BIOFOULING
https://doi.org/10.1080/08927014.2020.1782388

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A novel micellar formulation based on natural plant extracts enhances the efficacy of hydrogen peroxide against biofilms of *Staphylococcus* spp. and *Pseudomonas aeruginosa*

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ABSTRACT

The antibacterial efficacy of hydrogen peroxide encapsulated in micelles (mH_2O_2) against biofilms was compared with that of hydrogen peroxide alone and of three commercially available aqueous biocides. The activity of mH_2O_2 on 24-h biofilms of reference strains of *Staphylococcus* spp. and *Pseudomonas aeruginosa* was tested in a static microtiter plate model. The biofilms were incubated with mH_2O_2 (17% v/v H_2O_2, 2% lactic acid, 0.3% phytoextract, H_2O) and its individual ingredients and compared with three aqueous biocides at different concentrations and times of exposure. After 5-min exposure, 10% mH_2O_2 (corresponding to 1.7% v/v H_2O_2) achieved $>$ 8 $\log_{10}$ reductions against all the test strains, while 1.7% H_2O_2 achieved a maximum of 1.5 $\log_{10}$ reduction. After 5-min exposure, none of the commercially available biocides tested showed themselves to be capable of completely eliminating the test strains embedded in biofilms. Hydrogen peroxide encapsulated in micelles demonstrated enhanced activity against planktonic cells and biofilms of *Staphylococcus* spp. and *P. aeruginosa*.

ARTICLE HISTORY
Received 25 July 2019
Accepted 9 June 2020

KEYWORDS
biofilm; hydrogen peroxide; micellar-based formulation

Introduction

Bacterial cells within biofilms are less susceptible to antimicrobials and antiseptics than planktonic cells (Presterl *et al.* 2007, 2009; Krzyściak *et al.* 2017). Microbial biofilms play a major role in the pathogenesis of infections of implanted material. The leading pathogens of medical implant infections are *Staphylococcus aureus* and *Staphylococcus epidermidis* (Darouiche 2004; Holinka *et al.* 2012; Zatorska *et al.* 2018). The reduced susceptibility of bacterial cells in biofilms to antimicrobial compounds is multifactorial and due to differential mechanisms including bacterial quorum sensing, exchange of resistance genes, or the scavenger properties of the matrix or the metabolic altered state of the bacterial cells (Donlan and Costerton 2002; Fey 2010). Moreover, bacterial biofilms in the food production environment have been recognized as sources of food contamination with pathogenic bacteria (e.g. *Salmonella* spp. or *Bacillus cereus*) (Galié *et al.* 2018). Besides medicine, the offshore industry, in particular, is also combatting microbial biofilms. Such biofilms may harbour microorganisms expressing enzymes and metabolites that are corrosive to metal or other materials (Vigneron *et al.* 2016). Environmental bacterial biofilms also occur in hospitals and other healthcare buildings. Not only water and air-conditioning systems but also the respiration tubing and waterlines of medical devices (e.g. dental units) may be heavily infested with bacterial biofilms (Capelletti and Moraes 2016) including various bacterial species. The most prominent are *Pseudomonas aeruginosa* and *Legionella pneumophila*, which cause life-threatening pneumonia and death (Patil and Patil 2017; Demirjian *et al.* 2015). Healthcare-associated infections and outbreaks related to biofilms in the moist patient environment of hospitals have been reported: In a recent review, almost all the 23 outbreaks of clinical infection caused by multi-resistant microorganisms (i.e. carbapenem-resistant bacteria such as *P. aeruginosa*), were associated with sinks (Carling 2018).

To date, the most efficient method of eradicating established biofilms is to eliminate embedded microorganisms using mechanical force (brushing), filtration, or high temperatures. However, these physical disinfection methods are not always feasible. Therefore, the most frequently used method of addressing the issue of bacterial contamination and biofilm formation in such environments is either the

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Supplemental data for this article can be accessed at https://doi.org/10.1080/08927014.2020.1782388.

continuous or the intermittent application of biocides into water. The development of novel and effective drug delivery strategies is required to kill bacterial cells in biofilm more effectively.

Recently, a European patent (PCT/EP2018/051620) claimed a novel method of enhancing the antimicrobial efficacy of antimicrobial compounds such as hydrogen peroxide or others by encapsulating them in micelles formed from modified plant extracts (Fürlinger 2018). These modified phyto-micelles consist of an assembly of surfactant molecules (fatty acids) supra-molecularly arranged to form a spherical structure with a hydrophobic core and a hydrophilic surface. Micellar formulations enhance the uptake of compounds into bacterial cells. Park *et al.* (2016) demonstrated in *in vitro* experiments that ferrocene-loaded PCAE micelles produce hydroxyl radicals able to kill *Escherichia coli* and *Pseudomonas aeruginosa* through membrane damage (Park *et al.* 2016). Gerola *et al.* (2019) showed that micelle-based delivery systems for chlorophylls potentiated photo-damaging effects against bacterial cells (Gerola *et al.* 2019).

Thus, micellar formulations of biocides may increase the antimicrobial effects of H_2O_2 against bacteria embedded in a biofilm by direct delivery into the cell wall. Further, H_2O_2 may be used at lower and less corrosive concentrations or at shorter contact times than an untreated H_2O_2 aqueous solution. In the present study, the activity of micellar H_2O_2 (mH_2O_2) was tested against biofilms formed by three major pathogens, *S. aureus, S. epidermidis,* and *P. aeruginosa*. The efficacy of mH_2O_2 was compared with H_2O_2 alone and with commercially available aquatic biocides at different concentrations and/or after different exposure times. The outcome parameters used were the biofilm optical density [OD], representing biofilm thickness, and the $\log_{10}$ reduction of bacterial cells representing biocidal efficacy.

Material and methods

Bacterial test strains investigated

All experiments were conducted with the biofilm forming reference strains *Staphylococcus aureus* strain ATCC 29213 (American Type Culture Collection), *Staphylococcus epidermidis* strain DSM 3269 (Deutsche Sammlung von Mikroorganismen und Zellkulturen/German Collection of Microorganisms and Cell Cultures; http://www.dsmz.de/), and *Pseudomonas aeruginosa* strain PAO1 (American Type Culture Collection). All test assays were done in quadruplicate and repeated four times, overall 16 assays for each test strain and each experiment.

Tested compounds

(1) HYDRO L.O.G.® (CuraSolutions GmbH, Wiener Neustadt, Austria) subsequently referred to as mH_2O_2 (ingredients: 17% v/v H_2O_2, 2% lactic acid, 0.3% phytoextract, H_2O); (2) HYDRO L.O.G.® without 2% lactic acid (17% v/v H_2O_2, 0.3% phytoextract, H_2O); (3) H_2O_2 from a 30% v/v stock solution; (4) 2% v/v lactic acid; (5) 0.3% phytoextract; (6) sterile H_2O (B. Braun, Germany); (7) Nalco ®7330 (Nalco Company, Illinois, US) (ingredients: 1.1% w/w 5-chloro-2-methyl-4-isothiazolin-3-one; 0.4% w/w 2-methyl-4-isothiazolin-3-one; 1-5% w/w magnesium nitrate); (8) Dentosept PL/P® (Dentsply Sirona Deutschland GmbH, Germany) (ingredients: H_2O_2 >1.2%-1.6% v/v + silver ions Ag+); and (9) Halamid® (Axcentive SARL, France) (ingredients: 0.2% v/v tosylchloramidnatrium). The concentration of these commercially available biocides was adjusted to the manufacturers' recommendations for the use of environmental disinfection (Nalco ®7330 and Dentosept PL/P® were used undiluted and Halamid® was used at a 0.2% concentration). For negative controls, untreated biofilms incubated with Brain Heart Infusion Broth (BHI; Sigma-Aldrich, St Louis, USA) were used in all experiments.

Biofilm model and determination of biofilm mass

Biofilm was studied using a modified microtiter plate model as described by Christensen *et al.* (1985). Test strains were prepared at a concentration of 0.5 McFarland and diluted 1:10 with BHI. The wells of a 96-well polystyrene flat-bottom micro-titre plate (Corning, Costar; Corning Life Sciences, Tewksbury, MA, USA) were filled with 100 µl of the diluted bacteria and incubated for 24 h at 37 °C under agitation (100 rpm; ambient air). Next, the supernatants, including the non-adherent planktonic bacteria, were removed. The biofilms were then fixed using 4% glutaraldehyde and air-dried for 10 min. The biofilms were stained using 100 µl of 1% crystal violet (CV) for 10 min. The dyed biofilms were washed twice with 300 µl of phosphate buffered water (PBS) and air-dried. The wells were then visually checked for the presence or absence of a biofilm, based on the presence of staining at the bottom of the well. The CV in the stained biofilms was dissolved in absolute ethanol and measured using a FLUOstar1 Omega microplate

reader at 590 nm wavelength. The mean optical density (OD) recorded was considered to correspond to the attached biofilm mass (O'Toole 2011).

Antimicrobial efficacy against bacteria embedded in biofilm (time-kill curve)

To test the anti-biofilm effects of the compounds, mature biofilms were incubated with 100 µl of the respective test solution for 5, 15, 30 and 60 min at 37 °C ambient air. Four wells per isolate were tested for each concentration investigated and subsequent substances. For calculation of the decrease in the biofilm OD, a ratio of the biofilm OD of the isolate incubated with each compound, respectively, to the biofilm OD of the same isolate without the compound (control) was calculated. This OD ratio (ODr = OD of the treated biofilm/OD of the untreated biofilm) was used to measure changes in the thickness of the biofilms over time. The ODr of the untreated biofilms was 1 (Presterl *et al.* 2007).

The time kill curves of the compounds tested were generated using the viable colony count (VCC) method. For this purpose, the mature biofilms were not stained but scraped off and resuspended in PBS. The viable count of the tested reference strains exposed against the respective tested compounds was measured in serial dilutions: 10 µl of each dilution were plated onto Columbia agar + 5% sheep blood plates (bioMerieux SA, Marcy l'Etoile, France) and examined for growth. The plates were incubated at 37 °C in ambient air and read after 48 h. The antimicrobial efficacy was expressed as a bacterial $\log_{10}$ reduction, which is the ratio of pre-values (number of colony-forming units sampled before exposure with the test compound) and post-values (number of colony-forming units sampled after exposure with the test compound) expressed by the decimal logarithm. A neutralizer (30 g l^{-1} of polysorbate 80, 30 g l^{-1} of saponine, 3 g l^{-1} of lecithin, 1 g l^{-1} of histidine, and 5 g l^{-1} of sodium thiosulphate) was used to neutralize the antimicrobial efficacy of the test compounds. The neutralizer had been validated according to the European Standard EN 13697:2015.

Confocal laser scanning microscopy (CLSM)

Live/Dead Baclight bacterial viability stain (Molecular Probes, Oregon, USA) was used in this study. It consists of a mixture of two nucleic acid-binding stains: Syto 9 and propidium iodide. Syto 9 stains all viable bacteria of fluorescent green, while propidium iodide stains bacteria whose membranes have been damaged (non-viable bacteria) of fluorescent red (Live/dead stain). Biofilms of the three test strains were grown on an Ibidi 1 µ Slide 8 well (ibidiTreat, Martinsfeld, Germany) for 24 h. After bacterial colonization for 24 h, the supernatant and the non-adhering cells were removed by washing three times with PBS. The live/dead stain mixed according to the manufacturer's instructions, and 200 µl of 2 x stock solution were applied directly to the surface of each sample. After 15 min in dark incubation, the stain surplus was removed. Immediately after, samples were analysed with an LSM 780 confocal microscope system (Zeiss, Oberkochen, Germany).

To assess the biofilm matrix, 1 ml of each reference strain (diluted in Brain Heart Infusion Medium 1.5×107), was cultivated in single 24-well Ibidi µ-Dishes (Ibidi Treat 1, 5 polymer coverslip, tissue culture treated; Ibidi GmbH, Planegg/Martinsried, Germany). Biofilms were grown at 37 °C for 24 h on an orbital shaker. Biofilms were then washed twice in PBS and fixed with 4% glutaraldehyde. Propidium iodide was used to observe the dense DNA of the bacterial cells (Molecular Probes®; Thermo Fisher Scientific). Polysaccharides, representing the most characteristic fraction of the extrapolymeric substances, were stained using concanavalin-A (ConA) (Sigma-Aldrich Corp, St Louis, MO, USA).

Statistics

The Mann-Whitney U test was used for the significance of differences. A general linear model for repeated measurements was calculated to determine the changes due to the different biocides and isolates. All tests were performed using SPSS for Windows, release 24.0 (SPSS). A p-value of < 0.05 was considered significant.

Results

First, to prove the principle action of mH_2O_2, a series of preliminary experiments was conducted to test the activity of all single compounds on the three bacterial test strains: The antibacterial efficacy of undiluted mH_2O_2 and each individual ingredient of undiluted mH_2O_2 (1) water, (2) 17% v/v H_2O_2, (3) 0.3% phytoextract and (4) 2% lactic acid, all after various application times against the three test strains embedded in the biofilm, were tested. Both, 17% v/v H_2O_2 and undiluted mH_2O_2, which contains H_2O_2 at a concentration equivalent to a 17% H_2O_2,

Table 1. Ratio (ODr) of the biofilm optical density (OD) of isolates of three bacterial test strains (means ± SD) after exposure to various formulations at different exposure times to the biofilm OD of the same unexposed isolates (controls).

Test compound	*S. aureus*				*S. epidermidis*				*P. aeruginosa*			
ODr	5 min	15 min	30 min	60 min	5 min	15 min	30 min	60 min	5 min	15 min	30 min	60 min
Water	0.61 ± 0.11	0.64 ± 0.20	0.61 ± 0.19	0.65 ± 0.25	0.69 ± 0.26	0.76 ± 0.28	0.72 ± 0.32	0.69 ± 0.30	0.84 ± 0.49	0.70 ± 0.39	0.68 ± 0.42	0.69 ± 0.46
17% (v/v) H_2O_2	0.52 ± 0.17	0.51 ± 0.18	0.55 ± 0.17	0.51 ± 0.18	0.72 ± 0.27	0.77 ± 0.32	0.81 ± 0.37	0.80 ± 0.30	0.64 ± 0.20	0.64 ± 0.26	0.55 ± 0.20	0.40 ± 0.16
HYDRO L.O.G.®	0.79 ± 0.38	0.75 ± 0.35	0.87 ± 0.33	0.89 ± 0.31	0.99 ± 0.30	1.04 ± 0.38	1.16 ± 0.43	1.17 ± 0.41	0.81 ± 0.18	0.82 ± 0.24	0.75 ± 0.32	0.66 ± 0.27
0.3% Phyto-extract	0.66 ± 0.14	0.65 ± 0.14	0.68 ± 0.14	0.90 ± 0.57	0.77 ± 0.26	0.82 ± 0.26	0.82 ± 0.28	0.87 ± 0.29	0.79 ± 0.23	0.71 ± 0.22	0.71 ± 0.16	0.76 ± 0.34
2% Lactic acid	1.10 ± 0.58	1.09 ± 0.46	1.13 ± 0.47	1.08 ± 0.46	1.05 ± 0.36	1.14 ± 0.37	1.15 ± 0.43	1.15 ± 0.60	1.17 ± 0.40	1.06 ± 0.25	1.09 ± 0.29	1.07 ± 0.41

ODr of untreated biofilms = 1.

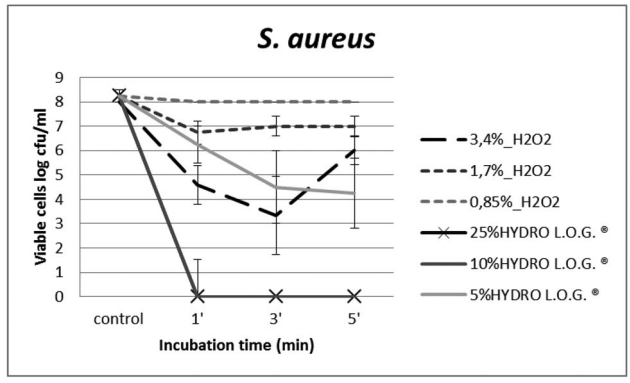
aqueoussolution (v/v), showed strong antibacterial efficacy against bacteria in biofilm, with a reduction > 7 $\log_{10}$ after a 5-min exposure. The individual ingredients of mH_2O_2 water and 0.3% phytoextract did not reduce the bacterial count. After a 15-min exposure, 2% lactic acid alone achieved a 3 $\log_{10}$ reduction for *S. epidermidis* and after 5-, 15- and 30-min exposures for *P. aeruginosa,* and a 5 $\log_{10}$ reduction after 30- and 60-min exposures for *S. epidermidis* (data shown in Supplementary material in Table S1). Incubation with 17% H_2O_2 led to a biofilm with significantly lower ODs than those of the untreated biofilms ($p > 0.0002$). The same was true for the biofilms of *S. aureus* incubated for 15 min with mH_2O_2 and the biofilms of *P. aeruginosa* incubated for 5, 15, 30 and 60 min with mH_2O_2 ($p = 0.01$-0.0002). The corresponding biofilm OD ratios are shown in Table 1.

After 5-, 15-, 30- and 60-min exposures to undiluted mH_2O_2, the OD ratios increased in biofilms formed by *S. epidermidis* (Table 1). After 5-, 15-, 30- and 60-min exposures to 2% lactic acid, the OD ratios increased in biofilms formed by all the bacterial strains tested.

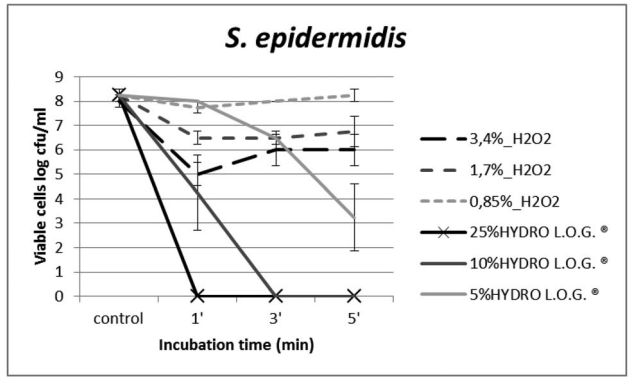
To demonstrate the action of mH_2O_2, H_2O_2 and the commercially available biocides over time, mH_2O_2 at 5%, 10% and 25% aqueous solutions (v/v), H_2O_2 at the equivalent concentrations of 0.85%, 1.7% and 3.4% as the equivalent concentration of mH_2O_2 at 20% aqueous solution (v/v) were tested at differential times of incubation. The commercially available biocides were tested at their standard concentrations as used in practice. Biofilms of *S. aureus*, *S. epidermidis,* and *P. aeruginosa,* grown for 24 h, were incubated with mH_2O_2, H_2O_2 and the commercially available biocides for 1, 3 and 5 min.

The resulting killing curves of *S. aureus*, *S. epidermidis,* and *P. aeruginosa* biofilms comparing H_2O_2 alone and mH_2O_2 are illustrated in detail in Figure 1. After 1- and 5-min exposure, H_2O_2 at a concentration of 1.7%, which is equivalent to a 10% solution of mH_2O_2, (v/v) achieved a maximum $\log_{10}$ reduction of 1.5 for all reference strains tested.

(a) *S. aureus*



(b) *S. epidermidis*



(c) *P. aeruginosa*

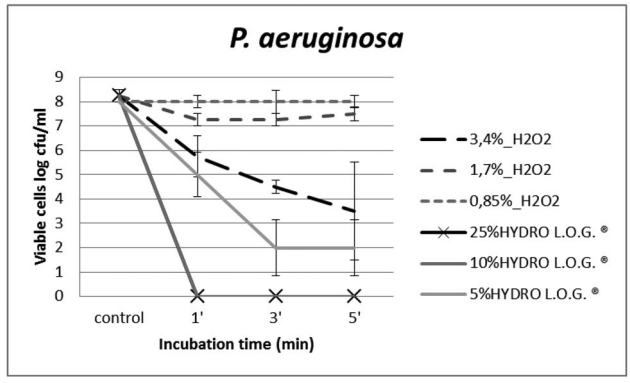
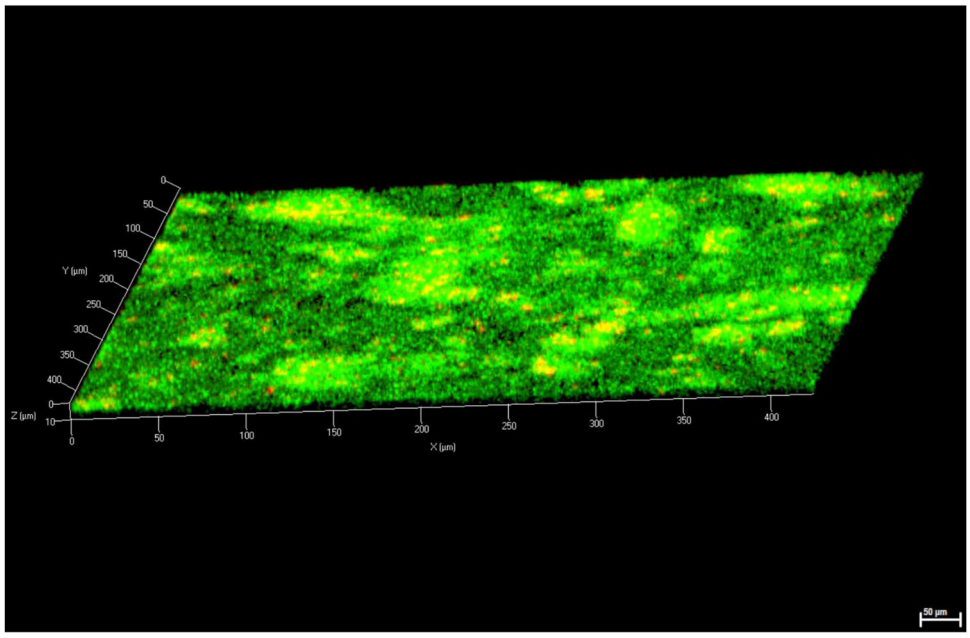


Figure 1. Killing curves (means ± SEM) of (a) *S. aureus*, (b) *S. epidermidis* and (c) *P. aeruginosa* biofilms grown for 24 h comparing different concentrations of H_2O_2 alone with micellic H_2O_2 (HYDRO L.O.G.®).

(a1)



(a2)

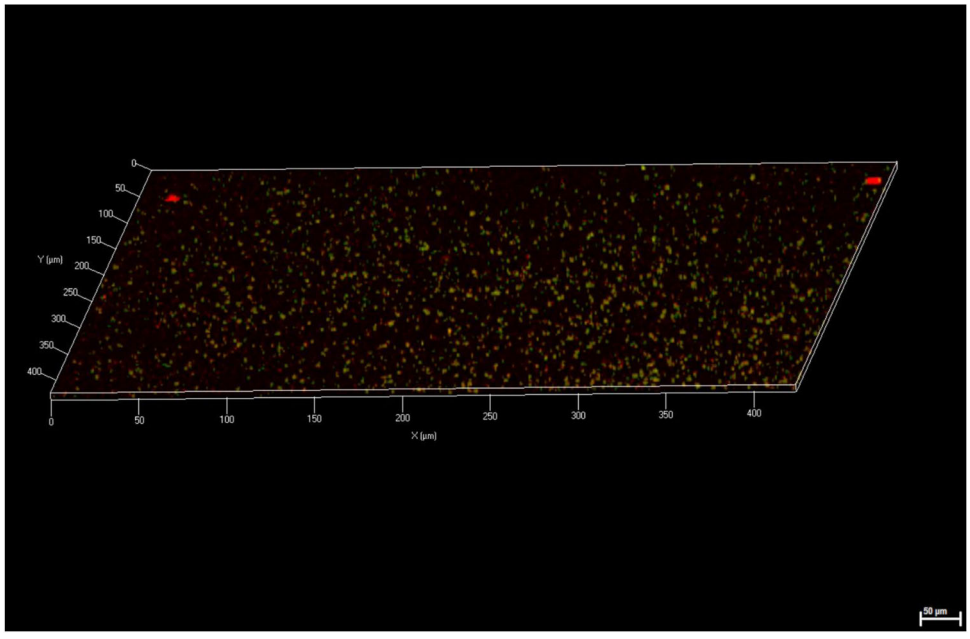
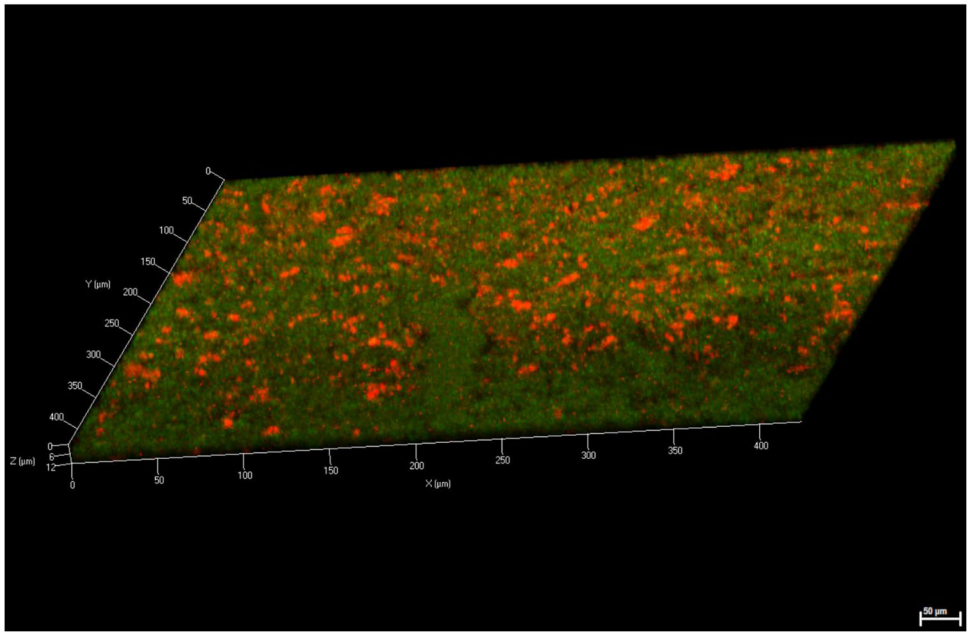


Figure 2. Untreated and treated 24- h biofilms of (a1-2) *S. aureus*, (b1-2) *S. epidermidis* and (c1-2) *P. aeruginosa* observed with CLSM after a 5-min exposure to 10% micellic H_2O_2 (HYDRO L.O.G.®). Live/dead staining SYTO9 was used to visualize viable bacteria (green color) and propidium iodide for dead cells (yellowish/red color). Scale bar = 50 µm.

Micellar H_2O_2 at a concentration of 10% yielded a reduction of 4.0 $\log_{10}$ in biofilm formed by *S. epidermidis*, $a > 8$ $\log_{10}$ reduction in biofilms of *P. aeruginosa* and of *S. aureus* after a 1-min exposure. However, after a 5-min exposure 10% mH_2O_2 achieved $a > 8$ $\log_{10}$ reduction in the bacterial count in biofilms of all the bacterial species tested.

Ten % mH_2O_2 yielded a reduction of 4.0 $\log_{10}$ against *S. epidermidis*, and > 8 $\log_{10}$ reduction against *P. aeruginosa* and *S. aureus* after a 1-min exposure,

(b1)



(b2)

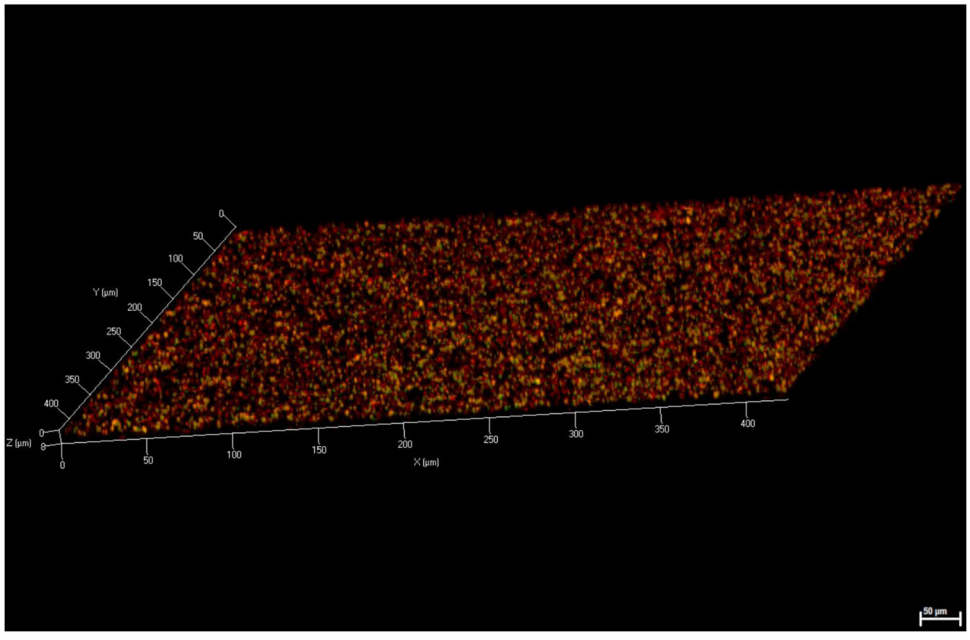


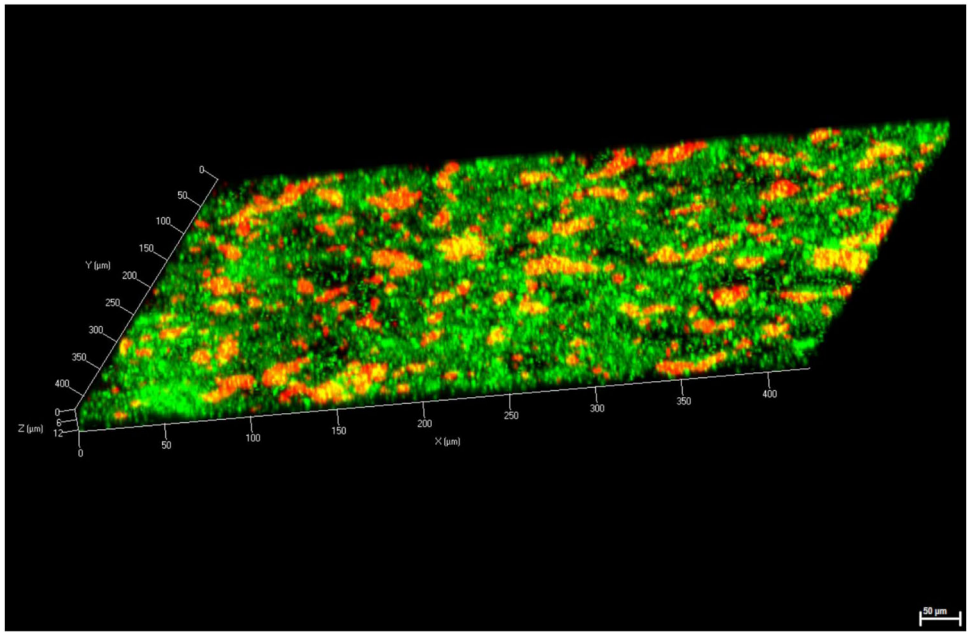
Figure 2. Continued

and $>$ 8 $\log_{10}$ reduction against all three test strains after a 5-min exposure time (Figure 1).

Figure 2 visualizes the bactericidal effect of 10% micellar H_2O_2 (HYDRO L.O.G. ®) on 24-h biofilms of all three test strains after a 5-min exposure and Figure 3 shows the effect on 24-h biofilms of *S. epidermidis*. There is a clear preponderance of viable cells (green) on the left of Figure 2 (2a1, 2b1, 2c1) in the untreated biofilm, while on the right of Figure 2 (2a2, 2b2, 2c2), dead cells (yellowy-red) prevail after exposure to the micellar biocide. In Figure 3, mH_2O_2 leads to the destruction of bacteria (red) and an alteration in the biofilm matrix visualized by the change of structure and colour from green (Figure 3a) to yellow, and the biofilm thickness was reduced from up to 16 µm to an average of 6.5 µm (Figure 3b).

In summary, the micellar formulation exhibited significantly enhanced antibacterial efficacy against

(c1)



(c2)

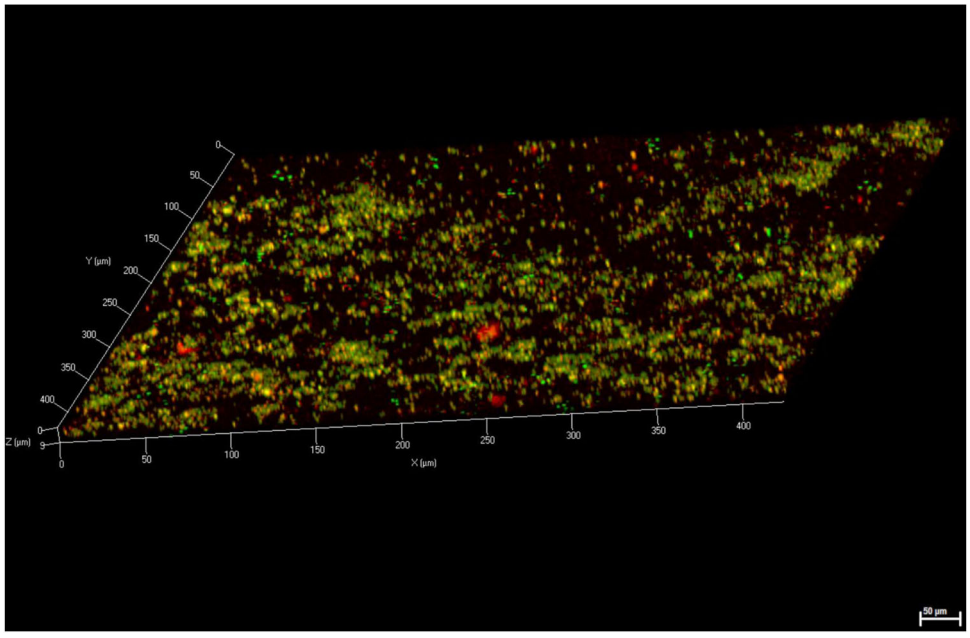
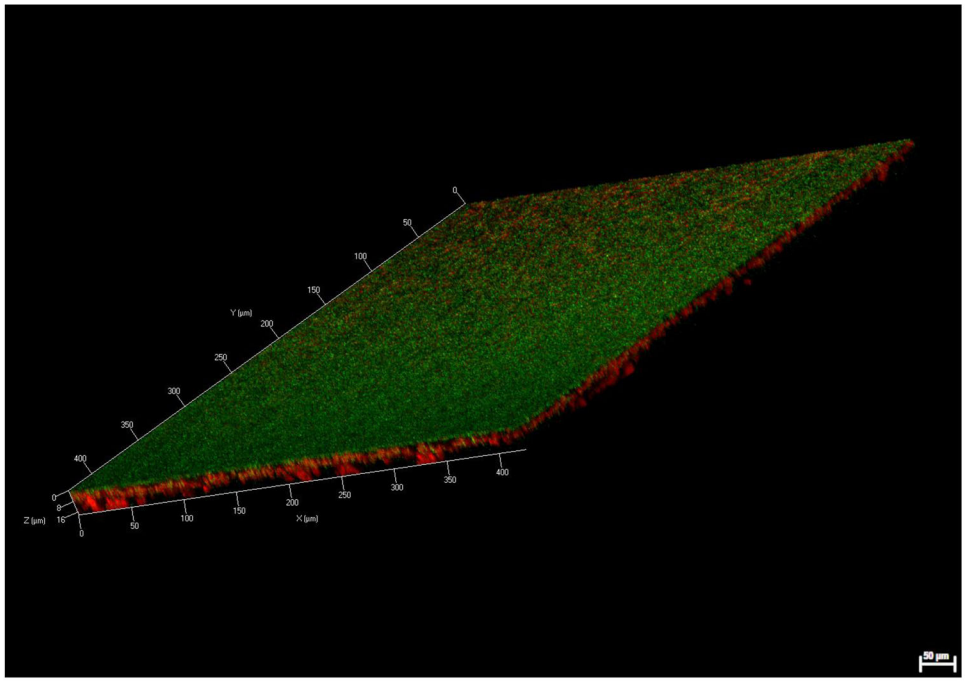


Figure 2. Continued

biofilms. This effect, however, was dependant on the presence of 2% lactic acid (data shown in Supplementary material Figure S1).

The results of mH_2O_2 tested against three commercially available biocides frequently used in the waterworks of cooling towers are depicted in Figure 4. Starting at an initial inoculum of 8 $\log_{10}$ colony-forming units (CFU) of *S. aureus, S. epidermidis* and *P. aeruginosa* in biofilms, Nalco ®7330 showed strong antibacterial efficacy against *P. aeruginosa*, but not against staphylococci, while Halamid® achieved a maximum $\log_{10}$ reduction of 5.9 in staphylococcal biofilms but < 1 $\log_{10}$ reduction against *P. aeruginosa* biofilms. Dentosept® showed a maximum of 1 log reduction against all the bacterial biofilms tested, while 10% mH_2O_2 showed complete bacterial eradication of the biofilms of all the bacterial strains tested after a 5 min exposure.

(a)



(b)

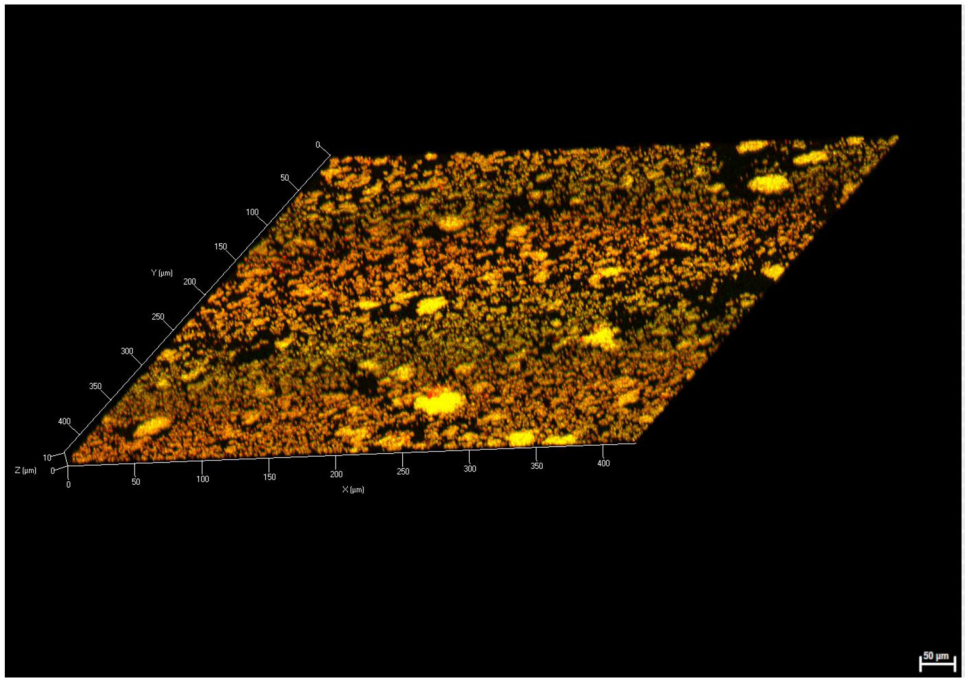


Figure 3. Biofilm matrix of 24- h biofilms of *S. epidermidis* (a) untreated and (b) after a 5 min exposure to 10% micellic H_2O_2 (HYDRO L.O.G.®) visualized by CLSM using propidium iodide and concanavalin-A staining. Propidium iodide was used to visualize bacteria (red) and concanavalin-A to visualize biofilm matrix (green/yellowish). Scale bar = 50 µm.

Discussion

Hydrogen peroxide exhibits concentration-dependent antimicrobial activity. It is commonly used as a standard biocide at concentrations of 3-5% and has a broad-spectrum efficacy against viruses, bacteria, yeasts, and -dose-, time-, and application-dependant-bacterial spores (Diab-Elschahawi *et al.* 2010). H_2O_2 is one of the most environmentally friendly biocides because it is non-toxic to humans and animals, and rapidly degrades to water and oxygen without toxic leftovers. However, it is unstable, difficult to store and

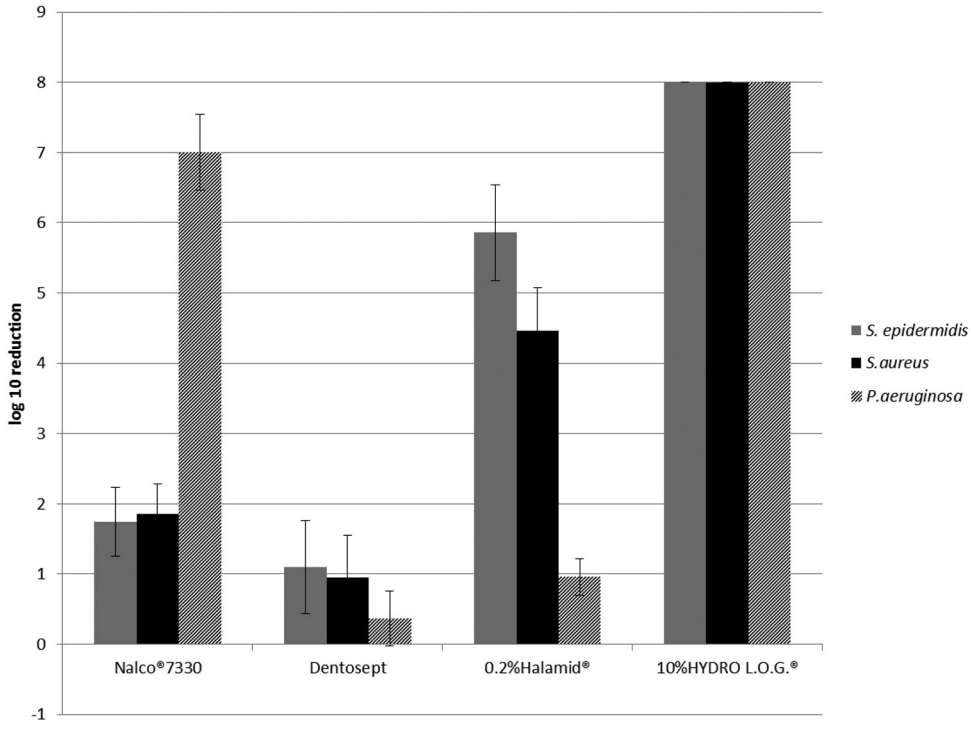


Figure 4. Mean log_{10} reduction* of viable bacteria in biofilms after a 5-min exposure, comparing three commercially available biocides (Nalco® 7330 undiluted, Dentosept® undiluted, 0.2% Halamid®) to the micellar H_2O_2 formulation (10% HYDRO L.O.G. ®).
* Mean log_{10} reductions are based on 4 replicates and rounded to full log_{10} values. Bars indicate the SEM of the mean.

to transport, and its antimicrobial action is highly dose- and time-dependant. Furthermore, H_2O_2 exhibits an exactable antimicrobial efficacy chiefly against planktonic microorganisms but a reduced activity against microbes embedded in biofilm. Exposing 12 bacterial strains isolated from dental unit waterlines to H_2O_2, Yoon and Lee (2019) reported a minimum bactericidal concentrations (MBC) of 0.0078 – 0.0156% against these environmental bacterial strains in their planktonic form. However, the MBC of H_2O_2 to eradicating the identical strains in biofilm ranged from 0.0312 to > 4%. In biofilms of *Sphingomonas echinoides*, the MBC was 128 times higher than in the planktonic state.

Micellar formulations enhance the uptake of compounds into bacterial cells and can help to increase their bactericidal activity (Gerola *et al.* 2019). Important advantages of micellar carriers include the enhanced solubility of hydrophobic drugs in aqueous solution, the protection against drug degradation, and the possibility of drug targeting to a preferred site. However, when bacterial biofilms are exposed to micellar biocide formulations, it is pivotal that the micelles reach the bacterial cells embedded in an extracellular matrix (composed of polysaccharide, eDNA and proteins) which acts as the natural barrier against various agents.

Undiluted mH_2O_2 and H_2O_2 alone in identical concentrations (17% v/v H_2O_2 each) and application times led to a significant reduction in bacterial growth within bacterial biofilms. The micellar formulation did not inhibit the action of H_2O_2 (data shown in the Supplementary material in Table S1). While 17% H_2O_2 also reduced the OD ratios of biofilms, as reported previously (Presterl *et al.* 2007), the mH_2O_2 formulation behaved differently. For *S. epidermidis* biofilms, in particular, there was an increase in the OD ratios after exposure to undiluted mH_2O_2 (see Table 1). Further investigations demonstrated that this increase in biofilm OD ratios was induced by 2% lactic acid, which is one of the components of the mH_2O_2 preparation. This increase in biofilm thickness after incubation with antimicrobial agents has already been described and attributed to an accumulation of decaying bacterial cells (Presterl *et al.* 2007). In the case of mH_2O_2, it may be due to a reaction of lactic acid with the proteins in the biofilm layer resulting in its fixation of the biofilm in the static biofilm model, as described earlier for alcohols. Nevertheless, alcohols had an excellent bactericidal effect even on established biofilms, although the ODr was increased (Presterl *et al.* 2007). Biofilm formation is differential among the bacterial species and regulated by different pathways (e.g. the AtlE pathway in *S. epidermidis* or the

las pathway in *P. aeruginosa*) and by environmental factors (e.g. temperature or nutrients) (Costerton *et al.* 1999; Wimpenny *et al.* 2000; Costerton *et al.* 2005). Thus, it is likely that biocides have a differential effect on the biofilms of different bacterial strains. Moreover, although there was no significant decrease in the biofilm ODr as a surrogate marker for biofilm mass, there was a decrease in biofilm thickness (Figure 3) and biofilm structure. The micellar formulation obviously reaches the bacterial cells in a biofilm to deliver H_2O_2 to kill the embedded cells acting synergistically by possibly deforming biofilm matrix compounds and consequently altering matrix structure, as shown exemplarily for *S. epidermidis* (Figures 2 and 3).

Next, the anti-biofilm activity of the novel mH_2O_2 compound compared with H_2O_2 alone was investigated. The most important finding of this study was that 1.7% H_2O_2 alone achieved a maximum of 1.5 $\log_{10}$ reduction even after a 5-min exposure to bacteria embedded in a biofilm, while the same low concentration with 1.7% H_2O_2 encapsulated in a plant micelle yielded $>$ 8 $\log_{10}$ reductions at identical exposure times. Hence, this plant micelle preparation can enhance the antimicrobial efficacy of H_2O_2 against bacteria in a biofilm. This improved antibacterial effect of the micellar formulation could also be due to an increased local H_2O_2 concentration against which bacteria are exposed by direct delivery into the cell or cell wall. H_2O_2 may therefore, be used in lower and less corrosive concentrations or at shorter contact times than an untreated H_2O_2 aqueous solution. The enhancement of antibacterial efficacy was also dependent on the presence of 2% v/v lactic acid. In the present experiments, 2% lactic acid alone showed only very little antibacterial efficacy at an exposure time of 5 min (data shown in Supplementary material Table S1) and nearly none at an exposure time of 1 min. Nevertheless, a difference between the effect of micellar H_2O_2 formulation with and without 2% lactic acid was observed. Thus, the observed enhanced antibacterial effect of mH_2O_2 seems to be due to the incorporation of the active compound (H_2O_2) into micelles and possibly an additional synergistic effect with 2% lactic acid.

Finally, the efficacy of the antimicrobially enhanced mH_2O_2 formulation against the efficacy of three commercially available biocides frequently used in the waterworks of cooling towers was tested. In this experimental setting, only the 10% dilution of mH_2O_2 (corresponding to 1.7% v/v H_2O_2) showed complete bacterial elimination of biofilm against all the test strains embedded in a biofilm after a 5-min exposure (see Figure 4).

In order to eliminate nutrient limitation and bacterial waste accumulation, the authors have established biofilms over recirculating batch culture and growth over 24 h. Since biofilms, however, may become less susceptible and provide increased protection as they mature, testing the antimicrobial efficacy of mH_2O_2 on biofilms that are 24 h old may be a limitation of the study, and future research should address the action on more mature biofilms.

In conclusion, H_2O_2 embedded in a novel plant micelle mixture showed enhanced broad-spectrum activity against bacteria in planktonic and biofilm forms, rapidly eliminating them. Moreover, low MIC, rapid bactericidal activity, degradation to non-toxic compounds and thus little possibility for triggering emergence of resistance are positive characteristics for the potentially improved application of H_2O_2 biocides. However, further biocompatibility studies will be necessary to establish the applicability of this formulation in other clinical settings (e.g. wound care or the antimicrobial coatings of medical surfaces).

Acknowledgements

The authors want to thank Mrs Doris Moser, PhD of the Department of Oral- and Maxillofacial Surgery, Medical University of Vienna, Austria for her valuable support during this study.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was supported by the research fund of the Medical University of Vienna and by Cura Solutions GmbH, Austria.

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