

## High frequency ultrasound imaging of a single-species biofilm

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#### ABSTRACT

*Objective*: This study evaluated the feasibility of a high frequency ultrasound scan to examine the 3D morphology of *Streptococcus mutans* biofilms grown in vitro.

Methods: Six 2-day S. mutans biofilms and six 7-day biofilms were grown on tissue culture membranes and on bovine dentine discs. A sterile growth medium on the membrane and disc were used as controls. Surfaces were rinsed and then immersed in sterile saline. High-frequency ultrasound imaging system was used to scan these surfaces at 55 MHz, and a computer program calculated the average thickness of the biofilm layer from the 3D images. *Results:* 3D pictures of the biofilm layers were obtained. Different cross-sections and plains are easily demonstrated. The average thickness of the 7-day biofilm was significantly bigger than the 2-day on both the membranes and dentinal discs. No structures were observed on the sterile membrane or disc.

Conclusion: Three-dimensional structural imaging in situ is possible without damaging the biofilm layer in a quick and easy manner and can therefore be used to evaluate biofilms longitudinally as a function of time.

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#### 1. Introduction

Biofilms are communities of microorganism growing on a surface or interface.<sup>1</sup> One of the best studied biofilms is dental plaque, which forms on tooth surfaces and causes dental caries.<sup>2,3</sup> It is a multi species biofilm dominated by *Streptococcus* species.<sup>4</sup> Biofilm structure has a crucial role in its function and resistance. It has a circulatory system and mixed microbial community that is highly integrated in terms of nutritional needs and output.<sup>5</sup> Knowledge of the 3D structure of a biofilm is important in order to understand its special characteristics as well as to monitor the efficacy of procedures to eliminate or

chemically remove it.<sup>6</sup> Although various successful attempts to quantify biofilm structure were made,<sup>7</sup> producing a clear image of the biofilm or obtaining reliable data about its thickness, density or biomass still presents a challenge.<sup>8,9</sup> Laser scanning microscopy is the method most frequently employed for 3D biofilm imaging but its disadvantages are the toxicity of fluorescent markers used, depth of penetration and resolution.<sup>10</sup> Recently, nuclear magnetic resonance (NMR) was suggested as a non-invasive method to image live biofilms.<sup>11</sup>

Ultrasound imaging has been extensively used as a noninvasive diagnostic tool in medicine for over 50 years, and in the majority of applications, it operates in the frequency range

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of 1–5 MHz. Ultrasound imaging as a possible diagnostic dental tool has been investigated as far back as the 1960s. Lees and Barber<sup>12</sup> were the first to describe a sonic echogram of incisor teeth, and later discussed the difference of echoes received from the dentine-amalgam interface and demineralized dentine.<sup>13</sup> They were able to estimate the enamel and dentine thickness at various points, but these measurements could not be representative for the whole tooth. The researchers also neglected to take into account the phase change that occurs when acoustic waves pass through interfaces between materials of different acoustic impedances, thus allowing an error to arise in their measurements.<sup>14</sup>

More recently, relevant clinical dental applications of ultrasonic scans were discussed,<sup>15,16</sup> such as the detection of caries and cracks in the enamel. These studies were imaging hard-tissues, while ultrasound imaging has an excellent capacity to image soft tissues as well. Higher frequency ultrasound systems (20–100 MHz) have lately been developed for specialized biomedical applications, often imaging soft tissues, and offer improved spatial resolution for situations where the imaging probe (transducer) can be brought to within <10 mm from the region of interest.<sup>17</sup>

The aim of the present study was to evaluate the feasibility of a high frequency ultrasound imaging system to examine the 3D morphology of biofilms. In particular, single-species *Streptococcus mutans* biofilms on the order of 100–300  $\mu$ m were non-invasively imaged.



#### 2.1. Growth of S. mutans biofilm

S. mutans biofilms were prepared: a frozen stock of S. mutans C180 was plated on Brain Heart Infusion (BHI) agar and grown under anaerobic conditions in gas packed jars for 2 days in 37 °C. One colony was inoculated in 25 ml BHI solution and stored in 37 °C overnight.<sup>18</sup> The next morning 0.5 ml of the bacterial suspension was transferred to 25 ml of half strength BHI with pipes buffer (50 mM). 0.1 ml sterile sucrose solution 0.2% was added. This bacterial suspension was pipetted into cell culture inserts (BD Biosciences San Jose, CA, USA) which have a thin (12  $\mu$ m) membrane on which the biofilm was grown and maintained in anaerobic conditions. The supernatant was carefully removed every day and exchanged with a fresh sterile growth medium containing sucrose. A white layer was visible on the insert's membrane after 24 h of incubation, indicating biofilm formation. Four different plates of six inserts each were used to generate different biofilms or environments: in plate A (six inserts), biofilms were grown for 7 days before they were ultrasonically scanned, while in plate B (also six inserts) biofilms were grown for only 2 days before scanning. Two other sets of plates were prepared with dentine discs: in plate C, six sterile bovine dentine discs (diameter: 5 mm, uniform thickness of 2 mm) were placed on the insert's membrane. They were inoculated with 4 ml of bacterial culture and growth medium like the previous plates and the procedure was repeated as in group A. In plate D biofilms were grown on the dentine discs for 2 days. The control plate contained four inserts with

sterile growth medium supplemented with sucrose, two of which contained a sterile bovine dentine disc in it.

#### 2.2. Ultrasound scan

A commercially available high frequency ultrasound system, designed primarily for use with small animal imaging, was employed in this study (Visualsonics Vevo 770, Toronto, Canada). The 55 MHz probe (#708) was used, which has a single element focused transducer with an aperture of 2 mm and a focal length of 4.5 mm. The estimated 6 dB beam width of this transducer was 62  $\mu$ m. The nominal bandwidth of the pulses was reported to be 100%, giving pulse duration of approximately 0.02 ms. This type of transducer mechanically scans laterally over a region of interest, and a 2D image is built up using echoes from a series of 384 adjacent transducer beam locations (one pulse per location). 2D images were acquired at 20 frames per second and for an 8 mm lateral image distance, such as that used in this study, the spacing between pulses was 21  $\mu$ m on average. At a given beam location, the received ultrasound echo is converted into a brightness scale (logarithmically compressed) that is then displayed as one line on the image. The transducer was mounted to the proprietary positioning system, which permitted manual control of the transducer orientation and location relative to an imaging platform. 3D imaging capabilities were enabled with a motorized position axis, which scans the imaging probe through a series of 2D imaging planes to acquire volumetric data.

Before scanning, growth medium was carefully removed and the culture inserts were rinsed and filled with saline, taking care no air bubbles were formed. The inserts were immediately placed on a stable imaging platform and the membrane or dentine surface was manipulated to be in the focal zone of the transducer, and perpendicular to the transducer beam. The configuration is shown in Fig. 1, which indicates that the transducer beam travels through saline before encountering the biofilm. The saline is required as a coupling medium to permit the ultrasound energy to travel into the biofilm. The presence of air, which creates larger echoes, would prohibit ultrasound imaging of the sample.

An image of 8 mm  $\times$  8 mm  $\times$  10 mm in the x–y–z directions (Fig. 2) took approximately 10 s to scan. Taking into account



Fig. 1 – Tissue culture insert, and the setting of the scan. A transducer is located inside saline perpendicular to the biofilms' surface.



Fig. 2 – Ultrasound scan output screen: (A) cube view, 3D reconstruction, (B) cross view, (C) transverse view, (D) sagittal view.

the beam width of the transducer, each location was exposed to a maximum of 4 pulses during an individual 2D plane scan and 12 pulses during the 3D scan. The transmit amplitude was set to 10% of the maximum setting. The specific pressure levels employed in this study were not available, but the manufacturer indicated that the peak negative pressure measured using the maximum transmit amplitude for another unit of the same type was 3.3 MPa.

The resulting data was presented as a series of standard 2D ultrasound 'b-scan' (brightness) images. These data sets then underwent two forms of processing. First, the 3D images were rendered and examined using the internal Vevo 770 system software. This resulted in a 3D visualization of the biofilm surface, which permitted inspection of the biofilm morphology. The images could also be manipulated to look at userselected slices within the volumetric data sets. Second, to perform quantitative analyses, the data sets were transferred to a personal computer and processed using custom software written in a Matlab<sup>TM</sup> environment (Mathworks Inc., Natick, MA, USA).

Differences between thicknesses of 2-day and 7-day biofilms were statistically analyzed with the Mann–Whitney

tests (SPSS, version 12.0.1, Chicago, IL, USA). The level of significance was set at  $\alpha = 0.05$ .

#### 3. Results

The 3D output shows four different views (Fig. 2). A specific sagittal or transverse image could be generated at the specific plane determined by the cross-view. Sterile dentine discs and membranes immersed in sterile growth medium showed no biofilm growth at all. The membrane itself appeared as a very bright line resulting from a strong echo. In the experimental groups, biofilm structures were clearly visible (Figs. 2-4) as heterogeneous layers above the membrane or dentine discs. The upper boundary of the biofilm could be delineated due to presence of "scattered" echoes from within the biofilm and the absence of echoes from the overlying saline. The high amplitude membrane reflection could be readily used to identify the lower boundary of the biofilm. Using these boundaries, the average thickness of the biofilm was calculated for both substrates: the membrane and the dentine discs (Table 1). Seven-day biofilms were significantly thicker



Fig. 3 – Seven-day-old Streptococcus mutans biofilm grown on tissue-culture membrane with mm-scale.

than 2-day biofilms both on the membrane and dentine discs (P = 0.08, P = 0.016, respectively).

#### 4. Discussion

This study was a preliminary attempt to visualize S. *mutans* biofilms with an ultrasound imaging system and to assess its validity as a new method in the research of these structures. The assessment of biofilm was conducted at different growth stages, providing an illustration of its compatibility for

| Table 1 – Average thickness of biofilms grown on        |  |  |  |  |
|---|--|--|--|--|
| different substrates (membrane in tissue culture plates |  |  |  |  |
| and dentine discs) of 2 and 7 days old                  |  |  |  |  |

| Substrate     | Biofilm<br>age days | Average<br>thickness (μm) | S.D. |
|---------------|---------------------|---------------------------|------|
| Membrane      | 2                   | 102                       | 11   |
|               | 7                   | 266                       | 56   |
| Dentine discs | 2                   | 92                        | 13   |
|               | 7                   | 126                       | 16   |

longitudinal studies. The approach employed was to use a commercially available instrument which has rapid scan time (10 s per sample) and could immediately produce 3D visualization results. These factors are appealing from the perspective of increasing the accessibility of ultrasound techniques to the wider dental biofilm research community.

Ultrasound imaging is widely used for a range of medical applications.<sup>19</sup> A short burst of ultrasound is emitted from a transducer and directed into tissue. Echoes are produced as a result of the interaction of sound with tissue, and some of these travel back to the transducer. By timing the period elapsed between the emission of the pulse and the reception of the echo, the distance between the transducer and the tissue can be calculated and an image formed. Echoes are generated when the propagating ultrasound wave encounters an object with different acoustic impedance (product of density and speed of sound). When the 'object' is much smaller than the acoustic wavelength, such as in the case of cells within tissue, echo is generated through scattering. This is the origin of the echoes within the biofilm. If the wave encounters an interface that is relatively large with respect to the wavelength, then reflection occurs. This is the situation for the substrate/biofilm interface.



Fig. 4 – Streptococcus mutans biofilm growth on tissue-culture membranes (A–C) and on dentine discs (D–F): (A) sterile membrane (negative control), (B) 2-day-old biofilm, (C) 7-day-old biofilm, (D) sterile dentine disc (negative control), (E) 2-day biofilm, (F) 7-day biofilm.

The biofilms thickness estimates were made based on an assumed ultrasound velocity in biofilm of 1540 m/s.<sup>20</sup> It is important to note however that the velocity of sound in biofilm was not measured and although expected to be within the relatively narrow range of velocities found in soft tissues,<sup>21</sup> a calculation of this value will allow more precise measurements within the biofilm itself and not only its borders.

According to the results of this experiment, a 7-day biofilm is thicker than a 2-day biofilm regardless of the substrate used. This is in accordance with previous reports, where an increase in dental plaque thickness was observed, that did not conform to simple growth pattern models.<sup>22</sup> The current results however show that the 7-day biofilm was significantly thicker when grown on the membrane. This could be explained by the method applied to grow the biofilm on the dentine discs. Since the discs were not attached to the membrane below them, they constantly moved during the growth medium refreshment every day and it may have dislodged bacteria layering over the disc. Since this problem was not present in the membrane group, a thicker biofilm was able to grow.

There have been two other recent reports of using high frequency ultrasound to image biofilms. Holmes et al.23 imaged non-specific biofilms created in water ponds with a custom 50 MHz system. Placing an immersed transducer below a membrane and detecting reflections from an air/ biofilm interface allowed imaging the surface morphology of these biofilms. The use of air provided a very large reflection signal due to the difference in acoustic properties between air and biofilm. This strong echo, in combination with the signal processing methods applied, permitted the measurement of films of thickness on the order of 10 µm. Unfortunately, this approach has the potential drawback of altering the properties of the biofilm and making longitudinal studies difficult. Furthermore, such an approach may not be possible to implement if the substrate is dentine rather than a thin membrane. Good et al.<sup>24</sup> reported biofilm visualization of bacteria relevant to water pipes using 50 MHz ultrasound. The scanning approach was to discretely move the transducer through a grid, while stopping at each point to acquire a series of signals. Custom software was then used to form 3D images and measure thicknesses. It was also concluded that sound energy is an effective way to investigate biofilms noninvasively.

Microscopy is a highly valuable approach to biofilm investigations, and especially confocal scanning laser microscopy can create accurate 3D reconstructions. However, it cannot be used to visualize unstained (non-fluorescent) material or any other matrix which was not specifically treated.<sup>25</sup> Furthermore, the ultrasonic scan of a biofilm takes a few seconds to complete which makes it far easier and more convenient than previous methods. The ultrasound scan can provide an additional tool as a non-invasive method which could be applied repeatedly on the same sample after subjecting is to different treatments or materials without the need of any staining or fixing procedure.

As the number of pulses exposing any given location of the biofilm was very low (<12 over 150 ms) and each pulse was of short duration (<20 ns) and at 20% of the maximum transmit power, it is not expected that any heating, radiation pressure, or bioeffects would be induced during measurements. Cucevic et al.<sup>26</sup> used a Visualsonics system (40 MHz) and found temperature rises only when exposing at maximum power with longer pulses (0.4  $\mu$ s) at high repetition rates (10–20 kHz) sustained over many seconds at the same location.

While in this study we have focused only on morphology and thickness, it should also be noted that the received ultrasound signal may contain more useful information. For example, it has been shown that high frequency ultrasound signals can be sensitive to both tissue structure and cells undergoing apoptosis.<sup>27</sup> It may therefore be useful to examine ultrasound signals coming from different biofilm types or during growth and after therapy. Another avenue of investigation would be to examine the use of ultrasound molecular imaging probes which are typically comprised of targeted micro-bubbles.<sup>28,29</sup>

#### 5. Conclusion

The present study demonstrated a high frequency ultrasound imaging system that was successfully used to generate 3D computer images of S. *mutans* biofilm. The biofilm thickness could be calculated from these images. Biofilms on bovine dentine discs were also imaged, proving the method as an effective way to investigate biofilms for dental research.

#### R E F E R E N C E S

- Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM. Microbial biofilms. Annual Review of Microbiology 1995;49:711–45.
- Kinniment SL, Wimpenny JW, Adams D, Marsh PD. Development of a steady-state oral microbial biofilm community using the constant-depth film fermenter. *Microbiology* 1996;142:631–8.
- Marsh PD, Bradshaw DJ. Dental plaque as a biofilm. Journal of Industrial Microbiology 1995;15:169–75.
- 4. ten Cate JM. Biofilms, a new approach to the microbiology of dental plaque. Odontology 2006;**94**:1–9.
- Kolenbrander PE, Andersen RN, Blehert DS, Egland PG, Foster JS, Palmer Jr RJ. Communication among oral bacteria. Microbiology and Molecular Biology Reviews 2002;66:486–505.
- Wood SR, Kirkham J, Marsh PD, Shore RC, Nattress B, Robinson C. Architecture of intact natural human plaque biofilms studied by confocal laser scanning microscopy. *Journal of Dental Research* 2000;**79**:21–7.
- Heydorn A, Nielsen AT, Hentzer M, Sternberg C, Givskov M, Ersboll BK, et al. Quantification of biofilm structures by the novel computer program COMSTAT. Microbiology 2000;146:2395–407.
- Beyenal H, Donovan C, Lewandowski Z, Harkin G. Threedimensional biofilm structure quantification. *Journal of Microbiological Methods* 2004;59:395–413.
- Beyenal H, Lewandowski Z, Harkin G. Quantifying biofilm structure: facts and fiction. Biofouling 2004;20:1–23.
- Neu TR, Lawrence JR. One-photon versus two-photon laser scanning microscopy and digital image analysis of microbial biofilms. *Methods in Microbiology* 2005;34: 89–136.
- Majors PD, McLean JS, Pinchuk GE, Fredrickson JK, Gorby YA, Minard KR, et al. NMR methods for in situ biofilm metabolism studies. *Journal of Microbiological Methods* 2005;62:337–44.

- 12. Lees S, Barber FE. Looking into teeth with ultrasonics. *Science* 1968;**161**:477–8.
- 13. Lees S, Barber FE. Looking into the tooth and its surfaces with ultrasonics. Ultrasonics 1971;**9**:95–100.
- Culjat M, Singh RS, Yoon DC, Brown ER. Imaging of human tooth enamel using ultrasound. IEEE Trans Med Imaging 2003;22:526–9.
- Ghorayeb SR, Valle T. Experimental evaluation of human teeth using noninvasive ultrasound: echodentography. IEEE Transactions on Ultrasonics Ferroelectrics and Frequency Control 2002;49:1437–43.
- Culjat MO, Singh RS, Brown ER, Neurgaonkar RR, Yoon DC, White SN. Ultrasound crack detection in a simulated human tooth. Dentomaxillofacial Radiology 2005;34:80–5.
- 17. Foster FS, Pavlin CJ, Harasiewicz KA, Christopher DA, Turnbull DH. Advances in ultrasound biomicroscopy. Ultrasound in Medicine and Biology 2000;**26**:1–27.
- Luppens SB, ten Cate JM. Effect of biofilm model, mode of growth, and strain on Streptococcus mutans protein expression as determined by two-dimensional difference gel electrophoresis. Journal of Proteome Research 2005;4:232–7.
- 19. Kremkau FW. Diagnostic Ultrasound: Principles and Instruments. Philadelphia: Saunders; 2005.
- 20. Duck FA. Physical Properties of Tissue: A Comprehensive Reference Book. London: Academic Press; 1990.
- 21. Greenspan M, schiegg TC. Tables of the speed of sound in water. Journal of the Acoustical Society of America 1959;**31**:75–8.

- Sissons CH, Wong L, Cutress TW. Patterns and rates of growth of microcosm dental plaque biofilms. Oral Microbiology and Immunology 1995;10:160–7.
- Holmes AK, Laybourn-Parry J, Parry JD, Unwin ME, Challis RE. Ultrasonic imaging of biofilms utilizing echoes from the biofilm/air interface.. IEEE Transactions on Ultrasonics Ferroelectrics and Frequency Control 2006;53:185–92.
- Good MS, Wend CF, Bond LJ, Mclean JS, Panetta PD, Ahmed S, et al. An estimate of biofilm properties using an acoustic microscope. IEEE Transactions on Ultrasonics Ferroelectrics and Frequency Control 2006;53:1637–48.
- Palmer Jr RJ, Sternberg C. Modern microscopy in biofilm research: confocal microscopy and other approaches. *Current Opinion in Biotechnology* 1999;10:263–8.
- Cucevic V, Brown AS, Foster FS. Thermal assessment of 40-MHz pulsed Doppler ultrasound in human eye. Ultrasound in Medicine & Biology 2005;31:565–73.
- Czarnota GJ, Kolios MC, Vaziri H, Benchimol S, Ottensmeyer FP, Sherar MD, et al. Ultrasonic biomicroscopy of viable, dead and apoptotic cells. Ultrasound in Medicine & Biology 1997;23:961–5.
- Dayton PA, Ferrara KW. Targeted imaging using ultrasound. Journal of Magnetic Resonance Imaging 2002;16:362–77.
- 29. Goertz DE, van Wamel A, Frijlink ME, de Jong N, van der Steen AF. High frequency nonlinear imaging of targeted microbubble contrast agents. *Proceedings of IEEE Ultrasonic Symposium* 2005;4:2002–6.

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