Staphylococcus Aureus Biofilm Evolution and Povidone-Iodine (PVP-I) Treatment Efficacy

mingli chen (drchenmingli@163.com)
The Affiliated Southeast Hospital of Xiamen University https://orcid.org/0000-0002-8662-4871
Zhi-da Chen
909
Qing-quan Wu
909
Li-lin Dai
909
Jin Wu
909
Wen-rong Zeng
909

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Abstract

Objective The aim of this study is to observe the evolution changes of staphylococcus aureus biofilm on the surface of titanium alloy and evaluate bacterial changes of these biofilm after 0.5%g/L povidone–iodine treatment through the in vitro experiment. We have done research on this to provide some clinical guidance.

Methods First, each period of 7days, 14days, 21days and 28days staphylococcus aureus biofilm models were established on the surfaces of titanium alloy and the biofilm morphology were detected by the confocal laser scanning microscope and scanning electron microscope. Then, morphological structure and the effect of 0.5%g/L povidone–iodine were further evaluated by viable count method after These biofilm were soak in the0.5%g/L povidone–iodine for5 min and 10 min respectively.

Results 1. the extracellular polymer of biofilm was increasing with the Among them, the most obvious change and maturity occurred at the 21days and 28days respectively. With the process of biofilm maturation, the number of bacteria was decreased but the bacteria close to 90,000 and there was no significant difference (P>0.05). 2. The effect of 0.5%g/L povidone–iodine sterilization was significant,0.5%g/L povidone–iodine sterilization ten minutes group was better than PBS group(no intervention group) (P<0.05),0.5%g/L povidone–iodine sterilization five minutes was better than PBS group too (P<0.05). However, with the maturation of the biofilm, but not maturity 0.5%g/L povidone–iodine sterilization effect weakened but the 21days0.5%g/L povidone–iodine sterilization ten minutes was better than five minutes(P<0.05).there was no significant difference in maturity(P>0.05).

Conclusion 1. The maturation of the biofilm is bacteria and outside then formation of extracellular polymeric substance and they formation micro environment together and the 21days is the time critical point for distinguishing young biofilm and mature biofilm, the most difference of which is the extracellular polymers and micro-environment.2. Early Implant related biofilm 0.5%g/L povidone–iodine treatment for 10 min is better than 5 min, but there is no extension meaning of static after maturity. So for early clinical implant related infections0.5%g/L povidone–iodine sterilization 10 min is accepted.

Introduction

Joint replacement has a favourable impact on end-stage arthropathy, and the number of domestic and international joint replacements has been increasing. Peripheral prosthesis infection is a disastrous complication after joint replacement; treatment is difficult, of long duration, and expensive. The infection rate is 0.3%-1.7% after hip replacement and 0.5%-2% after knee replacement[1, 2]. Currently, prosthesis retention is used to treat acute hematogenous disseminated prosthesis infections and early postoperative infections. Clinical success rates of 20%-90% have been reported, averaging around 50–60%. It is generally assumed that earlier treatment inventions have higher success rates and better overall effects. It is generally thought that antibiotic therapy is not successful for treating prosthesis infection because the formation of bacterial biofilm on the prosthesis surface is refractory [3]. Because of changes in the physiological status and morphology of biofilm bacteria, bacterial antibiotic resistance is enhanced, 10-1000 times more than that of floating bacteria[1, 4–6].Therefore, a greater understanding of the evolution and structural changes of bacterial biofilms on joint prosthesis surfaces is urgently needed and could guide the clinical treatment of periprosthetic infections.

Povidone-iodine (PVP-I), a non-antibiotic antiseptic and disinfectant, is widely applied in clinical practice because of its antibacterial efficacy and few side effects [7, 8]. Luo et al. found that bacterial biofilm, cultivated for 24 hours, could be completely eradicated after treatment with 0.5% PVP-I for 10 min, but not 5 min[10]. Further, Shirai et al. showed promising effects of applying iodine coating to orthopedic external fixed screws used to treat patients with chronic fracture infections[9]. The in vitro biofilm cultivation time is generally reported to be several hours to several days, however, there have been no reports on the evolution of bacterial biofilms in vitro over 28 days. Additionally, the impact of iodine in this setting is unknown. Here, we investigated changes in bacterial biofilms over 28 days and their sensitivity to iodine, with the aim of providing future clinical guidance.

1. Materials And Methods

1.1 Materials

We used standard strains of staphylococcus aureus (ACTT25923 Microbiology Laboratory of Fuzhou General Hospital of Nanjing Military Command),titanium-alloy plates (Ti6Al4V, diameter 12.7 mm, thickness 4.7 mm, Shenzhen Smarl Equipment Co., Ltd.),phosphate buffer (PBS), 24-well tissue culture plates, standard agar culture dishes, an electronic confocal laser scanning microscope (CLSM; FVl000,Olympus Corporation), CLSM professional dishes, and a Quanta 450 scanning electron microscope (SEM; Thermo Fisher Scientific).

1.2 Experimental Methods

1.2.1 Preparation of Bacterial Suspension

Standard staphylococcus aureusstrains were grown on slants in 10 ml trypticsoy broth (TSB) and were placed in a constant temperature incubator(37°C, 5%CO2) for overnight anabiosis. A trace amount of bacteria solution was taken, grown in blood plate medium using the four-zone streaking method, and cultivated to obtain bacterial colonies. A single bacterial colony was dissolved, resulting in a turbidity of 0.5 (measuring using aturbidimeter). One ml of bacteria solution was diluted in 149 ml of sterile TSB medium, yielding afinal concentration of 1×106CFU/ml, which was used for further applications.

1.2.2 Preparation of Bacterial Biofilms (BBF) on a Titanium Alloy Surface

The titanium alloy plates were pretreated by sterilization using the moulding method[11]. They were then put into 24-well plates and 1mlof bacterial suspension at a concentration of 1×106CFU/ml was added to each well. The plate was placed in a constant temperature incubator to cultivate for 7,14,21, or 28d.

1.2.3 Treatment of Biofilms with PVP-I

After surface biofilm growth, the titanium alloy plates were washed 3 times with PBS to remove non-adherent floating bacteria and then put into a new sterile 24-well plate. One ml of 0.5% PVP-I solution was added to each well and incubated at 37°C for 5min, or10min. Additionally, some plates were treated with PBS only.

1.2.4 Experimental Groups

A total of 480 titanium alloy plates were used to establish biofilms in 4 phases (120 plates at each time point). The cultivated biofilm in each phase was randomly divided into 3 treatment groups with 40 plates per group. After PVP-I treatment for 0, 5,or 10 min, 10 plates from each group were randomly selected for scanning electron microscopy, 20 plates were imaged using CLSM, and 10 plates were used for CFU counts.

1.2.5 CLSM Imaging of Bacterial Biofilm Structure

After different cultivation times, titanium alloy plates with surface biofilm were washed with PBS. 40 plates were selected randomly from each cultivation phase group and treated with PVP-I for 5 min or10 min. Another 20 plates from each group were treated with PBS only. 10 plates from each PVP-I treatment group (30 plates per cultivation phase) were selected randomly, stained using fluorescein isothiocyanate-conjugated concanavalinA (FITC-ConA) in the dark for 30 min, and then stained using propidium iodide (PI) in the dark for 15 min. The other 10 plates from each PVP-I treatment group were stained using PI and SYTO™ 9 green fluorescent nucleic acid stain(SYTO 9) in the dark for 20 min. After staining, the plates were washed with PBS, dried, and placed in an observation dish. The biofilm morphology and bacteria vitality were imaged used CLSM.

1.2.6 SEM Imaging

After different cultivation times, titanium alloy plates with surface biofilm were washed with PBS. 20plates were selected randomly from each cultivation phase group and treated by PVP-I for 5 min or10 min. Another 10 plates from each group were treated with PBS only. The plates were fixed in glutaraldehyde for more than2 hours and then dehydrated with different concentrations ofethyl alcohol. The plates were then dried using aK850 critical point dryer and placed into a Q 150R S/E/ES sample preparation system for metal spraying. The morphological structure of iodine-treated bacterial biofilms indifferent cultivation phases was then observed using SEM. Random fields were selected for imaging.

1.2.7 Viability Counts

After different cultivation times, titanium alloy plates with surface biofilm were washed with PBS. 20 plates were selected randomly from each cultivation phase group and treated by PVP-I for 5 min or 10 min. Another 10 plates from each group were treated with PBS only. The plates were washed again using PBS and then placed into a culture flask with 10 ml of PBS. The plates were then treated with an ultrasonic cleaning machine and vortex oscillator and diluted with eluent. 100μlof eluent was added dropwise to the center of a general nutrition agar plate and applied homogeneously using a triangle push rod. The plate was placed in a constant temperature incubator at5% CO2and 37°C for 48 h, and then CFU counts were performed.

1.3 Statistical Analysis

We performed a normality test of the CFU counts using SPSS20.0 software and found that the test results were not normally distributed. Thus, we performed a Kruskal-Wallis H non-parametric test on multiple independent samples and a Mann-Whitney U test for comparison among groups. The test level was set to 0.05 and $P<0.05$ was considered statistically significant.

2. Results

2.1 Determination of biofilm morphology using CLSM and SEM

We used CLSM and SEM to observe the morphological structure of bacterial biofilms grown on titanium plates for 1 week to 1 month. We stained the biofilms using SYTO9(green fluorescence) and PI(red fluorescence) and imaged them using CLSM. We found that plates with 7-day old biofilms had predominantly green fluorescence with a small amount of red fluorescence (Fig. 1-1a), and the biofilm structure was not patchy. After 14 days of growth, the green fluorescence became denser and stronger and patchy areas emerged, with some areas locally connected in series and combined with dense areas (Fig. 1-2a).After 21 days of growth, the distribution of green fluorescence was continuously patchy, fine, and smooth(Fig. 1-3a). After 28 days of growth, the titanium plate was almost entirely covered by green fluorescence. The local distribution had uneven thickness and alternating high and low density areas(Fig. 1-4a).We also stained the biofilms using FITC-ConA (green fluorescence) and PI(red fluorescence).We found that after 7 days of growth, the green fluorescence was sparse and dim, and overlapped with the red signal(yellow areas, Fig. 1-1b).We found that the red fluorescence signal was dominant after 14 days of growth(Fig. 1-2b).After 21 days of growth, the green fluorescence signal became brighter, wider, and unevenly distributed (Fig. 1-3b).After 28 days of growth, a large area of green fluorescence showed a mountain-like stereo structure with high and low levels(Fig. 1-4b). Overall, we found that the biofilms changed most significantly from day 21 to day 28($P < 0.05$, Fig. 4-D1).

We next performed SEM and found that, after 7 days of growth, a large number of cocci were loosely stacked and deformed, formed unequal sized protuberant granule, and had several different pore sizes (Fig. 2-sb,sc, and sf, red arrows).After 14 days of growth, the fibrin was interconnected between cocci like a spider web(Fig. 2-se, red arrow).After 21 days of growth, the extracellular slime substance(ESS) enveloped the bacteria and fused into pieces. The innerpores were interlaced. The cocci surface spots were larger and denser, and they were linked together by coarse fibrin. The cocci displayed locally wrapped parts(Fig. 2-sg,sh, and si, red arrow).By 28 days of growth, a biofilm with a certain thickness and spatial stereo structure had formed. There were distinct membrane layers, and the pores formed an interconnected network that was structurally distributed. Extracellular polymeric substance(EPS) formed stable structures with the cocci. We observed that the EPS appeared to drift away, fall off, and form nest-like structures(Fig. 2-sj,sk, and sl, red arrow and black and red circles).

2.2 Efficacy of PVP-I treatment on superficial bacterial biofilms grown ontitanium alloy for 1 week to 1 month.

We examined the fluorescence signals and grayscale ratios of SYTO9 and PI staining after PVP-I treatment. We found that the green fluorescence significantly decreased after PVP-I treatment for 5 min (Fig. 1-1c,2c,3c, and 4c). The green fluorescence was further reduced after treatment for 10 min (Fig. 1-1d,2d,3d, and 4d).There was a statistically significant difference in fluorescence after PVP-I treatment (as compared to no PVP-I treatment, $P < 0.05$,Fig. 3- Ta, Tb, Tc, and Td). The decrease was more obvious after5minthan 10 min. These results were consistent with the fluorescence grayscale ratios (Fig. 4-D2).PVP-Itreatment had the greatest effect on biofilms grown for 21 days(Fig. 4-D2).There were no statistically significant differences after 7, 14,or 28 days of growth ($P > 0.05$); however, after21 days of growth, there was a statistically significant difference between biofilms treated with PVP-I for 5minversus 10 min($P < 0.05$). The bacterial biofilm CFU was around 90,000 after each of the four growth periods and was not significantly different ($P > 0.05$). Comparing the biofilms grown for 7 versus 21 days, 14 versus 21 days, 7 versus 28 days, and 14 versus 28 days, we found that PVP-I treatment for 10 min had statistically significant effects($P < 0.05$). Of note, we observed no significant differences by SEM after PVP-I treatment for 5minor 10 min.

3. Discussion

3.1 Bacterial Biofilm Structure

The treatment of orthopedic implant infections is a great clinical challenge. These implant infections are caused by the formation of bacterial biofilms on the implant surfaces. Bacterial biofilm structure has been widely reported in the literature. The process of biofilm maturation has been studied in vitro and has, to some extent, helped to understand and treat relevant implant infections in clinical practice. Here we showed that the structure of biofilms grown on titanium plates was loose and dominated by single bacterium after 7 days of growth. We found limited morphological changes to the bacterial films, with few extracellular slime substances and no lamellate structure. The internal porous development was simple and incomplete. The features mentioned above suggest that a young biofilm had formed, which has strong self-renewal capability[12]. The change in the BBF curve(Fig. 4-D1)was the most remarkable. The biofilms could be observed using an electron microscope under different growth phases, and we found that biofilm formation was a dynamic process during which the biofilm was continuously depolymerized and rebuilt(Fig. 2-si, red arrow). A relatively ripe bacterial biofilm[13] was formed after 21

days. After 28 days, an increase inextracellular polymeric substances, incrassation of fibrous protein, integration of bacteria, deep inner porous microstructure, and a netlike distribution were observed. The outline of the bacterial biofilm was a ridge-like three-dimensional structure with inhomogeneous appearance[14]. This kind of spatial structure is conductive to metabolism and information exchange between intramembranous bacteria[15]. Quorum-sensing(QS) has been shown to play an important role in the establishment of a biofilm[16, 17]. The bacteria that we observed to be dissociated from the biofilm and the nests formed by bacterial shedding were consistent with a remaining vesicular biofilm shell after dissociation of floating bacteria[18–19]. Stoodleyet al. reported a clinical case of recurrent infections after elbow joint replacement, demonstrating that the patient's clinical symptoms were consistent with bacterial biofilm depolymerization[20]. It is thought that the process of biofilm formation is as follows: extracellular polymeric substances are gradually formed after thalli and cell membrane maturation untilfinally the entirety has formed a ripe biofilm[21–22]. Early biofilms become ripe biofilms around 21 days. The difference between an early biofilm and a ripe biofilm is that an early biofilm is limited in its change of bacteria, bacterial film, and extracellular polymeric substances. However, a ripe biofilm has a significant extracellular matrix, changes in extracellular polymeric substances, greater stability, and an improved bacterial biofilm microenvironment.

3.2 Effects of PVP-I on Bacterial Biofilms

In this study, living bacteria were found to be dominant in early biofilms, with rare exopolysaccharide(EPS) formation. Small numbers of bacteria formed the biofilms, the bacteria's self-protection was weak, and PVP-I was obviously effective. There was a statistically significant difference between 5 min and 10 min of PVP-I treatment on biofilms grown for21 days($P < 0.05$). This suggested that the osmotic force and sterilizing effects of PVP-I increased with longer treatment time. This is consistent with previous reports[7, 8, 23]and shown in Fig. 1. Even though the extension of bacterial cultivation time decreased the efficacy of PVP-I, it was still more effective after 10 min of treatment compared with 5 min[10]. Comparing the efficacy of 10 min of PVP-I treatment, we found a statistically significant difference between biofilms grown for 7 versus 21 days, 14 versus 21 days, 7 versus 28 days, and 14 versus 28 days($P < 0.05$). The greater efficacy of prolonged incubation time suggests increased formation of bacterial extracellular mucous and thickened biofilm ($P < 0.05$)[7, 8].Changes in microenvironment and bacterial membrane phenotype, and biofilm maturation weaken the ability of PVP-I to infiltrate the biofilm. The formation of spore-like features also decreased the PVP-I efficacy[5].Compared with biofilms grown for 7 days and treated with PVP-I for 10 min, biofilms grown for 14 days had intramembranous bacteria protection, and the intramembranous bacterial survival rate increased. We found that biofilms grown for 21 days had the maximum protection from PVP-I treatment. In 21-day old biofilms, the PVP-I treatment duration is key for its efficacy. After 28 days of growth, the biofilm is mature and the intramembranous bacterial survival rate begins to decline. From this point, the amount of intramembranous bacterial is relatively stable. There was no statistically significant difference between treating the 28-day old biofilms for 5 min versus 10 min. This suggests that an intrinsic biofilm factor is dominant and the destructive effects of external factors is relatively small. Thus, we speculate that PVP-I treatment duration is key. In the early stages of biofilm formation, bacteria can produce a large number of neutral lipids, which can cause increased resistance to drugsand bactericidal substances[24].PVP-I treatment for 10 min can kill biofilm bacteria; however, PVP-I cannot simply kill the bacteria in ripe biofilms.

4. Conclusion

Biofilm maturation occurs sequentially from the inside cell membranes to the outside cell membranes, untilan organized, structured, and specialized microenvironment and social system is formed. From our experimental results, we can speculate that early biofilms become ripe biofilms after 21 days. The major difference is the formation of extracellular polymeric substances and microenvironment maturation. Treatment of early biofilms with PVP-I for 10 min is more effective than treatment for 5 min, but this difference is not observed with ripe biofilm, indicating that earlier treatment is better for implant-related infections. Further, treatment with PVP-I for 10 min is recommended.

5. Limitations

This research was performedin vitro, which differs greatly from the in vivo environment. Therefore, the biofilms formed in these two environments may differ. Further, clinical, wild bacteria are different from the bacteria used here, and there are often mixed infections of multiple strains of bacteria in clinical cases. Finally, various bacteria have been found in clinical prosthetic joint infections, and the ability of each species to form biofilms is different.

Abbreviations

BBF
bacterial biofilm
CFU
colony forming unit

CLSM
confocal laser scanning microscopy
EPS
exopolysaccharide
FITC-ConA
fluorescein isothiocyanate-conjugated concanavalin-a
PVP-I
povidone iodine
PI
propidium iodide
PBS
phosphate-buffered saline
S.aureus
staphylococus aureus
SYTO-9
green fluorescent nucleic acid
SEM
scanning electron microscopy
TSB
trypticsoy broth

Declarations

Ethics approval and consent to participate

This study was a prospective and clinical study and was approved by the Xiamen University Institutional Review Board.

Consent for publication

All authors are in agreement with the content of the manuscript and have approved the manuscript for submission.

Competing interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Authors' contributions

MC contributed to the writing of the paper, data analysis, collection. ZC contributed to the writing of the paper and data analysis. QW contributed to the data collection. LD contributed in searching the related articles and data and collection. WZ contributed to searching the related articles and data analysis. All authors have contributed significantly and are in agreement with the content of the manuscript.

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Authors' information

1 Department of Orthopaedics, the 909th Hospital of PLA, the Affiliated Southeast Hospital of Xiamen University, Orthopaedic Center of People's Liberation Army, No. 279 Zhanghua Road, Zhangzhou 363000, Fujian, People's Republic of China

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Tables

Table 1 Statistical Results of 0.5% PVP-I Action (0min, 5min, 10min) on Four Groups of Superficial Bacterial Biofilm of Titanium Alloy with Different Incubation Time Md（p_{25}，p_{75}）

Groups	Incubation Time of Staphylococcus Aureus（Days）			
	1[a] (7day)	2[b] (14day)	3[c] (21day)	4[d] (28day)
PBS G[@]	110000(97500,140000) 27182.51	75000(50000,110000)	60000(17500,167500)	50000(37500,92500)
5min G[#]	0(0,0)	0(0,0)	300(275,425)	500(375,700)
10min G[*]	0(0,0)	0(0,0)	100(100,125)	250(175,400)

1.$^{\#a}p<0.05$vs$^{@a}p$, $^{*a}p<0.05$vs$^{@a}p$; $^{\#b}p<0.05$vs$^{@b}p$, $^{*b}p<0.05$vs$^{@b}p$; $^{\#c}p<0.05$vs$^{@c}p$, $^{*c}p<0.05$vs$^{@c}p$, $^{*c}p<0.05$vs$^{\#c}p$; $^{\#d}p<0.05$vs$^{@d}p$, $^{*d}p<0.05$vs$^{@d}p$;

2. $^{\#a}p<0.05$vs$^{\#c}p$, $^{\#a}p<0.05$vs$^{\#d}p$, $^{\#b}p<0.05$vs$^{\#c}p$, $^{\#b}p<0.05$vs$^{\#d}p$; $^{*a}p<0.05$vs$^{*c}p$, $^{*a}p<0.05$vs$^{*d}p$, $^{*b}p<0.05$vs$^{*c}p$, $^{*b}p<0.05$vs$^{*d}p$.

Table 2 Kruskal-Wallis H Rank-sum Test Results

Statistical Results	7days	14days	21days	28days	PBS G	5min G	10min G
X^2	27.514	26.064	25.753	23.510	7.070	28.410	34.296
P	<0.05	<0.05	<0.05	<0.05	0.070	<0.05	<0.05

Table 3 Kruskal-Wallis H Rank-sum Test Results of Superficial Bacterial Biofilm of Titanium Alloy with the Third Week Incubation Time

Statistical Results	5min G vs PBS G	10min G vs PBS G	10min G vs 5min G
Z	2.614	5.074	2.460
p	0.027	<0.05	0.042

Table 4 Gray Scale Ratio (G/R) of Green Fluorescence and Red Fluorescence of 4 Groups of Superficial Bacterial Biofilm of Titanium Alloy with Different Incubation Time

Incubation (Days)	Groups			
	PBS G	5min G	10min G	BBF G
7	1.028	0.942	0.905	0.907
14	1.090	0.998	0.918	0.993
21	1.120	0.995	0.960	1.044
28	1.125	1.072	1.026	1.369

Note: Gray Scale Ratio (G/R) just suggests the tendency of survival rate

Figures

Figure 1

.CLSM images of ACTT25923 after being cultured for different amounts of time(×200).Panels labeled a, c, and d were stained with SYTO9 (green, live bacteria stain) and PI(red, dead bacteria stain).Panels labeled ‘b’ were stained with FITC-ConA (green, biofilm stain) and PI (red).

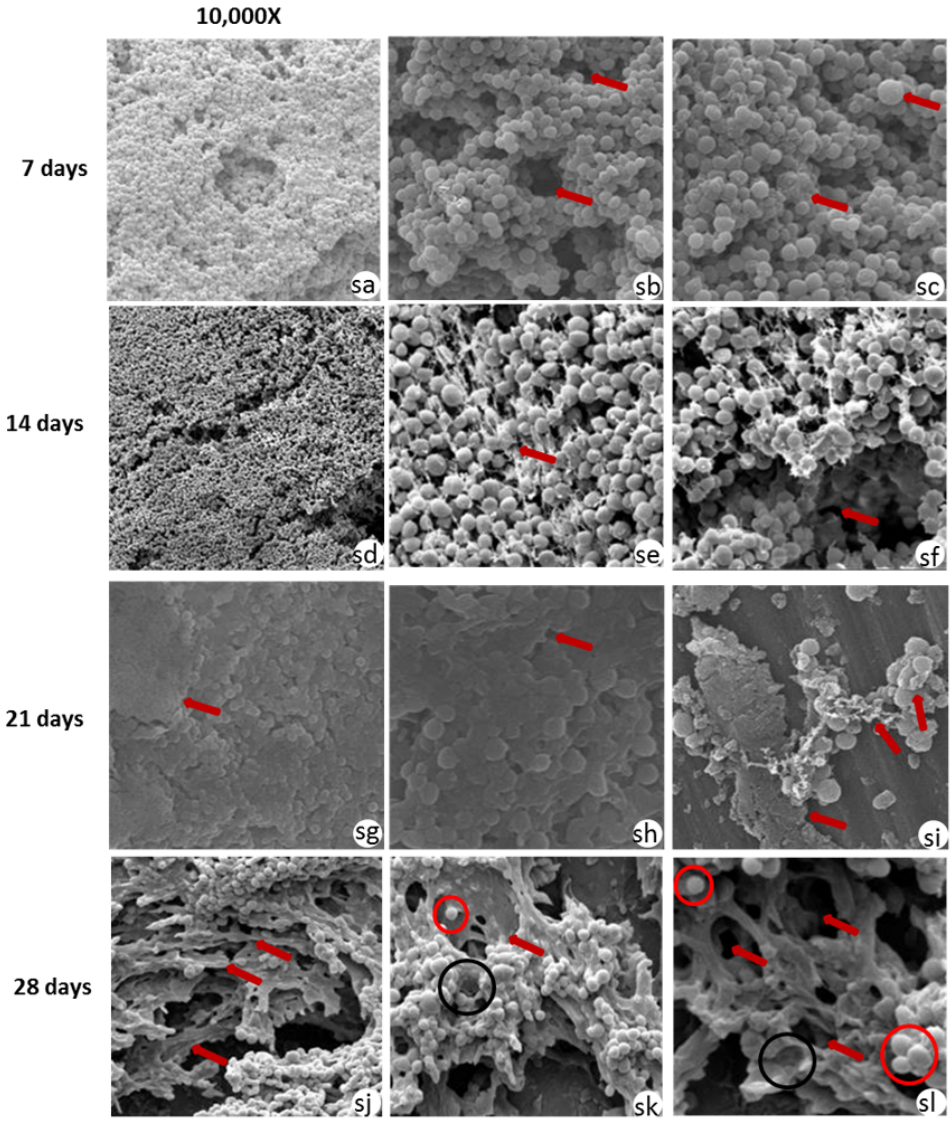

Figure 2

SEM images of ACTT25923 after being cultured for different amounts of time (sb, sc: 20,000×, se, sf: 25,299×,sh:10,000×,sj,sk:14,000×, sl:25,642×). Red arrows indicate the following: sb, pores of uneven sizes; sc, budding protuberance; se, filamentous fibers; sf, deep voids; sg, cells fused into blocks; sh,channels between the biofilm; si,different stages of biofilm; sj, the deep layers of biofilm are of distinct gradation; sk, extracellular matrix; sl, inner biofilm channels. Red circles indicated epolymerized free bacteria. Black circles indicate the cavity left by the depolymerized and dissociated bacteria.

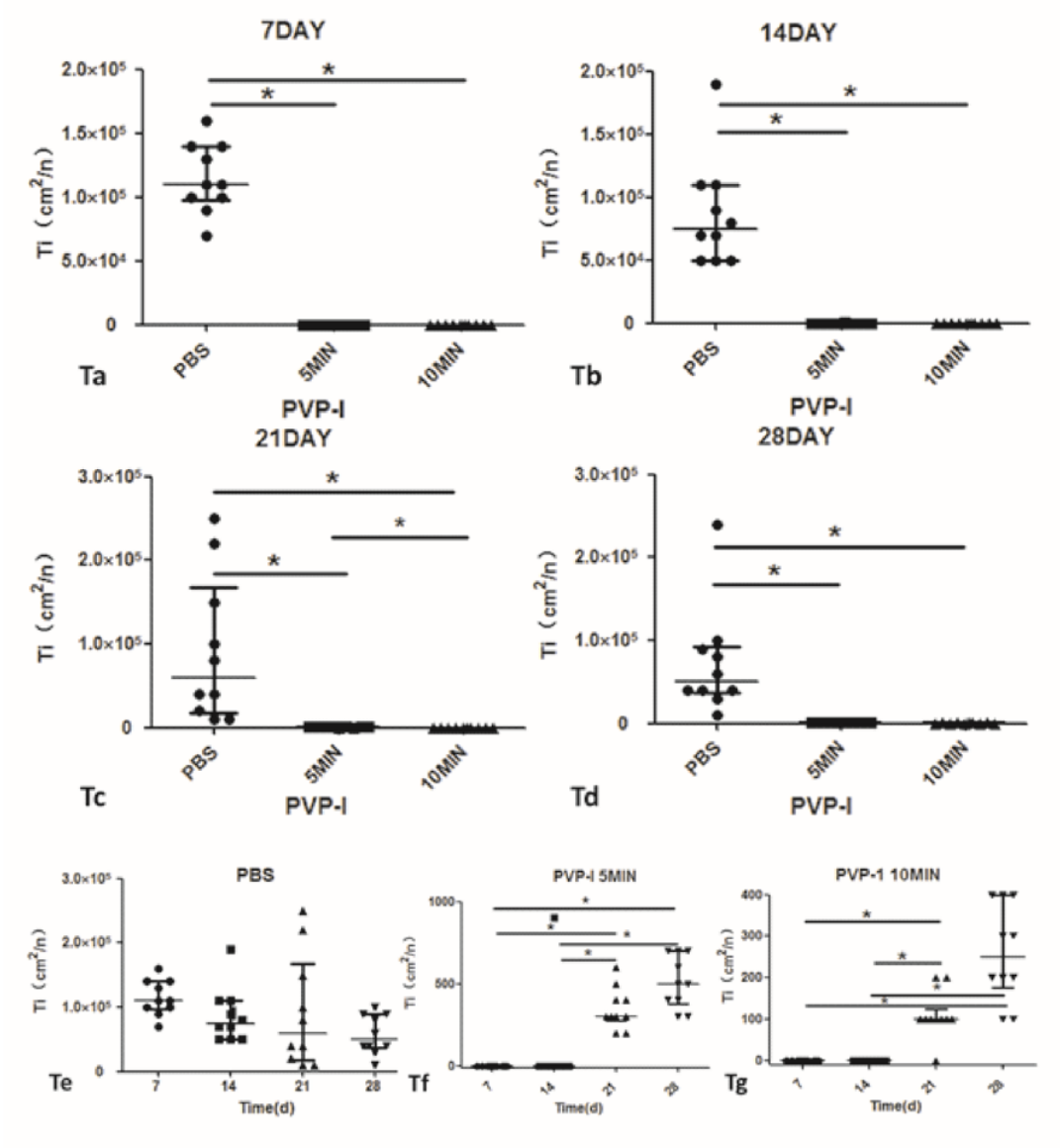

Figure 3

Panels a-dcomp are the CFU results from biofilms grown for 7, 14, 21, or 28 days after treatment with PBS, PVP-I for5min, and PVP-I for 10min. Panels e-g compare the CFU results after treatment with PBS, PVP-I for 5 min, or PVP-I for 10 min across biofilms at different growth stages. (* $p<0.05$).

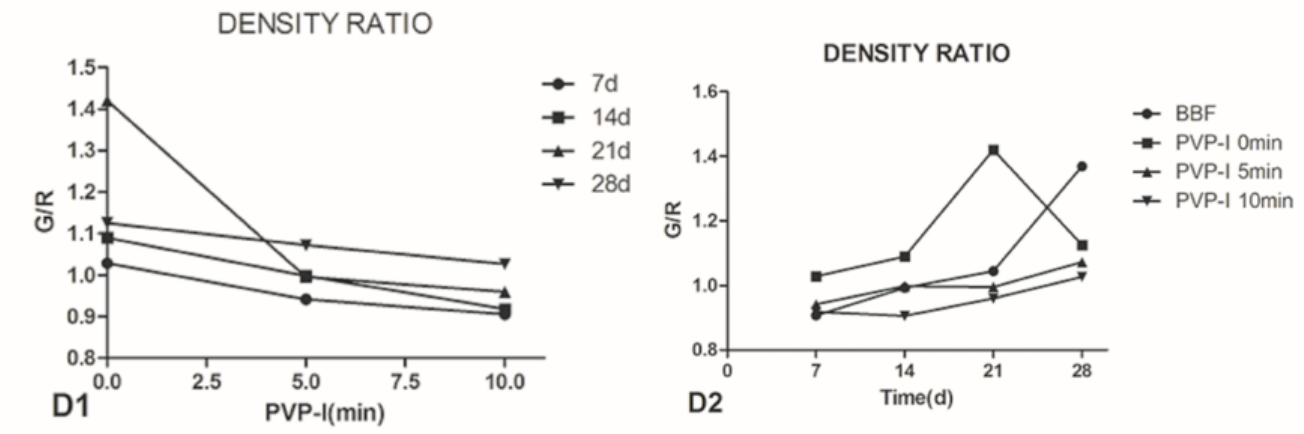

Figure 4

.1) The gray scale ratio of green and red fluorescence signals (G/R) from CLSM images from the BBF group, the PBS group, the PVP-I 5 min treatment group, and the PVP-I 10 min treatment group after different treatment times. 2) The gray scale ratio of green and red fluorescence signals (G/R) from CLSM images of the different treatment groups after different amounts of biofilm cultivation time.