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# Red fluorescence of dental biofilm as an indicator for assessing the efficacy of antimicrobials

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**Abstract.** The study aimed to determine whether the red fluorescence (RF) of a dental microcosm biofilm as measured with quantitative light-induced fluorescence (QLF) technology is useful for assessing the efficacy of antimicrobials. Dental microcosm biofilms were formed on bovine enamel discs and grown under 0.3% sucrose challenge and treated with chlorhexidine (CHX) solutions at different concentrations (0.05%, 0.1%, and 0.5%) plus a negative control [sterile distilled water (DW)] twice daily for 7 days. The biofilms were photographed using a QLF-digital system to evaluate the RF by calculating the red/green ratio, and pH values of the medium were measured daily. After 7 days, the bacterial viability of the biofilm was assessed by measuring the counts of viable total bacteria and aciduric bacteria, and the percentage surface microhardness changes (%SHC) was evaluated. The RF and cariogenic properties were compared for the different concentrations of CHX, and their correlations were examined. The RF and its increase rate were much lower for CHX-treated biofilms than for DW-treated biofilms. The RF after 7 days of maturation decreased significantly with increasing CHX concentrations ( $p < 0.001$ ) and was from 31% (for 0.05% CHX) to 46% (for 0.5% CHX) lower than that of the DW group. Strong correlations were reported between the RF of the 7-day-maturation biofilms and cariogenic properties, such as the number of total bacteria ( $r = 0.93$ ), number of aciduric bacteria ( $r = 0.97$ ), supernatant pH ( $r = 0.43$ ), and %SHC ( $r = 0.98$ ). In conclusion, the RF of dental biofilms as measured with QLF technology can be used to nondestructively assess and monitor the effect of antimicrobials against biofilm. © 2018 Society of Photo-Optical Instrumentation Engineers (SPIE) [DOI: 10.1117/1.JBO.23.1.015003]

Keywords: antimicrobials; dental biofilm; quantitative light-induced fluorescence; red fluorescence.

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## 1 Introduction

The dental biofilm is a type of microbial film formed on the surface of teeth that represents a diverse microbial community.<sup>1</sup> The microbiota shift as the biofilm changes from a health state to pathogenic states through interaction with bacterial activity and environmental conditions, resulting in the biofilm becoming pathogenic.<sup>2,3</sup> The degree of metabolic activity depends on the level of biofilm pathogenicity, which induces bacteria-mediated oral diseases, such as dental caries and periodontitis and influences their severity.<sup>4</sup> The frequent intake of fermentable carbohydrates increases acidogenic and aciduric species within the dental biofilm. These shifts in microflora and their metabolism induce a cariogenic biofilm and the initiation of dental caries.<sup>4,5</sup> Therefore, we need to focus on the detection and inhibition of the biofilm cariogenicity rather than a single specific pathogen for prevention of dental caries.

Recent studies have investigated red autofluorescence of pathogenic biofilms and its relationships with biofilm properties. It has been reported that oral biofilms formed on the teeth or tongue surface can be observed as red fluorescence (RF) using quantitative light-induced fluorescence (QLF) technology that uses a narrowband blue light source (centered at 405 nm) obtained by modifying the filter set.<sup>6,7</sup> This phenomenon of autofluorescence is known to be caused by bacterial

metabolites, such as an endogenous metal-free fluorescent porphyrin.<sup>8,9</sup> The porphyrin derivatives have previously been held responsible for fluorescence emission in the red spectral region of dental biofilm and caries lesions when excited with 405 nm violet light.<sup>10</sup> The porphyrin concentration is high in Gram-negative oral bacteria, which indicates the observed RF was produced by mature and pathogenic biofilms. Also, previous studies have reported that the intensity of RF increased with the biofilm maturation and its pathogenicity.<sup>8,11,12</sup>

Previous *in vitro* and *in vivo* studies reported that the biofilm fluorescence as observed using the QLF technology is related to pathogenicity associated with the risk of dental diseases, such as caries, gingivitis, and oral malodor.<sup>11-14</sup> Biofilm-related RF as observed in the oral cavity was reported to be due to mature biofilm, and hence the RF can be considered an indicator of oral hygiene in the clinical situation.<sup>15</sup> In addition, correlations between the RF of the biofilm and inflammatory response were reported in a recent clinical study, which indicates that the RF is related to the gingivitis and periodontitis, as well as dental caries.<sup>13</sup>

Recent studies have reported that the RF emitted from a dental microcosm biofilm to be significantly associated with its cariogenic properties, suggesting that biofilm fluorescence can be used to identify high-risk areas with a high cariogenic potential in the oral cavity.<sup>11</sup> It has been shown that the RF intensity of the

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microcosm biofilm grown in the presence of a cariogenic challenge increases with its maturation as well as its cariogenicity, based on experiments involving different sucrose concentrations and frequencies of application.<sup>12,16</sup> Previous studies have focused on the changes and differences in the RF induced by applying the factors that are pathogenic to a biofilm but none of them have evaluated the RF response after exposure to factors that inhibit the pathogenicity to the biofilm or have determined the effect of inhibiting factors on the RF of oral biofilms.

While various antimicrobial agents have been widely used to prevent dental diseases, no methods were previously available to assess not only their efficacy on biofilm formation but also its pathogenic activity nondestructively. A better approach to study the effects of antimicrobial treatments on microcosm biofilms would be based on bacterial interactions rather than simply identifying the quantity of specific causative bacteria, because biofilm pathogenicity is determined by the bacterial metabolism of the entire bacterial community in the biofilm.<sup>17</sup> In addition, existing microscopic techniques such as confocal laser scanning microscopy and scanning electron microscopy can focus on the physiological properties of the biofilm, but they do not assess the pathogenic properties of the entire biofilm. For these reasons, it is necessary to use an assessment tool for monitoring changes in pathogenicity of the biofilms nondestructively after applying sequential treatments with agents acting against cariogenic biofilm formation. If the RF as an indicator of biofilm assessment could be used to monitor the efficacy of antimicrobial agents, the effect of specific antimicrobials on a dental biofilm could be predicted noninvasively and monitored continuously by simply evaluating the RF of the biofilm.

The aims of this study were to measure the RF responses of dental microcosm biofilms to chlorhexidine (CHX), which is still considered a gold-standard antimicrobial agent for prevention of oral diseases, and to determine the correlation between the RF and biofilm properties by comparing treatments with CHX at various concentrations in the presence of cariogenic challenges.

## 2 Materials and Methods

### 2.1 Experimental Design

Dental microcosm biofilms were formed using the model described in detail by Lee et al.<sup>11</sup> In brief, the model applied basal medium mucin (BMM) supplemented with sucrose to induce cariogenic challenges. Using enamel specimens, the model is used to assess the effects of different treatment on enamel caries. In this study, the BMM with 0.3% sucrose was used as a growth medium to produce the optimal cariogenic biofilm for the assessment of anticariogenic activity of antimicrobials based on the results of preliminary study. To form an

environment with different antimicrobial levels, the biofilms were treated with CHX solutions at different concentrations (0.05%, 0.1%, and 0.5%) plus a negative control [sterile distilled water (DW)] twice daily for 7 days after 24 h of incubation (Fig. 1). The microcosm biofilm that formed on enamel specimens was photographed using a quantitative light-induced fluorescence-digital (QLF-D Biluminator™, Inspektor Research Systems BV, Amsterdam, the Netherlands) daily to obtain fluorescence images that were used to assess the RF, as described previously.<sup>11</sup> The supernatant pH was also measured on each day before replacing the growth medium. At 7 days after inoculation, the biofilms were collected to determine the total and aciduric bacteria counts, and the supernatant pH was measured to analyze their cariogenic properties. The changes in surface hardness of the enamel discs were evaluated by calculating the percentage surface microhardness change (%SHC). The experimental procedures are summarized in Fig. 1 briefly.

### 2.2 Enamel Specimen Preparation

Enamel discs (8 mm in diameter and ×3 mm thick) were obtained from the labial surface of bovine incisors. The discs were ground in a wet state using water-cooled abrasive sand papers (600, 800, 1000, and 1200 grit) to obtain plane-parallel surface, and they were embedded in circular acrylic molds at 1 mm below the surface to allow biofilm accumulation. Prior to the biofilm formation, the baseline enamel surface hardness [quantified as the Vickers microhardness number ( $VHN_{\text{baseline}}$ )] of each enamel disc was measured by making four indentations (200 g, 10 s; JT Toshi, Tokyo, Japan) at the center of the surface and were spaced 100 μm from each other. The mean value of the four measurements was calculated for each specimen as the baseline hardness.

### 2.3 Dental Microcosm Biofilm Formation

A microcosm biofilm model was used as described previously.<sup>11</sup> Human stimulated saliva was used as an inoculum to produce microcosm biofilms. The fresh saliva was collected from a healthy female donor, who had abstained from performing oral hygiene for 24 h before saliva collection. The donor had no active caries, periodontal diseases, or/and no history of antibiotic use within the previous 3 months. Ethical approval for the collection of human saliva was granted by the Ethics Committee of Yonsei Dental Hospital, Korea (approval number 02-2015-0051). The collected saliva was filtered through sterilized glass wool (Duksan Chemicals, Ansan, Korea) and then diluted in sterile glycerol to a final concentration of 30%. The final glycerol stock was stored at -80°C, and the same batch of frozen saliva was used as an inoculum for each set of experiments. The prepared saliva (1.5 ml) was inoculated onto each specimen

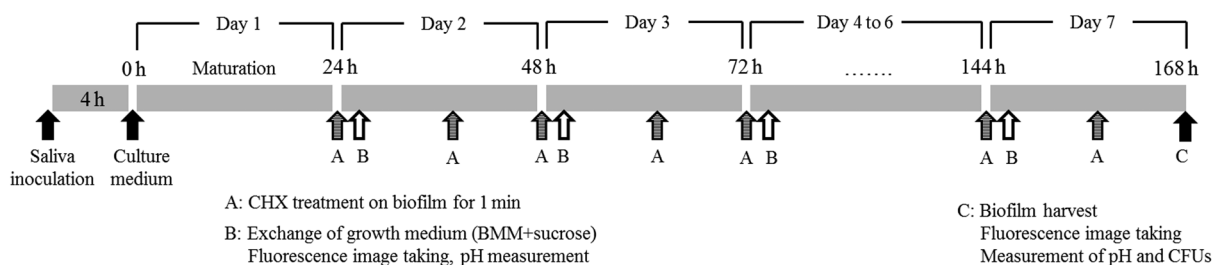


Fig. 1 Flow diagram of the experimental procedures.

in wells of 24-well cell culture plates, and the plates were incubated anaerobically at 37°C for 4 h. The inoculated saliva was gently aspirated from the bottom of each well, and 1.5 ml of growth medium that contained 0.3% sucrose and BMM was added to the well. The growth medium was replaced daily by placing the enamel block in a new well containing fresh medium after the first CHX treatment performed on each day (Fig. 1). The biofilms were incubated in an anaerobic condition under an atmosphere of 80% N<sub>2</sub>, 10% CO<sub>2</sub>, and 10% H<sub>2</sub> for up to 7 days.

## 2.4 Chlorhexidine Treatment

The biofilms were first treated with CHX at 24 h after the initial biofilm formation, with CHX treatment then continued for up to 7 days (Fig. 1). The biofilms that formed on the enamel discs were immersed in a new plate containing 1.5 ml of CHX solutions (0.05%, 0.1%, and 0.5%) and DW (negative control) twice daily for 1 min. The CHX concentrations were determined within the range of the proven efficacy *in vivo* against biofilm (from 0.02% to 2%).<sup>18,19</sup> Since the efficacy of 0.1% to 0.2% CHX mouth rinses in the prevention of dental biofilm has been widely accepted, the 0.1% CHX was selected and then determined to use 0.05% (lower than 0.1% CHX) and 0.5% (higher than 0.1% CHX) so as to confirm the dose-response effects on the dental biofilm. After each treatment, the discs were rinsed for 1 min with cysteine peptone water (CPW) to remove excess CHX solution before being returned to the growth medium.

## 2.5 Fluorescence Image Acquisition and Image Analysis

The QLF-D Biluminator™ system was used to obtain fluorescence images each day of the biofilms that formed on the specimens (from 1 day to 7 days after saliva inoculation). This system is equipped with a “Live View”-enabled digital full-sensor single-lens reflex camera (model 550D, Canon, Tokyo, Japan) with blue and white LED lights with a peak wavelength of 405 ± 7 nm and modified filter set (D007; Inspektor Research Systems, the Netherlands). The QLF-D images were captured at the following settings: shutter speed of 1/60 s, aperture value of 7.1, and ISO of 1600 while ensuring that the distance between the light sources and biofilms was consistent. For the image analysis, an area of interest (AOI) was drawn on the same area of biofilms in the fluorescence images, and then red and green values for every pixel within the AOI were obtained using image analysis software (Image-Pro PLUS 6.0, Media Cybernetics, Rockville, Maryland). The average red and green ratio (R/G value) for every pixel was calculated for each fluorescence image to quantify RF intensities of the biofilm. The R/G value was used to evaluate the changes in RF over time and for different CHX concentrations. The procedures used to obtain and analyze the images have been described in detail elsewhere.<sup>11</sup>

## 2.6 Microbial Composition of a Dental Microcosm Biofilm

After allowing biofilm maturation with CHX treatments applied for 7 days after inoculation, the enamel blocks with biofilms were rinsed in 1 ml of CPW to remove loose bacteria, and the biofilms were transferred into tubes with 2 ml of CPW. The biofilms were harvested by 1 min of sonication and

vortexing for 1 min. The resulting bacterial suspensions were then serially diluted in CPW and plated in duplicate on a 5% tryptic soy blood agar plate to determine the total bacterial counts. The suspensions were also plated onto a brain heart infusion agar plate adjusted to pH 4.8 for aciduric bacteria. The numbers of colony-forming units (CFUs) of total and aciduric bacteria were determined after the plates were incubated anaerobically at 37°C for 72 h.

## 2.7 Biofilm Acidogenicity

The medium collected daily from each well (before replacing the specimens into the fresh medium) was analyzed for biofilm acidogenicity as a pH value. The supernatant pH was determined using a pH electrode (Orion 4-Star, Thermo Scientific, Waltham, Massachusetts) to measure the acidogenicity of the biofilms.

## 2.8 Surface Microhardness Changes of Enamel Specimen

After the 7 day-maturation, biofilms were harvested from the enamel blocks, and their surface microhardness was measured by making four indentations (200 g, 10 s) separated by 100 μm before (VHN<sub>baseline</sub>) and after (VHN<sub>after</sub>) each experimental phase as described in Sec. 2.2. The changes in surface hardness of enamel were expressed as %SHC = 100[(VHN<sub>baseline</sub> - VHN<sub>after</sub>)/VHN<sub>baseline</sub>]. The procedure was carried out by a single blinded and trained examiner.

## 2.9 Statistical Analysis

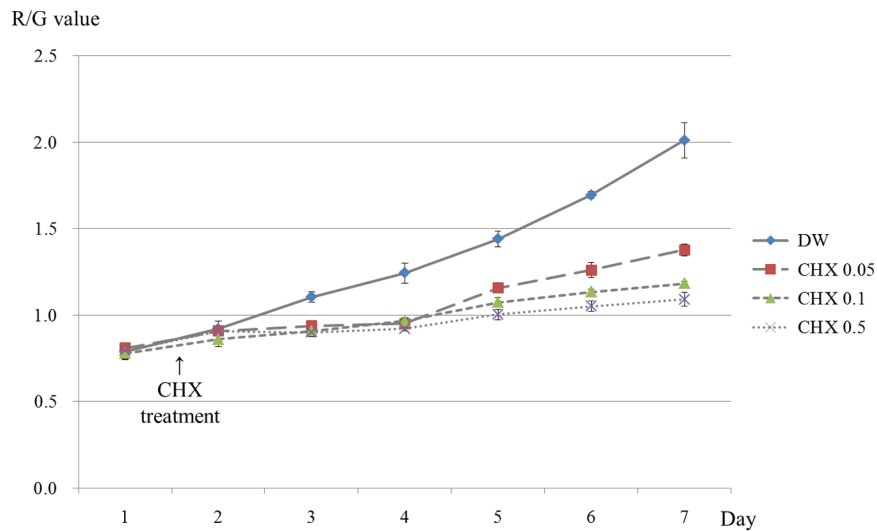
The variations of the measured variables of the microcosm biofilms were analyzed according to were analyzed according to the CHX concentrations [0% (i.e., DW), 0.05%, 0.1%, and 0.5%], using one-way analysis of variance, and Tukey's *post-hoc* test to identify statistically significant differences between the groups. Also, the correlations of the RF intensity of CHX-treated biofilms with the cariogenic properties (total bacteria CFUs, aciduric bacteria CFUs, supernatant pH, and %SHC) were assessed using Pearson's correlation test. All statistical analyses were performed using PASW Statistics (version 20.0 SPSS, IBM Corporation, Somers, New York), and the cutoff for significance was set at  $\alpha = 0.05$ .

## 3 Results

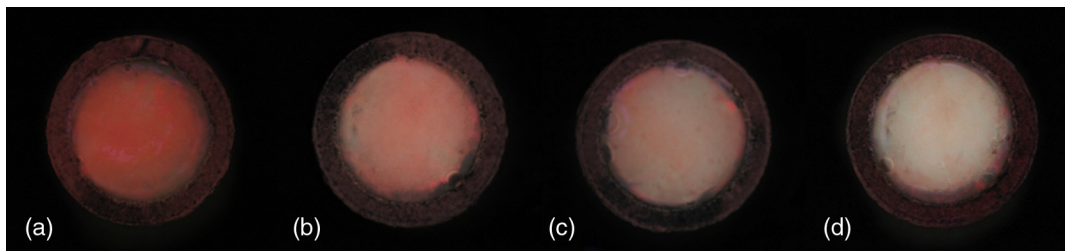
The DW-treated biofilm started to emit RF (R/G value = 1.16) at 3 days after the inoculation, whereas the CHX-treated groups first exhibited RF after 5 days and their RF intensity consistently increased over time (Fig. 2). The increase rate of RF of CHX-treated biofilms was slower than that of DW-treated biofilms during the maturation period. Regardless of the CHX exposure and its concentration, the R/G value increased with the maturation period (Fig. 2).

Figure 3 shows fluorescence images of 7-day mature biofilms for different concentrations of CHX, which indicate that the R/G value decreased significantly with increasing CHX concentration ( $p < 0.01$ ). The R/G value was lowest for the 0.5% CHX group, at ~46% lower than that of the DW group. A negative linear dose-response relationship was evident between the RF of biofilm and CHX concentration ( $p < 0.01$ ).

The R/G values of the 7-day mature biofilm showed significant correlations with all of the cariogenic properties measured



**Fig. 2** Changes in RF (quantified as red/green ratios) of microcosm biofilm grown in the presence of antimicrobial treatment with CHX at different concentrations and DW according to the maturation time.



**Fig. 3** Fluorescence images of dental microcosm biofilms that formed on the enamel discs after 7 days according to treatment with CHX at different concentrations: (a) 0% (DW), (b) 0.05%, (c) 0.1%, and (d) 0.5% CHX.

in this study (Table 1). Strong correlations were identified between the R/G values and the number of total bacteria ( $r = 0.93$ ,  $p < 0.001$ ), the number of aciduric bacteria ( $r = 0.97$ ,  $p < 0.001$ ), and the %SHC ( $r = 0.98$ ,  $p < 0.001$ ). The total bacterial counts and aciduric bacterial counts were decreased significantly with increasing CHX concentrations ( $p < 0.001$ , Table 1).

The counts of total bacteria and aciduric bacteria were significantly lower for the biofilms treated with CHX than for those in the DW group. Also, the changes in surface hardness of enamel was significantly higher in the DW group than in all of the CHX treatment groups ( $p < 0.001$ ) demonstrating that %SHC was higher for a lower concentration of CHX ( $p < 0.001$ ).

**Table 1** Mean (SD) values of R/G values and cariogenic properties of 7-day-maturation microcosm biofilms grown in the presence of antimicrobial treatments with CHX at different concentrations.

Groups	R/G value (ratio)	Total bacteria CFUs ( $\log_{10}$ CFUs)	Aciduric bacteria CFUs ( $\log_{10}$ CFUs)	Supernatant pH	%SHC
DW	2.01 (0.03) <sup>a</sup>	8.72 (0.19) <sup>a</sup>	8.01 (0.25) <sup>a</sup>	7.12 (0.33) <sup>a</sup>	71.46 (2.55) <sup>a</sup>
CHX 0.05	1.38 (0.10) <sup>b</sup>	7.40 (0.12) <sup>b</sup>	6.17 (0.17) <sup>b</sup>	6.93 (0.29) <sup>a,b</sup>	30.29 (1.74) <sup>b</sup>
CHX 0.1	1.18 (0.05) <sup>c</sup>	6.80 (0.10) <sup>c</sup>	5.14 (0.28) <sup>c</sup>	6.81 (0.17) <sup>a,b</sup>	25.79 (2.33) <sup>c</sup>
CHX 0.5	1.09 (0.08) <sup>d</sup>	6.31 (0.19) <sup>d</sup>	4.62 (0.21) <sup>d</sup>	6.68 (0.10) <sup>b</sup>	17.47 (1.38) <sup>d</sup>
<i>r</i>	—	0.93*	0.97**	0.43*	0.98**

Note: Different letters within the same column indicate significant differences between groups by Tukey's *post-hoc* analysis at  $\alpha = 0.05$ .

R/G values mean the ratios of red pixels to green pixels in fluorescence images captured by the QLF-D.

CFUs mean colony-forming units.

%SHC is the percentage of surface microhardness change calculated as  $\%SHC = 100(VHN_{baseline} - VHN_{after})/VHN_{baseline}$ .

*r* is the Pearson's correlation coefficient between R/G values and each variable related the biofilm cariogenicity.

\* $p < 0.01$

\*\* $p < 0.05$

## 4 Discussion

This study confirmed the potential that RF detected by the QLF technology is useful for assessing and monitoring the efficacy of antimicrobial treatments against biofilm. Based on the relationship of RF with biofilm properties imposed by treatment with CHX at different concentrations, it can be suggested that the RF is indicative of the level of antibacterial and anticariogenic activity of antimicrobials. Furthermore, based on the dose-response effect of CHX, the present cariogenic microcosm biofilm model involving QLF technology could be used to estimate the effect of antimicrobials on biofilms and the tooth surface.

This study reported that the RF and its increase rate were lower for CHX-treated biofilm than for the DW group (Fig. 2). This indicates that the pathogenicity of the CHX-treated biofilms took longer to be identified and was lower than for untreated biofilms, which is supported by the finding that the RF intensity was strongly correlated with the cariogenic properties of 7-day mature biofilm. These results are consistent with previous findings of the RF intensity of microcosm biofilms increasing with the level of its cariogenicity induced by cariogenic challenges using sucrose supplementation.<sup>11,12</sup> However, the culture condition of the biofilms in this study differed from that in the previous studies. The maximum level of the biofilm cariogenicity was reduced by applying CHX, which is considered as a gold-standard antimicrobial agent to prevent biofilm growth. The confirmed relationships between the RF of the biofilm and its properties under the antimicrobial treatments mean that the RF intensity can be used to assess the pathogenic level of a dental biofilm as well as predict its cariogenic potential by monitoring the RF intensity.

We reported dose-response effects for the RF intensity relative to the CHX concentration, with the anticariogenic levels of the biofilms increasing with the CHX concentration. This is consistent with previous studies utilizing dental microcosm biofilms that cause mineral loss and CFU counts finding dose-response effects of CHX treatments.<sup>20</sup> It has been reported that RF intensity emitted from the biofilms is strongly associated with their maturity and imposed cariogenicity.<sup>11,12,16</sup> The present results therefore indicate that the magnitude of the effect of antimicrobial substances on biofilm growth and pathogenic levels could be reflected in differences in the RF intensities of the biofilms. This suggests that the rate of biofilm accumulation and the subsequent demineralization response can be predicted by assessing the RF intensity.

Differences in the RF intensity of 7-day-maturation biofilms for different CHX concentrations could be related to their microbial composition and their metabolic activity induced by antimicrobial agents. Previous studies have reported that CHX exhibits a broad antibacterial spectrum against Gram-negative and Gram-positive bacteria, with the counts of total anaerobic and aerobic bacteria in dental biofilms reducing after treatment with CHX, with particularly large changes in *Streptococci*, *Actinomyces*, *Bifidobacterium*, and *Veilonellae* species.<sup>21,22</sup> In addition, the levels of caries-related species and anaerobes within cariogenic microcosm biofilms tended to be lower after exposing them to CHX.<sup>23</sup> The bacterial species identified previously could show that the RF depends on the growth conditions under the QLF lighting conditions,<sup>24</sup> which is attributed to the production of porphyrin compounds. Based on these results, it can be assumed that decreases in the RF intensity for an increasing CHX concentration could be due to the decrease in the proportion of the red fluorescing species

in entire biofilm. It indicates that these species of the CHX-treated biofilm produce relatively low amounts of red-fluorescing metabolites from bacterial interactions. This hypothesis is consistent with CHX, reportedly interfering with the metabolism of oral bacteria by inhibiting the production of enzymes and metabolic products of cariogenic and periodontal pathogens.<sup>25</sup> Identifying the exact mechanism underlying RF differences related to bacteria will require the microbial profiles and their overall concentrations of metabolites within CHX-treated biofilms to be investigated using molecular biology analysis.

The observed differences in RF intensities could also be due to the physical properties of the biofilms. Previous studies have reported that the thickness, density, and volume of a biofilm can affect the detected fluorescence intensity.<sup>16,26</sup> The physical properties of the biofilms were not quantified directly in this study, but it can be speculated that the degree of biofilm formation differed between the groups so as to result in differences in the amount of polysaccharide matrix, biomass concentration, and bacterial cell density. A pattern of dose-response effects with decreasing biofilm formation and increasing CHX dose has been reported previously.<sup>18</sup> Differences in RF could, therefore, have been at least partially due to differences in the amount of biofilm accumulation resulting from the used of different CHX concentration in this study.

When measuring the RF of biofilms treated with antimicrobials, it should be considered that the RF response to exposure of the antimicrobial could depend on the mechanisms of antimicrobial action against dental biofilms. It has been reported that chemotherapeutic agents exert different effects on the bacterial composition of oral biofilms and their metabolic activity, which could be due to differences in the relative proportions of the specific bacteria in the biofilm and the its amounts of metabolites produced according to different types of antimicrobial agents.<sup>22</sup> Furthermore, antimicrobial agents might affect the physicochemical properties of a biofilm, such as the pH, and the relative proportions of different metabolites, such as porphyrins, which could affect the fluorescence properties of biofilms treated with antimicrobials.<sup>27</sup> The RF, therefore, does not necessarily indicate the level of the antibacterial activity or a clear pattern that is the same as that for CHX treatment.

The methodological approach adopted in this study of combining microcosm biofilm model with the QLF technology made it possible to assess an inhibitory effect of antimicrobials on the biofilm formation and the entire cariogenic potential by sequential application during biofilm development. This assessment model could be used to evaluate the efficacy of antibacterial agents against biofilm pathogenicity according to treatment time, frequency, and application methods, which could also be useful in testing formulations of antimicrobials and determining effective treatment regimens in preclinical stage. Furthermore, by observing RF changes, this model can be a useful tool for monitoring the antibacterial and anticariogenic potentials of antimicrobials nondestructively and in real time.

## 5 Conclusions

The RF intensity emitted from CHX-treated biofilms was reported to decrease significantly with increasing CHX concentrations. Based on the strong association between the RF intensity of the biofilm and its anticariogenic activity induced by sequential CHX treatment, it can be concluded that the RF measured with the QLF technology can be used to evaluate the

efficacy of antimicrobial agents against biofilm formation and cariogenic potential.

### Disclosures

Inspektor Research Systems BV provided the salary for author Elbert de Josselin de Jong (EdJdJ) but did not have any additional role in the study design, data collection, analysis, decision to publish, or preparation of the paper. EdJdJ's involvement in this research was under the auspices of his status as adjunct professor at Yonsei University College of Dentistry supported by Brain Pool Program and BK21 PLUS Project. The specific role of EdJdJ was to provide for his expertise regarding the fluorescence technology. This does not alter the author's adherence to the *Journal of Biomedical Optics* policies on sharing data and materials. EdJdJ holds several patents with respect to QLF technology. The remaining authors declared no conflicts of interest.

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