Monitoring the maturation process of a dental microcosm biofilm using the Quantitative Light-induced Fluorescence-Digital (QLF-D)

Young-Seok Kim, Eun-Song Lee, Ho-Keun Kwon, Baek-Il Kim *

Department of Preventive Dentistry and Public Oral Health, BK 21 Plus Project, Yonsei University College of Dentistry, 50 Yonsei-ro, Seodaemun-gu, Seoul, Republic of Korea

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ABSTRACT

Objective: The aim of this study was to investigate whether Quantitative Light-induced Fluorescence-Digital (QLF-D) could monitor the degree of maturation of dental microcosm biofilms by observing the red fluorescence emitted from the biofilms.

Methods: Dental microcosm biofilms were grown on bovine enamel discs. They were initiated from human saliva, and then grown in 0.5% sucrose growth media for 10 days. On days 1, 2, 3, 7, and 10 after the inoculation, fluorescence images of the biofilms were captured using the QLF-D and the red fluorescence intensity was quantified by calculating the red/green ratio (R/G value). Total and aciduric bacteria within the biofilms were counted, and the degree of demineralization was evaluated by measuring the percentage of surface micro-hardness change (ΔVHN) and lesion depth in the enamel.

Results: The R/G values of the biofilms assessed by the QLF-D increased significantly over time up to 7 days after inoculation (p < 0.0001). The R/G values showed significant positive correlations with the total bacterial CFUs (r = 0.74, p = 0.001), aciduric bacterial CFUs (r = 0.85, p = 0.001), ΔVHN (r = 0.65, p = 0.001), and lesion depth in the enamel (r = 0.82, p = 0.001) according to the maturation time.

Conclusions: The red fluorescence detected by the QLF-D increased according to biofilm maturation and was significantly associated with the cariogenicity of the biofilm. Therefore, this device could be used to monitor the degree of biofilm maturation by observing the red fluorescence emitted from cariogenic biofilms.

Clinical significance: The QLF-D enables the detection of a mature dental plaque and monitoring of its cariogenic status by observing the plaque fluorescence non-destructively, in real time.

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1. Introduction

Dental biofilm formation is a continuous process initiated by the attachment of planktonic bacteria to a surface of dental tissue and leads through different stages into a mature, structurally complex biofilm.1,2 In particular, as the cariogenic biofilm becomes more mature, more number of acidogenic and aciduric bacteria become dominant in the biofilm, which are capable of demineralizing the enamel and increasing the risk of dental caries.3-4 Therefore, early detection of a mature biofilm and changes in its pathogenic activity that is directly
associated with disease progression are important for prevention of dental caries.

For these reasons, some researchers have sought to develop novel systems for more sensitive plaque assessment. In clinical fields, it is common to use disclosing agents containing erythrosine to stain colourless plaque and make them visualized. However, the patients encounter discomfort during disclosing, and it is also inconvenient for the clinicians to perform this method in the clinical practice. In addition, the disclosing method only enables to detect plaque distribution, which makes hard to consider the method as an appropriate method for determining exact pathogenic status of plaque.

Hence, we need to focus on developing methods that are able to detect differences in plaque properties more sensitively and conveniently.5

The Quantitative Light-induced Fluorescence-Digital (QLF-D) system, which is called the QLF-D Biluminator™ (Inspectro Research Systems BV, Amsterdam, The Netherlands), is a novel optical device in dentistry, which can detect dental biofilms by its red fluorescence.6-8 It has been demonstrated that this phenomenon is caused due to the generation of fluorescence from endogenous porphyrins in bacteria when irradiated with 405 nm of visible blue light.6 This device can visualize the dental biofilm more clearly by the red fluorescence than the previous QLF system and can provide high-resolution images under appropriate lighting conditions.6,7 Therefore, the QLF-D can be used to detect the distribution of the undisclosed plaque more sensitively, and it offers many advantages such as reduction in the time and efforts required for biofilm analysis. In a previous study, it has been proposed that this device would enable accurate quantitative analysis of the plaque and assessment of progression of treatment.7 Also, the red fluorescence was found to be due to the intrinsic characteristics of the biofilms.5 A recent study reported that this device can detect the cariogenic biofilm and has the potential to assess the cariogenic levels of dental plaque based on the intensity of red fluorescence.10 According to these findings, if this device could quantify the changes in cariogenic characteristics of the dental biofilm, it would enable to assess the early caries risk and to provide continuous, effective treatment for successful prevention of caries by obtaining biofilm images at different time points. However, to date, no research has focused on assessing the changes in red fluorescence according to the pathogenic status of biofilms induced by the maturation process.

In a preclinical dental research similar to the present study, the use of a good dental biofilm model which can demonstrate the real microbial interactions in an in vivo plaque is needed.11 Previous reports have suggested that the dental microcosm biofilms initiated from the human saliva can replicate the variability and heterogeneity of bacterial population in an in vivo plaque, thus simulating the natural oral environment.12,13 Therefore, it was possible to observe the changes, which were induced by the maturation process, in response to microbial interactions reflecting the diversity, complexity, and heterogeneity of an in vivo plaque to determine the potential of QLF-D for plaque assessment.13

Therefore, the aim of this study was to evaluate whether the QLF-D can quantitatively monitor the maturation process of dental microcosm biofilms by observing the red fluorescence emitted from the biofilm.

2. Materials and methods

2.1. Enamel block preparation

Bovine incisors without cracks and white spots were selected. To make enamel specimens, the labial surface of bovine incisors were sectioned (8 mm × 3 mm) using a low-speed saw with a diamond disc. All of the specimens were embedded in circular acrylic moulds, and ground using 600–1200 grit abrasive papers (SiC Sand Paper, R&B Inc., Daejeon, South Korea) in a water-cooled polishing unit to obtain a flat enamel surface. After measuring the surface microhardness as the Vickers microhardness number (VHN), all of the specimens were re-embedded in acrylic moulds, 1 mm shorter than the mould surface to make the biofilm easier to accumulate.

2.2. Growth medium preparation

Biofilm was formed in Basal Medium Mucin (BMM) supplemented with 0.5% sucrose.10,11 The BMM artificial saliva medium contained 2.5 g/l porcine mucin (Type III, Sigma Chemicals, MO, USA), 10.0 g/l protease peptone, 5.0 g/l trypti-case peptone, 5.0 g/l yeast extract, 1 mmol/l urea, 1 mmol/l arginine, 2.5 g/l KCl, and 1 mg/l menadione (pH 7.0).

2.3. Dental microcosm biofilm formation

Biofilms were produced in a caries-related microcosm biofilm model as described by Lee et al.10 Stimulated saliva was collected from a healthy male donor, who had been refrained from oral hygiene for 24 h and had no active caries or periodontal disease. Ethical approval (02-2012-0030) was granted by the Ethics committee of the Yonsei Dental Hospital, Korea. The saliva was filtered through sterile glass wool and diluted in sterile glycerol (final concentration 30%). The mixture of saliva and glycerol was stored at −80 °C. The prepared saliva (1.5 ml) was inoculated into each specimen, which was placed in wells of 24-well cell culture plates (SPL Life sciences, Pocheon, Korea). After 4 h, the saliva was gently aspirated from the bottom of the wells, and 0.5% sucrose growth media were added (1.5 ml). The plates were then incubated in an anaerobic hood in an atmosphere of 80% N2, 10% CO2, 10% H2 for up to 10 days at 37 °C. The BMM growth medium in each well was replaced daily by placing the enamel block in a new well containing 1.5 ml of fresh medium. This whole procedure was repeated three times.

2.4. Analysis of the dental microcosm biofilm

2.4.1. Red fluorescence of the dental microcosm biofilm

The red fluorescence of the biofilm was evaluated by Quantitative Light-induced Fluorescence-Digital (QLF-D Biluminator™, Inspectro Research systems BV, Amsterdam, The Netherlands) 1, 2, 3, 7, and 10 days after inoculation as previously described by Lee et al.10 Normal white light images and sequential fluorescence images of all enamel specimens were captured with a ‘Live View’-enabled full-frame sensor
digital SLR camera (model 550D, Canon, Tokyo, Japan) using the following settings: shutter speed of 1/45 s, aperture value of 3.2, and ISO speed of 1600. The distance between the lens of the camera and the biofilm on the specimen was kept constant. Proprietary software (C3 v1.16, Inspektor Research Systems BV) was used to capture and store all of the digital images on a PC automatically. The fluorescence images were analyzed using an image analysis software (Image-Pro PLUS, Media Cybernetics, MD, USA). The red and green values in the same selected area of fluorescence images and the average red and green ratio (R/G value) were calculated for each fluorescence image in order to observe the changes in red fluorescence over time. All of the analyses were performed by a single trained examiner.

2.4.2. Microbial composition of the dental microcosm biofilm
1, 2, 3, 7, and 10 days after inoculation, the specimens with biofilm growth were rinsed in 1.5 ml cysteine peptone water (CPW) to remove loose bacteria, and then transferred into a conical tube containing 2 ml CPW. The biofilms were dispersed using sonication and vortexing for 1 min. The resulting bacterial suspension was serially diluted (10⁰ to 10⁻⁹) in CPW, and plated in duplicate onto a 5% tryptic soy blood agar (for total bacterial count) and brain heart infusion which were adjusted to pH 4.8 (for aciduric bacterial count). All plates were incubated in an anaerobic incubator for 72 h, and the number of colony-forming units (CFUs) was then determined by a trained operator.

2.5. Analysis of the enamel specimen

2.5.1. Surface microhardness of the enamel
Hardness testing of the enamel specimen was performed by making four indentations using a Vickers Hardness (VH) tester with a load of 200 g for 10 s (JT Toshi Inc., Tokyo, Japan). The surface microhardness was measured before the inoculation (VHNbaseline) and 1, 2, 3, 7, 10 days after the inoculation (VHNafter). The percentage of surface microhardness change was calculated with the following formula: \( \% \text{VHN} = \frac{100(\text{VHNbaseline} - \text{VHNafter})}{\text{VHNafter}} \). This procedure was carried out by a single, blind and trained examiner.

2.5.2. Lesion depth in the enamel
After measuring the VHN, the specimens were cut in the direction perpendicular to the enamel surfaces. A 300 μm thick section was cut (Techcut™, Allied High Tech Products, Inc., California, USA), and then ground (800 grit, SiC Sand Paper, R&B Inc., Daejeon, South Korea) to a thickness of approximately 130 μm. The thickness of the samples was tested using a digital micrometre with a precision of 1 μm (ID-C125B, Mitutoyo Corporation, Kawasaki, Japan). The specimens were mounted on a glass slide. The slabs were placed in deionized water, and the lesion depth in the enamel was measured under a polarized light microscope (PLM, CX31-P, Olympus, Tokyo, Japan). The images were captured at a 100× magnification. The lesion depths were quantified by measuring the depth from the surface to the dark layer using the image analysis software (Image-Pro PLUS, Media Cybernetics, MD, USA).

2.6. Statistical analysis
Each variable representing the characteristic of the biofilm were analyzed according to the maturation period using repeated measure ANOVA, followed by Bonferroni post hoc test to confirm statistically significant differences between groups according to different maturation time periods. Also, correlations between the characteristics and the red fluorescence (R/G values) of the biofilm were tested by Pearson’s correlation analysis. All of the statistical analysis was conducted using PASW Statistics 18.0 (SPSS, IBM Corporation, Somers, NY 10589, USA), and the level of significance was set at \( \alpha = 0.05 \).

3. Results

When the white light images of the dental microcosm biofilms were observed, it was noted that the biofilms were formed after 24 h of inoculation, and they became thicker and denser over time. The red fluorescence was detected in QLF-D fluorescence images over a small portion from day 2 onwards. The microcosm biofilms were cultured for 3 days; the whole surface of biofilms evenly emitted red fluorescence, and the intensity of red fluorescence was increased over time (Fig. 1).

![](image)

**Fig. 1** – White light images (upper line) and QLF-D fluorescence images (lower line) of dental microcosm biofilms formed on the bovine enamel discs 1 day, 2 days, 3 days, 7 days, and 10 days after inoculation.
When the colour analysis was performed, the R/G values of the biofilms increased significantly from day 1 (0.83) through day 7 (2.07), and the R/G value of the 7-day biofilm was the highest and the value was saturated (p < 0.0001). This R/G value was not significantly different from that of the mature 10-day biofilm (Table 1). Also, the R/G value of the biofilm matured over 3 days was significantly higher compared to that of the 1-day and 2-day biofilms (p < 0.0001).

According to the results of bacterial counts in the microcosm biofilms depending on the maturation time, the total bacterial CFUs and aciduric bacterial CFUs were significantly increased from day 1 through day 7 (p < 0.0001). However, there was no difference in the total and aciduric bacterial CFUs between day 7 and day 10. The ratio of aciduric bacterial count to total bacterial count was significantly increased over time, from 88% on day 1 to 98% on day 10 (p < 0.0001).

Fig. 2 represents the depth of enamel lesion beneath the microcosm biofilm measured on different days (on days 1, 2, 3, 7, and 10), indicating that the severity of demineralization increased with biofilm maturation. The mean values of ΔVHN and lesion depth in the enamel were significantly increased with the maturation time (p < 0.001). It was observed that the demineralization caused by the 10-day biofilm resulted in a 69% increase in ΔVHN and an 80% increase in the lesion depth compared to that caused by the 1-day biofilm.

Regarding the correlation coefficients, the R/G values of the microcosm biofilms observed by the QLF-D showed significant correlations with all of the variables measured in the present study (Table 2). Strong correlations were identified between the R/G values and the total bacterial CFUs (r = 0.74, p = 0.001), and between the R/G values and the aciduric bacterial CFUs (r = 0.85, p = 0.001) over time. With regard to the variables associated with the mineral content, ΔVHN (r = 0.65, p = 0.001) and the lesion depth (r = 0.82, p = 0.001) of enamel showed significant, strong correlations with red fluorescence intensity of the biofilm with increasing biofilm maturity.

### Table 1 – Red fluorescence (R/G value) and cariogenicity variables of dental microcosm biofilms according to the maturation time.

<table>
<thead>
<tr>
<th>Days</th>
<th>R/G values (ratio)</th>
<th>Total bacterial CFUs (log10 CFUs)</th>
<th>Aciduric bacterial CFUs (log10 CFUs)</th>
<th>ΔVHN (%)</th>
<th>Lesion depths (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.83 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.31 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.45 ± 0.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>96.5 ± 33.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.5 ± 10.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>0.99 ± 0.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.67 ± 0.48&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.92 ± 0.36&lt;sup&gt;b&lt;/sup&gt;</td>
<td>230.0 ± 36.3&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>46.6 ± 12.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>1.35 ± 0.22&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.68 ± 0.34&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.26 ± 0.45&lt;sup&gt;c&lt;/sup&gt;</td>
<td>252.0 ± 12.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>79.1 ± 11.8&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>7</td>
<td>2.07 ± 0.29&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9.10 ± 0.57&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8.48 ± 0.13&lt;sup&gt;d&lt;/sup&gt;</td>
<td>268.4 ± 25.7&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>117.6 ± 13.4&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>10</td>
<td>2.04 ± 0.29&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8.55 ± 0.40&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8.38 ± 0.51&lt;sup&gt;d&lt;/sup&gt;</td>
<td>309.8 ± 25.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>124.7 ± 23.1&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

All values are expressed as mean ± standard deviations. Different letters within the same column indicate significant differences between groups by Bonferroni’s post hoc analysis at α = 0.05. R/G values represent the ratios of red pixels to green pixels in red fluorescence images of biofilms captured by the QLF-D. CFUs indicate colony-forming units.

### Table 4. Discussion

The present study evaluated the continuous changes in red fluorescence observed by the QLF-D and the cariogenic level of dental microcosm biofilm during 10 days. It was possible to assess the ability of QLF-D to quantitatively monitor the properties of microcosm biofilms according to the maturation process by observing a sequence of fluorescence images of the biofilms.

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**Fig. 2 – Polarized light microscope (PLM) micrographs (X100) of the bovine enamel beneath dental microcosm biofilms taken 1 day (A), 2 days (B), 3 days (C), 7 days (D), and 10 days (E) after inoculation.**
According to the results of the present study, there was an increase in the red fluorescence intensity of the biofilm over time depending on its level of cariogenicity. It could be confirmed that more mature biofilms emitted a greater degree of red fluorescence than the biofilms at an earlier stage of development. In this study, in white light images, dental microcosm biofilms were formed on the enamel block 1 day after inoculation when observed with naked eye. However, the red fluorescence detected in fluorescent images captured by the QLF-D was emitted evenly by the mature biofilms cultured for 3 days or more. When observed under the QLF-D lighting conditions, it was noted that all of the biofilms did not always emit red fluorescence, which implies that the biofilms emitting red fluorescence were more mature, more cariogenic and had been cultured for at least more than 3 days.

The results of the present study were in accordance with the results of earlier studies, which reported that a mature, relatively old biofilm on teeth under existing QLF conditions emitted a red fluorescence and its colour changed with plaque maturity, indicating that obligate anaerobes and secondary colonizers producing porphyrins were increased in the biofilm community, and the red fluorescence emitted by a mature biofilm was due to the metabolic products of the biofilm rather than a single species. It has been suggested that changes in the red fluorescence and its intensity are associated with the changes in microbial composition of the biofilm that harbours more cariogenic species as the biofilm matures, which implies the role of general properties of the biofilm rather than that of a single pathogenic species.

Our results demonstrated that the red fluorescence emitted from the biofilms continuously increased up to 7 days, but after 7 days, there was no increase in the red fluorescence. A similar tendency was observed in other variables, and there was no significant difference between the cariogenicity variables of 7-day and that of 10-day mature biofilms, indicating that there was a threshold level for cariogenicity of the biofilm. Based on this study, we postulate that there is a threshold for the red fluorescence no matter how long the maturation time is, thus supporting the claim that there is a significant correlation between the red fluorescence and the cariogenic potential of the biofilm. Especially, the fluorescence of the biofilm might be due to the bacterial metabolites and its intensity may be affected by the changes in microbial composition of the biofilm as demonstrated by the association between the red fluorescence and the microbial composition in the biofilm.

Statistically significant correlations were observed between the cariogenic properties and the red fluorescence according to the maturation process. This result corroborates the findings of the previous study, suggesting that the biofilm that emits red fluorescence is associated with a high caries risk. So far, we have not confirmed the exact correlation between the biofilm fluorescence and dental caries, and are only assuming that there is a correlation between the two based on our knowledge of biofilm maturation and identification of caries-related bacteria in a carious lesion. The result about this association corresponds to the previous finding that the red fluorescence intensity detected by the QLF-D increased according to the cariogenic level of the biofilm, which was induced by supplying the sucrose at different concentrations. Therefore, it could be assumed that the red fluorescence is associated with cariogenic biofilms and caries process, which might enable us to use this device to assess caries risk and estimate demineralization status by analyzing red fluorescence intensity of the biofilms non-invasively.

Considering the reliability of the basic principle of the QLF system, when conducting longitudinal QLF measurements, it is important to maintain standardized measurement conditions for observing changes in fluorescence intensity. Because the fluorescence observed by the QLF system is influenced by several factors including camera geometry, focal distance and surrounding lighting conditions. Also, the fluorescence intensity of an object might depend on its hydration and morphological status. Furthermore, especially for monitoring the biofilm, the type and surface status of the underlying materials should be considered. In the present study, all factors that have been identified to affect were controlled, and the conditions were identically maintained for each measurement to eliminate these effects.

Compared to the actual maturation process, as the 0.5% sucrose concentration induced a very high cariogenic condition, it can be assumed that a highly cariogenic biofilm was formed at a relatively rapid rate in this study. Although, this did not matter when observing the continuous changes in biofilms, it is difficult to directly compare the red fluorescence and the levels of cariogenicity of an in vivo plaque at a certain time point. Also, as porphyrin synthesis in bacteria could vary under different culture conditions, further in vivo studies are needed to confirm the usefulness of the QLF-D system as a plaque assessment device in the clinical field.

This study provided a rationale for using the QLF-D as a new plaque assessment device. The results might make it possible to identify the cariogenic biofilms and estimate the caries severity in real time. In patients, in whom there is easy accumulation of a mature plaque, especially orthodontic patients who are considered as the high caries risk group due

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| Table 2 - Correlations (r values) between the red fluorescence (R/G value) and the cariogenicity variables of biofilms according to the maturation time. |
|-----------------|-----------------|-----------------|-----------------|
| R/G             | Total bacterial CFUs | Aciduric bacterial CFUs | ∆VHN | Lesion depths |
|                 |                  |                      |      |              |
| r               | 0.74             | 0.85                | 0.65  | 0.82         |
| p               | 0.001            | 0.001               | 0.001 | 0.001        |

r indicates the Pearson correlation coefficient. p < 0.01.
to brackets, this novel system could be used to examine oral hygiene status and development of an early caries lesion. If we could detect the changes in red fluorescence emitted from the biofilms by capturing images at different visits using the QLF-D in clinical practice, we might be able to determine the individual risk of caries development and provide appropriate preventive treatments based on the results. This new device will make it possible to provide more active preventive care by detecting and monitoring the pathogenic status of dental biofilms.

5. Conclusion

It can be concluded that the red fluorescence detected by the QLF-D increased according to the biofilm maturation, and it was significantly correlated with the cariogenicity of the biofilm. Therefore, the QLF-D could be used to quantitatively monitor the maturation process of cariogenic microcosm biofilms by observing the red fluorescence emitted from the cariogenic biofilms in real time.

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