TÜBİTAK

Turkish Journal of Chemistry

http://journals.tubitak.gov.tr/chem/

Research Article

Turk J Chem
(2019) 43: 323 – 334
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doi:10.3906/kim-1803-81

Plasma polymerized linalool (ppLin): an antimicrobial and biocompatible coating

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Received: 30.03.2018 • **Accepted/Published Online:** 10.12.2018 • **Final Version:** 05.02.2019

Abstract: Bacterial infections in medical devices and drug resistance of bacteria can cause chaos in the world due to loss of lives in addition to the cost of device revisions, quarantine, disinfection of infected areas, and patient treatment. Antibacterial coatings of essential oils on medical devices can prevent bacterial attachment and reduce costs. Linalool is an antibacterial constituent of essential oils. Herein, we examine for the first time the fabrication and characterization of radio frequency (RF) plasma polymerized hydrophilic thin films from linalool (ppLin) by varying deposition parameters (RF power and deposition time) and the behavior of ppLin with two bacteria (Escherichia coli and Staphylococcus aureus) commonly related to microbial contamination of medical devices. While a dramatic reduction in E. coli and S. aureus attachment was observed on ppLin films, their hydrophilic surface was also bactericidal to S. aureus. Additionally, ppLin films were shown to be adherent and noncytotoxic to human fibroblast cells. ppLin can be potentially integrated into medical and other clinical devices as a promising low-cost biocompatible antimicrobial coating.

Key words: Linalool, plasma polymerization, antimicrobial coating

1. Introduction

Bacterial contamination of implanted or intravascular devices such as intraocular lenses, dentures, orthopedic prostheses, artificial valves, and urinary tract and cardiovascular catheters is a major problem in the biomaterials and healthcare sectors. In addition, uncontrolled use of antimicrobial drugs may cause drug-resistant bacterial strains, and new strains can be spread by medical devices. In Turkey, for example, a study on antimicrobial susceptibility of methicillin-resistant *Staphylococcus aureus* (MRSA) in 12 hospitals showed that only 8 of 397 isolates were susceptible to all antibiotics.[1] In another study, MRSA strains isolated from food producers were found to have low resistance to antibiotics but high rates of spreading.[2] Rapid proliferation and antibiotic resistance may make bacteria the biggest threat to life. Hence, materials with long-lasting biocidal properties to minimize bacterial attachment and bacterial growth on the surface of medical devices are urgently required. Antibacterial coatings have been generated with Cu and Ag nanoparticles and organic compounds such as chitosan and fatty acids.[3–8] However, to obtain a stable (long-lasting) organic thin film coating, the monomer

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material has to be polymerized onto the surface of a substrate. Otherwise, the effectiveness of the coating may diminish over time due to host immune system and/or environmental conditions. However, most monomer materials cannot be polymerized by conventional methods. Even if the molecule can be polymerized, its antibacterial activity can diminish due to a loss of functional groups during polymerization.

In recent years, the radio frequency (RF) plasma polymerization technique has been used to create antimicrobial surfaces. This technique allows for the polymerization of organic molecules that do not normally polymerize via conventional pathways on a solid substrate. Polymer thin films fabricated by means of RF plasma polymerization have been shown to retain structural similarity and inherit the bioactivity of the original monomer while gaining the mechanical advantages associated with the presence of the solid substrate. RF plasma polymerization also allows for the production of smooth and uniform polymer thin films from organic molecules. Therefore, plasma polymerization is a promising deposition technique to generate bioactive organic surfaces. Some thin films of essential oils have been shown to be effective alternative antimicrobial coatings. For example, cineole, terpinen-4-ol, and carvone, which are antimicrobial natural essential oils, were deposited on solid surfaces using a plasma polymerization technique and the coatings inherited the biocidal properties of their respective precursors.[9–11]

The goal of this study was to develop a novel organic polymer coating for the prevention of adhesion and growth of these two medically significant bacteria (gram-negative *Escherichia coli* and gram-positive *Staphylococcus aureus*) on a solid surface. For this purpose, plasma polymerized thin films of linalool (ppLin), which is a naturally antimicrobial essential oil,[12] were generated on a glass substrate under particular deposition conditions. Static contact angle (SCA), FTIR, SEM, and AFM analyses were performed for surface characterization, and antiattachment and antibacterial properties of the films against *E. coli* and *S. aureus* bacteria were investigated for the first time in this study. Additionally, the biocompatibility of ppLin films was tested on human fibroblast cells.

2. Results and discussion

A plasma polymerization route was used to fabricate thin films from linalool. The deposition conditions (e.g., power and frequency of the excitation signal, type and position of the substrate, geometry of the deposition chamber) affect thin film characteristics.[13] To obtain optimum deposition conditions, we changed only two conditions: RF power (15 and 20 W) and deposition time (15 and 30 min).

Linalool (Figure 1) belongs to the family of monoterpenes. Its solubility in water is low because of its hydrophobic nature. However, surprisingly, the SCA of ppLin thin films was measured as 38.65, 28.56, 28.17, and 23.17 degrees (Figure 2) for 15 W – 15 min, 15 W – 30 min, 20 W – 15 min, and 20 W – 30 min, respectively, indicating that ppLin has a hydrophilic nature. As seen, SCA values were affected by RF power and deposition time, and slightly decreased with an increase of both deposition time and RF power.

A lower contact angle suggests that atoms having high electronegativities, such as O, F, and Cl, are incorporated into the thin film. Linalool has an -OH group and two double bonds. Therefore, the presence of -OH groups can be expected in ppLin. Jacob et al. reported that FTIR spectra of ppLin film (25 W, 30 min) have -OH peaks.[14] Plasma polymerization involves the fragmentation, subsequent deposition of organic monomers, and etching simultaneously. Alcohol monomers generally lose their -OH groups during plasma polymerization in especially high RF power. When the ratio of discharge power (W) to flow rate (F) is decreased, there is less cleavage of -OH groups, which generates more hydrophilic thin films.[14] Therefore, we kept the plasma power

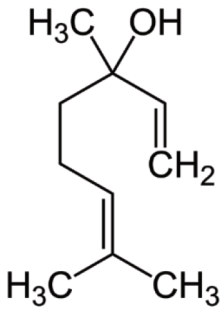

Figure 1. Chemical structure of linalool.

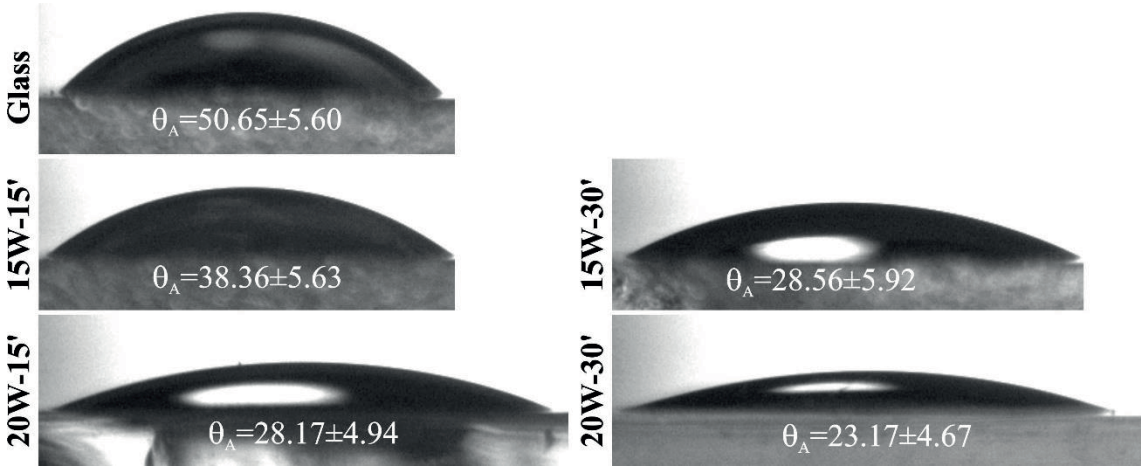

Figure 2. Static contact angles (SCAs) of ppLin. ?A: Average of contact angle measurements.

level low (15–20 W) in order to avoid excessive loss of -OH groups, and the resultant coatings were expected to have relatively low SCAs. Additionally, unsaturation in the monomer also influences the density of -OH groups.[15] Therefore, the two double bonds in linalool can be expected to keep the -OH groups in ppLin during polymerization. Interestingly, contrary to Jacob et al., the FTIR spectra of our ppLin samples do not have any -OH peaks (3200–3400 cm^{-1}) while they have strong alkoxy C-O peaks (1050–1150 cm^{-1}) (Figure 3). This finding implies that linalool loses -H atoms from C-OH groups of linalool and thus alkoxy C-O bonds are kept during plasma polymerization. However, O makes two bonds. Accordingly, it seems likely that the O should be a part of a C-O-C bridge. Newly formed alkoxy C-O-C bridges may be responsible for the low contact angle of ppLin. On the other hand, C-O-C should be a bridge between linalool residues during polymerization.

In another study, Jacob et al. also generated hydrophobic ppLin thin films whose SCA value was ~90 degrees, which was dramatically higher than our results (23.17°, 28.56°, 28.17°, and 38.65°).[16] On the other hand, the resultant films from terpene oil have been hydrophobic so far.[9–11,16] This means that we are the first to synthesis hydrophilic film (wettable surface) from linalool. The differences between the previously reported ppLin coatings and our ppLin are likely due to differences in the plasma reactors and application conditions that affect the chemical and physical properties of the resultant thin films. The distance between the electrodes was 13 cm and maximum RF power was 20 W in our study, while these were 10 cm and 25 W in the study reported by Jacob et al.[16] High resolution SEM images (Figure 4) demonstrate that small changes of deposition condition (RF power and deposition time) cause dramatic changes in the topology of the ppLin thin films. According

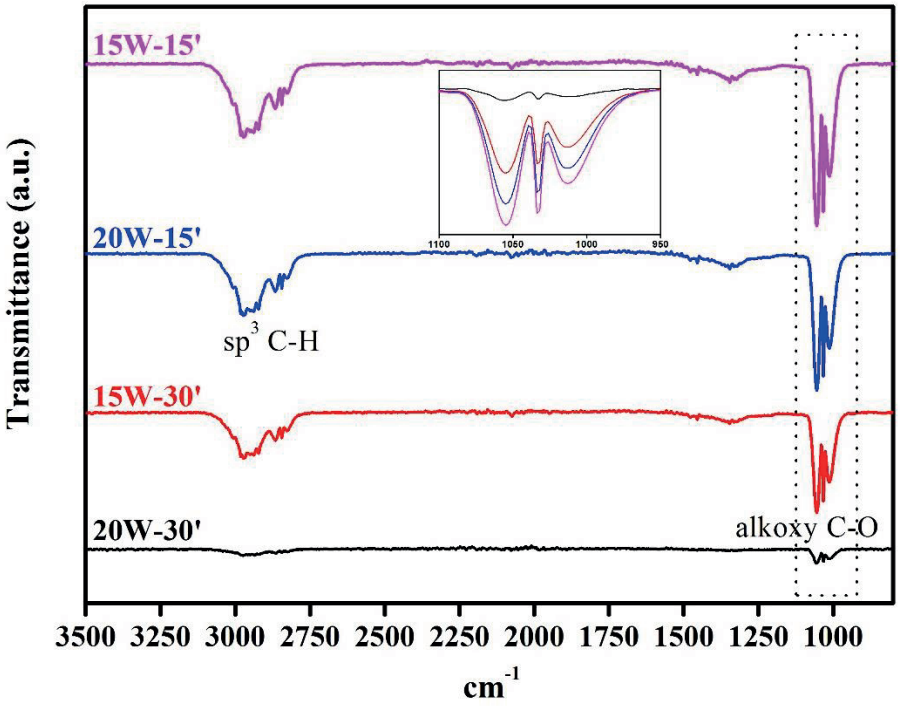

Figure 3. FTIR spectrum of ppLin.

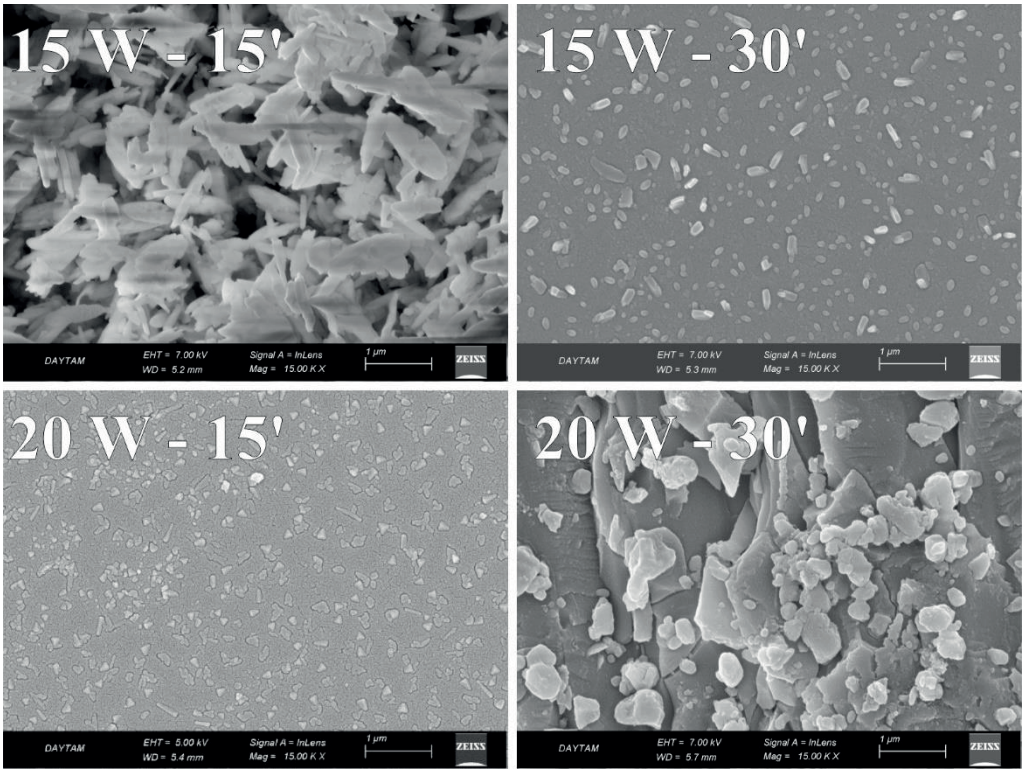

Figure 4. SEM images of ppLin.

to the SEM images with the same scale bar, elongated deposition time causes etching at 15 W while it causes deposition at 20 W. Therefore, it is quite normal that the ppLin thin films generated by Jacob et al. should be different from our ppLin.

Similar to SEM images, the AFM (Figure 5) studies demonstrated that RF power and deposition time alter the surface topology of the films. Figure 5 shows that small changes of deposition conditions cause dramatic

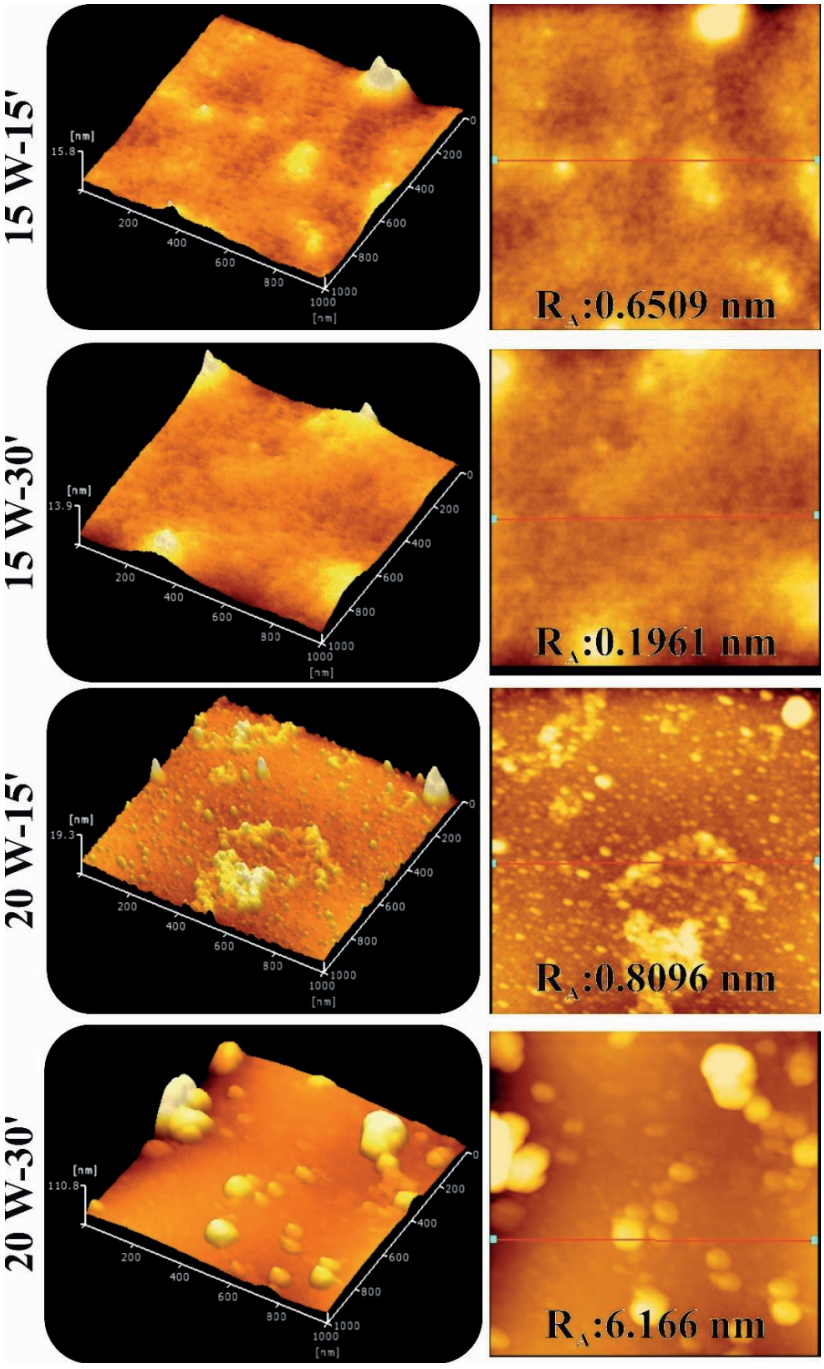

Figure 5. AFM images of ppLin. Typical surface morphology of linalool thin films deposited at 15 and 20 W RF fabrication power and application times of 15 and 30 min as inferred from 3D AFM images. RA: Average roughness.

changes of the ppLin thin film topology. Here, roughness increased with RF power. However, roughness slightly decreased from 15 to 30 min at 15 W RF power, while it increased with extension of deposition time at 20 W. As a result, roughness is not linear with SCA values of the films. The most deterministic factor for SCA is probably the functional groups on the surface of the thin films.

To investigate the antiattachment and antibacterial properties of ppLin films, *E. coli* and *S. aureus* were used. The behavior of ppLin (hydrophilic surface) was compared to clean uncoated glass surfaces (hydrophilic control). Both of the bacterial species can grow on and strongly attach to the glass substrate so that they cannot be removed from the surface by rinsing with water. However, a significant reduction of bacterial attachment was observed for ppLin films when compared to the glass controls (Figure 6). It can be seen from Figure 6 that attachment for both bacterial species decreased with RF power. However, attachment to ppLin increased with deposition time for only *E. coli*. Attachment of *S. aureus* on the ppLin surface decreased with both deposition time and RF power. These results show that the antiattachment properties of ppLin vary according to the type of bacteria, RF power, and deposition time.

Why did the bacteria detach from the ppLin surface? Is the death of the bacteria on the ppLin surface the reason for their detachment? To answer these questions, we performed antibacterial tests for both bacteria grown on ppLin surfaces for 4 days. Bacterial survivability was measured each day. By taking into consideration the optimal antiattachment test results, 20 W – 15 min ppLin for *E. coli* and 20 W – 30 min ppLin for *S. aureus* were chosen for antibacterial tests. As can be seen from the Table, ppLin showed a 10% bactericidal effect for *E. coli* while it was strongly bactericidal to *S. aureus* with an 87% bactericidal effect after 4 days of incubation. *E. coli*, which is a gram-negative bacterium, has an extra barrier (cell wall) and is flagellated in comparison to *S. aureus*, and thus these may keep and/or move away *E. coli* from the bactericidal character of the ppLin surface in liquid media. Similar to our result, Friedlander et al. showed that flagella decrease the adhesion of *E. coli* to hydrophilic model surfaces.[17] Friedlander et al. also concluded that some properties such as surface chemistry, topography, shear forces, pressures, osmolarity, ionic strength, and nutrients affect adhesion and thus the interaction between surface properties and bacterial physiology is complex and often unpredictable. However, *S. aureus*, which is a gram-positive bacterium, does not have an extra barrier (cell wall) or flagella and thus inevitably may be more exposed to the bactericidal effect of the ppLin surface. In a study of Yang et al. it was shown that essential oil from *F. koreana*, which is rich in linalool (10.68%), acted on the cytoplasmic membrane of bacteria, resulting in loss of integrity and increased permeability.[18] Similarly, the detachment of bacteria on the ppLin surface can be caused by the loss of bacterial cell membrane integrity. Hui et al. reported that the primary mechanism of essential oil components is disruption of the cell membrane through binding with the lipophilic parts of the membrane.[19] Unlike Hui et al., however, our ppLin is antimicrobial despite its hydrophilic nature. Further studies are required to identify the exact mechanism for the prevention of bacterial attachment observed on ppLin thin films.

Table. Antibacterial test results of ppLin against *E. coli* and *S. aureus*. 20 W – 15 min ppLin for *E. coli* and 20 W – 30 min ppLin for *S. aureus* were tested.

	1st day	2nd day	3rd day	4th day
E. coli Abs_{625}: 0.088	0.497 ± 0.01	0.558 ± 0.02	0.541 ± 0.01	0.503 ± 0.08
(McFarland 0.5)	6%	1%	10%	10%
S. aureus Abs_{625}: 0.093	1.036 ± 0.06	0.600 ± 0.5	0.357 ± 0.3	0.122 ± 0.07
(McFarland 0.5)	4%	50%	72%	87%

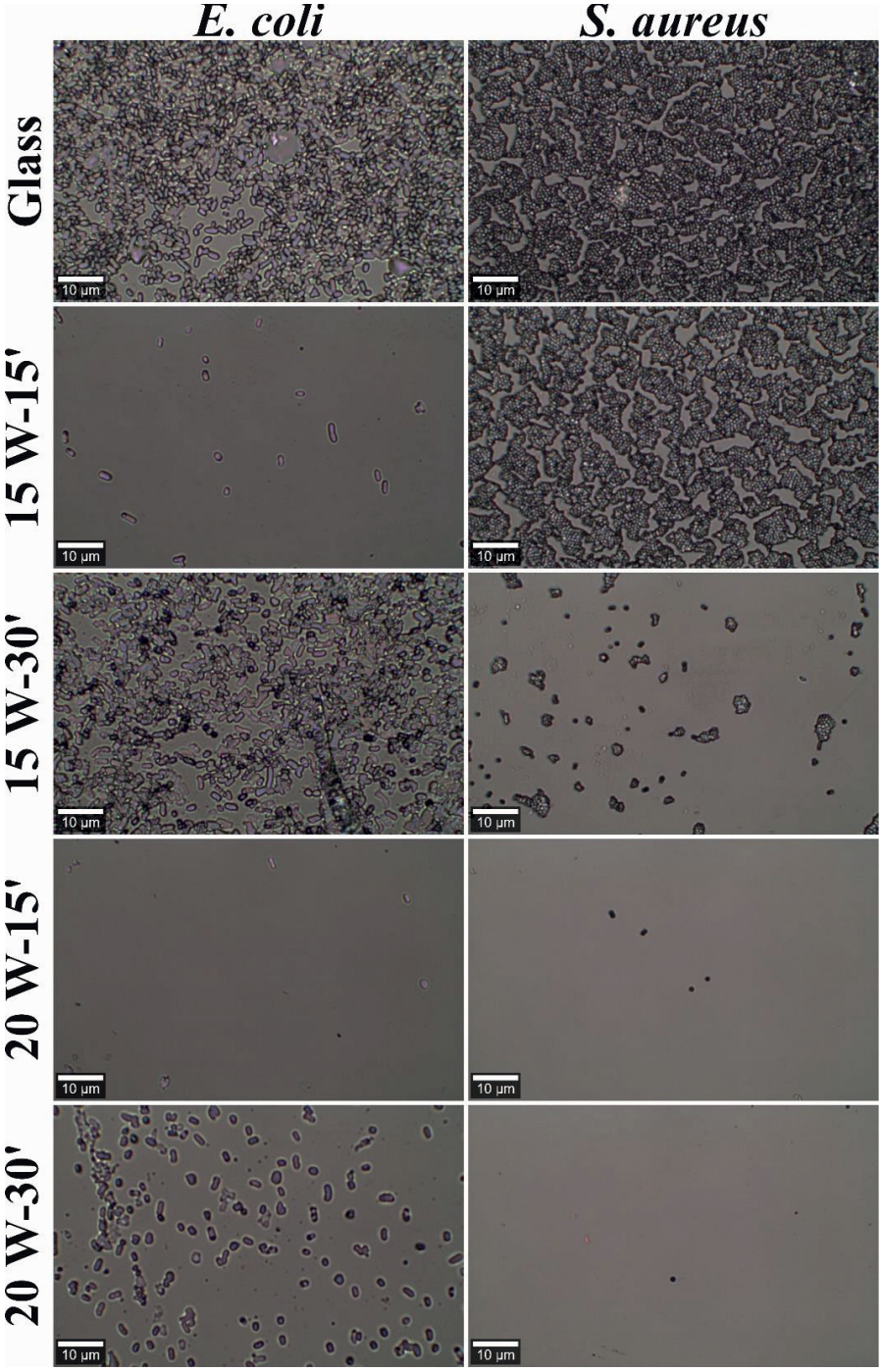

Figure 6. Results of antiattachment tests.

Our results showed that the presence of the ppLin thin films significantly altered the surface properties of the glass substrates, which resulted in a notable change in the adhesion behavior of both *E. coli* and *S. aureus* cells. However, the biocompatibility of materials is an important criterion for using them in clinics. Therefore, cytotoxicity effects of ppLin on human fibroblast cells were tested. As can be seen from Figures 7 and 8, ppLin films were shown to be adherent and noncytotoxic to human fibroblast cells.

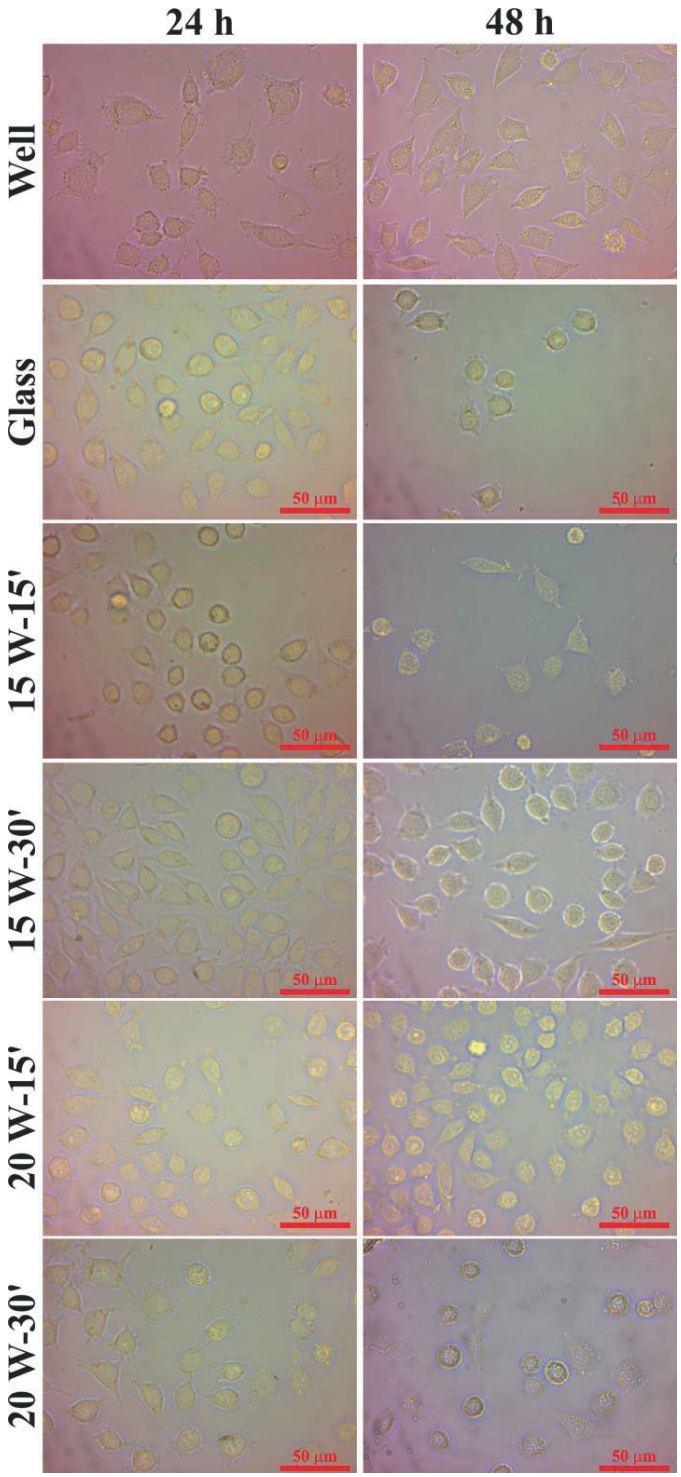

Figure 7. Images of human fibroblast cells grown on ppLin (40 X).

In conclusion, ppLin thin films obtained at 15 W/20 W – 15 min/30 min can be potentially integrated into medical and other clinical devices as promising low-cost biocompatible antimicrobial coatings.

3. Experimental

3.1. Plasma polymerization

A plasma polymerization system (PlazmaTek) was used to fabricate the thin films from linalool (Figure 1). The plasma polymerization setup included a chamber coupled to a vacuum system, RF amplifier, and a monomer holder. The schematic diagram of the experimental set up is shown in Figure 9. Glass and potassium bromide (KBr) glass were used as substrates in this study. Glass substrates were washed in hot distilled water and then cleaned in an ultrasonic bath before being placed in the vacuum chamber. The wet cleaning process for the KBr glass was not performed. After the cleaning process, the pressure of the medium was set to 1 mTorr by means of a vacuum pump, and surface cleaning of the substrates was repeatedly performed using argon gas at 1 mTorr. The monomer was released gradually into the chamber and the glow was maintained by controlling the monomer flow by means of a vacuum stop cock. Capacitively coupled copper electrodes were used to ignite the glow discharge from a 13.56 MHz RF generator with an output power of 15 or 20 W. Deposition was performed for 15 or 30 min at room temperature and a pressure of 500 mTorr, and the distance between the electrodes was adjusted to 13 cm in order to achieve optimal deposition conditions.

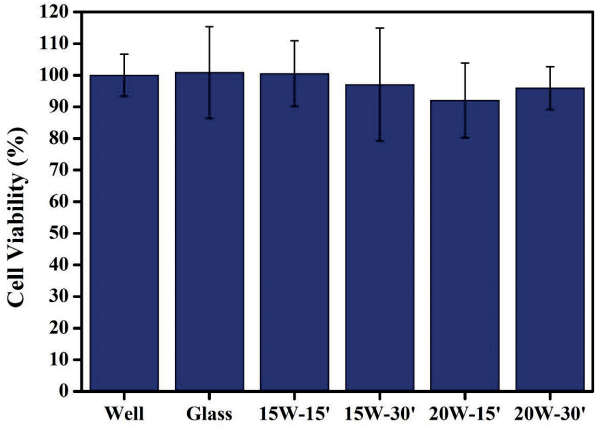

Figure 8. The viability of human fibroblast cells grown on ppLin.

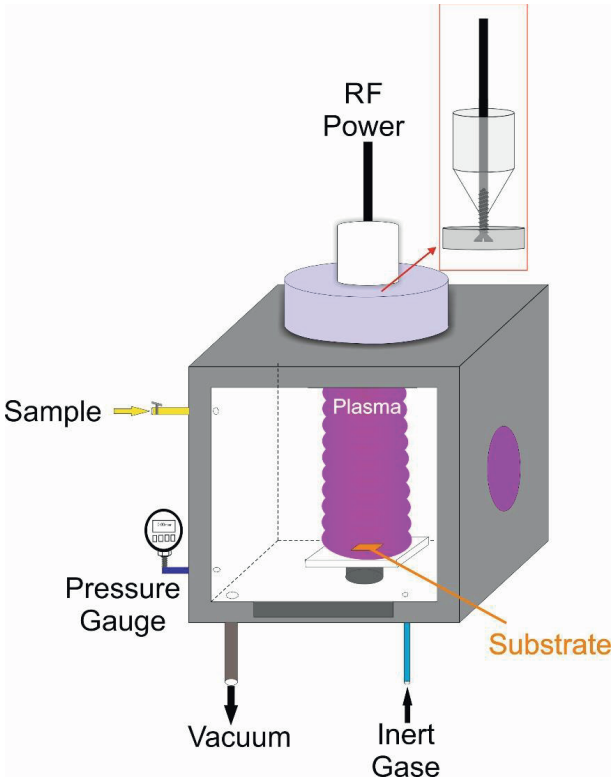

Figure 9. RF plasma polymerization system.

3.2. ppLin characterization

3.2.1. SCA measurement

The static contact angle measurement was performed using a CAM-101 optical contact angle analyzer (KSV Instruments, Finland). The contact angles were measured by using a goniometer, which used a water drop of 6 µL and took the Young–Laplace equation into account at the solid-liquid interface.[20]

3.2.2. FTIR spectrum

All ppLin samples were manufactured on KBr pellets. FTIR spectra of the samples were obtained using Vertex 80 & 80V (Bruker) spectrophotometer in the 400–4000 cm^{-1} range.

3.2.3. SEM analysis

All ppLin samples were coated with gold and then surface morphologies of the samples were examined by a Zeiss Sigma 300 model scanning electron microscope operating at 5.00 kV under vacuum.

3.2.4. AFM analysis

The surfaces of the resultant thin films were scanned using an atomic force microscope (Hitachi 5100N) in semicontact (tapping) mode to observe the surface topography and to quantitatively estimate the extent of surface roughness.

3.3. Bacterial attachment test

Escherichia coli (ATCC 25922) and *Staphylococcus aureus* (ATCC 29213) were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). *E. coli* ATCC 25922 grown overnight in LB broth (Luria Bertani Broth, Merck 110285) was spread on LB agar (Luria Bertani Agar, Merck 110283) with a swab, and the ppLin sample (deposition side of the glass substrate) was placed on the surface of petri plate (Figure 10). The initial numbers of the bacteria inoculated on ppLin were about 10^7 CFU/mL. The same procedure was applied for *S. aureus* ATCC 29213. The growth of *S. aureus* used TSB (Tryptic Soy Broth, Sigma Aldrich 22092) and TSA (Tryptic Soy Agar, Sigma Aldrich 22091). Then plates were incubated at 30 °C for 48 h. A sample without ppLin was used as a control in each treatment. After the end of incubation, the samples were taken from the plates, rinsed with sterile water, and then evaluated using an Alpha 300R (100 × NA/0.9 objective) microscope.

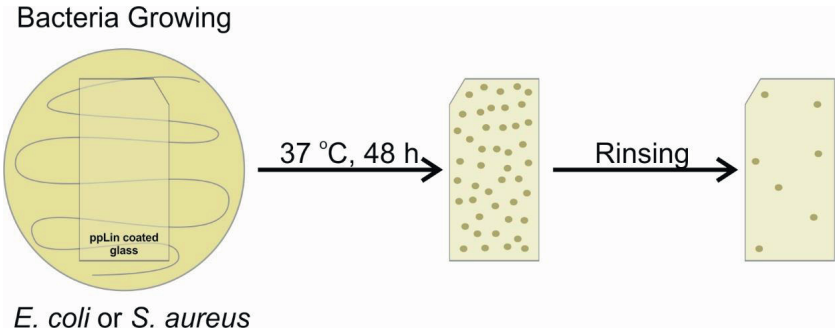

Figure 10. Bacterial attachment test.

3.4. Antibacterial test

Inoculum prepared from colonies on agar plates (TSA or LB agar) and adjusted to the turbidity of a 0.5 McFarland standard was used for the test and 100 µL of prepared inoculum was transferred onto the surface of the ppLin samples, which were exposed to 20 W for 15 min (the best result for *E. coli*) or 20 W for 30 min (the best result for *S. aureus*). The samples with inoculum were transferred to the relevant broths for each strain

and then inoculated tubs were incubated at 30 °C for 4 days (Figure 11). Absorbance values were determined at 625 nm every 24 h. The test was repeated three times.

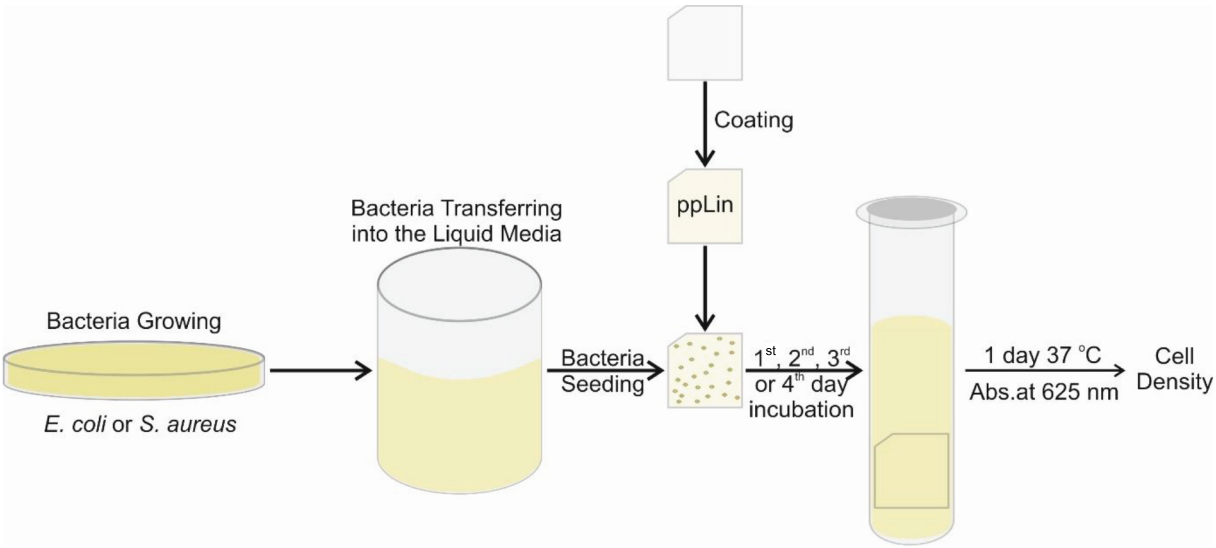

Figure 11. Antibacterial test.

3.5. Cytotoxicity test

Human fibroblast cells were grown in Dulbecco's modified Eagle medium high glucose media (GIBCO) containing 10% fetal bovine serum (GIBCO) and 1% penicillin-streptomycin (GIBCO). Cells were incubated at 37 °C with 5% CO_2 and 90% humidity (NUAIRE). ppLin was placed into the 6-well plate, sterilized with UV irradiation for 1 h, and washed with phosphate-buffered saline (PBS, Sigma). Cells were dyed by trypan blue, counted, and seeded onto ppLin coated glasses with 3000 cells/well density. For cytotoxicity effect of ppLin on fibroblast cells, the MTT cytotoxicity test was applied according to the kit supplier's protocol (TOX1, Sigma). After 72 h of incubation, 10% MTT solution was added to each well and incubated for 3 h. After the reaction was stopped by stabilization solution (TOX1, Sigma), absorbance values were measured by ELISA plate reader (Biotek/Epoch) at 570 nm. Cell images were taken (Axiomcam ERc 5s, Zeiss) at the end of the 24th and 48th hours.

Acknowledgments

The authors thank the East Anatolia High Technology Application and Research Center (DAYTAM) of Atatürk University for instrumental supports. The authors are grateful to Dr Demet İskenderoğlu for the SEM analysis. The authors are also grateful to Prof Dr Ahmet Gürses for the SCA measurements.

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