

The Efficacy of Tetrasodium EDTA on Biofilms

S.L. Percival and A-M. Salisbury

Abstract

The aetiology of delayed wound healing characteristic of a chronic wound is relatively unknown but is thought to be due to a combination of the patient's underlying pathophysiology and external factors including infection and biofilm formation. The invasion of the wound by the hosts' resident microbiome and exogenous microorganisms can lead to biofilm formation. Biofilms have increased tolerance to antimicrobial interventions and constitute a concern to chronic wound healing. Consequently, anti-biofilm technologies with proven efficacy in areas outside of wound care need evaluation to determine whether their efficacy could be relevant to the control of biofilms in wounds. The aim of this study was to assess the anti-biofilm capabilities of tetrasodium EDTA (t-EDTA) as a stand-alone liquid and when incorporated in low concentrations into wound dressing prototypes. Results demonstrated that a low concentration of t-EDTA (4%) solution was able to kill *Staphylococcus aureus*, methicillin-resistant *S. aureus* (MRSA), *S. epidermidis*, *Pseudomonas aeruginosa* and *Enterococcus faecalis* within in vitro biofilms after a 24-h contact time. The incorporation of

low levels of t-EDTA into prototype fibrous wound dressings resulted in a 3-log reduction of bacteria demonstrating its microbicidal ability. Furthermore, hydrogels incorporating only a 0.2% concentration of t-EDTA (at preservative levels) caused a small reduction in biofilm. In conclusion, these studies show that t-EDTA as a stand-alone agent is an effective anti-biofilm agent in vitro. We have demonstrated that t-EDTA is compatible with numerous wound dressing platforms. EDTA could provide an essential tool to manage biofilm-related infections and should be considered as an anti-biofilm agent alone or in combination with other antimicrobials or technologies for increased antimicrobial performance in recalcitrant wounds.

Keywords

Antimicrobial · Biofilm · EDTA · Infection · Wound Dressing · Wounds

1 Introduction

The formation of biofilms in medical devices or acute and chronic wounds is of great importance (Francolini and Donelli 2010, Percival et al. 2012, 2015; Percival 2017). Biofilms have been identified in chronic wounds using microscopy methods, and it has been demonstrated that these wound biofilms are polymicrobial in nature (Wolcott et al. 2013). The clinical significance of biofilms relates to their

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increased tolerance and recalcitrance to antimicrobial interventions. Biofilms are microorganisms that attach to biotic and abiotic surfaces, or themselves forming aggregates, and produce an encasing polymeric substance known as exopolymeric substance (EPS) (Wingender et al. 2012). The presence of metal ions in the biofilm is significant to its development, sustainability and maintenance (Donlan 2002). Such divalent cations such as magnesium and calcium have been shown to cross-link with the polymer strands in EPS to provide greater binding force in a developed biofilm (Donlan 2002). Many studies have shown that the treatment of biofilms with metal ion chelators such as ethylenediaminetetraacetic acid (EDTA) helps to reduce biofilm formation or aid the removal of established biofilm (Devine et al. 2007; Finnegan and Percival 2014; Kite et al. 2005; Moreau-Marquis et al. 2009). Iron permits bacterial differentiation and essential biofilm growth, and therefore biofilm formation can be suppressed by limiting available iron (Weinberg 2004; Che et al. 2009). The use of EDTA to control biofilm formation has also been well documented and has been shown to outperform heparin in the prevention of biofilm growth in catheter-related blood stream infections (Percival et al. 2005; Kite et al. 2004; Raad et al. 2008). Interestingly, the conventional use of heparin has been shown to stimulate *Staphylococcus aureus* biofilm formation (Shanks et al. 2005). Despite the suggested use of using EDTA in wound care dressings documented in 2001 (Kite and Hatton 2001), and in combination with other agents (Kite et al. 2005; Percival et al. 2017), its application has only relatively recently been utilised in wound dressings despite extensive safety and toxicology profiles (Anon 2004).

EDTA when combined with sodium exists in many different forms and at different pH ranges and different concentrations in medical devices and has differences in their antimicrobial and anti-biofilm properties (Kite and Hatton 2001; Kite et al. 2005). The use of tri- (tri-EDTA, pH 7 to 9) and tetrasodium EDTA (t-EDTA, above pH 9) as stand-alone antimicrobial and anti-biofilm agents, or as synergistic agents to other antimicrobials, is being explored and has been reviewed elsewhere (Finnegan and Percival 2014). Both tri-EDTA and t-EDTA have shown anti-biofilm capabilities in part

due to the chelation of metal ions including calcium, zinc, magnesium and iron, which affects the stability of the biofilm and can also potentiate and sensitise the cell walls of bacteria (Banin et al. 2006).

Therefore, the aim of this paper was to assess the effect of t-EDTA only, as we have shown it has very similar antimicrobial and anti-biofilm performance to tri-EDTA (data not shown), on in vitro biofilm models and, furthermore, whether the incorporation of t-EDTA into various wound dressing platforms at both preservative (0.2%) and potential therapeutic concentrations (2–8%) would be effective on in vitro biofilms.

2 Methods

2.1 Microorganisms

P. aeruginosa NCTC 10662, *Staphylococcus aureus* ATCC 25923, *S. aureus* ATCC 29213, methicillin-resistant *S. aureus* (MRSA) ATCC BAA-43, *S. epidermidis* ATCC 35984 and *Enterococcus faecalis* ATCC 29212 were tested in the Minimum Biofilm Eradication Concentration (MBEC) model. *P. aeruginosa* NCTC 10662 and *S. aureus* ATCC 29213 were further tested in the Centers for Disease Control and Prevention (CDC) bioreactor and filter biofilm models, and *E. faecalis* ATCC 29212 was tested in the confocal study.

2.2 Chemicals

Tetrasodium EDTA was obtained from Sigma-Aldrich (UK).

2.3 Wound Dressings

Multisorb (BSN Medical, UK) absorbent dressings (fibrous gauze dressing) were used as basic gauze dressings to incorporate t-EDTA formulations. Briefly, 5 cm × 5 cm pieces of fibrous dressings were soaked in 2% and 4% t-EDTA for 1 h, weighed and allowed to dry in a sterile environment. T-EDTA was incorporated into amorphous hydrogels with a final concentration of t-EDTA of 0.2%.

2.4 Direct Contact Antimicrobial Test

The direct contact method was adapted from British Standards BS EN 16756 Antimicrobial Wound Dressings - Requirements And Test Methods (Section H.6). Control dressings and t-EDTA-incorporated wound dressings were aseptically cut to 2.5 cm × 2.5 cm and placed into 50 mL sterile centrifuge tubes. An overnight culture of *S. aureus* and *P. aeruginosa* was diluted to 10⁸ CFU/mL, and 500µL was added of each microorganism to the dressings. Dressings were incubated for 24 h at 37°C +/- 2 °C before adding 10 mL of Tryptone Soya Broth (TSB) (Oxoid, UK). All tubes were vortexed and sonicated to remove attached bacteria from the wound dressings before performing serial dilutions and determining total viable counts (TVCs).

2.5 Biofilm Models

2.5.1 Minimum Biofilm Eradication Concentration (MBEC) Model

The method for the use of the MBEC biofilm model was adapted from ASTM E2799. Briefly, an overnight suspension of microorganisms was diluted to 1 × 10⁵ CFU/ml before inoculating the wells of the 96-well plate. The 96-peg lid was added to the plate and incubated for either 24 h (24-h biofilm) or 48 h (48-h biofilm) at 37 °C +/- 2 °C and agitated at 125 rpm in humidified conditions. Biofilms were washed in sterile 0.85% sodium chloride (Sigma, UK) solution and placed into a new plate containing 150µl of 4% t-EDTA. Biofilms or the plates were incubated for 24 h. All pegs were washed and sonicated for 30 min into sterile 0.85% saline solution before serially diluting for total viable counts (TVCs).

2.5.2 CDC Bioreactor Biofilm Model

A modified ASTM E2871–13, Standard Test Method for Evaluating Disinfectant Efficacy Against *Pseudomonas aeruginosa* Biofilm Grown In CDC Biofilm Reactor Using Single

Tube Method, was used. Briefly, Tryptone Soya Broth was inoculated with either *S. aureus* ATCC 29213 or *P. aeruginosa* NCTC 10662 to a concentration of 1 × 10⁸ CFU/ml, which was determined by optical density (at 600 nm) and total viable counts. Each CDC bioreactor (BioSurface Technologies, USA) contains eight polypropylene rods designed to hold three coupons. In this experiment, polycarbonate coupons were used. The CDC reactor was sterilised before aseptically adding 300 ml of sterile TSB through the inoculation port. Following this, 1 ml of the previously prepared 10⁸ CFU/ml inoculum was then added to the reactor. The reactor was placed on a magnetic stir plate, and the rotation speed was set to 125 ± 5 rpm. The CDC reactor was operated in batch mode at room temperature (21 ± 2 °C) for 48 h (48-h biofilms). Following incubation, the rods containing the polycarbonate coupons were removed and rinsed in sterile 0.85% sodium chloride solution to remove planktonic cells. Each coupon was released from the rods into individual sterile 50 mL centrifuge tubes. For the testing of liquid t-EDTA (stand-alone), each coupon was placed into 3 mL of 4% t-EDTA. For the testing of hydrogels, each coupon was treated with 3 g of either hydrogel (control) or hydrogel containing 0.2% t-EDTA. Treatments of the coupons were performed in triplicate. The coupons were then incubated at 37 °C for 24 h. Following incubation, 27ml of sterile distilled water was added to each tube and sonicated for 30 min, followed by mixing for 10 s using a vortex to ensure the bacterial cells were in suspension. The disaggregated biofilm samples were sampled and serially diluted for bacterial enumeration. Biofilm density was calculated Log₁₀ density for each coupon. The Log₁₀ density of each coupon was subtracted from the Log₁₀ density of the untreated control coupon to determine the Log₁₀ reduction value of each treated biofilm.

2.5.3 Bespoke Biofilm Filter Model

S. aureus or *P. aeruginosa* was cultured overnight in TSB at 37 °C before diluting to 1 × 10⁸ CFU/ml. Twenty microliters of the bacterial suspension was added to a sterile 0.2µm filter disc

that was placed on Tryptone Soya Agar (TSA) (Oxoid, UK). Bacteria were incubated at 37 °C/−2 °C in humidified conditions for 48 h (48-h biofilm). Following incubation, 2 cm × 2 cm gauze (control), gauze with 2% t-EDTA or gauze with 4% t-EDTA was hydrated with 600µl sterile distilled water and placed on top of the biofilm. Biofilms were incubated for a further 24 h before removing and discarding the dressings. The filter containing the biofilm was then placed in 10ml of sterile distilled water, sonicated on full power for 30 min and vortexed for 10 s to remove the biofilm. The resulting suspension was used to perform total viable counts (TVCs) using serial dilution and plating.

2.6 Confocal Analysis

Overnight cultures of bacteria were diluted to 10⁶ CFU/mL before adding 400µl of the inoculum to the wells of a Lab-Tek™ glass chamber slide. The chamber slides were incubated at 37°C ± 2°C in humidified conditions for 48 h. Following incubation, 48-h biofilms were washed twice in 0.85% sodium chloride solution to remove planktonic cells. Biofilms were then stained using the LIVE/DEAD BacLight stain kit (Thermo-Fisher, UK) for 20 min at room temperature. Biofilms were then washed once in 0.85% sodium chloride solution and images were taken (T0) using the Zeiss 710 confocal laser scanning microscope. Biofilms were then treated with 400µl of t-EDTA at 0.2%, 2% and 4%. Control biofilms were in TSB only. Biofilms were incubated for 24 h at 37°C ± 2°C in humidified conditions. Images were taken at T24 (24-h contact time) hours using the Zeiss 710 confocal laser scanning microscope. Images were processed using FIJI software.

2.7 Cytotoxicity

2.7.1 Indirect Cytotoxicity (Lysis Index)

A 3% solution of Agar (suitable for cell culture) (Sigma, UK) was made and autoclaved for 15 min at 121 °C. The autoclaved agar was put into a 45 °C

water bath and allowed to cool to 45 °C. DMEM was warmed to 45 °C in a water bath. Equal volumes of the DMEM and 3% agar were mixed and allowed to cool to approximately 39 °C. The medium from all acceptable cultures was removed and replaced with 2.0 mL of agar medium. The cultures were placed on a flat surface to solidify at room temperature. Sterile, 10 mm blank antibiotic susceptibility discs (Sigma, UK) were saturated in test solution or control solution before being placed in each dish in contact with the agar surface. Triplicate cultures for each test material and both positive and negative controls were performed. All cultures were incubated for 24 ± 1 h. The outline of the specimen was marked on the bottom of the culture dish with a permanent marker, and then the specimen was removed. Two millilitres of 0.01% neutral red solution was added to each dish and incubated for 1 h. Following incubation, the neutral red solution was removed and each culture was examined microscopically under and around each control and test specimen. The cell culture was deemed to show a cytotoxic effect if microscopic examination revealed malformation, degeneration, sloughing or lysis of the cells within the zone or a moderate to severe reduction in cell layer density. The lysis index (Table 1) measures the number of cells affected within the zone of toxicity.

Table 1 Qualitative lysis description

Lysis index	Description of zone	Reactivity
0	Discrete intracytoplasmic granules, no cell lysis, no reduction of cell growth	None
1	Not more than 20% of zone shows rounded cells, loosely attached and without intracytoplasmic granules or show changes in morphology	Slight
2	Not more than 50% of the cells are round, devoid of intracytoplasmic granules, no extensive cell lysis; not more than 50% growth inhibition observable	Mild
3	Not more than 70% of the cell layers contain rounded cells or are lysed; cell layers not completely destroyed, but more than 50% growth inhibition observable	Moderate
4	Nearly complete destruction of the cell layers	Severe

2.8 Statistics

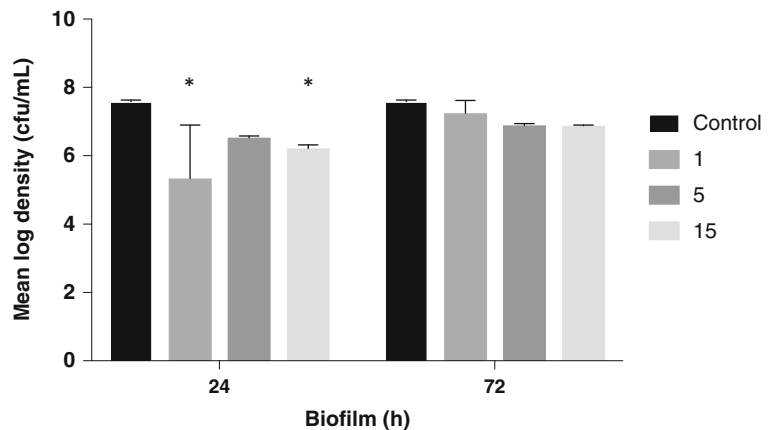
Statistical analysis was performed using GraphPad Prism 7 software. Statistical comparisons were performed using the two-way ANOVA. Results were interpreted as significant if the p value was ≤ 0.05 .

3 Results

3.1 Effectiveness of T-EDTA on Biofilms After Short and Long Contact Times

The treatment of 24-h and 72-h *S. aureus* and *P. aeruginosa* biofilms with a 4% t-EDTA solution for short contact times (1, 5 and 15 min) in the CDC bioreactor model resulted in a reduction in biofilms from 54.1% to 99.6% at the various short contact times (Figs. 1 and 2). Treatment of 24-h *S. aureus* biofilms for 1- and 15-min contact times demonstrated a significant reduction in cell density ($p = <0.04$). A significant reduction in cell density following treatment of 24- and 72-h *P. aeruginosa* biofilms was found at all contact times ($p = <0.0001$). The efficacy of 4% t-EDTA was demonstrated when exposing 24- and 48-h biofilms to t-EDTA for 24 h, whereby treatment caused 100% kill of *S. aureus*, MRSA, *S. epidermidis*, *E. faecalis* and *P. aeruginosa* biofilms (Fig. 3).

Fig. 1 Mean log density of 24- and 72-h *S. aureus* biofilms following short exposure to 4% t-EDTA. *S. aureus* biofilms were created using the CDC biofilm bioreactor and treated with 4% t-EDTA for 1, 5 and 15 min. Testing was done in triplicate. Error bars represent the standard deviation. A significant reduction in bacterial cell density compared to a control was found with some treatment groups at 24 h (* $p = <0.04$)



3.2 Effectiveness of Tetrasodium EDTA on Biofilms when Incorporated into Fibrous, Gauze and Hydrogel Prototype Platforms

The incorporation of t-EDTA at varying concentrations into fibrous prototype dressings was evaluated using a direct contact method (BS EN 16756) against *S. aureus* and *P. aeruginosa*. To test the effectiveness of t-EDTA-incorporated platforms against biofilms, 20 mg per gram (mg/g) (2%) and 40 mg/g (4%) of t-EDTA were added to the gauze and its efficacy was tested against a bespoke filter biofilm model. Results showed the gauze alone resulted in a small decline in cell density, which is likely due to mechanical disruption of the biofilm. Results showed 24-h treatment of *S. aureus* and *P. aeruginosa* biofilms caused moderate log reductions with gauze containing 4% t-EDTA causing greater log reductions than gauze containing 2% t-EDTA (Fig. 4). Incorporating 4% t-EDTA into the dressing resulted in a significant reduction of cell density against both strains in comparison to the control ($p = <0.002$) as well as the gauze only ($p = <0.04$) (Fig. 4).

Hydrogel only and hydrogel plus 0.2% t-EDTA resulted in a significant reduction of bacterial cell density against 48-h biofilms of *S. aureus*, MRSA, *P. aeruginosa*, *S. epidermidis* and *E. faecalis* ($p = <0.035$) with the exception

Fig. 2 Mean log density of 24- and 72-h *P. aeruginosa* biofilms following short exposure to 4% t-EDTA.

P. aeruginosa biofilms were created using the CDC biofilm bioreactor and treated with 4% t-EDTA for 1, 5 and 15 min. Testing was done in triplicate. Error bars represent the standard deviation. A significant reduction in the bacterial cell density of 24- and 72-h biofilms compared to a control was found with all treatment groups (* $p = <0.0001$).

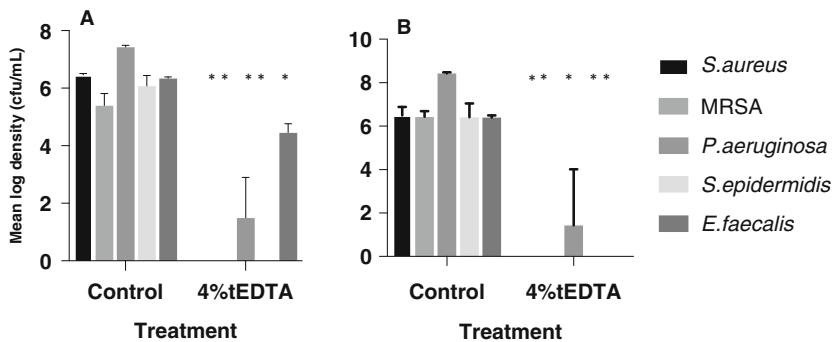
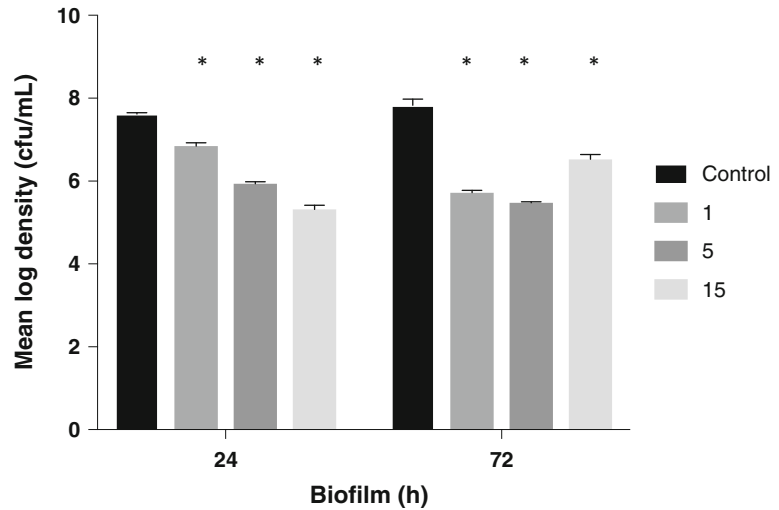


Fig. 3 MBEC results for treatment with t-EDTA. 24- (A) and 48- (B) hour biofilms were formed using MBEC plate method and treated with 4% t-EDTA for 24 h. Testing was done in triplicate. Error bars represent the

standard deviation. A significant reduction in biofilm cell density was found following treatment with 4% t-EDTA in comparison to a control group with all 5 strains (* $p = <0.0006$).

of hydrogel only against *S. epidermidis*. The reduction of cell density with hydrogel only is likely due to disruption of the biofilm upon application. A significant reduction in cell density was found with hydrogel plus 0.2% t-EDTA with all 5 strains when compared to hydrogel alone ($p = <0.013$) (Fig. 5).

3.3 Confocal Microscopy

Treatment of the *S. epidermidis* biofilm with all concentrations of t-EDTA resulted in a reduction in viability and changes in biofilm architecture.

Treatment of *S. aureus*, MRSA and *E. faecalis* (Fig. 6) with t-EDTA for 24 h reduced the viability of attached cells. The reduction in biofilm formation appeared to be dose-dependent based in all confocal images analysed.

3.4 Cytotoxicity of Tetrasodium EDTA

In the lysis index, it was shown that 2%, 4% and 8% of t-EDTA were non-cytotoxic, with grades 2, 1 and 2, respectively (Table 2).

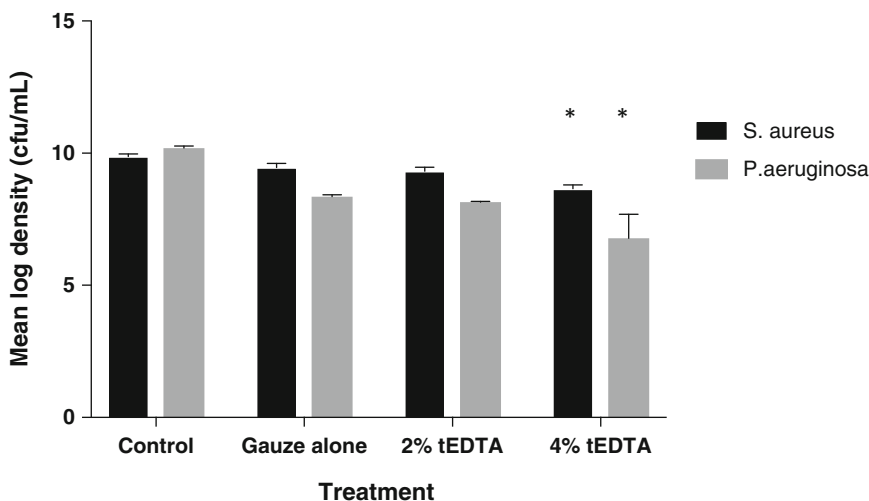


Fig. 4 The antimicrobial activity of t-EDTA incorporated into fibrous dressing against *S. aureus* and *P. aeruginosa* using the direct contact method. Testing was done in triplicate. Error bars represent the standard deviation. Results showed that the gauze

alone had an effect on the biofilm density, so t-EDTA treatment groups were compared to the gauze alone to determine a significant reduction in cfu/mL (* $p < 0.04$)

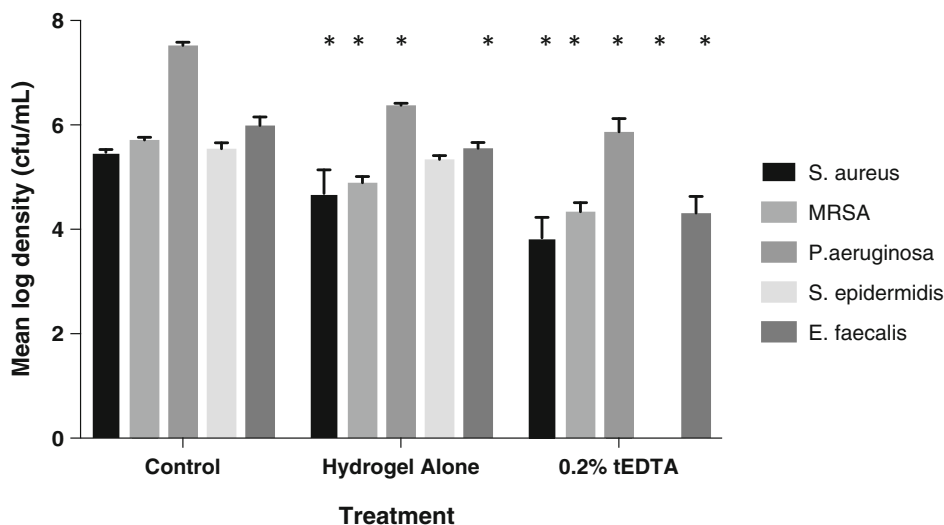


Fig. 5 Mean log density of 48-h biofilms following treatment with hydrogel alone and hydrogel with 0.2% t-EDTA in the CDC model. Biofilms (48 h) were exposed to treatment for 24 h at 37 °C ± 2 °C. Samples

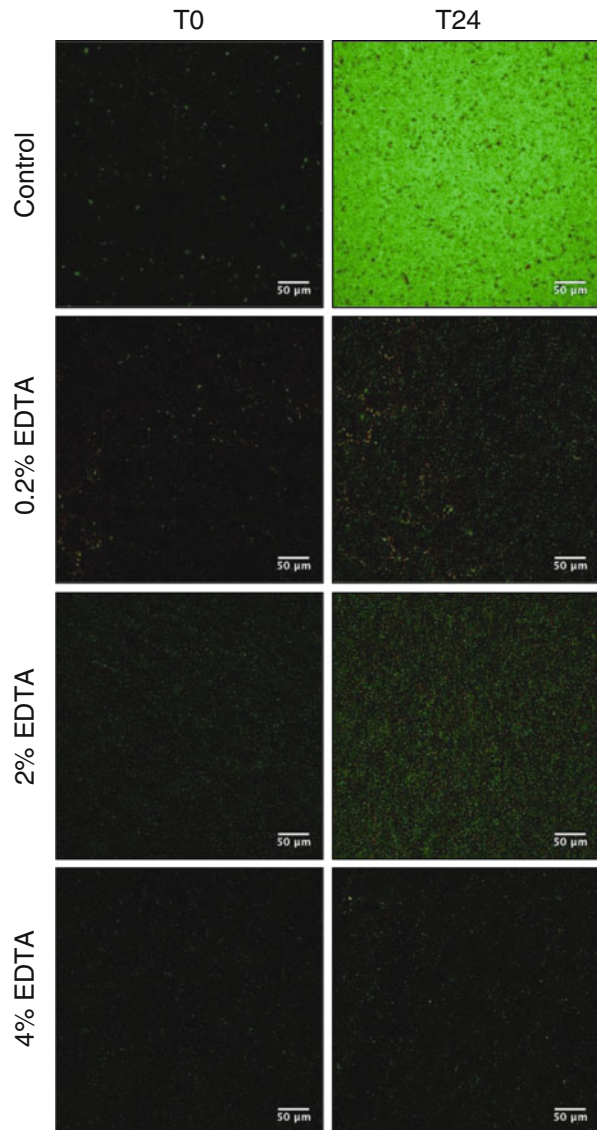
were run in triplicate. Error bars represent standard deviation. A significant log reduction in biofilm density was found in both treatment groups compared to the control (* $p = < 0.035$)

4 Discussion

Within this study we have shown that tetrasodium EDTA, as a solution, and within certain wound prototype dressing formats helps

to remove established biofilm and kill microorganisms that had been growing within the biofilm. Interestingly, the ability of 4% t-EDTA to remove established biofilm varied in the different models utilised in this study. The

Fig. 6 Treatment of *E. faecalis* biofilms with tetrasodium EDTA. Biofilms were treated with 0.2%, 2% and 4% tetrasodium EDTA for 24 h. Control biofilm was in Tryptone Soya Broth. Green fluorescence represents live cells. Red fluorescence represents dead cells. Images were taken using Zeiss confocal laser scanning microscope at x20 magnification with Z-stacks. Scale bar represents 50 μ m



MBEC model showed 24-h treatment with 4% t-EDTA is effective in significantly reducing 24- and 48-h biofilm cell density to below detectable levels against the majority of 5 strains tested. In comparison, although 4% t-EDTA reduced the bacterial cell density of biofilms in the CDC bioreactor, this was to a lesser extent. The difference observed between the 2 models could be due to a higher bacterial cell density being achieved in the CDC bioreactor or due to differences in biofilm formation in each model.

For example, the plates used in the MBEC are polystyrene, whereas the coupons used in the CDC bioreactor model are polypropylene. Different materials can affect the ability of bacterial cells to attach to a surface and form biofilms. The difference in results from the MBEC and CDC bioreactor experiments shows the importance of utilising different biofilm models when determining the anti-biofilm properties of a test agent. Differences in the anti-biofilm efficacy of the hydrogel test dressing and tetrasodium EDTA

Table 2 Zone index and lysis index for indirect cytotoxicity test

Sample	Lysis index	Interpretation
DMEM	0	Non-cytotoxic
Water	0	Non-cytotoxic
Phenol	3	Cytotoxic
EDTA 2%	2	Non-cytotoxic
EDTA 4%	1	Non-cytotoxic
EDTA 8%	2	Non-cytotoxic

Zone index measures the clear zone in which cells do not stain with neutral red. The lysis index measures the number of cells affected within the zone of toxicity. All samples were tested in triplicate

alone (2% and 4%) were due to a lower concentration of tetrasodium EDTA in the hydrogel formulation (0.2%). However, low levels of t-EDTA were added at low concentration to see if there was a preservative effect in the hydrogel. Further work continues at higher concentrations of tetrasodium EDTA.

The incorporation of tetrasodium EDTA into fibrous prototype platforms resulted in at least a 3-log reduction in the direct contact method demonstrating a microbicidal effect according to international guidelines. It has been previously shown that the use of liquid-only tetrasodium EDTA ((40 mg/mL, 4%) and (20 mg/ml, 2%)) as a catheter lock solution is effective in the reduction of biofilm in a catheter model (Percival et al. 2005; Kite et al. 2004). Similarly, tetrasodium EDTA has been reported to help with the partial removal of biofilm from polymethyl methacrylate (PMMA) and toothbrushes (Devine et al. 2007). Other studies have also assessed the effects of EDTA in biofilm prevention and control, which resulted in positive biofilm prevention in a dose-dependent manner; however the reduction of pre-formed biofilms was minimal with only a 31% reduction recorded (Ramage et al. 2007). EDTA in conjunction with other metals and solutions has demonstrated significant efficacy on biofilms. Banin and colleagues also determined that EDTA in Tris buffer was a thousand times more effective in killing *P. aeruginosa* biofilm

than gentamicin alone. Furthermore, the excess of divalent cations, magnesium, calcium and iron, protected the biofilm from anti-biofilm EDTA activity, supporting the importance of these divalent ions in the protection of the biofilm (Banin et al. 2006).

Within this study we have demonstrated that tetrasodium EDTA showed moderate to no cytotoxicity in the indirect contact method. Other studies support this finding whereby neutral and alkali tetrasodium EDTA cause moderate to severe cytotoxicity in L929 cells in a dose-dependent manner (Koulaouzidou et al. 1999). EDTA is a strong organic acid that is approximately 1,000 times stronger than acetic acid and does not appear to occur naturally. Toxicological data shows that the oral lethal doses of disodium EDTA and trisodium EDTA salts in rat are 2000 mg/kg and 2,150 mg/kg, respectively (Anon 2004; Kimmel 1977; Schardein et al. 1981). Extensive reviews of EDTA have determined a low toxicity profile for humans and no risk to human health (Grundler et al. 2005).

Overall we have shown that it is possible to incorporate tetrasodium EDTA into various wound dressing prototype materials such as gauze (fibrous material) and hydrogels. The use of tetrasodium EDTA as an effective microbicidal, antimicrobial and anti-biofilm agent has been demonstrated in this study indicating that concentration levels and the concentrations incorporated into wound dressings, and the amounts eluting from the dressing, are important and can affect anti-biofilm ability. As is the case for all microbicidal technologies, the efficacy of t-EDTA on planktonic microbes, sessile microbes and biofilms is dose-dependent. Consequently, the use of tetrasodium EDTA as a destabilising agent against biofilms should be considered and may aid in the removal of biofilms in the wound ecosystem (Percival et al. 2017) and represents an important component of a programme for patients undergoing biofilm-based wound management (Rhoads et al. 2008).

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