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Research Article

Bacterial Biofilms and Ethylenediamine-N,N'-disuccinic acid (EDDS) as Potential Biofilm Inhibitory compound

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Abstract: Bacterial biofilms are populations of microorganisms that stick to some surfaces and are embedded in self-secreted extracellular polymeric matrixes. These synergistically controlled sessile cells exhibit different proteome than their planktonic counterpart and are often difficult to treat. They make a different mechanism to escape the antimicrobial activity and host defenses. Biofilms on indwelling prosthetic devices are a major problem in the medical field because they could lead to recurrent and systemic infections. Biofilms also result in bio-corrosion in industries and their remediation often causes environmental problems. The goal of this study is to find antimicrobial and biofilm inhibition activity of non-toxic and biodegradable economical iron-chelating compound EDDS against *Staphylococcus aureus* SA113 and *Pseudomonas aeruginosa* PAO1 strains. Broth microdilution assay was performed to find the inhibitory concentration of EDDS and biofilm assay performed in the micro-well plate to determine the biofilm inhibitory concentration of EDDS against SA113 and PAO1. The results show that a higher concentration of EDDS is required to suppress the growth of SA113 a gram-positive bacterium compare to gram-negative PAO1 strain. EDDS successfully inhibited the biofilm formation of both SA113 and PAO1 at very low concentration and significant biofilm inhibition ($p<0.05$) was observed at 0.78 mM and 1.56 mM EDDS concentration for PAO1 and SA113 respectively. Biodegradable ion-chelator EDDS is a good biofilm inhibitor compound for both *S. aureus* SA113 and *P. aeruginosa* PAO1 strains.

Keywords: Biofilm inhibition, EDDS, ion chelation, *Staphylococcus aureus*, *Pseudomonas aeruginosa*.

1. INTRODUCTION

Metabolic co-operation and self-association of cells could be helpful to sustain the communal survival in otherwise single, hostile, and hazardous conditions. Many microbial cells like bacteria adopt this lifestyle using biological phenomenon, called biofilm formation. Biofilms are a well-organized association of microscopic cells in which cells are linked to each other in the self secreted polymeric matrix on solid inert or living surfaces [1]. This phenomenon was first noticed by Anton Von Leeuwenhoek and described in 1978 [2, 3]. Cells in biofilm exhibit an altered phenotype compared to planktonic cells [4].

Biofilms are formed to protect the bacteria from the host cell immunity, antibacterial agents, and harsh environmental conditions [4, 5]. It is known that biofilm's tolerance to antimicrobials and escapes from the immune system is achieved by low metabolic rate, persister cells, and expression of specific genes for antimicrobial resistance produced by the cells [6]. Consequently, the bacterial cells at high cell density in biofilms show enhanced resistance to antimicrobial agents and account for ~80% of the infection particularly in the hospital environment and health care units [5-8]. In nature, biofilms can either be formed by the synergistic association of different microbial species or similar type of bacterial strain [1].

Adherence to the solid substratum is a prerequisite to making the clump or aggregates of microbial cells which activates the expression of several genes within minutes like *algC* gene in *P. aeruginosa*, responsible for the synthesis of alginate (exopolysaccharide) necessary for the

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biofilm matrix development [9]. The colonization of bacteria to the polymeric surface of the medical devices like intravenous catheters, heart valves, and others facilitates the growth of cells in several layers to form a biofilm and cause life-threatening infections by many opportunistic bacterial like Staphylococci (Coagulase-negative Staphylococcus and *S. aureus*) and Pseudomonads [10, 11].

One of the important biological phenomena involved in biofilm formation is quorum sensing (QS) [12]. The QS process involves multiple signaling molecules like Acyl-Homoserine Lactones (AHL), Auto-Inducing Peptide (AIP), and the Auto-Inducer-2 (AI-2) either in gram-negative and gram-positive bacteria or both, respectively [10]. The signaling molecules achieve the co-ordination for making up the architecture of biofilm and its detachment in a specific cell-density dependent manner [13].

Several quorum sensing inhibitory compounds are known to control bacterial biofilms [14, 15]. Chelating agents like Ethylenediaminetetraacetic acid (EDTA), trisodium citrate (TSC), ethylene glycol tetraacetic acid (EGTA) and ethylenediamine-N,N'- disuccinic acid (EDDS) are known to control biofilm and used as biocides to enhance the antibacterial activity of other compounds [16, 17].

EDTA is a synthetic metal chelator [18] and is known to increase the sensitivity of planktonic proteobacteria [19] and adhered biofilms on catheters to different antimicrobial agents [20, 21]. However, the persistent nature of EDTA in the environment makes it less suitable for antimicrobial applications.

In comparison, S, S-EDDS (292.24 MW), a natural isomer of EDTA from *Amycolatopsis orientalis*, exists in biodegradable configuration with low toxicity (Fig 1). It was first detected due to its ability to inhibit Zn^{2+} -dependent phospholipase C activity [22]. Several other bacteria like Brevundimonas, Pseudomonas, Acidovorax, and Sphingomonas are known to produce EDDS.

Since EDDS is a natural cation chelator, many microorganisms have probably developed mechanisms to degrade it to make it less threatening for eco-friendly organisms like algae in the environment [23-25].

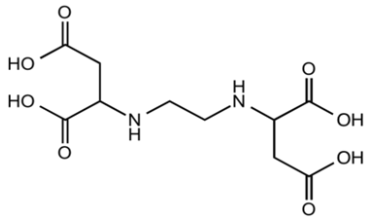

Fig. 1. Structure of (S , S) - Ethylenediamine - N , N'-disuccinic acid (EDDS).

Several studies show that EDDS gets degraded within days in the natural environment [26, 27] hence it is proposed as an environmentally friendly alternative to persistent EDTA for heavy metal remediation and decontamination [26]. The biological activities studied suggest EDDS is not toxic to human cells and the toxicity of metal-EDDS complexes is dependent upon the nature of metal [28]. It has also been shown that metal complexes of ligand EDDS are biodegradable and exhibit antimicrobial activity against fungi. For example, the complex of EDDS with cadmium (Cd^{2+} (EDDS)) is more toxic to fungus than in its free form [24, 29]. Some studies have reported the biological activities of this chelator, such as its inhibitory effect on *Xylella fastidiosa* [30], cytomegaloviruses [31], and tumor cells [28].

Some studies described the antibacterial activity of EDDS conjugates against some microbes by agar well method and fluorescent label assay [17, 19]. The anti-biofilm activity of EDDS is determined against sulfate-reducing bacteria [16, 32]. However, detailed antibacterial activity by broth dilution method against SA113 and PAO1 as well as anti-biofilm activity was not described before.

We, therefore, focused our investigation on the effect of chelating agent S, S-EDDS on microbial growth vs biofilm formation by SA113 and PAO1. These bacteria are a leading cause of infections associated with colonized intravenous catheters.

2. MATERIALS AND METHODS

2.1 Stock Solutions

Tri-sodium salt of S,S-Ethylenediamine-N,N'-disuccinic acid (EDDS-Mol.wt 292.2) was used for the assay prepared in 1M potassium hydroxide. It is a biodegradable ion chelator which does not produce persistent metabolites [26]. Gallidermin

and (Z-) - 4 - Bromo - 5 - (bromomethylene) - 2 (5H) - furanone (Sigma-Aldrich) were prepared in Milli - Q H_2O. All solutions were filter sterilized using 0.22 µm pore size filters and stored at -80°C.

2.2 Bacterial Culture

Bacterial strains. *S. aureus* SA113 (ATCC®35556™) which is restriction mutant and *agr*- [33] and *Ps. aeruginosa* PAO1 a wildtype strain [34] were used in this study. Bacteria were grown in Basic Medium (BM: soy peptone 1%, yeast extract 0.5%, NaCl 0.5%, K_2HPO_4 0.1%, glucose 0.1%, pH 7.4 (Gibco Life Technologies GmbH, Germany).

2.3 Growth Inhibition by EDDS

The growth inhibitory concentration of EDDS against SA113 and PAO1 were determined by the microdilution method using Basic medium broth (BM broth) following the method described earlier with little modification [35]. Stock solutions of EDDS, gallidermin, and furanone were diluted in 96-well microtiter plate (U-bottom transparent polystyrene) (Greiner Bio-One GmbH, Germany) in BM broth medium. EDDS solution is diluted to concentrations ranging from 50, 25, 12.5, 6.25, 3.125, 1.56 and 0.78 mM in 100 µl volume in each well in BM broth. Gallidermin and furanone solutions were diluted to concentrations 8 µg/ml and 2 µg/ml in BM broth volume in respective wells. One hundred microliter BM broth bacterial suspension with OD_{578} 0.05 (~10^6 CFU/ml) was mixed in each well and incubated at 37°C for 24 hours. The bacterial cultures without any compound were considered as control.

After incubation, the optical density (at 578nm) was measured using a Tecan Infinite M 200 plate reader (Tecan, Groedig, Austria) and minimum growth inhibitory concentration was determined as the lowest concentration that completely inhibited bacterial growth. The experiments were made in triplicates. The un-inoculated BM broth is considered as blank.

2.4 Biofilm Formation Assay

Assay for inhibition of biofilm formation was performed as described before [35]. Different Concentrations of EDDS, Gallidermin, and Furanone were adjusted in 96 microtiter well plate as described above in growth inhibition assay. In control wells, 100 µl BM broth was added. Overnight bacterial culture diluted to OD_{578} 0.05 in BM broth and 100 µl in each well. SA113 culture was incubated at 37°C and PAO1 at 35°C for 24 hours. The cultures were discarded and plates were washed 3 times with 20 mM tris buffer and air-dried. Adherent cells were stained with 200µl 0.1% Crystal violet (CV) for 30 minutes at RT. Washed with dH_2O three times and air-dried. The stain was extracted with 200 µl 95% ethanol and the absorbance of CV at 590 nm was measured with Tecan infinite M200 plate reader as a measure of biofilm formation. Cultures without any compound were considered as control and un-inoculated BM broth was taken as blank. Gallidermin and furanone were used as a positive control agent.

2.5 Data Analysis

All of the experiments for growth and biofilm inhibition were performed in triplicate. Data were analyzed and values of mean, standard deviation, and standard error were calculated in Microsoft Office Excell 2010. To calculate the significant difference between two independent means t-test was performed at $p < 0.05$.

3. RESULTS

3.1 Chelating Agents Has Inhibitory Effect On The Growth Of SA113 And PAO1

The ion chelator EDDS at different concentrations were tested to know the antimicrobial activity against the *S. aureus* SA113 and *P. aeruginosa* PAO1. The growth inhibitory effect of EDDS was determined on the planktonic cells of SA113 and POA1 in broth dilution assay. Gallidermin inhibit the growth of *S. aureus* at 8 µg/ml and the growth of PAO1 was inhibited by furanone at 25 µg/ml in BM broth medium as previously described [35, 49]. The growth of SA113 was inhibited at higher concentration of EDDS (12.5 mM) than PAO1 (6.25 mM) as indicated by optical density (OD_{578} nm) of the cultures after incubation (Fig 2).

3.2 Chelating Agent Inhibits The Biofilm Formation By SA113 And PAO1.

The biofilm inhibitory effect of EDDS was determined on the cells of SA113 and POA1 in

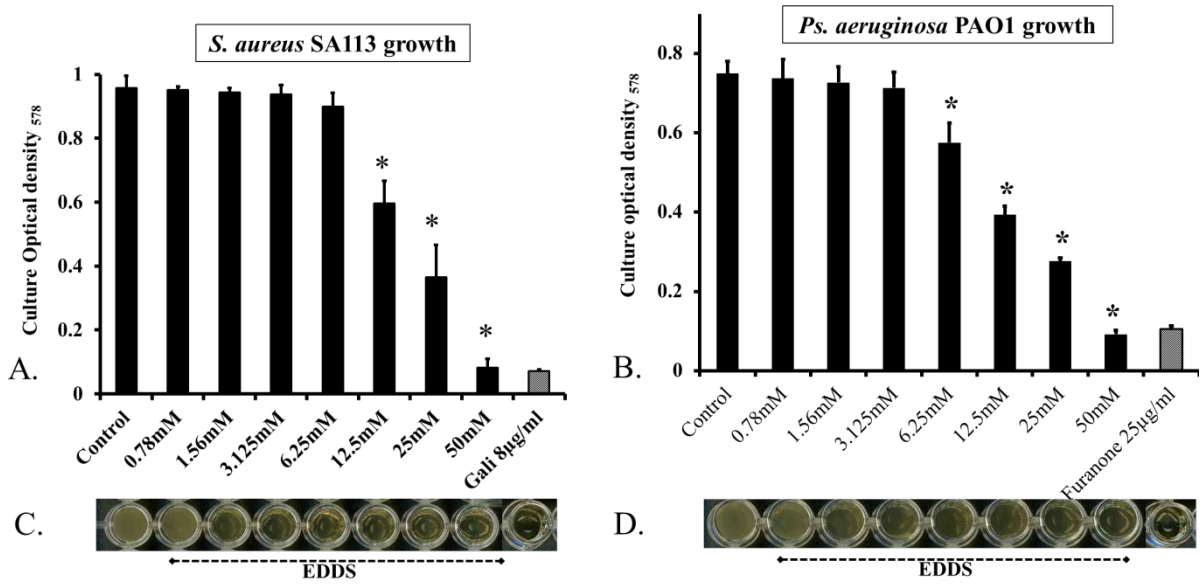

Fig 2. Effect of EDDS on the growth of *S. aureus* SA113 and *P. aeruginosa* PAO1. The effect of EDDS was determined by broth dilution assay in micro-well plate. Bacterial cultures of SA113 (A,C) and PAO1 (B,D) were grown with various molar concentrations of EDDS in BM broth. The optical density of the cultures were determined after 24 hours at 578 nm. Gallidermin and furanone were used a positive control agent. The control well contained no compound in the culture. Error bar indicates standard deviation from mean. Asterisks (*) indicates significant difference at p value <0.05

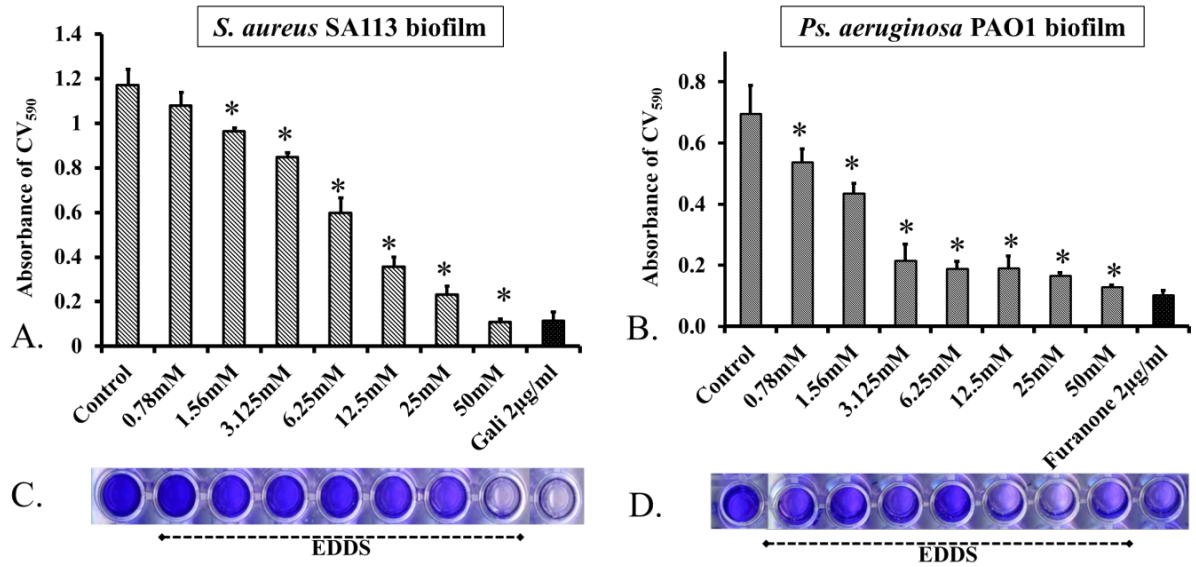

Fig 3. Effect of EDDS on biofilm produced by *S. aureus* SA113 and *P. aeruginosa* PAO1. The biofilm assay was carried out in microtiter plate using different concentrations of chelating agent EDDS (50 – 0.78mM) against SA113 and PAO1 in BM broth. Galliderm in and furanone were used as positive control agent against SA113 (A, C) and PAOI respectively (B, D). The bar diagram shows the CV absorbance (at 570nm) of stained cells attached to the well surface (A, B) and corresponding pictures of crystal violet staining of biofilm (C, D). The control well contained no compound in the culture. Error bar indicates standard deviation from mean. Asterisks (*) indicates significance at p value <0.05.

the micro-well plate assay [35]. The absorbance of crystal violet stain from the adherent cells extracted with 95% ethanol was measured (at 590nm) as a biofilm formation activity.

A dose-dependent decrease in absorbance of CV was observed with an increase in EDDS molar concentration in both SA113 and PAO1 strains as indicative of biofilm inhibition. A significant difference (t-test $p < 0.05$) between the mean of control and test compound treated cells was observed at 1.56 mM EDDS for SA113 and 0.78 mM for PAO1 strain.

4. DISCUSSION

In comparison to the single or planktonic living state, bacterial growth in the form of biofilms is of important clinical concern due to its tendency to accumulate on the indwelling or implanted medical devices and industrial installations which are difficult to eradicate. The infections associated with biofilms on devices are persistent and difficult to treat due to their increased tolerance towards antimicrobial compounds. Therefore, inhibition of the biofilm formation on indwelling/implanted medical devices and in industries is a prerequisite [5].

Several antimicrobial compounds are known to inhibit the biofilm formation including chelators with antimicrobial properties, used in medicine, and in industries as anti-biofilm agents and biocides [10, 17].

EDTA and EDDS are well-known ion chelating compounds to inhibit bacterial growth and biofilms. EDTA could kill gram-negative bacteria by chelation of Mg^{2+} ions, destabilize the lipopolysaccharide permeability, and enhance the antibiotic mediated killing of the cell [36].

EDTA widely used in industries to decontaminate medical devices and as a biocide enhancer [17]. Raad et al patented the idea of using the iron chelator as a biocide enhancer [16, 20, 21].

EDDS is also a cation chelator and known to sequester iron and calcium ions required for the maintenance of the biofilm matrix [37, 38]. Metallic cations such as Fe^{2+}, Ca^{2+}, and Mg^{2+} play a role in microbial adherence, biofilm formation, and bacterial growth. These divalent cations can stimulate cell-cell adhesion and aggregation through their interactions with cell-wall teichoic acid. Therefore, the removal of free cations from the milieu reduces intercellular adhesion and biofilm formation [38-40]. Furthermore, chelating agents can reduce biofilm formation by inhibiting the production of the Staphylococcal polysaccharide intercellular adhesin poly-N-acetylglucosamine [41, 42].

The use of EDDS is preferred over EDTA because it is not hazardous, non-toxic, and can be bio-degraded [26]. It is widely used as biocides to control the growth of Sulfate Reducing Bacteria (SRB) [16]. Biofilms by SRB are considered a nuisance in various industries. SRB biofilms produce hydrogen sulfide gas which causes souring in the reservoirs in the oil and gas industry and water cooling system [43]. The oxidized product of sulfuric acid is highly corrosive also causes pollution in the potable water system. EDDS increases the permeability of SRB outer membrane, as a result, they become more susceptible to biocide mediated killing [44, 45].

The complex of EDDS with Cd2+ and Hg2+ was observed to be more toxic than free metal ions for *S. aureus* showing the "Trojan Horse" effects where EDDS facilitate the delivery of the metal ion. It also enhances the antibacterial activity of fatty acids [24, 46].

The biological activities studied suggest that EDDS is not toxic to human cells. Metal-EDDS complexes also displayed some anti-proliferative activity where Leukemia cell line K562 cells were more sensitive to M-EDDS toxicity than peripheral blood mononuclear cells in all cases which offer for the development of new metallodrugs for cancer chemotherapy [24].

The bacterial natural zincophore EDDS is a potent Metallo-beta-lactamase (MBL) inhibitor and in combination with imipenem overcomes MBL-mediated carbapenem resistance in vitro and in vivo [47].

In this study we have tested the antibacterial and anti-biofilm activity of EDDS over *S. aureus* strain, SA113, and *P. aeruginosa* strain, PAO1.

The previous study has shown that at 10 mM concentration of EDDS inhibits the growth of *P. aeruginosa* while no growth inhibition of S. simulant was indicated even at 40 mM EDDS concentration using agar well diffusion assay [19]. Another study mentions that EDDS at 2000 ppm concentration (~6.8 mM) alone did not inhibit the SRB growth [16]. In our study, we found a significant growth inhibition of SA113 at 12.5 mM and PAO1 at 6.25 mM EDDS (Fig 1) concentration which is lower than that is mentioned in other studies [17, 19]. However, the assay methods used in those studies were different than it is used in our study. While testing the EDDS to inhibit the biofilm formation we observed that the biofilm formations of SA113 is inhibited at 1.56 mM and PAO1 at 0.78 mM concentration of EDDS (Fig 2), indicating antimicrobial and anti-biofilm potency of EDDS for gram-negative strain is greater as compared to the gram-positive strains. We used Furanone as a positive control agent for growth and biofilm inhibition of PAO1 and Gallidermin for SA113 and found similar results as previously stated [35, 48, 49]. The present study provides the foundation to use further tests to describe the antimicrobial efficacy of EDDS for the indigenous clinical isolates with the tendency of biofilm formations.

5. CONCLUSIONS

From the present study, we can suggest EDDS as a promising biofilm inhibitory compound against the pathogenic strains of *P. aeruginosa* (PAO1) and *S. aureus* (SA113). Significant biofilm inhibition of PAO1 at 0.78 mM and SA113 at concentration 1.56 mM concentration was observed, indicating EDDS an effective, biodegradable, and comparatively stable ion chelator compound against both gram-positive and gram-negative pathogenic bacteria.

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