the microscope programmed to track randomly selected microcolony. Throughout of the experiment laser intensity and sensitivity of photo multipliers were kept constant. Ar/Kr laser at

488 nm line was used for image acquisition for detection of GFP. Simulated 3D images and sections were generated with the ImageJ software [111] running on an ArchLinux workstation. The images were acquired from random positions on the surface of stainless discs.

CLSM images were analyzed by use of the ImageJ. Thresholds for the different image stacks were determined automatically and connected volume filtration was used in the analysis.

2.6.1 CSLM on SS substrate

Stainless steel implants coated with furanone and control samples were sterilized in 0,9 % sodium hypochlorite overnight, and washed in sterile distilled water before use. 10 implants were placed in 250 ml shake flask with 50ml of cultures of the *Pseudomonas aeruginosa*. Cultures were prepared by centrifuging (5000 g) cultures in Luria-Bertani (LB) medium. Cultures were diluted in 0,9 % NaCl to an OD600 of 0,05. The steel implants were incubated in the flasks for 20h at 37 ºC and shaking at 110 rpm for adhesion.

2.7 Experimental Design of *P. aeruginosa* Infection In a Mouse Intraperitoneal Foreign-Body Infection Model

All mouse experiments were approved by Dyreforsøgstilsynet (The Danish Ministry of Justice for Animal Experiments Inspectorate). Healthy female BALB/C mice weighing 20-25 g and aged 8 weeks were put in pathogen-free animal rooms for 5 days. Before the operation, the mice were anesthetized with subcutaneous injection of a mixture of etomidate and midazolam 1:1 ratio. The abdominal zone was dissinfected with ethanol and shaved with a razor. An incision of approximately 1.5 cm was made in the right groin area straight into the peritoneal cavity. 10mm thickness, 2 mm coated and uncoated SS-SC substrates were inserted into the peritoneal cavity with use of steril opthalmic forceps. SS-SC, PAA and FU2 coated substrates were used in this experiment. Before insertion, SS-SC coupon and PAA, FU1 and FU2 coated coupons incubated with *Pseudomonas aeruginosa* PAO1 saline solution at 37 °C for 20 h with shaking at 110 rpm for cell attachment and biofilm formation. The solution was prepared by dissolving bacteria diluted in 0,9 % NaCl

to an OD600 of 0,05. The incision was sutured with silk and healed without any complications.

The mice were euthanized at 24th hour by intraperitoneal injection of 20 % pentobarbital at a dose of 2 mL/kg body weight. An uninfected SS-SC implant was also included as a negative control. Mice were observed regularly for functional behaviour and abdominal symptoms.

For bacteriological analysis, the substrates were removed from the peritoneal cavities of the mice and were analyzed by standard CFU counting method as previously described.

3. RESULTS AND DISCUSSION

3.1 Surface Characterization

3.1.1 FTIR Analysis

Surface modification of stainless steel (SS-SC) was analyzed by FT-IR spectroscopy. As can be seen from Figure 3-1, 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 APTES was completely absorbed onto the surface. The weak band at 935 cm^{-1} showed that there were some silanols in the film.

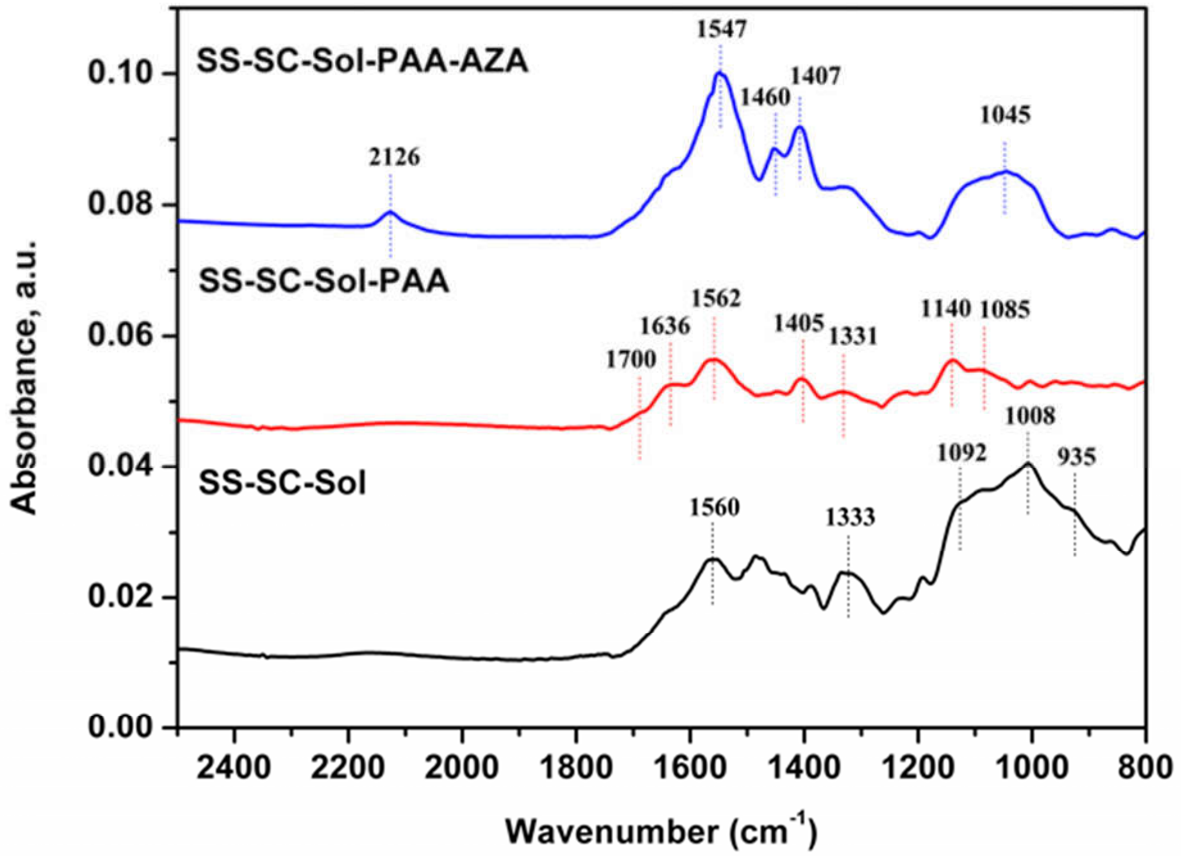


Figure 3.1 : FTIR spectra of SS-SC-Sol, SS-SC-Sol-PAA and SS-SC-Sol-PAA-AZA

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 3-1). 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 have appeared. 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 attachment of azidoaniline to carboxyl groups of PAA was evidenced by the presence of asymmetric stretching of azide at 2126 cm^{-1} (Figure 3.2).

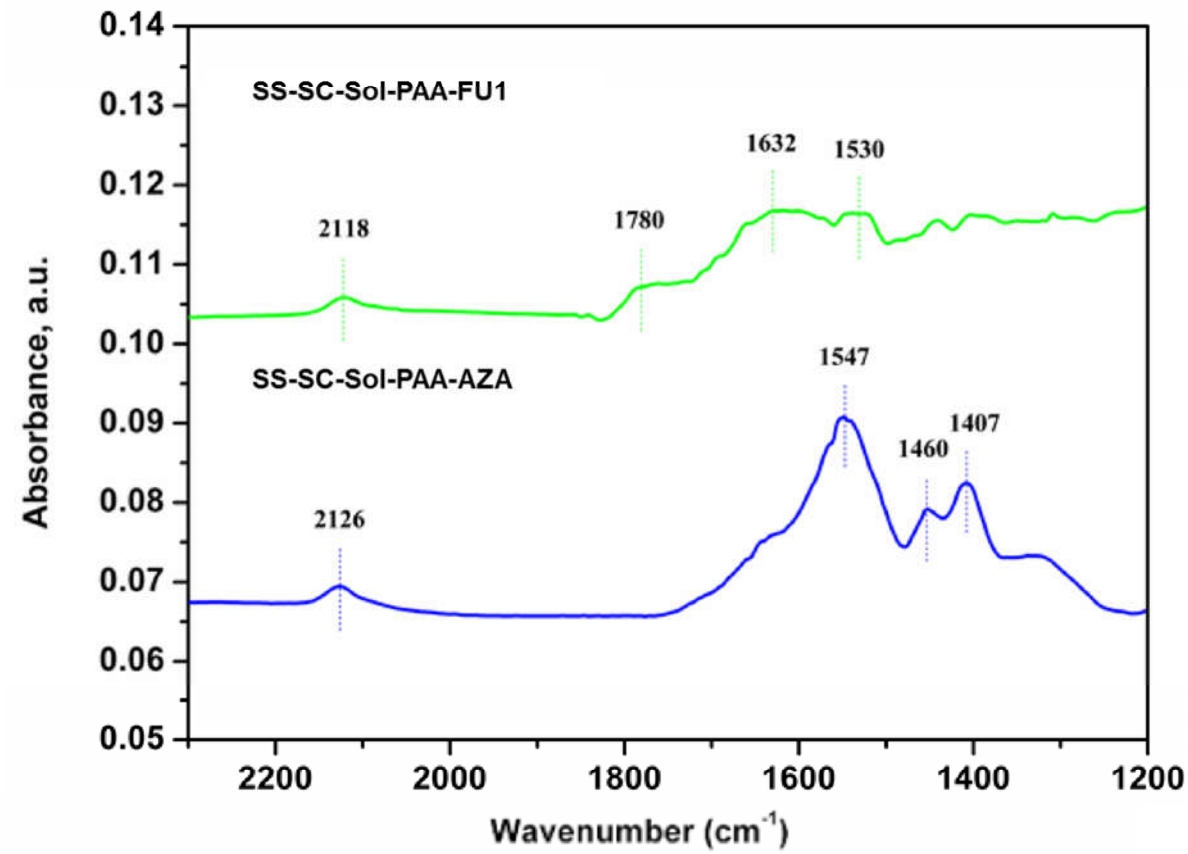


Figure 3.2 : FTIR spectra of SS-SC-Sol-PAA-AZA and SS-SC-Sol-PAA-AZA-FU1

The immobilization of two different furanone compounds (FU1 and FU2) was monitored by FTIR (Figure 3.2 and 3.3). The corresponding azide peak at 2126 cm^{-1} slightly decreased in the case of FU1, while a complete disappearance of azide was observed for FU2. It is known that the nitrene radical generated by UV irradiation of the azide group can react with various parts of furanone molecules. When the structures of the furanones used in this work are compared, it could be clearly seen that FU2 has a longer alkyl substituent compared to FU1 and that makes it more reactive towards azide.

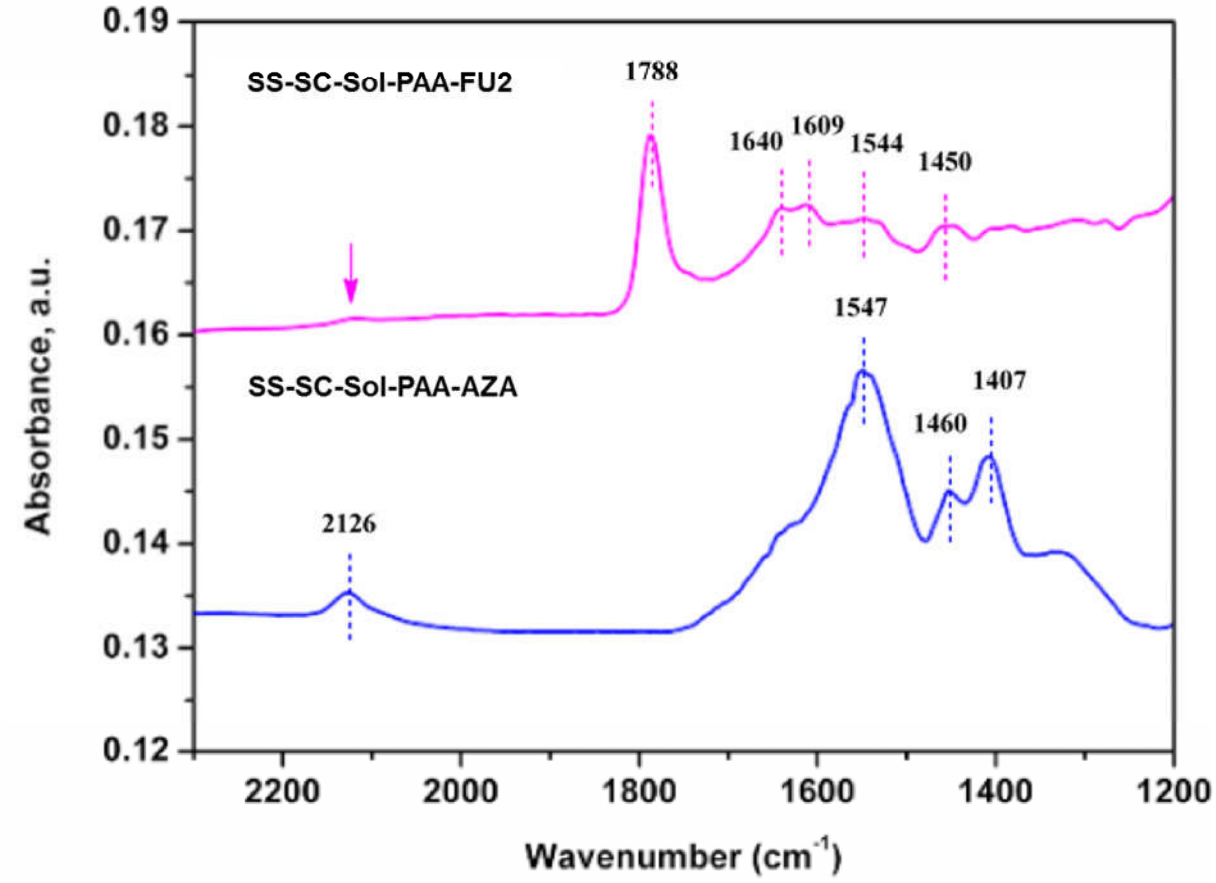


Figure 3.3 : FTIR spectra of SS-SC-Sol-PAA-AZA and SS-SC-Sol-PAA-AZA-FU2

3.1.2 AFM Analysis of Coating Steps and Furanone Immbolized Substrates

AFM analysis was performed to study the alteration of the surface topography and the Root Mean Square (RMS) roughness and total surface area values were calculated for each step of the immobilization process. Figure 3.4 shows the AFM height images of different coating steps.

Polished and sulfochromic acid treated SS discs are excellent support for AFM, has an RMS roughness of 2,059 ± 0,05 nm over 1,00 x 1,00 μm^2 scan area. RMS roughness of MTMOS/APTMOS treated SS-SC decreased to 0,71 nm which is consistent with previous reports [112, 113]. It can be argued that the sol-gel coating has a flattening effect on the surface and since RMS roughness proportionally correlated with surface area, there will be less space in sol-gel coated material for the attachment of bacteria. These results are in agreement with FTIR data and it can be argued that AFM results indicate a good sample

coverage and silane coating generates a uniform layer on the surface of the discs (Figure 3.4).

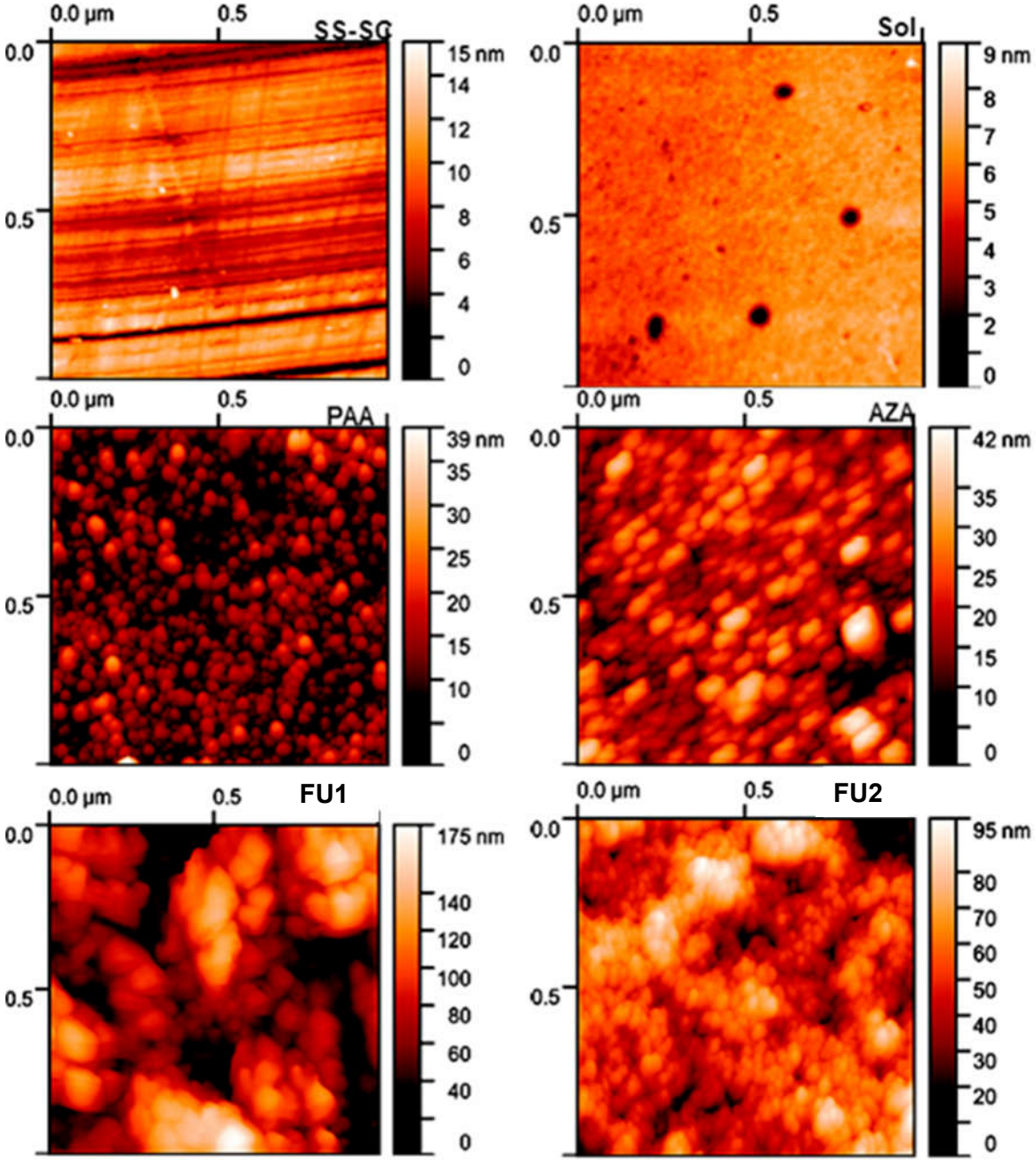


Figure 3.4 : AFM results of furanone coating steps.

Thin silane film-coated discs were further used for covalent immobilization of PAA layer onto the surface. Silanization allowed functinalization of the sample surface with amino groups. The same scale was used for all AFM measurements to allow a direct comparison of the samples. After grafting the PAA layer, RMS roughness value of the surface increased to 6,821 ± 0,75 nm, indicating that the surface underwent macroscopic modifications during

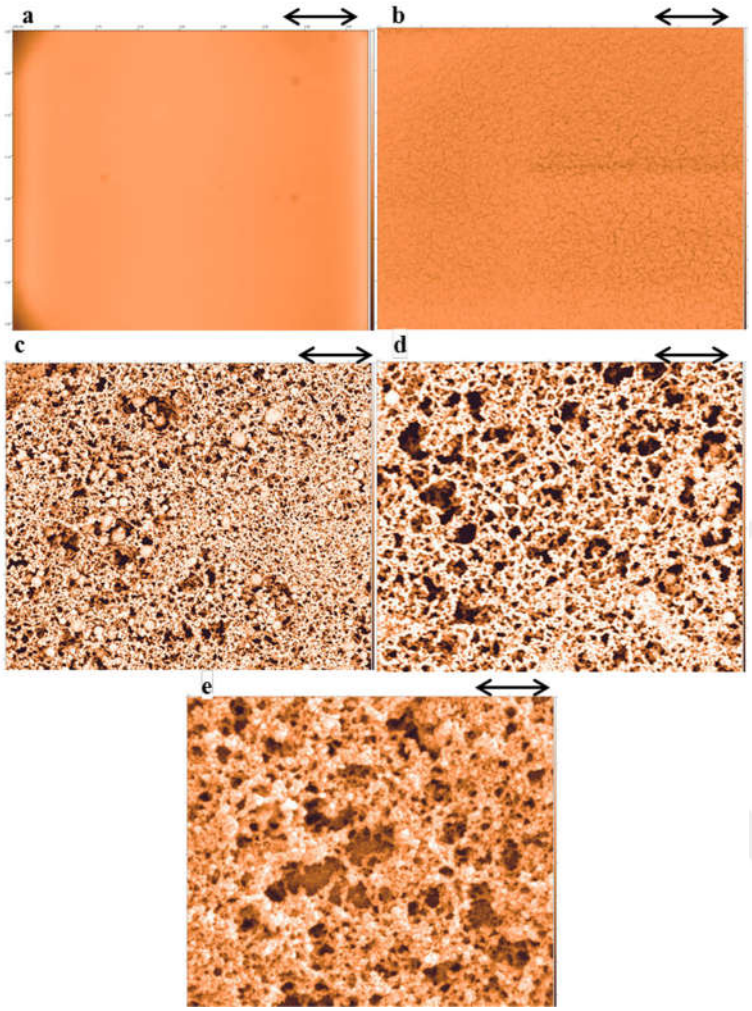
PAA grafting step [75]. The RMS roughness value and obtained AFM image of PAA immobilization step suggest that PAA polymer covers the entire surface, however, due to the shrinkage of PAA polymer in dry state results in the increase of surface roughness [86]. Surface topography of PAA coated matrix entirely changed due to immobilization of PAA and AFM results suggest good sample coverage with a monolayer of PAA molecules. Brush like the shape of PAA layer dramatically expanded surface area and created niche areas for attachment of the bacteria.

AZA coated sample topography is similar to PAA coated matrix and has similar RMS roughness with 5,648 nm ± 0,47 nm. Azidoaniline is a small molecule and has 170 g/mol molecular weight. Therefore its effect on surface topography was expected to be minor.

After covalent attachment of furanone molecules onto PAA-AZA grafted surface, the RMS roughness value increased to 13,696 ± 1,21 nm (FU2). The molecular weight of furanone is only 112 Da and one might compare with PAA (250 kDa) and it might be speculated that reaction between PAA and furanone changed spatial structure of PAA. These results are coherent with SEM results. Furanone molecules are uniformly immobilized onto sol-gel-PAA grafted surface with the appearance of peaks with the same height.

3.1.3 SEM Analysis

The size and shape of the coating materials and degree of distribution were obtained from SEM photographs. Glass substrate for flow cell studies was flat as expected (Figure 3.5a). Sol-gel layer had formed an even distribution on the glass surface as in Figure 3.5b. Small-scale “cracks” were observable as in Figure 3.5c. The obvious difference in the morphology was observed for PAA samples (Figures 3.5d and 3.6). The SEM image of the PAA sample shows a porous structure. As shown in the figure, size of porosity for this coating is in the range of nanometer scale (20-90 nm). AZA attachment onto PAA substrate had slightly changed the spatial distribution. Furanone coated substrate has larger pores as depicted in Figure 3.5e. All SEM results are coherent with AFM results since larger pores increase surface roughness and surface roughness increase through coating steps.



Şekil 4.1 SEM photographs of the prepared materials; scale bars denote 1µm. (a) glass substrate (b) Sol, (c) PAA, d., AZA, e FU1

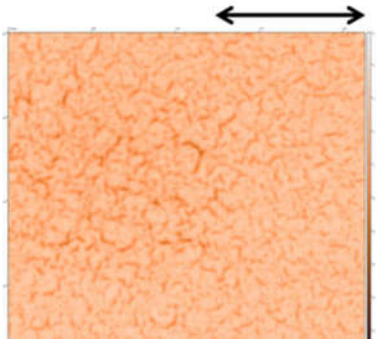


Figure 3.5 : Magnified view of Sol coating. Scale bar denotes 1µm

3.2 Cytotoxicity of Furanone Compounds

Uncoated steel samples show no significant change in L929 cell viability results. They were 97 % viable for 12h and 98 % viable for 48 h of incubation (Figure 3.7 & 3.8). When compared with negative controls, cell viability of furanone coated samples decreased 17 % at same time points. Therefore furanone compounds exhibited a slight cytotoxic effect on L929 cells.

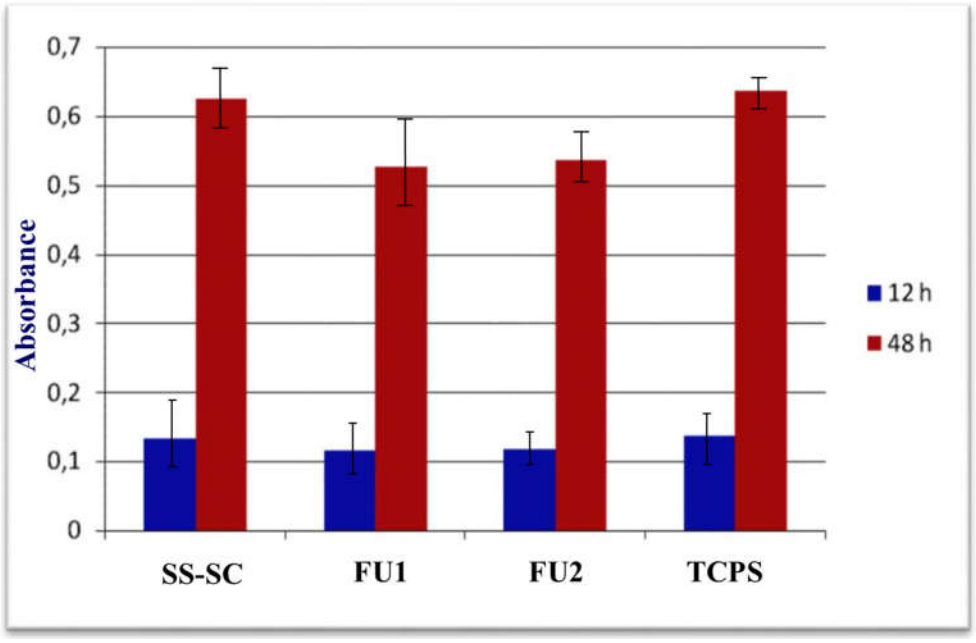


Figure 3.6 : Absorbance versus samples graph for 12- and 48-h.

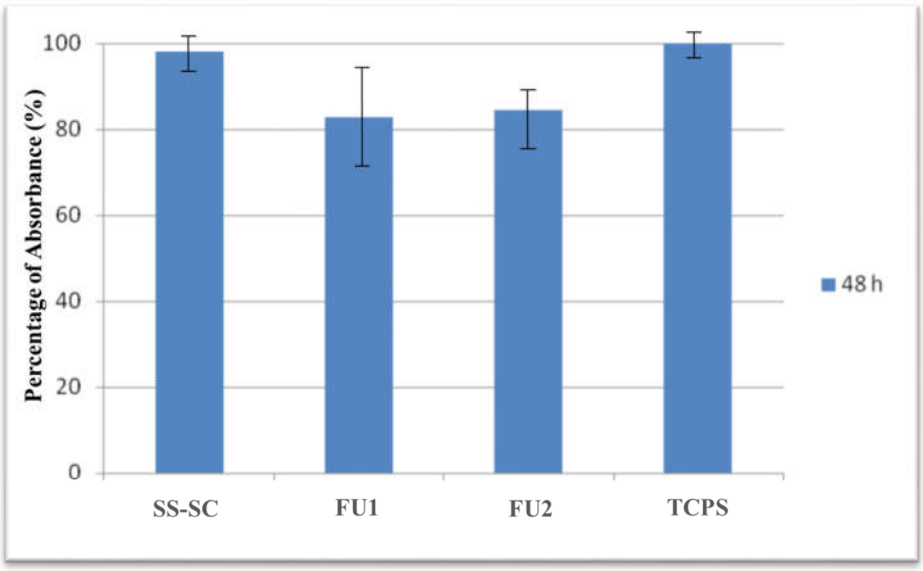


Figure 3.7 : The percentage of absorbances after 48 hours of incubation.

There were three different samples compared with cells on TCPS as the negative control for 12- and 48 h. SS-SC; uncoated steel samples were not cytotoxic at both time points. Uncoated SS-SC samples have no significant effect on cell proliferation. While furanone coated samples; FU1 and FU2 showed same trend and both are slightly cytotoxic.

3.3 Antibiofilm Activity of Furanone Coated Substrates

3.3.1 CSLM, Image Acquisition and Analysis

Furanone compounds were tested for their activity after immobilization onto SS-SC-PAA-AZA layer. Uncoated SS-SC and each step of coating were also tested for process control. Each substrate was coated with a specific furanone compound. FU1, FU2 and 4-Z are derivatives of naturally occurring 2-5(H) furanone compound.

The microalga *D. pulchra* produce furanone compounds which have been known for quorum sensing inhibitory properties as well as interfering complex surface-dependent interactions such as mobility and biofilm formation of *Serratia liquefaciens* [3, 87]. Natural furanones have a limited anti-biofilm effect on *P. aeruginosa* when tested individually [88]. Since natural QSI compounds can be modified by combinatorial chemistry, it is possible to generate a large number of analogues for screening their activity. Three such synthetic furanone compounds, 4Z, FU1 and FU2, which contain bromine substitution at their functional groups and a natural furanone compound have been tested for their effect on biofilm formation.

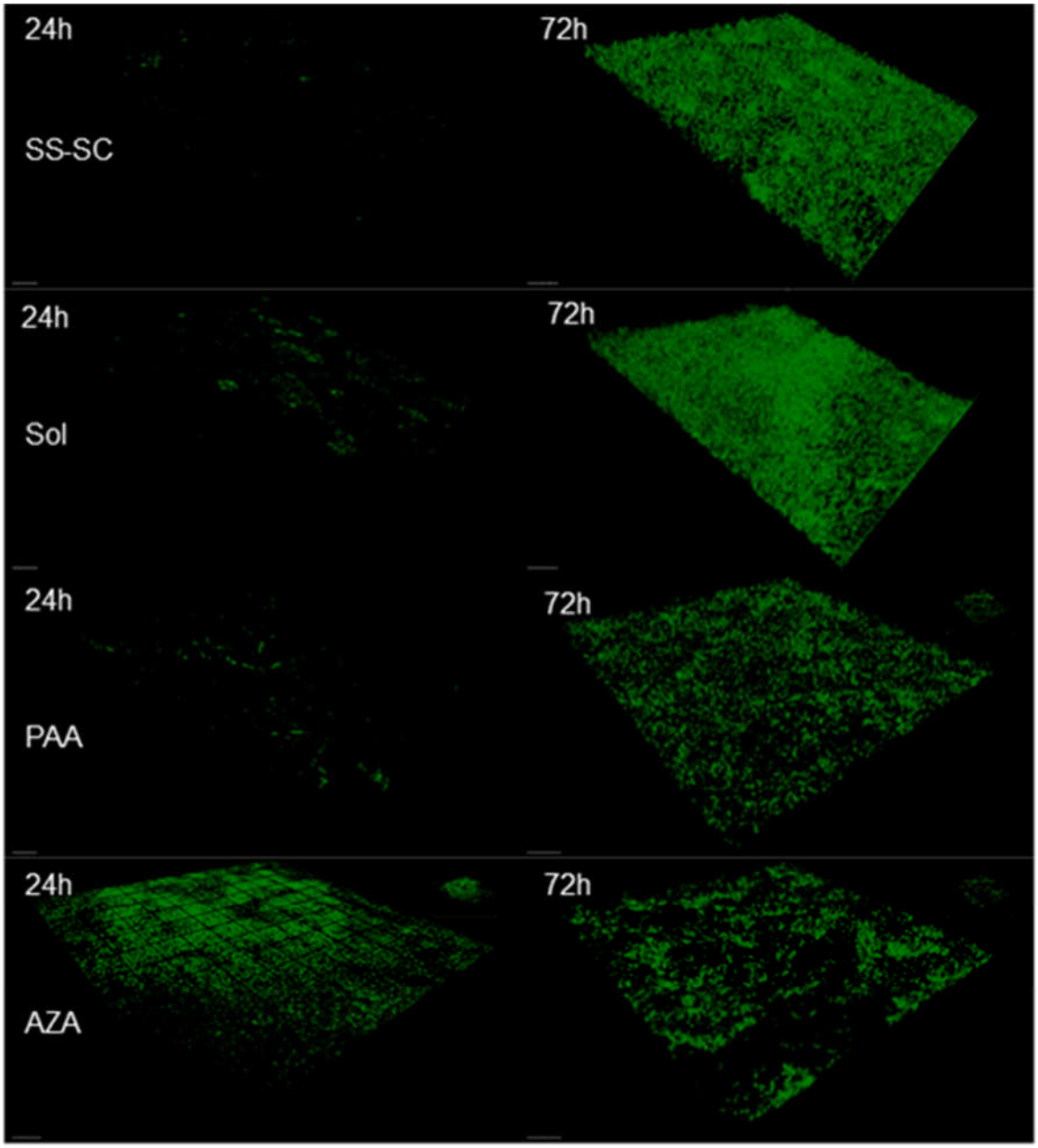


Figure 3.8 : Uncoated SS-SC and intermediated steps of coating had been visualised at 24h and 72h by CSLM.

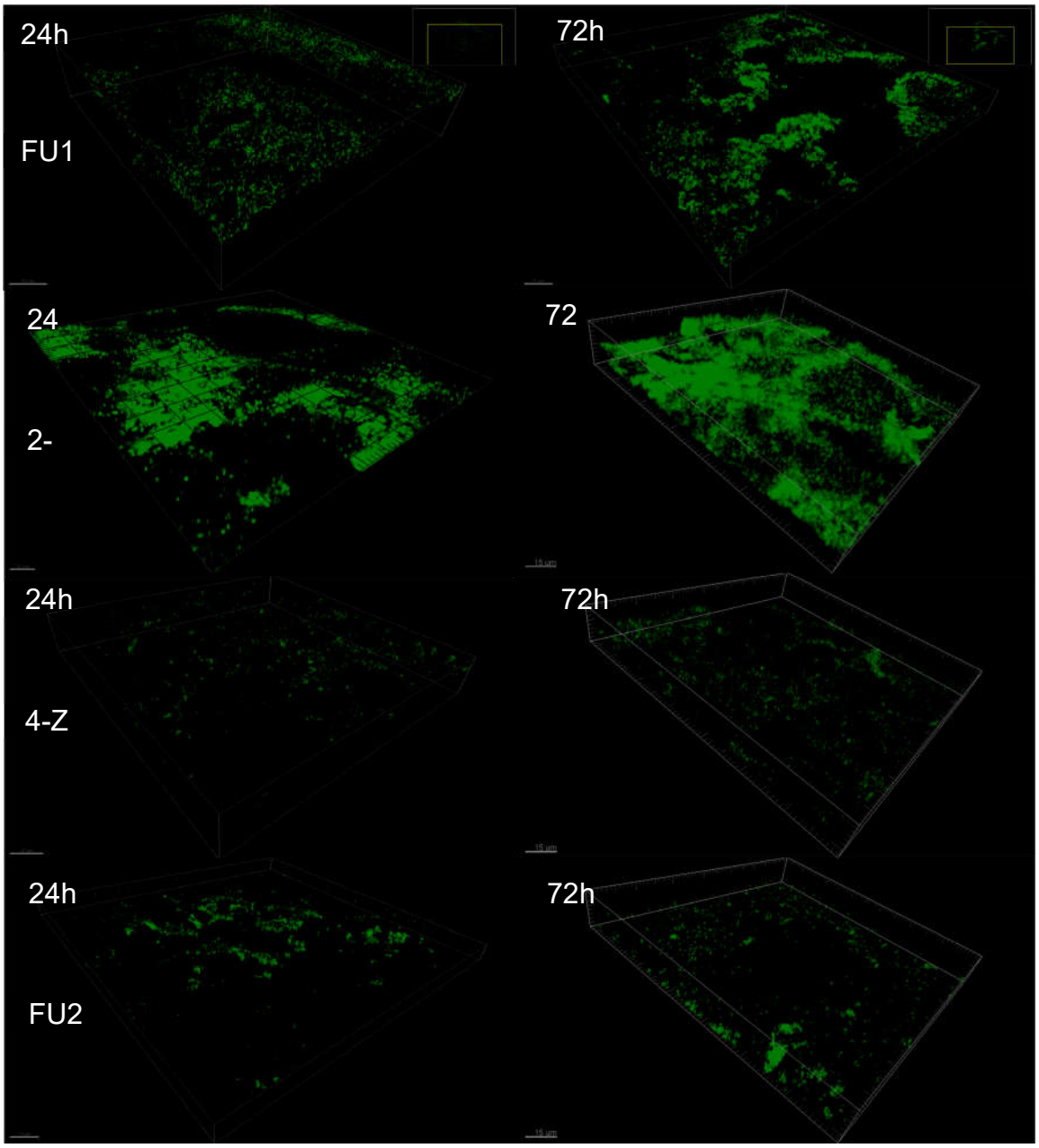


Figure 3.9 : Antibacterial activitiy of furanone derivatives at 24th and 72nd hours.

To assess the impact of covalently bound furanone molecules on the composition and biomass of *P. aeruginosa* biofilms, uncoated and partly coated samples were co-cultured in parallel flow cells. On uncoated and intermediate coating steps, numerous bacterial aggregates were observed (Figure 3.9). By contrast, biofilms on furanone coated surfaces were relatively thin, sparsely distributed (Figure 3.10). Bacteria were clearly more abundant on SS-PAA surfaces than any other coating steps. Quantification of cells by CFU counting demonstrated

significant reductions on furanone coated surfaces compared with non-furanone coated substrates after 72h (Figure 3.11).

Figures 3-9 and 3-10 represent images obtained from SS-SC coupons coated with furanone molecules and intermediate steps before furanone coating after 24 and 72 hours of incubation in ABt media. Spatial distribution of microcolony based biofilm on implants was distinctly different from the highly structured and mushroom-shaped biofilms which develop under certain growth conditions *in vitro* flow-cell system [114]. It is clearly visible that bacterial attachment onto FU2 and FU1 coated substrates are reduced when compared with rest of other furanone derivatives. CSLM imaging and analysis CFU counts indicates FU1 and FU2 are effective against biofilm formation and therefore rest of the studies continued with these furanone derivatives.

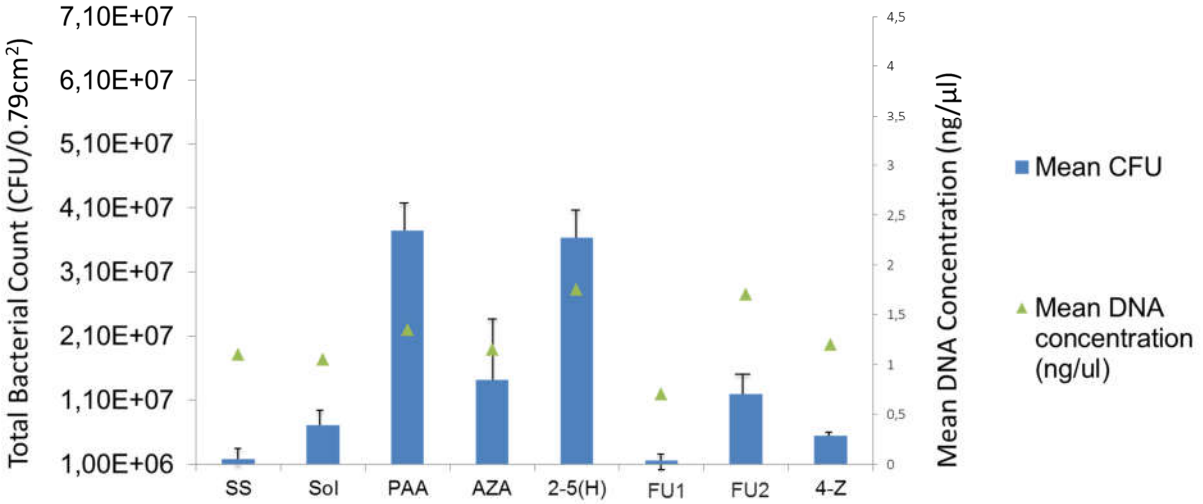


Figure 3.10 : Bacterial adhesion and mean DNA concentrations of uncoated, intermediate steps and furanone coated substrates

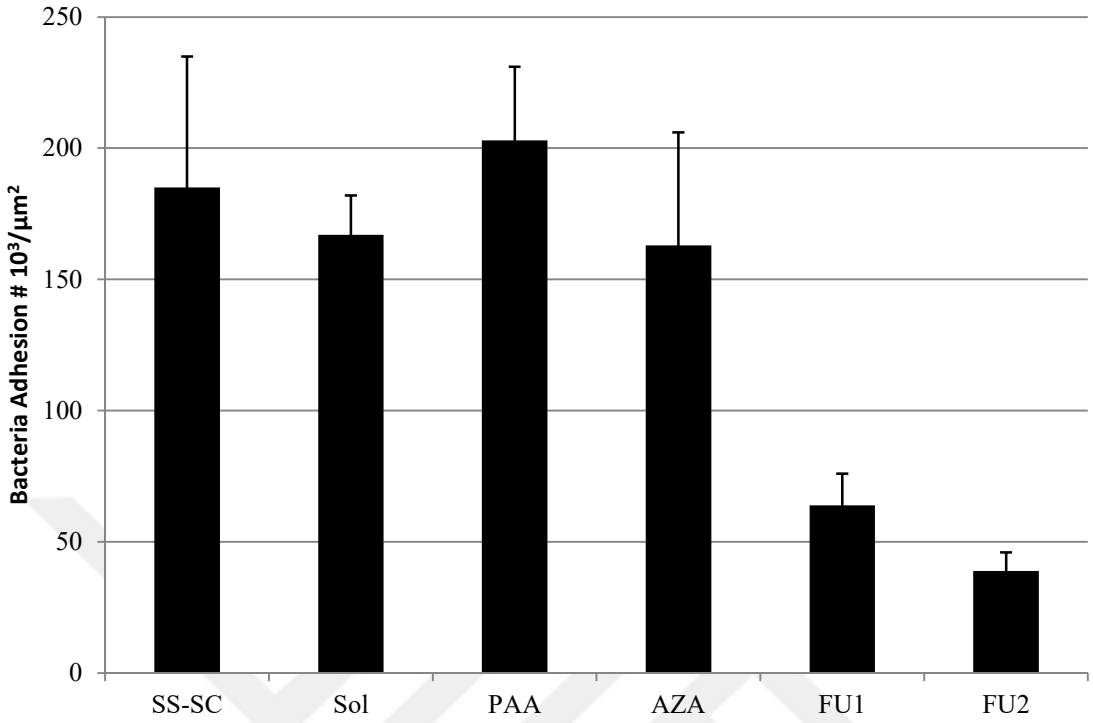


Figure 3.11 : Bacterial adhesion on uncoated, intermediate steps and furanone coated substrates without tobramycin under static growth for 72h at 37°C.

Bacterial adhesion to the SS and various coating steps was assessed under static condition and evaluated by the bacteria counts per unit area. Among the furanone coated surfaces, FU1 and FU2 significantly ($p<0,05$) reduced (78,9 % for FU2 and 65,4 % for FU1 when compared with SS-AZA coated substrate) bacterial adhesion as compared with control groups (Figure 3.12). To determine whether the reduction in cell numbers on furanone coated surfaces was associated with the capacity of furanone to inhibit bacterial attachment and observe the synergic effect of antibiotics and anti-biofilm surface, tobramycin was added to samples at sub-inhibitory concentrations (Figure 3.13). FU1 and FU2 coated substrates in combination with tobramycin added samples dramatically reduced bacterial adhesion as compared with tobramycin untreated samples (96.3 % reduction, $p<0,01$ for FU2 and 79,4 % reduction $p<0,05$ for FU1), thereby indicating a synergistic antibiofilm activity by the combination of furanone derivatives and tobramycin. Bacterial adhesion reduction rate as percentage has been shown in Figure 3.14. Asterisks in the graph represent a statistically significant decrease in

bacterial adhesion relative to Tobramycin added samples, with one asterisk denoting p <0,05 and two asteriks denoting p<0,001 (Figure 3.14).

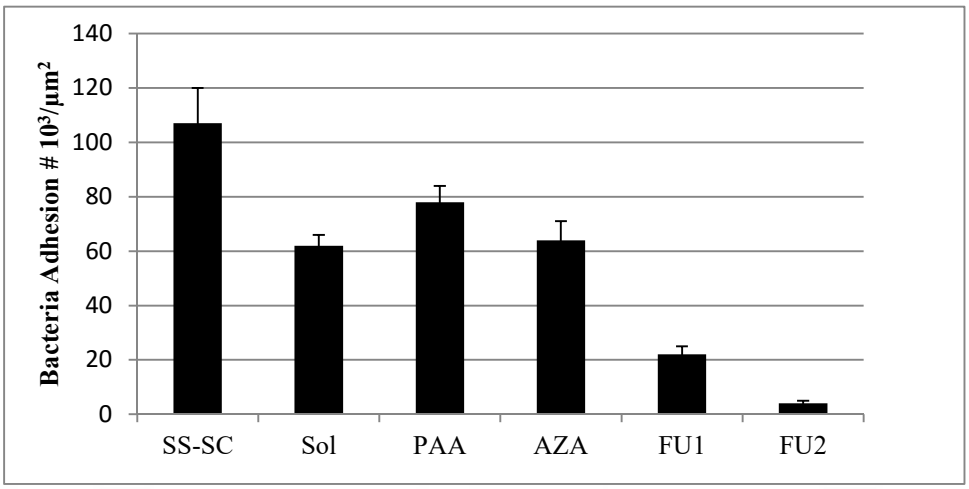


Figure 3.12 : Bacterial adhesion on uncoated, intermediate steps and furanone coated substrates in the presence of tobramycin under static growth for 72h at 37 °C.

Azidoaniline is a toxic chemical (contains N_3) and it reduces the number of viable cells on the disks. Therefore, bacterial attachment on SS-AZA sample was lower than any other coating steps.

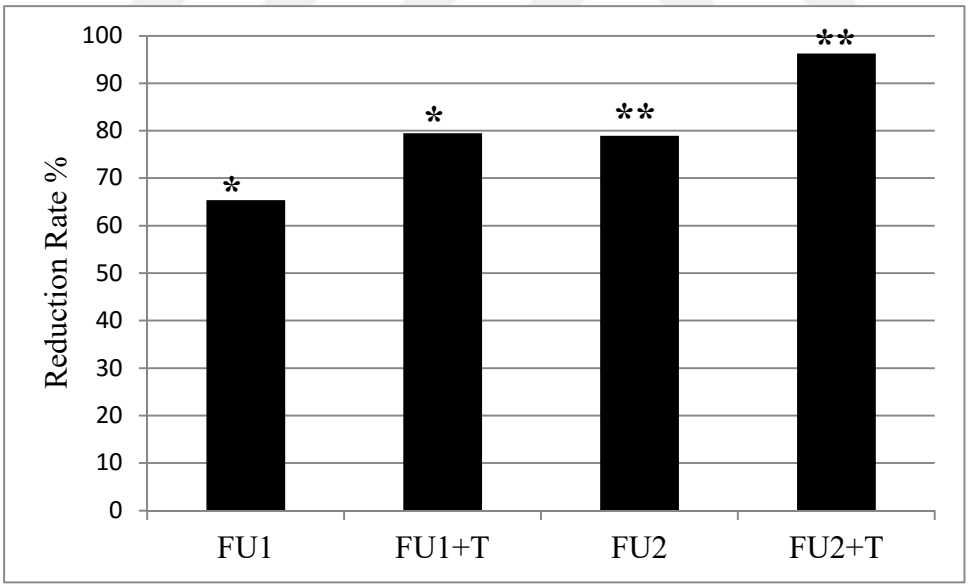


Figure 3.13 : Bacterial adhesion reduction rates as percentage in the presence of tobramycin and without tobramycin (+T denotes to tobramycin added samples)

The reducing effect of coatings on biofilm formation and growth of *P. aeruginosa* on substrates were presented in Figure 3-9 and 3-10. Figure 3.11 shows the bacterial counts of biomaterials at 72 h and indicates the total bacterial load associated with the surface. These

results clearly show that while 4-Z and 2-5(H) were ineffective to reduce bacterial load, FU2 and FU1 had reduced the number of attached bacteria. These observations are in agreement with the CSLM observations. The reduction in bacterial load was 4 times higher than AZA coated sample and 5 times higher than the uncoated SS-SC sample.

Combination treatment of *P. aeruginosa* resulted in a significantly lower CFU per implant as compared with single-treatment group (furanone coated substrates). As clearly indicated in other studies, brominated and natural furanones have synergistic antimicroabial efficacy against *P. aeruginosa* biofilm in both foreign-body infection model and in vitro tests [110, 115]

In one study FU1 was non-covalently attached onto glass surfaces and assessed for its antibacterial efficacy against *P. aeruginosa* biofilm [116]. It has been found that the attachment of *P. aeruginosa* on the FU1 coated surface was found to be significantly lower than the control, with reduction of 72,7 ± 1,7 %. Although this study applies non-covalent attachment procedure, similar reduction rates approves antimicrobial activity of furanone molecules.

3.4 Effects of FU2 against *P. aeruginosa* infection in a mouse intraperitoneal foreign-body infection model

Based on previous in vitro findings demonstrating the effect of FU2 on *P. aeruginosa* biofilm formation, covalently bound furanones were further investigated in a foreign-body infection model in vivo. In this pilot study, the activity and pharmacokinetic characteristics of covalently bound furanone molecules were assessed. The relationship between eDNA concentration and CFU also assessed with this experiment.

Unfortunately, 68 % of mice implanted with the substrates died before 24th hour and furanone coated substrates had more attached bacteria when compared with control substrates (Figure 3.15). The bacterial population on substrates differed substantially between samples and over time during biofilm development. In the Figure 3.15, each bar represents different mice subject. Furanone coated substrates had a higher number of CFU counts and therefore we couldn’t be able to assess the antibiofilm activi ty of furanone compounds in this foreign-body infection model study. The eDNA accumulated on samples did not reflect its

significance to biofilm formation and it had been found that eDNA concentration is proportional to cell density. Green triangles represent mean eDNA concentration while blue bars indicate CFU counts. Each bar represents a mice.

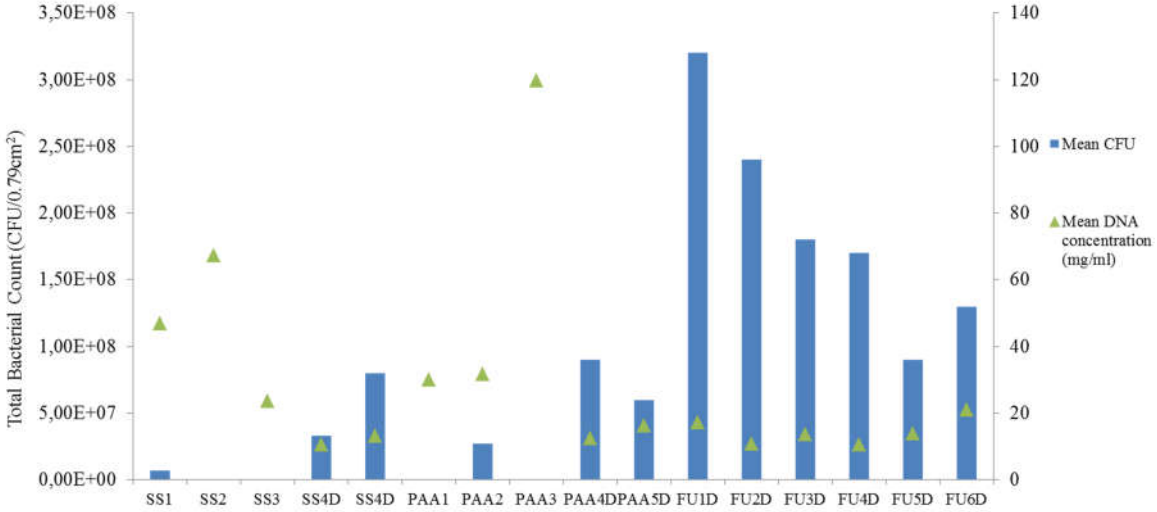


Figure 3.14 : Foreign-body infection model. Green triangles represent mean eDNA concentration while blue bars indicate CFU counts. Each bar represents a mice.

The coupons used in this study were 10mm in diameter, 2mm in thickness. The coupons could be too big for the foreign body infection model. Indeed many mice died a few hours after the surgical operation. Another reason could be that *P. aeruginosa* is a difficult-to-treat microorganism that tends to adhere and persist on foreign bodies, explaining the high rate of treatment failure.

4. CONCLUSION

This thesis aimed to develop a covalent coating of furanone derivatives which are effective against biofilm formation of *P. aeruginosa*. Chemical composition, molecular structure and properties of furanone coatings had been characterized by using AFM, SEM and FTIR. The antibacterial effectiveness of coated surfaces was also investigated by using flow cell and CSLM.

Stainless steel substrates and glass substrates were coated with furanone molecules and each immobilization step characterized visually and spectroscopically. AFM, SEM and FTIR analyses indicated successful covalent immobilization of furanone molecules via sol-gel and azide/nitrene chemistry by the presence of specific functional groups and surface morphology. Furthermore, azide peak has been decreased after furanone attachment and there was no decrease when the azide groups were not photolytically activated. Thus, furanone molecules were attached by covalent bonds, not only by physisorption.

Hydrogel like interlayer PAAC was deposited in order to produce a coating with a hydrophilic character. PAAC deposited layer formed a brush-like structure in a hydrated state and carboxylic groups dispersed throughout the whole surface and azidoaniline molecules bound throughout this interlayer not just on the outermost surface. Due to the existence of the N3 group, azidoaniline may exhibit toxic effect and reduces the number of viable cells on the disks. It might be speculated that furanone molecules followed the same distribution of azidoaniline molecules and they are present throughout the whole PAAC layer. UV radiation created nitrene radicals and they can react with furanone compounds. The reaction between furanone compounds and nitrene radical is unspecific and nitrene can react with lactone ring or C-H bond of furanone compound. If nitrene molecule reacts with the active site of the furanone molecule (lactone ring) antibacterial activity of the furanone molecule might be suspended. Therefore it is expected that some immobilised molecules are not effective against antibacterial attachment.

Four synthetic brominated furanone compounds were successfully immobilised by using azide/nitrene chemistry. Azide/nitrene chemistry has the advantage of compatibility with all synthetic furanone compounds and in this strategy, there is no need any functional group on the molecule for immobilization of target compound. Furanone compounds lack any functional group and they have liability under both acidic and alkaline conditions due to lactone ring and thus preventing us to use more chemically selective immobilization method.

Tapping mode of AFM analysis for each layer of furanone immobilization showed uncoated and sol-gel coated surface was smooth and PAAC coated surface had bigger surface area compared to uncoated and sol-gel coated surfaces. These analyses also showed that these sol-gel film, PAAC and subsequent applications were stable and survived during the procedure.

Control steps of coated samples contain the similar quantity of cells compared to the number of cells on SS-SC substrate. Since PAA has Mw of 250 kDa, bacteria might have found more niches to «attach» on the substrate. Azidoaniline and furanone coating steps have minor effects on surface topography and can be omitted. Bacterial adhesion onto PAA surface is slightly higher than adhesion onto SS-SC substrate.

Covalently attached furanone molecules on glass substrate had diverse antibacterial activity against biofilm formation and growth on bacteria. The highest activity against biofilm growth was by FU2. Cell viability test revealed that furanone molecules have slight toxic effect at the concentrations tested, however, most of them showed dose responses. Natural furanone (2-5(H)) and 4Z seems to be no effect on cells.

CFU counts and CSLM images showed that activities of the furanone molecules were different after surface immobilization. These results showed that the activity of the coatings mainly depends on the type of furanone compound, not the type of interlayer.

Covalently bound furanone molecules FU2 and FU1 exhibited anti-biofilm effect and reduced bacterial attachment to 78,9 % and 65,4 % respectively on the substrate. Bacterial adhesion to the furanone coated substrates further reduced by the addition of tobramycin when compared with control groups (96,3 for FU2 and 79,3 % for FU1), indicating a synergistic effect of covalently bound furanone molecules and

subinhibitory concentration of tobramycin. Obtained results in this study provide insights into chemical structure, composition and antibiofilm activity of furanone compounds effective against *P. aeruginosa* biofilm.

UV illuminated azidoaniline reacts with the C-H moiety or lactone ring of furanone molecules and FU2 has an alkyl group which creates a potential attachment site to azidoaniline. Alkyl group provides a projectile as an attachment site and keeps BR-C-H and lactone, functional groups further away from the reaction. Therefore it can be speculated that FU2 has higher anti-biofilm activity when compared with FU1 due to loss of activity of some immobilized FU1 molecules.

eDNA produced by biofilm-forming microorganism constitutes an important functional property to biofilm matrix by facilitating twitching modility-mediated biofilm formation, cell-to-cell interconnecting, as a nutrient source during starvation. eDNA production and density controlled by QS system and it might be speculated that by using QSI compounds concentration of eDNA should be decreased. In the study, eDNA concentration found to be proportional to the density of the microbial population.

Although synthetic furanone compounds FU2 and FU1 inhibited bacterial quorum-sensing in vitro, no reliable result obtained from *P. aeruginosa* foreign-body infection model in vivo. Unfortunately, 68 % of mice died after the surgical operation and results obtained from this experiment didn't reflect the results obtained from in vitro antibiofilm activity of furanone molecules. The mass ratio between coupon used for foreign body infection and mice is 5,0 %±1. Coupons could be too big to be used for surgical operation and this study can be repeated with smaller size samples.

For longevity and durability of medical applications further optimisation is necessary for specific attachment of furanone compounds onto the surface. This thesis can provide a baseline and a methodology for further biomedical applications against biofilm formation. Furthermore, furanone coatings need to be investigated under in-vivo conditions to obtain activity and durability in a real biological environment for comparison of in-vitro and in-vivo results.

The importance of eDNA has become clear from research on biofilm-related infections [117]. Only a few studies have aimed to determine whether the quantity of

eDNA produced can be linked to biofilm mode of growth [118, 119]. Although in the literature eDNA concentration have been found to peak at the stationary phase of growth [120], there is a lack of research on the quantitative analysis of biofilm development and eDNA concentration on substrates.

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