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Biofilm inactivation by synergistic treatment of atmospheric pressure plasma and chelating agents

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ABSTRACT

In this study, we investigated the bacterial biofilm reduction by combined treatment of atmospheric pressure plasma (APP) and chelating agents. Many of hospital acquired infections (HAI) are related to biofilm infections. APP and chelating agents were reported to be effective in bacterial biofilm inactivation and eradication. It is believed that chelating agents disrupt the biofilm formation through bonding metal ions, while APP inactivates bacteria mainly through reactive species [1–3]. In our study, we used a surface micro-discharge (SMD) driven by sinusoidal power input of 2 kHz and peak to peak voltage of 9 kV to treat *Escherichia coli* (*E. coli*), *Enterococcus faecalis* (*E. faecalis*), and *Staphylococcus capitis* (*S. capitis*) biofilm on 316 L stainless steel and hopkins rod lens glass plates in combination with chelating agents of trisodium citrate (TSC), ethylenediaminetetraacetic acid (EDTA), egtazic acid (EGTA), and alizarin. Bacterial biofilm reduction was measured by means of colony count assay and BacTiter-Glo cell viability assay. The results of colony count assay showed that combined treatment of EDTA and TSC with plasma has synergistic effects on all three bacterial biofilms, while EGTA only on *E. coli* and none for alizarin. Experiments of BacTiter-Glo cell viability assay indicate that EDTA, EGTA, and TSC has synergistic effects on all three biofilms while also none for alizarin.

1. Introduction

APP have provided a new potential strategy for disinfection of medical devices such as medical endoscopes [1,4,5]. Compared to conventional moisture heat sterilization such as autoclaving and chemical sterilization methods, APP treatment provides disinfection with relatively high efficiency, low gas temperature, and low cytotoxicity [2,5,6]. APP are partially ionized gases which consist of neutral gas atoms, neutral molecules, ions and electrons. For plasma inactivation, it is generally believed that reactive species such as O_3 or NO, which are produced by plasma induced chemical reactions in ambient air, play a dominant role [2].

It is noteworthy that the inactivation efficiency by plasma also strongly depends on the bacterial state, for instance planktonic bacteria and biofilms. Previous studies demonstrated that inactivation of bacterial biofilms require much longer exposure time to plasma compared to planktonic bacteria [7–9]. Xu et al. [10] compared the sterilization efficiency of planktonic *Neisseria gonorrhoeae* and biofilms, which requires 12 and 20 min for sterilization, respectively. Bacterial biofilms are bacteria attached on surfaces and embedded in an extracellular matrix, and are considered to have much higher tolerance to environmental stress [11].

As it is estimated among all microbial and chronic infections, 65% and 80%, respectively, are associated with biofilm formation [12–14]. Furthermore, around 1 million cases of hospital acquired infections are related to infection of medical implants in the U.S. each year [15,16]. Accordingly, our motivation is to test the possibility to enhance biofilm inactivation efficiency by investigating the combination treatment of APP and chelating agents. Chelating agents, chelates, or chelators are compounds that bond with metal ions. Earlier studies reported that chelating agents, such as TSC, EDTA, EGTA, and alizarin, could effectively prohibit the formation of biofilms [3,17–19]. It was reported that combination of EDTA and gentamicin results in complete killing of *P. aeruginosa* biofilm while less than 2 $\log_{10}$ inactivation for gentamicin single treatment [3]. It has also been shown that 30% TSC could effectively prevent biofilm formation in catheter compared to conventional catheter lock solution, heparin [17,19]. In general, it is supposed that chelates prevent or disrupt biofilm formation by bonding calcium or magnesium ions [20,21]. Therefore, in order to enhance biofilm inactivation efficacy, in this study, we investigated the possible synergistic effects of APPs and chelating agents on bacterial biofilms.

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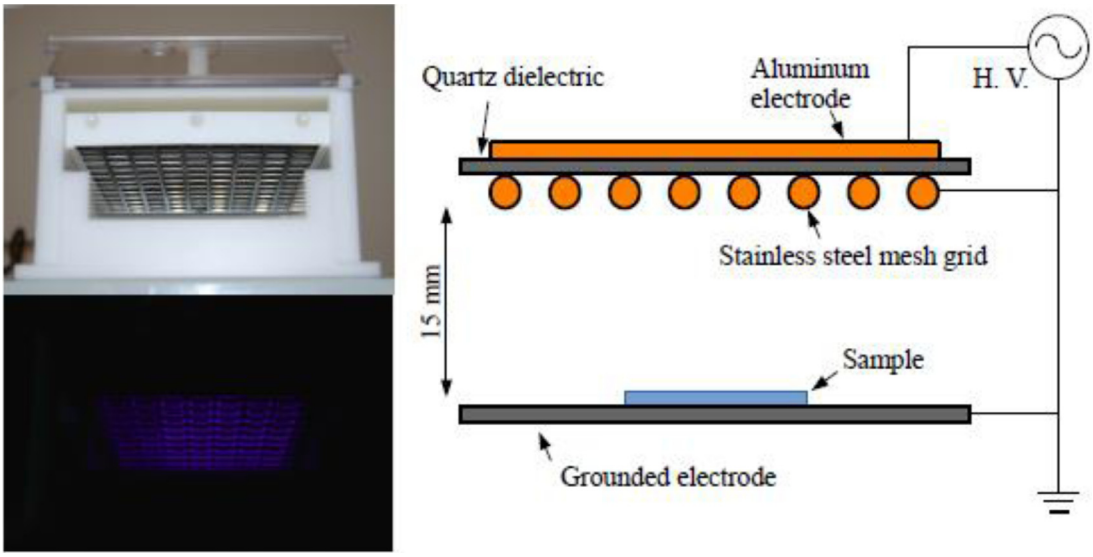

Fig. 1. (Left) Typical image of the SMD and (Right) Experimental schematic of the SMD. The three-layer discharge which consists of aluminum electrode, quartz dielectric and grounded stainless steel mesh were 15 mm above the samples.

2. Materials and methods

2.1. Surface micro-discharge (SMD)

The plasma source used in this study is a surface micro-discharge (SMD) based on the design from Max Planck Institute for Extraterrestrial Physics (MPE) which has a dielectric barrier discharge (DBD)-based three-layer electrode configuration (Fig. 1) [22]. The high voltage aluminum top layer and the grounded stainless steel mesh were separated by a 1.0 mm quartz plate as the dielectric. The mesh has a lattice width of 2.2 mm and a lattice distance of 10 mm. The electrodes were placed in the treatment chamber with a grip for vertical adjustment. The plasma was driven by 2 kHz and a peak to peak voltage of 9 kV sinusoidal high voltage power input by a function generator (HM8150, HAMEG Instruments GmbH) and an amplifier (10/40A-HS, TREK Inc.) throughout the study. The treatment distance was fixed at 15 mm and all treatments were completed under room conditions.

2.2. Biofilm growth and sample preparation

The bacteria strains applied in this study are Gram-negative *E. coli* K12 (EDCC 2009), a common model bacterium, and Gram-positive *E. faecalis*, (Symbioflor 1 DSM 16431 [23]), which is frequently found in endodontic infection, and *S. capitis* (EDCC 5473), which is common in infection of medical devices such as catheters [24–26]. Stationary phase *E. coli* and *S. capitis* were stored on Luria–Bertani (LB) (Carl Roth) agar dishes at 4 °C, while *E. faecalis* on Brain Heart Infusion (BHI) (Carl Roth) agar. We prepared biofilm on sample plates with a modified microtiter plate biofilm assay as described in earlier studies [27]. Briefly, the biofilms were either grown on 316 L stainless steel plates (10 × 10 × 1 mm) (AISI 316, Goodfellow GmbH) or Hopkins rod lens glass plates (D10 × 1 mm) (Fuzhou WTS Photonics Technology CO.) to imitate the materials of a medical endoscopes for possible applications. *E. coli* and *E. faecalis* were first incubated in LB broth at 37 °C until reaching the concentration of approximately 10^6 CFU/ml, while *S. capitis* was grown in BHI broth at 37 °C until 10^6 CFU/ml. The bacteria were incubated for 2 to 5 h and the concentration was estimated by measuring the optical density (OD) of 600 nm with a cell density meter (Ultrospec 10, Biochrom). Sample plates were covered with 100 µl of the 10^6 CFU/ml bacteria broth and incubated for 48 h at 37 °C. For incubation, each bacteria covered sample plate was placed in a D50 × 5 mm sterile glass bowl, and each glass bowl was then placed in 90 mm sterile petri dish containing 6 ml of distilled water in order to maintain a humid environment and prevent vaporization of the bacteria medium. After 48 h of incubation in an incubator (TIN-IN35, Phoenix Instuments), the sample plates were gently washed with distilled water to remove suspended planktonic bacteria and dried for 20 min. The samples immediately underwent different treatment which are described in details in the following sections. After all treatments, the sample plates were transferred into 10 ml glass vials containing 1 ml distilled water. The vials were ultrasonicated (RK100H, Bandelin Sonorex) for 40 min and vortexed for 1 min to detach the biofilm from the samples. The 1 ml sample suspensions were either examined by colony count assay or bacteria viability assay. Colony count assay was performed by serially diluting 100 µl of the sample suspension into 900 µl of distilled water in 1000 µl Eppendorf tubes. The dilution procedure was repeated until appropriate concentration. After vortexing the Eppendorf tubes, 100 µl of the suspension was cultured after plating on agar dishes and incubated for 16 h. We counted the CFU on agar dishes which the CFUs were in the range of 30 to 300 and recalculated the original concentration of the 1 ml sample solution. The viability assay will be descripted in Section 2.5.

2.3. Combined treatment of chelating agents followed by plasma

Inactivation effects of sole chelates and combined treatment of chelates followed by plasma (chelates + APP) were tested on all three bacterial biofilms and on both stainless steel and glass plates. *E. coli, E. faecalis* and *S. capitis* biofilm covered sample plates were immersed for 15 min into 10% (w/v) of TSC (Sigma-Aldrich), EDTA (Sigma-Aldrich), EGTA (EMD Millipore Corporation), alizarin (Sigma-Aldrich), and phosphate buffered saline (PBS, Carl Roth GmbH) as negative control. Afterwards, the sample plates were gently washed by distilled water and dried for 20 min. For chelates single treatment, samples were then immediately sonicated and vortexed as described above for colony count and viability assay, while for chelates + APP, the samples were then treated by plasma for 5 min followed by sonication, vortexing, and colony count assay or cell viability assay.

In addition, inactivation tests of different TSC concentrations with and without plasma were also applied. Stainless steel samples covered with *E. coli* biofilm were tested with 0%, 1%, 2%, 5%, 7.5%, 10%, and 15% of TSC followed by 5 min of plasma. Single treatment of the above mentioned TSC concentrations were also experimented. Both tests follow the same procedure of sole chelate and chelates + APP

treatment as described in the previous paragraph.

2.4. Treatment of different orders

Influence of treatment order was experimented on *E. coli* biofilm samples with 10% of chelating agents and APP on stainless steel plates. For chelates + APP treatment, the procedure is the same as described in 2.3, while for plasma followed by chelate treatment (APP + chelates), after removement of planktonic cells and drying, the samples were immediately exposed to plasma for 5 min and immersed in chelating agents for 15 min. Colony count assay were applied after sonication and vortexing.

2.5. Bacteria viability assay

Bacteria viability on stainless steel plates were tested on *E. coli, E. faecalis* and *S. capitis* biofilm samples with the 10% chelates + APP treatment procedure by BacTiter-Glo Microbial Cell Viability Assay (Promega GmbH). BacTiter-Glo Assay measures the viability of cells based on the Adenosine triphosphate (ATP) presented in the solution which indicates the metabolic activity of the cells. The experiments were performed following the assay protocol. After all treatments, sonication, and vortexing, 100 µl of the liquid samples were equilibrated with 100 µl of assay reagent in opaque 96-well plates for 5 min. The luminescence signal generated by the reaction of the luciferase-based reagent and ATP was recorded afterwards by a micro-plate reader (GloMax Discover, Promega GmbH) with the integration time of 0.3 s.

2.6. Statistical analysis

All tests in this study, including viability assay and colony count assay of different bacteria, sample materials, chelates, and treatment orders, were repeated three times with one test sample for each test condition per test run, and the data in the plots are presented as mean ± standard deviation (SD). Students *t*-test were applied by Python SciPy Statistical Functions for analyzing treatment of chelating agents and PBS treatment, between stainless steel and Hopkins rod lens glass plates as well as between treatment with different orders. Significant difference was considered when the *P* value was less than 0.05.

3. Results

3.1. Combination treatment of chelating agents followed by plasma

Comparison of single treatment of chelates and chelates + APP treatment were performed on all three bacterial biofilms (Figs. 2–4). Results showed that all of the single treatments of chelating agents did not cause significant reduction of the biofilm. For chelate + APP treatments, results indicate that, except alizarin and EGTA, higher inactivation efficiency is achieved by the treatment of 10% (w/v) of chelates + APP for all three bacteria strains, while EGTA only on Gram-negative *E. coli* and alizarin for none of the three. By comparing chelates + APP and PBS + APP treatment on *E. coli*, EDTA, EGTA, and TSC showed 1.0, 0.9, and 0.8 $\log_{10}$ higher inactivation, respectively. Furthermore, EDTA + APP and TSC + APP increased 1.2 and 0.7 $\log_{10}$ reduction on *E. faecalis*, and 0.9 and 0.5 $\log_{10}$ on *S. capitis* compared to PBS + APP, while no significant effects were observed on EGTA on both strains. Alizarin + APP is the only treatment with no significant differences compared to PBS + APP on all three bacteria. In addition, statistically, no significant difference was found between stainless steel and glass treatment.

3.2. Effect of TSC concentration

By changing the concentration of TSC, results show that single treatment of TSC with concentration lower than 5% has no significant effects on the bacterial biofilm, while 15% single treatment leads to 1.1 $\log_{10}$ bacteria reduction compared to no treatment (Fig. 5). For combination treatment of TSC and plasma, the most interesting result is that although 5%, 7.5%, and 10% TSC single treatment shows no effect on the biofilm, combined treatment induces 0.9, 1.1, and 1.3 $\log_{10}$ enhancement of inactivation rate, respectively.

3.3. Change of treatment order

By changing treatment order, generally, there were no significant enhancement for all treatment of plasma followed by chelators (APP + chelates) compared to APP + PBS treatment found in *E. coli* biofilms (Fig. 6). By comparing chelates + APP and APP + chelates treatment, no significant difference was found in alizarin treatment, while a P value less than 0.1 was observed in the case of EDTA and EGTA. Significant difference was found in TSC treatment with 0.6 $\log_{10}$ higher inactivation.

3.4. Bacteria viability assay

The recorded luminescence signal of the BacTiter-Glo assay indicates the metabolic activity of the bacteria cells. Significant enhancement of inactivation was observed in all three bacteria strains by EDTA + APP, EGTA + APP, and TSC + APP treatment (Fig. 7). Alizarin were not applied since the strong color interfered with the luminescence measured. Results of EDTA + APP treatment showed decrease of 2.5×10^5, 1.4×10^5, and 2.1×10^5 RLU on *E. coli, E. faecalis* and *S. capitis* biofilm compared to PBS + APP, while 1.8×10^5, 0.8×10^5, and 0.8×10^5 RLU for EGTA + APP treatment and 1.9×10^5, 1.3×10^5, and 1.4×10^5 RLU for TSC + APP treatment. In average, EDTA + APP treatment has the highest efficiency followed by TSC and EGTA.

4. Discussion

The aim of this study is to investigate the possible synergistic effects of chelating agents and APP treatment on bacterial biofilm. Combination of chemical solutions and APP has been shown to have synergistic enhancement of inactivation in previous studies. Gupta et al. [28] combined chlorhexidine (CHX) with non-thermal plasma and showed increase of killing *Pseudomonas aeruginosa* biofilm. Koban et al. [29] also found synergistic effects of APP and disinfecting agents in dentistry on dental biofilms. On the other hand, synergistic enhancement of chelating agents and antimicrobials were also observed in other studies. By combining NaOCl and EDTA, Soares et al. [24] believes that EDTA could disrupt biofilm structure and enhance penetration of NaOCl. Sherertz et al. [30] reported the significant reduction of colonization of *Staphylococcus epidermidis, Staphylococcus aureus*, and *Candida albicans* by combining treatment of EDTA and minocycline.

In our results of colony count assay, the synergistic effects were found in EDTA + APP, TSC + APP, EGTA + APP treatments. EDTA + APP and TSC + APP showed significant effects on all three biofilms, while EGTA only on *E. coli*. EDTA has been widely applied for application such as DNA extraction and is one of the most used chelating agents. Compared to EDTA, EGTA has weaker affinity for magnesium and is referred to as calcium specific chelate [31]. Earlier studies mentioned that both EDTA and EGTA causes strong detachment of biofilms, yet EDTA has a stronger inactivation due to chelating magnesium associated with the lipopolysaccharide [3,32,33]. Mulcahy et al. [34] showed that low concentration of EDTA could promote biofilm formation due to limitation of magnesium ions, yet they also stated that concentration higher than 1 mM could cause lethal effects. In our study, sole treatment of EDTA did not result in inactivation although we used a relative higher concentration of 10%. One should also keep in mind that we treated 2-days biofilm for a relative short time of

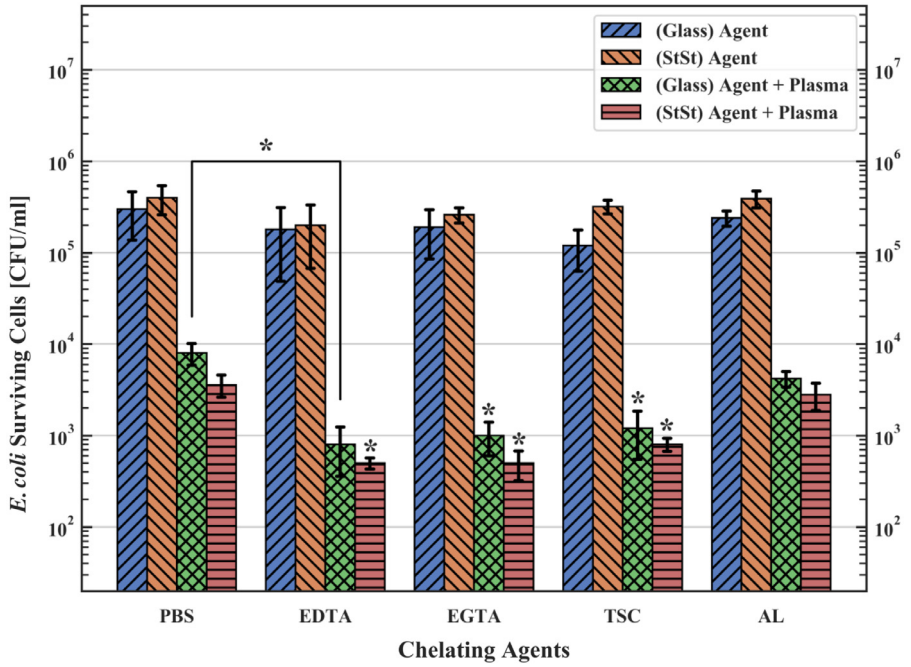

Fig. 2. Combined treatment of 15 min of 10% chelating agents followed by 5 min of plasma on *E. coli* biofilm on 316 L stainless steel plates (StSt) and Hopkins rod lens glass plates (Glass). *$P < 0.05$ versus PBS within the same colored columns.

15 min which is possibly not sufficient for significant inactivation. We assume that the higher inactivation efficiency of EDTA + APP compared to EGTA + APP is possibly caused by the disruption (but not inactivation) of cell membrane or biofilm integrity by the high concentration of EDTA [35]. Moreover, both TSC and alizarin have relative low cytotoxicity compared to EDTA and EGTA, while TSC are common food additives and are already applied for hemodialysis catheter locking [18,19,36,37]. The killing efficiency of TSC + APP are lower than EDTA + APP, especially for *E. faecalis* and *S. capitis* which both are around 0.4 to 0.5 $\log_{10}$ lower. Shanks et al. [38] demonstrated that similar to EDTA, concentration of sodium citrate lower than 0.5% could promote biofilm formation while higher than 0.5% could strongly inhibit *staphylococcal* biofilm formation. In the same report it was also shown that higher concentration of citrate is required to inhibit coagulase-negative *staphylococci* (CNS) biofilms. This is in agreement with our results, as the inactivation of *S. capitis*, which is a CNS, is less effective compared to the other two strains for TSC + APP treatment. For alizarin, earlier studies mentioned that alizarin could increase cell aggregation but decrease biofilm formation, and though higher concentration but the relative low treatment time in our study could be the cause of lower inactivation efficiency [18].

No significant difference between treatment on stainless steel and hopkins rod lens were found. We choosing these two materials initially, as we assumed that surface properties, which could affect the attachment and detachment of bacteria, could potentially influence the experimental results. Rod lenses have lower hydrophobicity and surface

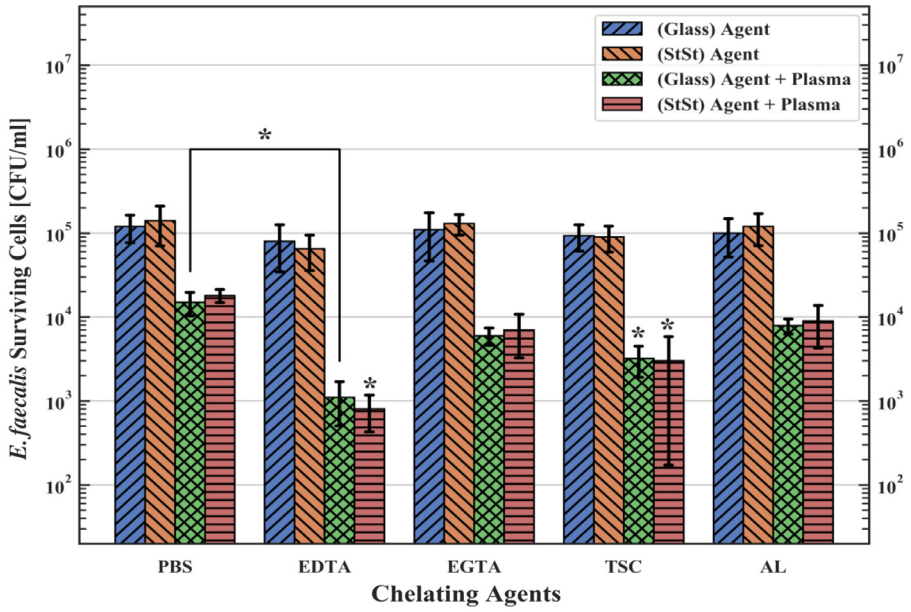

Fig. 3. Combined treatment of 15 min of 10% chelating agents followed by 5 min of plasma on *E. faecalis* biofilm on 316 L stainless steel plates (StSt) and Hopkins rod lens glass plates (Glass). * $P < 0.05$ versus PBS within the same colored columns.

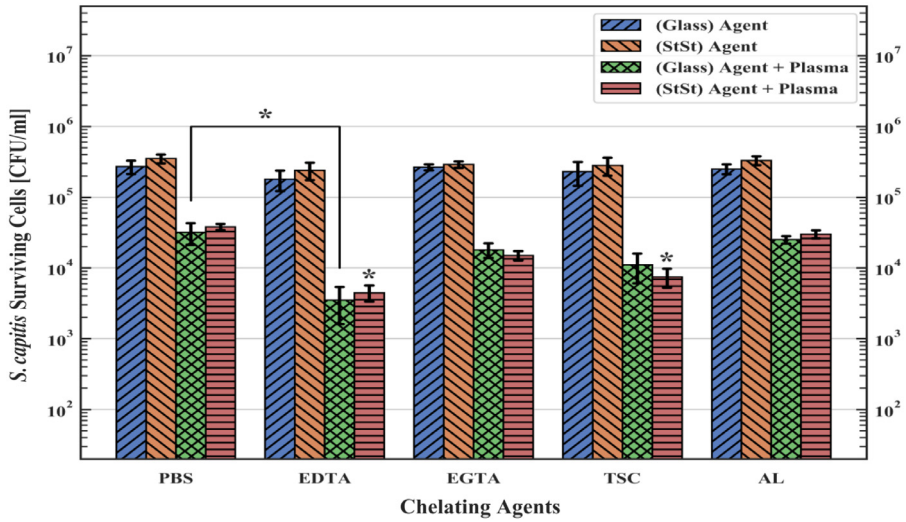

Fig. 4. Combined treatment of 15 min of 10% chelating agents followed by 5 min of plasma on *S. capitis* biofilm on 316 L stainless steel plates (StSt) and Hopkins rod lens glass plates (Glass). *$P < 0.05$ versus PBS within the same colored columns.

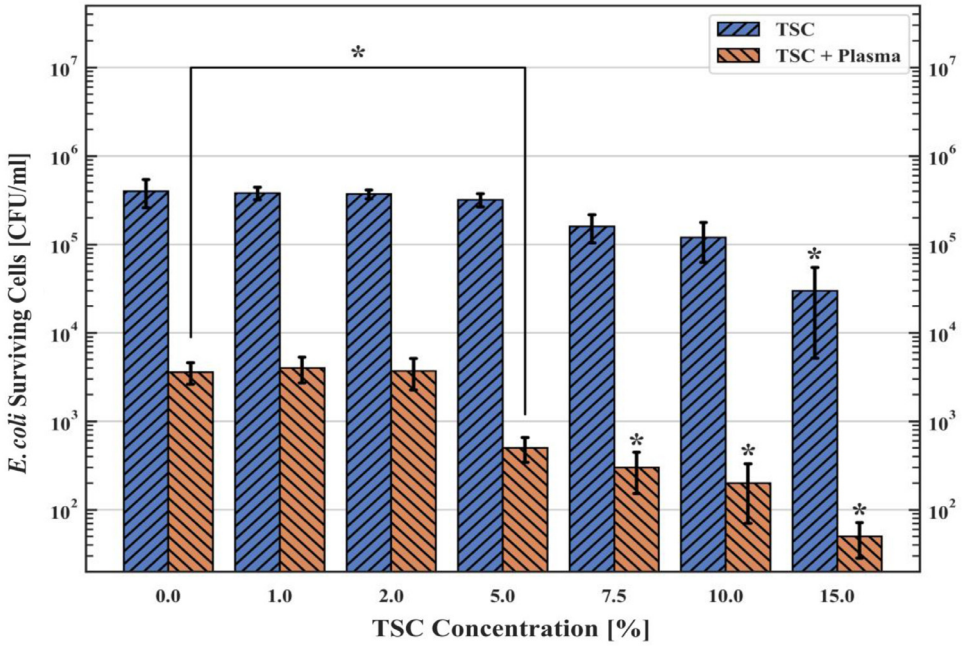

Fig. 5. Combined treatment of different concentration of TSC and plasma on *E. coli* biofilm on stainless steel plates. *$P < 0.05$ versus control samples within the same colored columns.

roughness compared to stainless steel [39]. As no clear difference was found, we believe that surface properties play a minor role in our inactivation process. Furthermore, it is also worth noting that the washing and drying process between chelating agents and plasma treatment were rather important and necessary. We assume the reasons are that the chelating agents applied in our study were weak alkalis and could impact the acidification caused by plasma, which were believed to be important in plasma inactivation of microbial, and eventually weaken the inactivation effects [40]. In addition, it is possible that the washing procedure could wash off detached bacteria especially after treatment of chelates. However, after culturing the distilled water which was applied in the washing process, no significant difference was found compared to the PBS samples (data not shown).

Treatment order affects the results which are in agreement with earlier studies using combined treatment of APP with other methods. Koban et al. [29] treated dental biofilms by combining kINPen 09 with dental disinfectants including EDTA. Their results were in agreement with ours as higher reduction were found in the EDTA + APP treatment compared to APP + EDTA. However, they also discovered reverse outcome for disinfectant such as hydrogen peroxide which they speculated that it is also possible that plasma destroyed the biofilm matrix and increase the effectiveness of antiseptic. Gupta et al. [28] also found a similar result as inactivation were much stronger when plasma was treated prior to disinfectant, chlorhexidine digluconate (CHX). They believe that APP disrupting the biofilm could enhance inactivation effects of CHX. In our case, we believe the chelating agents strongly affected the biofilm integrity and hence improved the penetration of APP to the bacteria cells in the extracellular matrix in the chelates + APP treatments. Compared to above mentioned studies, chelates are relatively weak in inactivation, which is also shown in our results of chelate single treatment. This hypothesis was also mentioned in the EDTA + APP results of [29]. In addition, one should also notice

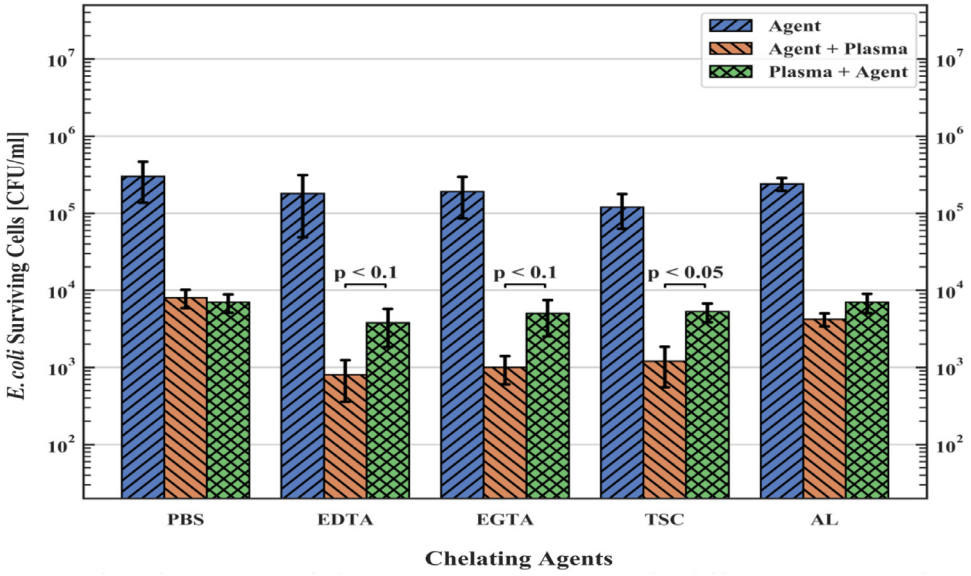

Fig. 6. Combined treatment of plasma and chelating agents by different treating order on *E. coli* biofilm on stainless steel plates.

that the plasma device used in [29] and [28] were DBD plasma jets driven by argon and helium gas, respectively. Both long and short living species, e.g. ozone and hydroxyl radicals, should be considered in their cases due to the direct contact of plasma afterglow with the samples, while, in our case, the dominant bactericidal species are long living reactive oxygen species (ROS), particularly ozone, according to previous reports [40,41].

In summary, since APP + chelates results in a less or no inactivation efficiency, we believe the synergistic effects of APP + chelates could begin with a disruption of biofilm integrity by the chelation of metal ions, which could then enhance the penetration of APP to the bacteria embedded in the extracellular matrix. Our results could be an idea for new treatment against hospital acquired infection related to bacterial biofilms. Though no disinfection or sterilization achieved, the synergistic effects of chelates and plasma could create new sterilization strategies. For instance, as TSC is already applied in catheter lock solutions and APP in sterilization treatment, combination of plasma treatment could potentially enhance efficiency of inactivation. Another possible treatment is to combine APP and TSC on wound cleaning. However, more studies are required as the whole procedure, especially the washing procedure, would be difficult to apply in current practical application and since we used in vitro 48 h monospecies biofilms, it is also not directly comparable with mature multispecies biofilms in natural habitats. Therefore, transferability of our results to other bacterial species and substances, e.g. human tissues, should also be considered carefully.

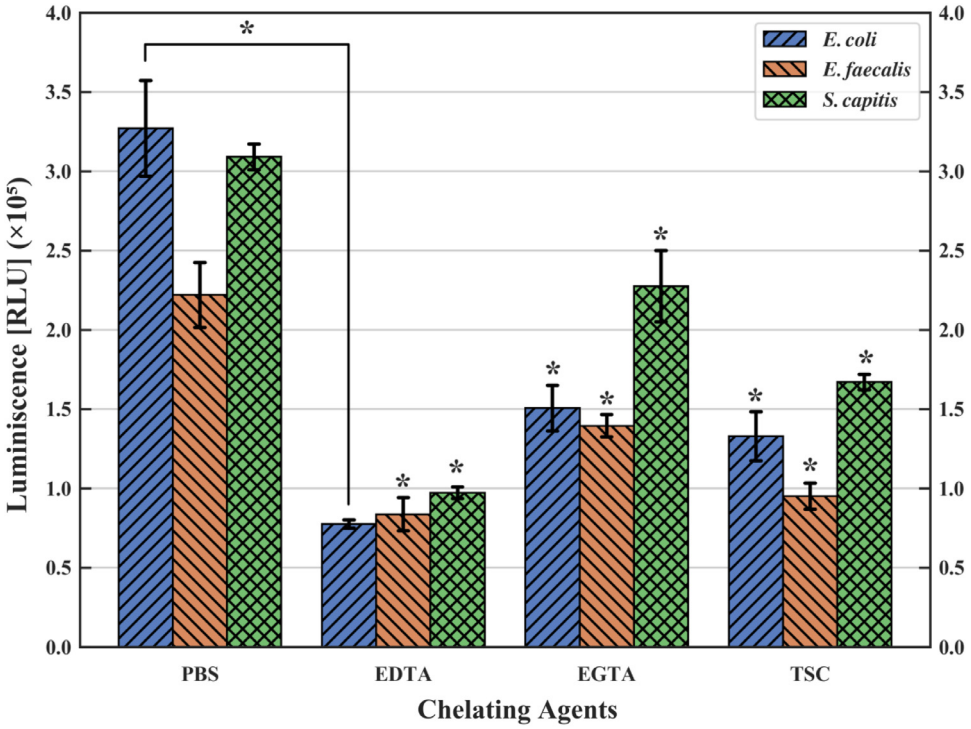

Fig. 7. Results of combined treatment (chelates + APP) by BacTiter-Glo cell viability assay. *$P < 0.05$ versus PBS within the same colored columns.

5. Conclusion

In this study, we demonstrated that combined treatment of chelating agents of EDTA, TSC, and EGTA with APP could enhance inactivation effects of bacterial biofilm compared to PBS + APP treatment. Results of colony count assay showed that EDTA + APP and TSC + APP have synergistic effects on *E. coli, E. faecalis*, and *S. capitis* biofilm, while EGTA + APP only on *E. coli*. Furthermore, we found that treatment orders are rather important, as chelating agents followed by plasma treatment shows less surviving bacteria. Last but not least, low concentration (< 5%) of TSC did not present any significant effects on biofilms of *E. coli*, while interestingly, combination of 5%, 7.5%, and 10% TSC and plasma treatment appears to have around 1 $\log_{10}$ higher reduction rate compared to sole TSC treatments. Considering the relative low cytotoxicity of TSC, we believe combined treatment of TSC and plasma could be a potential strategy for biofilm inactivation on medical devices such as medical endoscopes yet more studies are required for practical application.

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Conflict of interest

None.

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