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## Effects of endodontic irrigants on biofilm matrix polysaccharides

Tawakoli, P N; Ragnarsson, K T; Rechenberg, D K; Mohn, D; Zehnder, Matthias

**Abstract:** AIM To specifically investigate the effect of endodontic irrigants at their clinical concentration on matrix polysaccharides of cultured biofilms. **METHODOLOGY** Saccharolytic effects of 3% H<sub>2</sub>O<sub>2</sub>, 2% chlorhexidine (CHX), 17% EDTA, 5% NaOCl, and 0.9% saline (control) were tested using agarose (1-3 and 1-4 glycosidic bonds) blocks (n = 3) in a weight assay. The irrigants were also applied to three-species biofilms (*Streptococcus mutans* UAB 159, *Streptococcus oralis* OMZ 607 and *Actinomyces oris* OMZ 745) grown anaerobically on hydroxyapatite discs (n = 6). Glycoconjugates in the matrix and total bacterial cell volumes were determined using combined Concanavalin A-/Syto 59-staining and confocal laser scanning microscopy. Volumes of each scanned area (triplicates/sample) were calculated using Imaris software. Data were compared between groups using one-way ANOVA/Tukey HSD, alpha = 0.05. **RESULTS** The weight assay revealed that NaOCl was the only irrigant under investigation capable of dissolving the agarose blocks. NaOCl eradicated stainable matrix and bacteria in cultured biofilms after 1 min of exposure (P < 0.05 compared to all groups, volumes in means ± standard deviation, 10(-3) mm(3) per 0.6 mm(2) disc; NaOCl matrix: 0.10 ± 0.08, bacteria: 0.03 ± 0.06; saline control matrix: 4.01 ± 1.14, bacteria: 11.56 ± 3.02). EDTA also appeared to have some effect on the biofilm matrix (EDTA matrix: 1.90 ± 0.33, bacteria: 9.26 ± 2.21), while H<sub>2</sub>O<sub>2</sub> and CHX merely reduced bacterial cell volumes. **CONCLUSION** Sodium hypochlorite can break glycosidic bonds. It dissolves glycoconjugates in the biofilm matrix. It also lyses bacterial cells. This article is protected by copyright. All rights reserved.

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## Effects of endodontic irrigants on biofilm matrix polysaccharides

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**Keywords:** glycosidic bond, matrix, biofilm, NaOCl

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## Abstract

**Aim** To specifically investigate the effect of endodontic irrigants at their clinical concentration on matrix polysaccharides of cultured biofilms.

**Methods** Saccharolytic effects of 3% H<sub>2</sub>O<sub>2</sub>, 2% chlorhexidine (CHX), 17% EDTA, 5% NaOCl, and 0.9% saline (control) were tested using agarose ( $\alpha$  1-3 and  $\beta$  1-4 glycosidic bonds) blocks ( $n = 3$ ) in a weight assay. The irrigants were also applied to three-species biofilms (*Streptococcus mutans* UAB 159, *Streptococcus oralis* OMZ 607 and *Actinomyces oris* OMZ 745) grown anaerobically on hydroxyapatite discs ( $n = 6$ ). Glycoconjugates in the matrix and total bacterial cell volumes were determined using combined Concanavalin A-/Syto 59-staining and confocal laser scanning microscopy. Volumes of each scanned area (triplicates/sample) were calculated using Imaris software. Data were compared between groups using one-way ANOVA/Tukey HSD,  $\alpha = 0.05$ .

**Results** The weight assay showed that NaOCl was the only irrigant under investigation capable of dissolving the agarose blocks. NaOCl eradicated stainable matrix and bacteria in cultured biofilms after 1 min of exposure ( $P < 0.05$  compared to all groups, volumes in means  $\pm$  standard deviation,  $10^{-3} \text{ mm}^3$  per  $0.6 \text{ mm}^2$  disc; NaOCl matrix:  $0.10 \pm 0.08$ , bacteria:  $0.03 \pm 0.06$ ; saline control matrix:  $4.01 \pm 1.14$ , bacteria:  $11.56 \pm 3.02$ ). EDTA also appeared to have some effect on the biofilm matrix (EDTA matrix:  $1.90 \pm 0.33$ , bacteria:  $9.26 \pm 2.21$ ), while H<sub>2</sub>O<sub>2</sub> and CHX merely reduced bacterial cell volumes.

**Conclusion** Sodium hypochlorite can break glycosidic bonds. It dissolves glycoconjugates in the biofilm matrix. It also lyses bacterial cells.

## Introduction

Apical periodontitis is a biofilm-related disease (Nair 2004). The root canal system is complex, with small anatomical intricacies that cannot be reached by instruments (Peters *et al.* 2001). Consequently, infected root canals are treated chemomechanically, i.e. by a combination of instrumentation and the physicochemical action of root canal irrigants (Grossman 1943).

In terms of their proteolytic and thus-resulting effect on soft tissues, sodium hypochlorite (NaOCl) solutions are unique amongst commonly used endodontic irrigants (Naenni *et al.* 2004). Because infected root canals contain necrotic soft tissue remnants and bacteria-related proteins, NaOCl solutions are recommended as the main agents for chemical root canal cleansing (Zehnder 2006). However, NaOCl solutions per se do not suffice to predictably clean the root canal system, as they lack an effect on inorganic debris that can be contained therein (Koskinen *et al.* 1980). Ethylenediamine tetraacetic acid (EDTA) is the most commonly used decalcifying agent used for that purpose (Hülsmann *et al.* 2003). Furthermore, a sequential application of NaOCl with other disinfectants such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) or chlorhexidine digluconate (CHX) has been suggested to induce effervescence (Al-Ali *et al.* 2012) or add antimicrobial substantivity (Zamany *et al.* 2003), respectively.

In the context of chemical root canal debridement one key element has not been studied in detail: the effect of endodontic irrigants on the biofilm matrix. Instead, studies have focused on the proportion of live versus dead bacteria in the biofilm (Chavez de Paz *et al.* 2010, Stojicic *et al.* 2013). However, research has suggested that NaOCl solutions are uniquely effective against oral biofilms, and that they physically remove the biofilm structure, which contrasts with other common disinfectants (Müller *et al.* 2007, Del Carpio-Perochena *et al.* 2011). If or how NaOCl solutions affect the biofilm matrix has not been investigated in that

context. It has been claimed that EDTA has a disruptive effect on biofilm matrix (Banin *et al.* 2006). H<sub>2</sub>O<sub>2</sub> can also reduce biofilm mass under specific experimental conditions (Hijnen *et al.* 2012), whereas CHX appears to be ineffective (Del Carpio-Perochena *et al.* 2011).

The aim of the present study was to investigate whether common endodontic irrigants at their clinical concentration can dissolve agarose, a polysaccharide linked by  $\alpha$  1-3 and  $\beta$  1-4 glycosidic bonds. These are typically found in biofilm matrices (Strathmann *et al.* 2000; Jiao *et al.* 2010), including those of oral biofilms (Bowen & Koo 2011; Yang *et al.* 2014). In general, the glucan in dental plaque is insoluble, and is mainly composed of  $\alpha$  1-3-,  $\alpha$  1-4-,  $\alpha$  1-6-linked glucose (Bowen 2015). In a second step, the effects of these irrigants on a three-species anaerobic biofilm were investigated with a focus on glycoconjugates in the biofilm matrix and the physical presence of bacteria (dead or alive) in the biofilm after treatment.

## **Material and methods**

### **Solutions**

The solutions used in the current study were obtained from commercial sources and, if necessary, diluted to their end concentrations using ultrapure water. Percent values relate to weight per volume of water. A pure 0.9% saline (NaCl) solution (B.Braun, Melsungen, Germany) was used as an isotonic and inert control solution. The 3% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) solution was prepared from an analytical-grade 30% stock solution (Perhydrol, Merck, Darmstadt, Germany), the 2% chlorhexidine digluconate (CHX) solution from a 20% counterpart (Kantonsapotheke, Zurich, Switzerland). The 17% ethylenediamine tetraacetic acid (EDTA) solution was obtained from a specialized pharmacy (CareLand, St. Gall, Switzerland). The 5% sodium hypochlorite (NaOCl) solution was prepared from an 8% stock

solution (Fisher Scientific, Loughborough, United Kingdom). Osmolarity of the solutions was measured using a freezing-point osmometer (Model 110, Fiske, Norwood, MA, USA). This instrument has a linear range between 0 and 2000 mOsm/L of water. Based on the error of the individual measurement, results were rounded to 10 mOsm/L. All the solutions here could be measured at their used concentration, with the exception of 5% NaOCl. The osmolarity of 5% NaOCl had to be extrapolated based on a 1:1 dilution series in distilled water. Dilutions from 1.25% NaOCl and below were in the linear range ( $R^2 = 0.99$ ). The pH values of the solutions were measured using a calibrated electrode (827 pH Lab, Metrohm, Herisau, Switzerland).

### **Dissolution of simulated matrix polysaccharide**

A 5% (wt/vol of distilled water) agarose gel (Standard Agarose Type LE, Bioconcept, Allschwil, Switzerland) was used for this study. The gel had a molarity of 277 mmol/L. Since D-Galactose, the monomer of agarose, is non-dissociable, the fully dissolved gel would be almost isotonic (277 mOsm/L; an isotonic solution is 290 mOsm/L). Standard cylinders were punched out from the gel with a diameter of 14 mm and a height of 12 mm (Fig. 1). The cylinders were weighed in a precision balance (AT261, Mettler Toledo, Greifensee, Switzerland). Subsequently, they were immersed in 15 mL of the following solutions: 2% (w/v) CHX, 17% EDTA, 3% H<sub>2</sub>O<sub>2</sub>, 5% NaOCl, or 0.9% NaCl (saline) as the control treatment ( $n = 3$ , each). Immersion occurred without any agitation at room temperature (25°C) for 20 h. The agarose cylinders were then separated from the irrigants and washed with copious amounts of distilled water in a Buchner filter funnel containing a matching round filter paper (Blue Ribbon, pore size < 2 µm, Faust, Schaffhausen, Switzerland). The funnel was mounted on a flask connected to a vacuum pump. The filter paper had been moisturized in the washing apparatus and pre-weighed in the precision balance (AT 261, Mettler-Toledo).

After the washing step, the remaining weight of the agarose cylinders on the pre-weighed filter was determined. The outcome variable was the end weight in percent, i.e. the weight of the specimens after immersion divided by their weight before immersion multiplied by 100.

### **Biofilm formation**

A robust three-species biofilm model was used, which was validated in previous studies (Tawakoli *et al.* 2015). Bacterial strains were obtained from the Institute of Oral Biology, Section of Oral Microbiology and General Immunology, University of Zurich. Prior to biofilm formation, each strain (*Streptococcus mutans* OMZ 918, *Streptococcus oralis* OMZ 607, and *Actinomyces oris* (formerly known as *A. naeslundii*, OMZ 745) was propagated in culture media composed of 30% saliva supernatant solution and 70% modified fluid universal medium (Gmür & Guggenheim 1983) on a platform shaker at 37° under anaerobic conditions using gas packs (GENbox anaer and GENbag anaer, bioMérieux, Marci l'Etoile, France). The presence of *S. oralis* and *A. oris/naeslundii* was shown to influence *S. mutans* within the biofilm to increase the expression of matrix associated genes, such as for glucan synthesis and remodeling (gtfBC, dexA), and glucan-binding (gbpB) (Klein *et al.* 2012; Xiao *et al.* 2012). Fresh saliva was obtained from one healthy donor. To obtain saliva supernatant solution, the saliva was centrifuged two times for 30 min at 13400 rpm and the supernatant was diluted 1:2 in isotonic saline (0.9% NaCl, B.Braun) prior to sterile filtration (TPP syringe filters with 0.2 µm pores, Faust). After 7 h the bacterial solutions were adjusted to the optical density (OD<sub>550</sub>) of 1 and mixed in a tube as inoculum. To quantify the inocula per mL, 50 µL of each bacterial suspension were plated out on Columbia sheep blood agar plates (CSBA, bioMérieux) and incubated anaerobically for 2 d.

Thirty sterile sintered hydroxyapatite discs (Ø 9 mm, Clarkson Chromatography Products,

South Williamsport, PA, USA) were used in this study to facilitate a standardization of biofilms. The discs were incubated in 800  $\mu$ L of saliva supernatant solution (described above) for 4 h under gentle agitation (100 rpm at room temperature) to form a pellicle. For biofilm formation, pellicle-coated discs were then placed in 24-well polystyrene cell culture plates and incubated anaerobically with 1 mL of the prepared inocula under gentle agitation at 37°C for 7 d. Media were changed twice a day by transferring the specimens into new well plates containing fresh media. The transferring step accounted also as a dipping step to enhance bacterial adherence within biofilms. pH was controlled daily in the overnight medium using a calibrated pH meter (Easy Five, Mettler Toledo).

### **Treatment and staining of biofilms**

After 7 d, specimens were washed in phosphate-buffered saline (PBS, Invitrogen, Carlsbad, CA, USA; 3 washing cycles) to remove non-attached and loosely bound bacteria from the specimens. Specimens ( $n = 6$ , each) were then immersed passively for 1 min in 0.9% saline (NaCl) as the control treatment, 3%  $H_2O_2$ , 2% CHX, 17% EDTA or 5% NaOCl. Subsequently, specimens were washed in PBS and immediately fixed in 4% paraformaldehyde for 60 min at room temperature in the dark. Then, specimens were washed in PBS again and double-stained with Concanavalin A (ConA) conjugated FITC (Sigma-Aldrich, Buchs, Switzerland) and Syto 59 (Life Technologies, Zug, Switzerland). Briefly, aliquots of ConA stock-solution were prepared of 10 mg ConA FITC with 2 mL of 0.1 M sodium hydrogen phosphate and stored at -20 °C. Before use, the stock solution was centrifuged and the supernatant was added to a buffer, consisting of 1 mM  $CaCl_2$ , 1 mM  $MgCl_2$  and 1 mM  $MnCl_2$  in PBS (10  $\mu$ L stock solution/490  $\mu$ L buffer). The final staining solution consisted of 1  $\mu$ L of Syto 59 stock solution (5 mM in dimethyl sulfoxide) and 1000



μL of the ConA /buffer solution (Syto 59 end concentration of 5 μM). Each specimen was incubated in 500 μL staining solution for 15 min at room temperature in the dark. Subsequently, specimens were washed gently with PBS and mounted upside down on chamber slides using 10 μL Mowiol 4-88 (Sigma-Aldrich) for 6 h in the dark.

### **Confocal laser scanning microscopy (CLSM)**

Images were taken with a confocal laser-scanning microscope (SP5, Leica Microsystems, Heidelberg GmbH, Germany) using a 20x objective (numerical aperture: 1.25). For ConA FITC excitation an Argon laser at 488 nm wavelength was used and the fluorescence emission detected using a photomultiplier with a spectral detector between 510 – 540 nm. Matrix exopolysaccharides stained with ConA exhibited green fluorescence. For Syto 59 a 561 nm Helium laser was applied and emission also detected with a photomultiplier at 630 – 660 nm. All bacterial species labeled with Syto 59 fluoresced in a deep red. Each disc was examined at three random areas (triplicates/specimen each 0.6 mm<sup>2</sup> surface) with z step sizes of approximately 1 μm. The z step size stands for the third dimension and is a prerequisite to calculate the volume. The pixel size was 1.52 μm (512 x 512 pixels). A six-line average mode was used for image acquisition. The matrix and bacterial cell volumes were calculated using Imaris Software 7.7.2 (Bitplane AG, Zurich, Switzerland). The total volume of green (biofilm matrix) and red fluorescence (bacterial cells) of each scanned area was calculated in 10<sup>-3</sup> mm<sup>3</sup> and averaged per disc.

### **Data Presentation and analysis**

Data pertaining to agarose weight, matrix and bacteria volume were evenly distributed (Shapiro-Wilk test). Mean values per specimen were thus compared between groups using

one-way ANOVA/ Tukey HSD,  $\alpha = 0.05$ . For the biofilm-related measurements, averaged data per disc (see previous subchapter) were taken to calculate mean values for each group ( $n = 6$ ). Data are presented as means  $\pm$  standard deviation.

## Results

The solutions under investigation had a wide range in pH and osmolarity (Table 1). The 5% NaOCl and the 17% EDTA solutions were alkaline and hypertonic, whilst the 2% CHX was hypotonic (an isotonic solution has an osmolarity of 290 mOsm/kg). In the agarose weight assay, immersion for 20 h in the physiological saline solution, which was used as an inert control, resulted in an end weight of the agarose blocks of  $102 \pm 1\%$ . The end weight of these specimens did not differ significantly ( $P > 0.05$ ) from those immersed in  $H_2O_2$  ( $102 \pm 1\%$ ) or CHX ( $101 \pm 3\%$ ). EDTA caused a significant weight increase with an end weight of  $113 \pm 5\%$  ( $P < 0.05$  compared to all other groups). In the NaOCl solution, the agarose gel blocks were dissolved almost entirely, with an end weight of merely  $2 \pm 2\%$ . This effect could be appreciated visually (Fig. 1).

Glycoconjugates in the biofilm matrix were stained with Concanavalin A (488/500) and fluoresced in a green color. Living and dead bacteria were stained with Syto 59 (561/635) and appeared in deep red. Both images are shown in separate channels and combined in an overlay (Fig. 2). Measured volumes were calculated separately for each channel using Imaris software. The biofilm matrix volume of the  $H_2O_2$  and CHX groups did not differ significantly ( $P > 0.05$ ) from the matrix volume of the saline control treatment, while bacteria volume was significantly reduced (Table 2). Matrix volume of the EDTA group was significantly ( $P <$

0.05) reduced compared to CHX, H<sub>2</sub>O<sub>2</sub>, and the saline control, yet significantly higher than in specimens exposed to NaOCl (Table 2). NaOCl treated specimens showed significantly ( $P < 0.05$ ) lower matrix and bacteria volumes compared all other groups. The biofilm structure was almost entirely dissolved after 1 min of exposure to 5% NaOCl (Fig. 2).

## **Discussion**

Anti-biofilm properties of root canals irrigants are of core clinical interest. This study showed that in addition to its other oxidative properties, sodium hypochlorite is able to break glycosidic bonds. The NaOCl solution was unique among the currently tested endodontic irrigants in its combined effect on bacterial cells (regardless of their viability status) and extracellular polysaccharides/glycoconjugates.

This study highlighted a potentially important advantage of NaOCl solutions over other endodontic irrigants. However, this being a laboratory study, the current findings are merely basic, and no direct clinical conclusions should be drawn. The solutions investigated in this study are used for a vast array of applications and thus have been tested under various conditions (McDonnell & Russell 1999). Results can differ considerably. Nevertheless, it has been established that OCl<sup>-</sup> can cleave peptide and glycosidic bonds (Baker 1947), and promote fatty acid peroxidation (Carlin & Djursäter 1988). Biofilm matrix consists of extracellular polymeric substances (EPS), such as extracellular polysaccharides, proteins, nucleic acids, lipids and other biopolymers (Flemming & Wingender 2010). The core components of the EPS are exopolysaccharides, consisting of monosaccharides linked by glycosidic bonds (Bowen & Koo 2011). The weight assay used in this study to investigate

which of the irrigants cleaved  $\alpha$  1-3 and  $\beta$  1-4 glycosidic bonds, is simple yet reliable. It was used because concentrated irrigants with vastly different chemistry were assessed. Solutions such as NaOCl at their clinical concentration are so salty and reactive that they interfere with standard assays, such as the Galactose Assay Kit (abcam, Cambridge, UK), which oxidises galactose to produce colour. EDTA must not be present in this type of assay either. Weight assays, on the other hand, are less error-prone, and can thus be used to study such basic chemical effects of endodontic irrigants (Naenni *et al.* 2004). Instead of analysing the galactose that is dissolved, the current assay measured the weight of the remaining agarose. However, interpretation of the results is not free of doubt, and should be interpreted with care. While the current agarose assay clearly demonstrated a saccharolytic effect of the 5% NaOCl solution (Fig. 1), results obtained with 17% EDTA were less clear. Agarose is the polymer of D-Galactose. To dissolve agarose, the glycosidic bonds between its monomers need to be cleaved. It is more than unlikely that EDTA breaks glycosidic bonds, as it is a component of common agarose gels used for electrophoresis (Meyers *et al.* 1976). Instead, the effect of EDTA on biofilms has been shown to be related to the binding of divalent cations, thus dispersing bacterial cells from the matrix (Banin *et al.* 2006). In the current weight assay, the increase in sample weight had to be due to some binding effect of EDTA to the agarose, resulting in water uptake into the specimen. This water uptake had to occur during the washing step, because EDTA in the current concentration is hypertonic (Table 1) and thus will draw water from the agarose gel. Converse results have been reported with soft tissue samples, in which EDTA caused some weight loss (Grawehr *et al.* 2003). Nevertheless, EDTA as the sole irrigant except for NaOCl had some effect on the simulated matrix polysaccharide. This was confirmed in the second leg of this study, when NaOCl and EDTA but not H<sub>2</sub>O<sub>2</sub> and CHX reduced the matrix volume of the three-species biofilm. The effects of

the irrigants under investigation on bacterial volume as assessed by DNA/RNA staining (see below), on the other hand, appear to be related to various features of these irrigants, such as their ability to interfere with microbial life. Yet again, NaOCl was unique in that it apparently lysed the bacteria (Fig. 1). This is in line with a published report on the effects of endodontic irrigants on in-situ biofilms stained with acridine orange (Del Carpio-Perochena *et al.* 2011). In that study, a 2% CHX solution did not remove biofilm from dentine specimens, while NaOCl at concentrations of 1%, 2.5%, and 5.25% cleaned the contaminated dentine completely after 30 min of exposure.

Fluorescent staining procedures using CLSM enable a volumetric analysis of specific microenvironments within the biofilm (Heydorn *et al.*, 2000; Staudt *et al.* 2004; Chàvez de Paz 2009). Several endodontic studies used live/dead staining with CLSM to analyze bacterial viability within biofilms (Chavez de Paz *et al.* 2010, Stojicic *et al.* 2013). Most live/dead stains differentiate bacteria into viable or dead by means of their membrane integrity (Tawakoli *et al.* 2013). Calculations on the total biovolume of live and dead bacteria run the risk of an overestimation due to an overlapping of the double-stained bacteria. However, conclusions based on viable bacteria alone do not sufficiently represent treatment efficacies at all. In the present study, a different stain was used to assess the total cell volume. Syto 59 is a nucleic acid stain with DNA/RNA selectivity and binding affinity. It is not a stain that illustrates bacterial viability, which was not a target outcome of the current investigation. Instead, all bacterial cells were highlighted. Lectin staining with Con A was used to quantify matrix exopolysaccharides. This type of lectin represents a marker for glycoconjugates, which are major components of the extracellular polysaccharides, and bind selectively to  $\alpha$ -glucopyranosyl molecules such as glucans (Gupta *et al.* 1996).

Within the limitation of this study, it appeared that NaOCl has the unique property amongst

common endodontic irrigants in that it chemically reduces biofilm-related matter. In terms of biofilm matrix reduction, this property appears to be linked to its saccharolytic properties. It would appear that not only the high reduction-oxidation potential of endodontic NaOCl solutions, but also their high pH contribute to their saccharolytic effect (Hotz *et al.* 1972). On a similar note, alkaline capacity of NaOCl solutions can contribute to their proteolytic effect (Jungbluth *et al.* 2011). The specific effects of pH, alkaline capacity, and concentration on the antibiofilm properties of NaOCl solutions need to be singled out in future studies. Furthermore, it would be interesting to know whether the alternating usage of a sodium hypochlorite with a chelating agent such as EDTA would expedite the antibiofilm effects of the single components.

## **Conclusions**

Under the conditions of the current study, sodium hypochlorite displayed some unique features among the tested endodontic irrigants at their clinical concentration. The NaOCl solution broke glycosidic bonds. It dissolved glycoconjugates in the biofilm matrix. It also lysed bacterial cells.

## **Acknowledgments**

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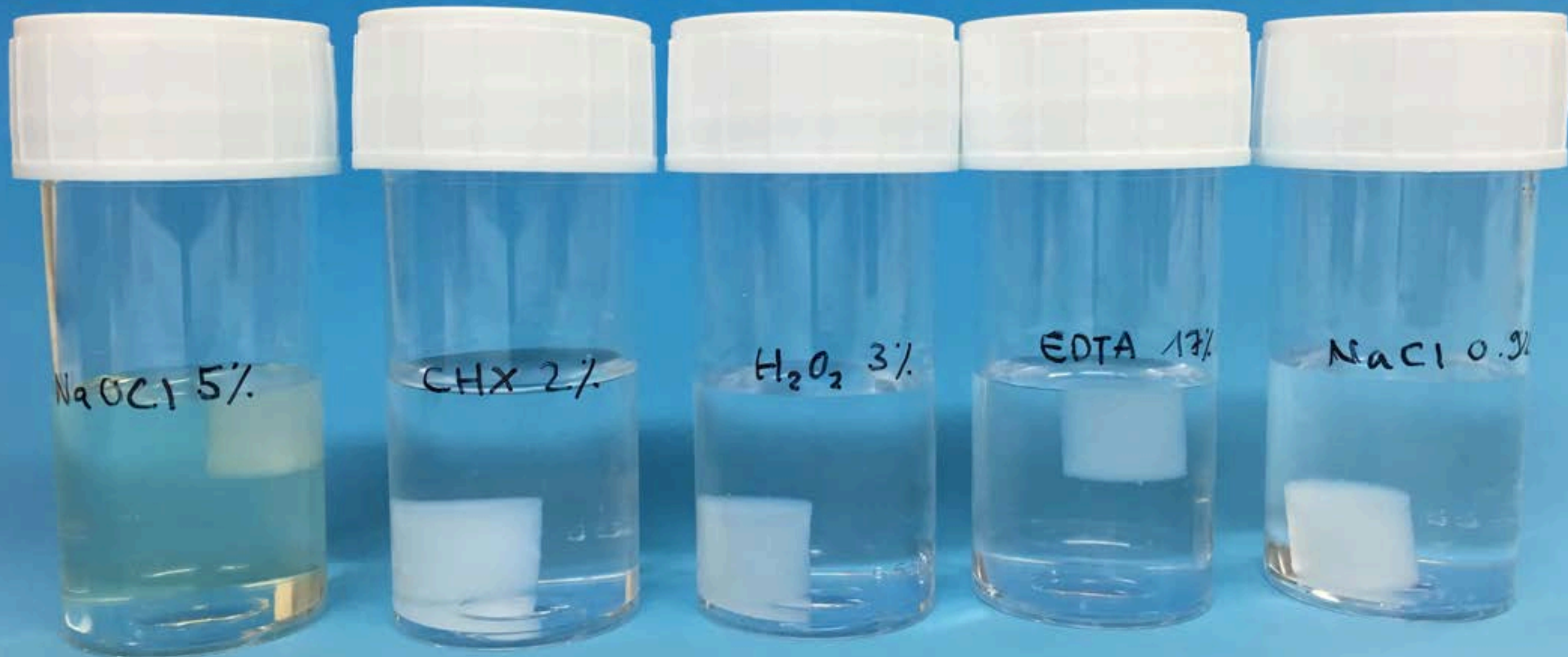
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## Figure Caption

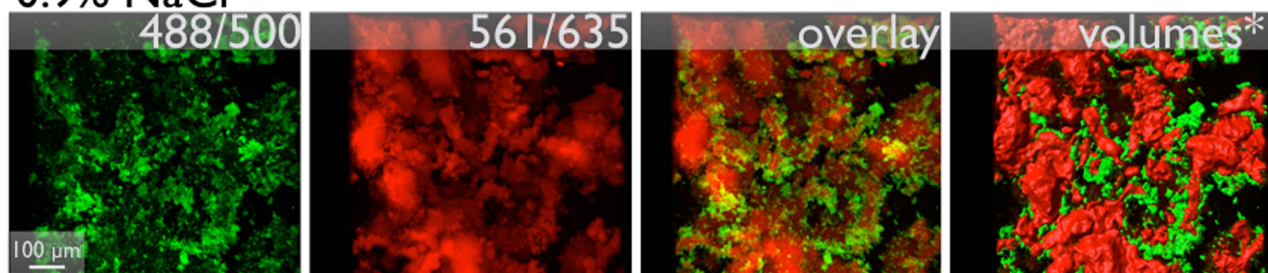
**Figure 1** Immersion of the standard agarose cylinders in the different solutions. Upper panel depicts situation immediately after adding 15 mL of the irrigating solutions. Seeming differences in specimen size are due to distortion effects of the cylindrical vials. Note that in the hypertonic solutions (5% NaOCl and 17% EDTA) the agarose cylinders had a tendency to swim, whilst they sank in hypotonic solutions (2% CHX and 3% H<sub>2</sub>O<sub>2</sub>). The lower panel depicts the same specimens after 20 h of non-agitated immersion. The 5% NaOCl solution completely dissolved the agarose, whilst the other solutions caused no visible effect other than that the specimen immersed in 17% EDTA sank to the bottom, suggesting an interaction between EDTA and the agarose.

**Figure 2** Appearance of the biofilms on the hydroxyapatite discs in CLSM images after cultivation for 7 d, exposure to test and control irrigants, and subsequent staining with Concanavalin A and Syto 59. Biofilm matrix (Concanavalin A staining, 488/500) and living and dead bacteria (Syto 59 staining, 561/635) are shown separately in original images and combined in an overlay. Measured volumes\* were calculated using Imaris software and averaged per disc. These values were used for statistical analysis.

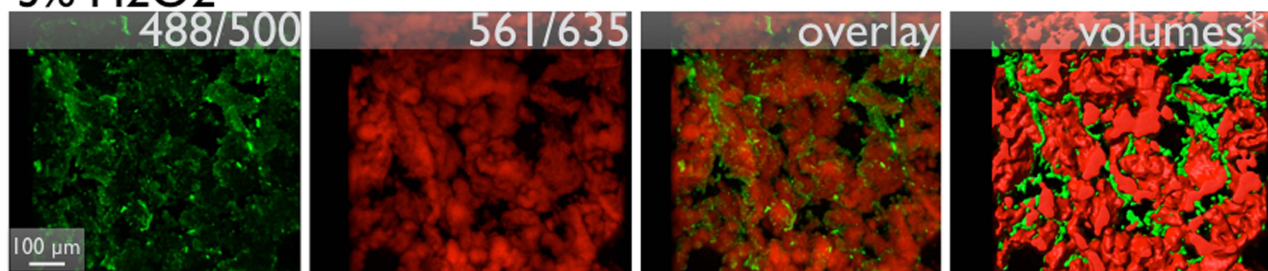




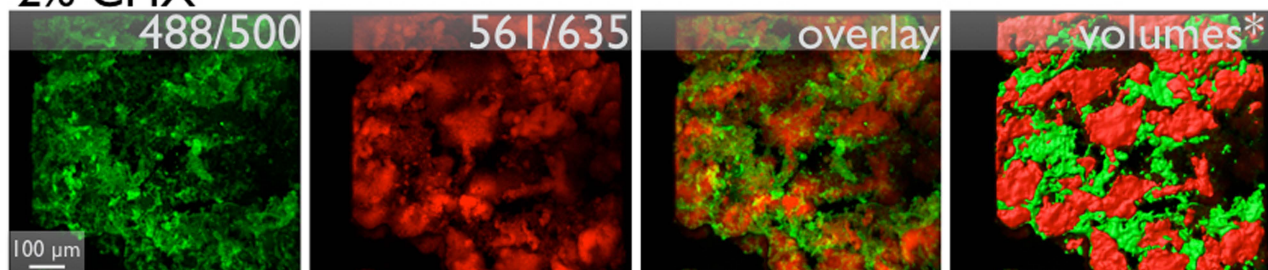
0.9% NaCl



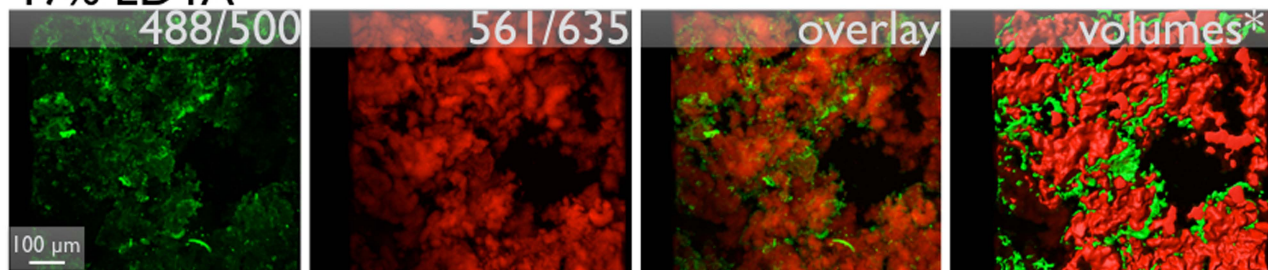
3% H<sub>2</sub>O<sub>2</sub>



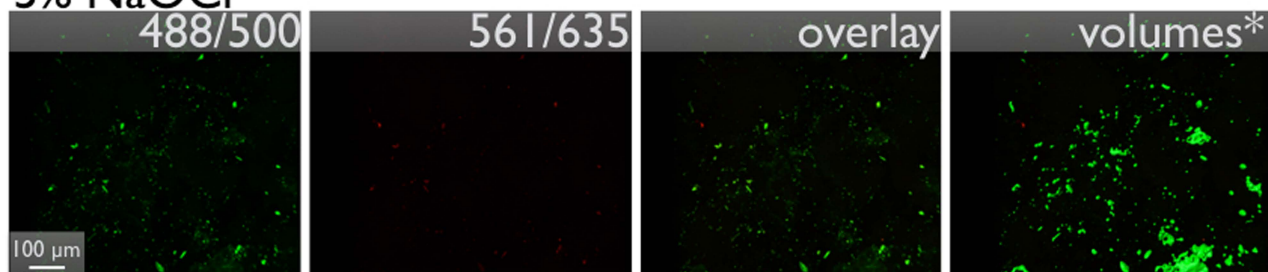
2% CHX



17% EDTA



5% NaOCl



**Table 1** Chemical specification of the irrigants used in this study. Mean values of triplicate measurements are presented, rounded according to the individual error of measurement

Irrigant	pH	Osmolarity* (mOsm/L)
0.9% NaCl (inert control solution)	5.0	290
3% H <sub>2</sub> O <sub>2</sub>	5.0	940
2% CHX	6.9	70
17% EDTA	8.0	1740
5% NaOCl	12.3	6920

\*Osmolarity was measured in the linear range of the assay. The value for 5% NaOCl was beyond the upper detection limit of the assay (2000 mOsm); it had to be extrapolated from the corresponding dilution series in pure water.

**Table 2** Volumes (means  $\pm$  SD,  $10^{-3}$  mm<sup>3</sup>) per 0.6 mm<sup>2</sup> hydroxyapatite disc ( $n = 6$ ) surface after 1 min of exposure to irrigant

Irrigant	Concanavalin A	Syto 59
0.9% NaCl (positive control)	$4.01 \pm 1.14^A$	$11.56 \pm 3.02^A$
3% H <sub>2</sub> O <sub>2</sub>	$3.87 \pm 0.91^A$	$8.05 \pm 1.49^B$
2% CHX	$3.32 \pm 1.13^{AB}$	$6.86 \pm 1.02^B$
17% EDTA	$1.90 \pm 0.33^B$	$9.26 \pm 2.21^{AB}$
5% NaOCl	$0.10 \pm 0.08^C$	$0.03 \pm 0.06^C$

Concanavalin A stained carbohydrates (biofilm matrix) and Syto 59 stained live and dead bacteria. Volumes were calculated using Imaris software.

Identical superscript letters indicate that there was no significant difference ( $P < .05$ , ANOVA and Tukey's HSD) for this measure between respective solutions.