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Covalent Immobilization of Furanone Derivatives for the Inhibition of Biofilm Formation on Implant Surfaces

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Abstract: *A biofilm is essentially composed of microbial cells attached to a surface and completely covered with an extracellular polymeric matrix produced by biofilm forming bacteria. Uncontrollable and undesirable accumulation of cell aggregates constitute serious problems in industrial processes and biomedical applications. There is a growing demand towards developing strategies to remove and protect the surfaces against biofilm formation. Natural and synthetic furanone compounds have been found to interfere with Quorum Sensing (QS) mechanism of many human pathogens and block this specific pathway. The aim of this study was to prevent biofilm formation on surfaces, especially medically important surfaces, through application of furanone derived coating material. Thus, the strategy for the present study was covalently linking furanone derivatives on surfaces to assess the effect of the immobilized furanone molecules on the adhesion strength of Pseudomonas aeruginosa (P. aeruginosa) biofilms. Results show that high molecular weight of Poly Acrylic Acid (PAA) has been increasing surface area of substrate and coated samples contain slightly higher number of cells compared to attached cells on stainless steel substrate. 76% of reduction has been found when furanone coated sample compared with 4-azidoaniline hydrochloride (AZA) coated substrate and 87% of reduction has been found when furanone coated sample treated with tobramycin compared to tobramycin treated AZA coated substrate. Therefore it can be said that covalently bound furanone molecules strongly inhibits attachment of P. aeruginosa on steel substrate.*

Keywords: *Biofilm, furanone, implant, covalent immobilization*

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I. Introduction

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 [1, 2]. Biofilm formation and metabolic activities within may cause serious community health risks. Most of these health risks are nosocomial infections of gastrointestine, eye, ear etc. Because of these reasons, there is a growing demand towards developing strategies to remove and protect the surfaces against biofilm formation. The benefits of biofilm formation to microorganism include increased protection against antimicrobials and defense mechanisms.

Several immobilized antibiotics such as cefazolin [3], minocycline-rifampin [4, 5], teicoplanin [6, 7], vancomycin [8] and antimicrobials like silver [9], chlorhexidine [10], salicylic acid [11], quaternary ammonium compounds [12, 13] 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 [14, 15]. Another study has been reported that chlorhexidine coated catheters resulted in anaphylaxis response [16]. Quaternary ammonium compounds and silver have been exhibited toxic effect to human cells [17, 18]. 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 [19-21].

A biofilm cannot be considered as a homogeneous distribution of cells but as 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. Furanone compounds have been found to interfere with quorum sensing mechanism of many human pathogens and block this specific pathway [22]. The aim of this study was to inhibit formation of bacterial biofilm using covalently attached furanone derivatives either for use in industrial or medical applications. In order to realize the project, furanone compounds were immobilized onto hydrogel-type interlayer, poly acrylic acid (PAA), using azide/nitrene chemistry for covalent attachment of the molecules. Presence of amine groups in the sol-gel

silicate network provides functional site for covalent bonding of PAA via the carbodiimide reaction.

Spectroscopic characterization of immobilization steps were performed using Atomic Force Microscopy (AFM), Fourier Transform Infrared Spectroscopy (FTIR) and Scanning Electron Microscopy (SEM). Cytotoxicity of furanone compounds were tested using MTT assay. The ability of covalently attached furanone compounds to prevent growth and biofilm formation of *P. aeruginosa* was assessed by using flow-cell system and Confocal Laser Scanning Microscopy (CLSM). *P. aeruginosa* was used as a model for biofilm forming microorganism. It is the most common bacterium found in life threatening infections of immunocompromised patient [23].

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. In order to have a long-lasting anti-biofilm effect, covalent attachment of furanone compounds onto the surface is a method of choice. Firstly, characterization of furanone compounds immobilized onto stainless steel (SS) substrate had been achieved. Characterized surfaces are then used as a substrate to test biofilm formation using *P. aeruginosa*. QS inhibition experiments will be performed in situ as previously reported [24, 25]. This project aims to find a solution to biofouling by covering the SS material that are commonly used for medical devices and equipments 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 [26]. The objectives of this study were to: (i) characterization of furanone coating steps on SS substrate, (ii) examine in vitro efficacy of furanone coated substrates compared with uncoated substrates and (iii) synergy of the combination of furanone and tobramycin in preventing colonization of *P. aeruginosa*.

II. Materials And Methods

2.1 Materials

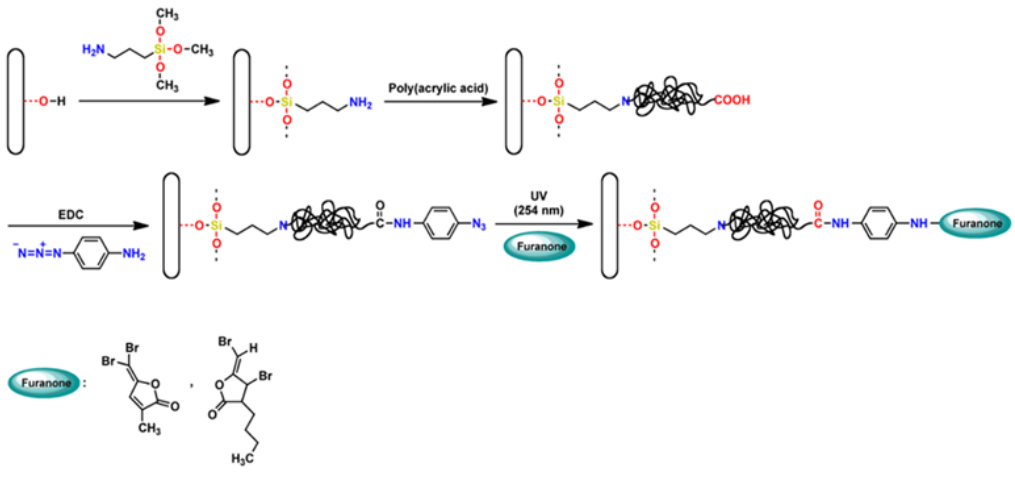
P. aeruginosa strain PAO1 (ATCC 15692) obtained from ATCC was revived according to American Type Culture Collection method from lyophilized strain ampoules and maintained on nutrient agar (1 g/L malt extract, 2 g/L yeast extract, 5 g/L peptone, 8 g/L sodium chloride and 20 g/L agar) at 37 °C.

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), poly(acrylic acid) solution (average Mw ~250.000, 35 wt. % in H2O) (PAA), 4-azidoaniline hydrochloride (AZA) was purchased from Sigma-Aldrich. 316 L SS coupons (1cm in diameter, 0.2 cm in thickness) were purchased from Gama Metallurgy.

2.2. Immobilization of furanone compounds

Coupons were cleaned and pre-conditioned according to literature methods [27, 28]. 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.

Summary of the immobilization protocol is given as shown in the scheme in Figure 1, followed by detailed description of each immobilization step.



2.1.a Sol-Gel 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 [29] in a small test tube at room temperature. Stock solutions were freshly prepared daily prior to the application of the spin coating. 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-Sol).

2.1.b Poly(acrylic acid) attachment onto sol-gel layer

Stainless steel surfaces were functionalized with amino groups of sol-gel matrix. In brief, PAA solution was deposited on the hydroxylated surface, which enabled attachment of azidoaniline molecules via carbodiimide chemistry. Covalent binding of furanone molecules were carried on dark room conditions due to the light sensitivity of azide groups. As shown in the figure 1, immediately after sol-gel coating, interfacial layer was applied by soaking the SS-Sol samples into 0.1 % aqueous solution of PAA at pH 4. 5mg/ml of EDC 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-PAA).

2.1.c Azidoaniline attachment onto SS-PAA layer

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

2.1.d Furanone immobilization onto SS-AZA layer

10mg/ml furanone compounds in acetone was applied evenly to the SS-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 repeated without UV illumination to the SS-AZA. Under this conditions furanone compound was expected to be washed off from the surface and confirmation of this study was performed with CSLM.

2.3 Surface Characterization

2.3.a AFM

AFM scanning of the samples were performed in air at room temperature using Ntegra Vita (NT MDT, Zelenograd, Russia). The device was operated in semi-contact mode and images were obtained using silicon cantilever Tap300 Al-G (BudgetSensors) with resonance frequencies of 200-400 KHz, force constant 40N/m. For each sample, several AFM scans (typically 1 x 1 µm) were made to check the surface uniformity. 2-D Fourier transforms of the images were obtained by using FFT analysis software tool 16 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.b FT-IR

FT-IR analyses were conducted using Perkin Elmer Spectrum One FT-IR spectrometer equipped with an attenuated total reflectance device. Coated steel surfaces and corresponding compounds for comparison were placed onto a ZnSe crystal with a 45° mirror angle. All analyses were performed with a resolution of 8 cm^{-1} using 400 scans.

2.3.c 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 [30]. 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 2-5kV accelerating tension. The magnifications used were x1.500 and x5000.

2.4 Biofilm Formation Assay

Biofilms were cultivated in continuous culture flow chambers perfused with sterile Abtrace minimal medium containing 0.3mM glucose. Pump speed was set to 3ml h-1. The cultures were prepared by centrifuging overnight grown cultures in Luria-Bertani (LB) media. Cells were resuspended in 0.9 % NaCl to an OD_{600} of

0.5. Flow cells were incubated at 37°C for 72h. Tobramycin (10µg/ml^{-1}) was added to medium when apporpriate.

2.5 Cell Cytotoxicity Assay

Mouse L929 fibroblastic cells which are well known model for the investigation of cytotoxicity were used in this study. The viable cell amounts were determined by using MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfofenyl)-2H-tetrazolium) 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 ^{o}C, 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.
Samples were SS-SC substrate and with two different immobilized furanone compounds (FU1 and FU2). 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 into each wells. Cells on tissue culture polystyrene (TCPS) surface of 24-well plates used as negative control, which considered to be ideal condition of cell growth. One sample without cells 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 of 5:1 according to manufacturer's instructions. 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^{o}C 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 based on cell viability were rated as severely for 30%, moderately for 30-60%, slightly for 60-90%, not cytotoxic for %90 cell viability.

2.6 CSLM, Image Acquisition and Analysis

All microscopic observations and image acquisitions were done with a Zeiss LSM510 confocal laser scanning microscope (CLSM) equipped with detectors and filter sets for monitoring of GFP. Images were obtained with a 63x/1.3 objective. Simulated 3D images and sections were generated and further processed with the ImageJ software [31]. 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.

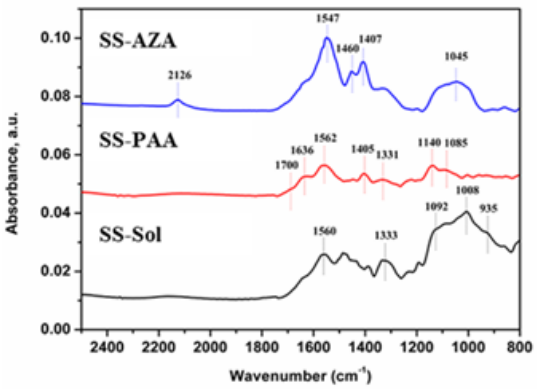
2.7. Determination of Bacterial Adhesion on Substrates

After incubation, biofilms were scraped off from SS, intermediate steps and furanone coated substrates and washed twice with sterile PBS. Cells were vortexed three times for 1 minute to disrupt biofilms and detach adherent cells from each other. Detached cells were serially diluted and plated onto LB plates (5gr/L yeast extract, 10gr/L tryptone, 10gr/L NaCl, and 15r/L agar). They were incubated at 37°C overnight and CFUs were counted. All experiments were performed in triplicate.

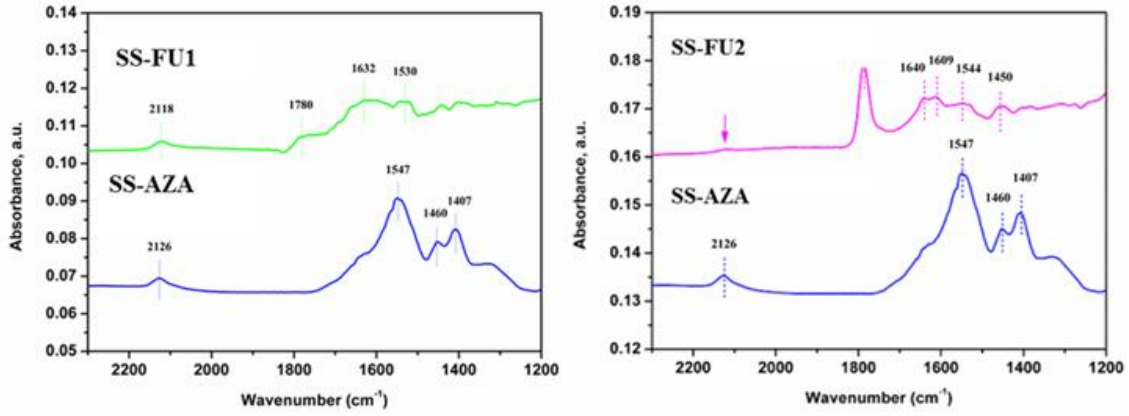
III. Results And Discussion

3.1 FT-IR Analysis

Surface modification of stainless steel (SS-SC) was analyzed by FT-IR spectroscopy. As can be seen from Figure 2, 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.



The absorption of Si-O-C and Si-O-Si stretching decreased a bit after PAA was grafted onto the SS-Sol surface (Figure 2). Also the N-H band at 1560 cm^{-1} disappeared while new bands appear at 1636 (stretching of C=O) and 1562 (bending of N-H) cm^{-1}. When PAA chains were grafted onto the SS-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} (Fig. 3).
Immobilization of two different furanone compounds (FU1 and FU2) were monitored by FT-IR (Figure 3 and 4). The corresponding azide peak at 2126 cm^{-1} slightly decreased in the case of FU1 (Figure 3), while a complete disappearance of azide was observed for FU2.



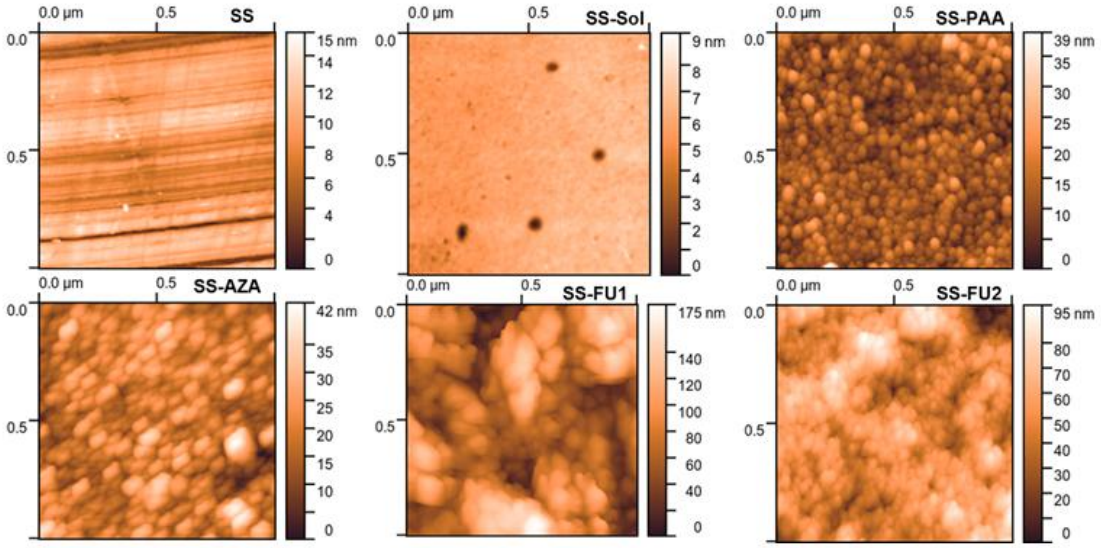
3.2 AFM Analysis

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 4 shows the AFM height images of different coating steps.
Polished and sulfochromic acid treated SS discs are excellent support for AFM, has 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 [32, 33]. It can be argued that sol-gel coating has flattening effect on 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 4).

Thin silane film-coated discs were further used for covalent immobilization of PAA layer onto the surface. Silanization allowed functionalization of sample surface with amino groups. 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 [34]. The RMS roughness value and obtained AFM image of PAA immobilization step suggest that PAA polymer covers the entire surface, however due to the shrinkage of PAA polymer in dry state results in increase of surface roughness [35]. 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 shape of PAA layer dramatically expanded surface area and created niche areas for attachment of the bacteria.

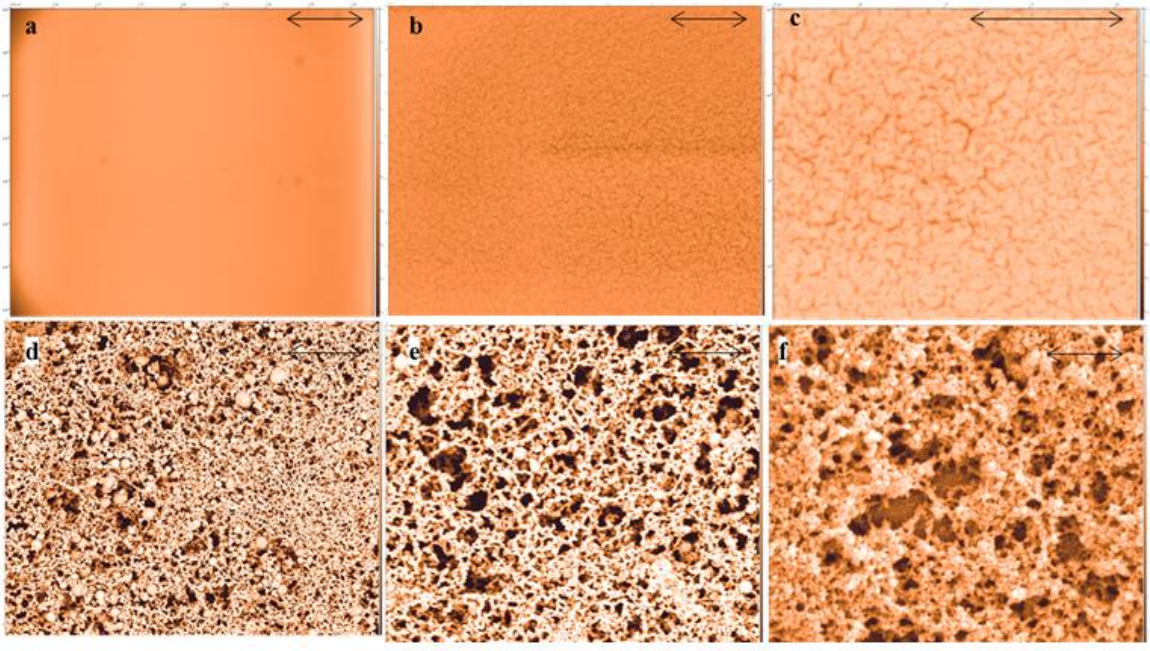
AZA coated sample topography is similar to PAA coated matrix and has similar RMS roughness 5.648nm ± 047nm. Azidoaniline is a small molecule and has 170g/mol molecular weight. Therefore, its effect on surface topography was expected to be minor.

After covalent attachment of furanone molecules onto SS-AZA grafted surface, the RMS roughness value increased to 13.696 ± 1.21 nm (FU1). 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 AFM results. Furanone molecules are uniformly immobilized onto sol-gel-PAA grafted surface with the appearance of peaks with the same height.



3.3 SEM Analysis

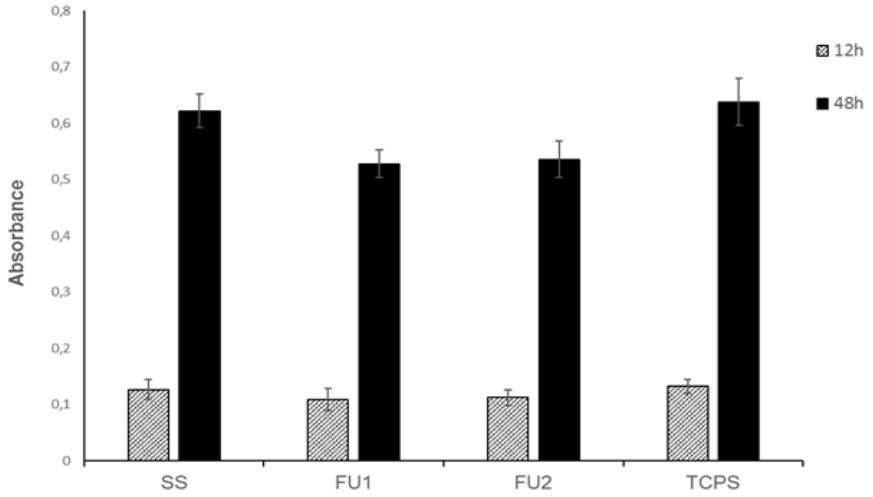
The size and shape of the coating materials and degree of distribution were obtained from SEM photographs. Glass substrate for flow cell studies was flat as expected (Fig 5a). Sol-gel layer had formed an even distribution on glass surface as in fig 5b. Small-scale "cracks" were observable as in fig. 5c. The obvious difference in the morphology was observed for SS-PAA samples (Fig. 5d&e). The SEM image of SS-PAA sample shows a porous structure. As shown in the figure, size of porosity for this coating is in the range of nanometer scale (20-90nm). AZA attachment onto PAA substrate had slightly changed spatial distribution. Furanone coated substrate has larger pores as depicted in figure 5f. All SEM results are coherent with AFM results, since larger pores increase surface roughness and surface rouhghness increase through coating steps.



3.4 Cytotoxicity of Furanone Compounds

Uncoated steel samples show no significant change on L929 cell viability results. They were 97% viable for 12h and 98% viable for 48h of incubation (fig 6). Cell viability of furanone coated SS-SC samples when compared with negative controls didn't change greater than 17% at both time points; which is considered as slightly cytotoxic.

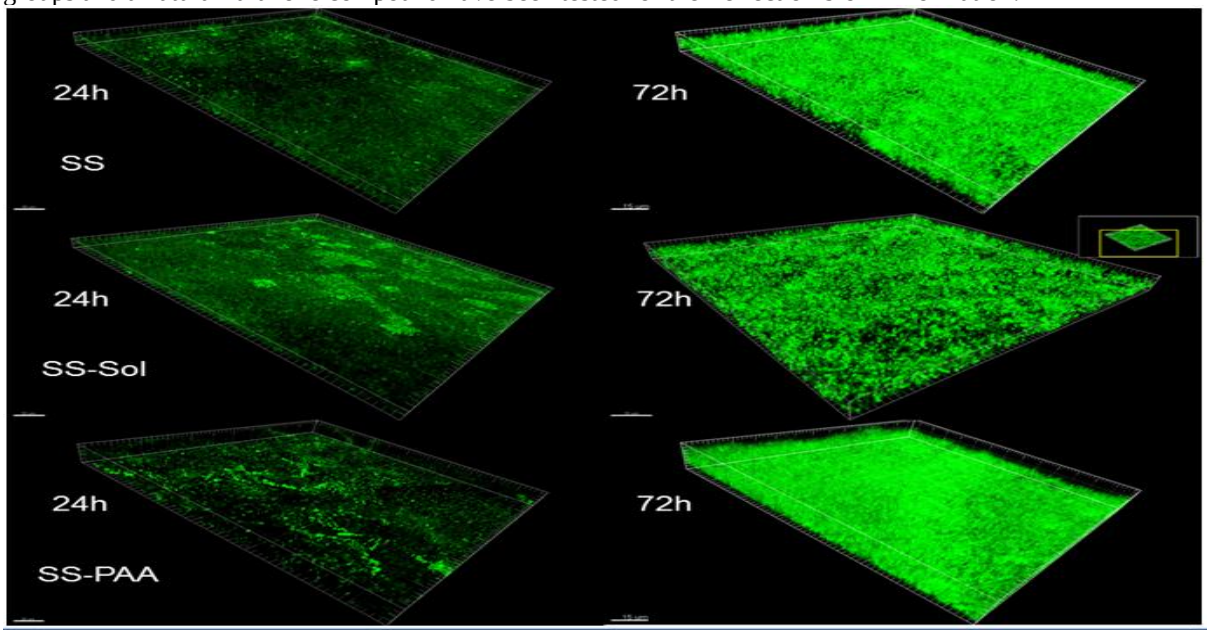
There were three different samples compared with cells on TCPS as negative control for 12- and 48 h. SS-SC; uncoated steel samples were not cytotoxic at both time points. Uncoated SS samples have no significant effect on cell proliferation. While furanone coated samples; FU1 and FU2 showed same trend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



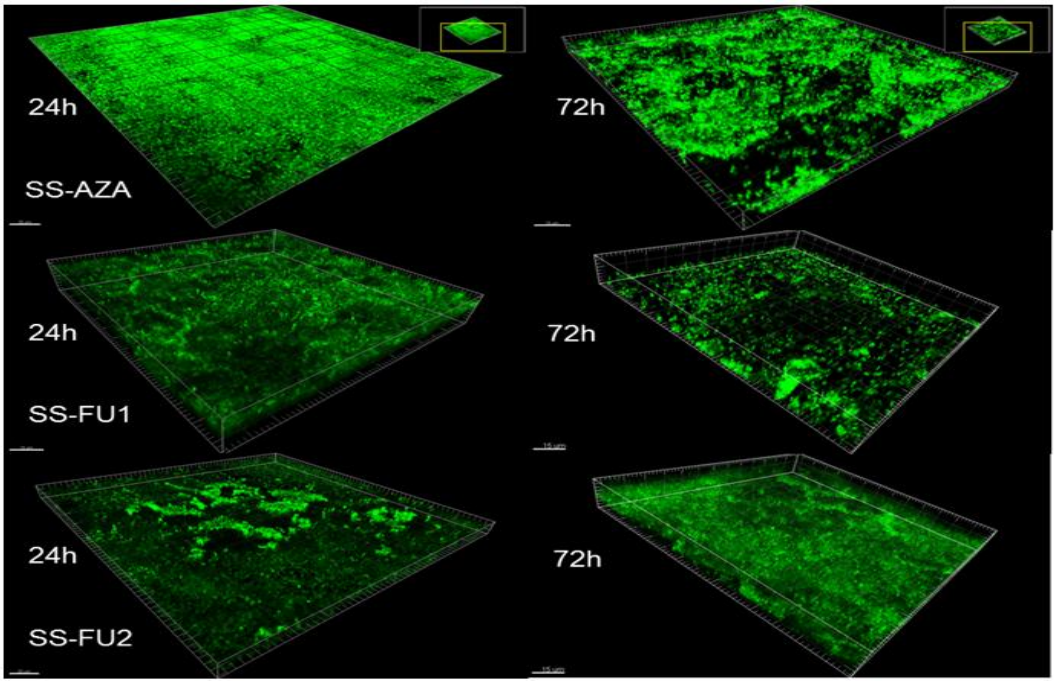
3.5 Confocal Imaging and Analysis

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

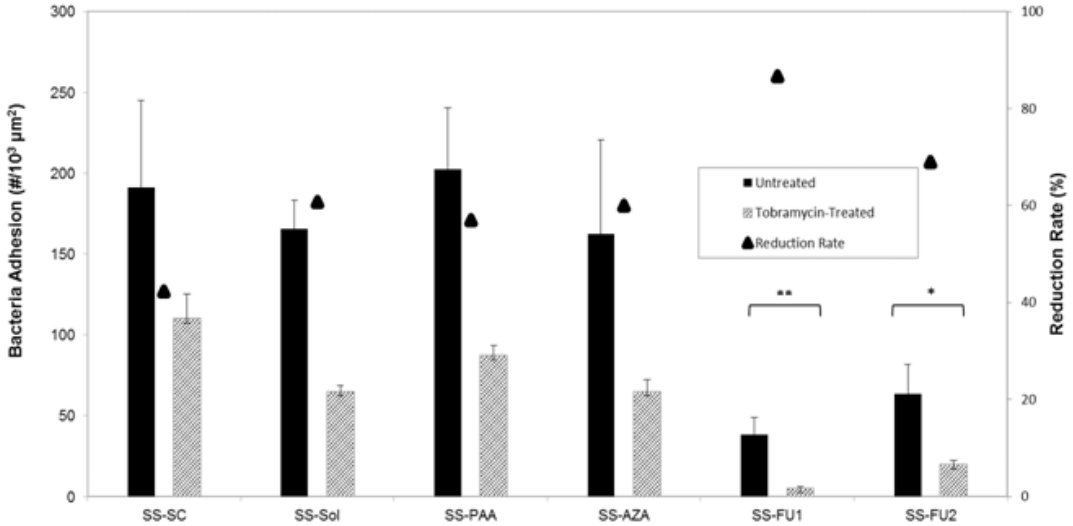
The microalga *D. pulchra* produce furanone compounds which has been known for quorum sensing inhibitory properties as well as interfering complex surface-dependent interactions such as mobility and biofilm formation of *Serratia liquefaciens* [36, 37]. Natural furanones have a limited anti-biofilm effect on *P. aeruginosa* when tested individiually [22] (Hentzer, 2002). Since natural quorum sensing inhibitory compounds can be modified by combinatorial chemistry, it is possible to generate a large number of analogues for screening their activity. Two such synthetic furanone compounds, FU1, FU2 which contain bromine substitution at their functional groups and a natural furanone compound have been tested for their effect on biofilm formation.



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. By contrast, biofilms on furanone coated surfaces were relatively thin, sparsely distributed (Figure 7 and 8). 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.



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 (76% for FU1 and 60% for FU2 when compared with SS-AZA coated substrate) bacterial adhesion as compared with control groups (Fig 9). 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 synergic effect of antibiotics and anti-biofilm surface, tobramycin was added to samples at sub-inhibitory concentrations. FU1 and FU2 coated substrates in combination with tobramycin added samples dramatically reduced bacterial adhesion as compared with tobramycin untreated samples (87% reduction, $p<0.01$ for FU1 and 69% reduction $p<0.05$ for FU2), thereby indicating a synergistic antibiofilm activity by the combination of furanone derivatives and tobramycin.



IV. Conclusion

Control steps of coated samples and as well as SS samples contain much higher number of cells compared to the number of cells on both furanone coated substrates. Bacterial adhesion was slightly higher value than SS-SC and SS-Sol, since PAA has Mw of 250kDa, bacteria might have found more niches to «attach» on substrate. Azidoaniline and furanone coating steps have minor effects on surface topography and can be omitted.

Covalently bound furanone molecules exhibited anti-biofilm effect and reduced bacterial attachment on substrate. Bacterial adhesion to the furanone coated substrates further reduced by the addition of tobramycin when compared with control groups, indicating a synergistic effect of covalently bound furanone molecules and subinhibitory concentration of tobramycin.

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.

Acknowledgments

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FIGURE CAPTIONS

Figure 1 Schematic diagram of the furanone coating sequence and molecular structure of FU1, FU2
Figure 2 FTIR Spectra of SS-AZA, SS-PAA, SS-Sol
Figure 3. FTIR Spectra of SS-AZA, SS-FU1 and SS-FU2
Figure 4. AFM images of SS, SS-Sol, SS-PAA, SS-AZA, SS-FU1 and SS-FU2
Figure 5 SEM images of the prepared materials; scale bars denote 1µm. (a) glass substrate (b) SS-Sol, (c) magnified view of SS-Sol coating, (d) PAA, (e) AZA, (f) FU1
Figure 6 Absorbance versus samples graph for 12- and 48-h. TCPS was used as negative control. Three repeats were done for each group.
Figure 7 Uncoated SS-SC and intermediate steps of coating had been visualized at 24h and 72h by CSLM
Figure 8 Antibacterial activitiy of furanone derivatives at 24th and 72nd hours. It is clearly visible that bacterial attachment onto BF11 coated substrate is reduced when compared with rest of other furanone derivatives
Figure 9. Bacterial adhesion and reduction rates (against without Tobramycin) on uncoated, intermediate steps and furanone coated substrates under static growth for 72h at 37°C. Asterisks in 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 $p < 0.001$

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