

A fiber optic biosensor (FOBS) to monitor mutans streptococci in human saliva

A. Kishen*, M.S. John, C.S. Lim, A. Asundi

Biomedical Engineering Research Center, Nanyang Avenue, Nanyang Technological University, Singapore 639798, Singapore

Received 9 May 2002; received in revised form 19 December 2002; accepted 9 January 2003

Abstract

A fiber optic biosensor (FOBS) to monitor mutans streptococci activity in human saliva is developed. The biosensor utilizes fiber optic evanescent wave spectroscopy to monitor a bacterial mediated biochemical reaction. To achieve this, a short length of the cladding is removed; the fiber core surface is treated and coated with a thin film of porous glass medium using sol–gel technique. The mutans streptococci mediated reaction with sucrose is monitored using a photosensitive indicator, which is immobilized within the porous glass coating. Spectroscopic analysis shows that the transmitted intensity at 597 nm increases conspicuously when monitored for 120 min. Two distinct phases are observed, one from 0 to 60 min and the other from 60 to 120 min. A negative correlation coefficient between the rate of increase in absorption peak intensity recorded by the FOBS and the decrease in pH measured using the pH meter, was calculated to be $\rho = -0.994$. This investigation highlights the potential benefits of this sensor to monitor mutans streptococci activity in saliva.

© 2003 Elsevier Science B.V. All rights reserved.

Keywords: Fiber optics; Biosensor; Evanescent wave; Sol–gel; Spectroscopy; Caries

1. Introduction

Dental caries is a multifactorial, bacterial disease, which is characterized by the demineralization of the inorganic portion and the destruction of the organic substance of the tooth (Tanzer, 1992). Earlier experiments with gnotobiotic animals have revealed that mutans streptococci are the main etiological microorganisms in dental caries (Fitzgerald and Keyes, 1960). A quantitative measure of Streptococcus is said to be a reliable measure to predict true caries activity (Alaluusua et al., 1984, 1987; Kohler et al., 1984, 1988). An investigation conducted on 235 children, aged 11–12 years with zero or moderate caries experience, suggests that salivary tests can elucidate true risk, especially in cases in which dentists underestimated the caries risk (Kneist et al., 1998). Early recognition of high caries activity enables dental professional to formulate and incorporate preventive treatment plan for such patients.

The bacterial content of human saliva is estimated to approach 10^9 bacteria per milliliter (ml) (Bowen, 1996). Although saliva can act as a selective medium for bacterial growth, repeated swallowing results in clearing of bacteria (Bowen, 1996). Earlier studies have shown that acidogenic bacteria such as Streptococcus mutans and Lactobacilli at 10^5 Colony Forming Units per ml are predictors of caries risk (Kneist et al., 1998). A significant correlation has also been demonstrated between the numbers of mutans Streptococci in saliva and their prevalence in dentition (Bowen, 1996; Duchin and van Houte, 1978; Gronroos, 2000).

A chair-side method used for the detection and the enumeration of mutans streptococci called Dentocult SM has been developed in 1989 (Alaluusua et al., 1989). The Dentocult SM dip slide (Orion Diagnostica, Espoo, Finland), consist of a special slide, coated with mitis salivarius agar containing 20% sucrose (Alaluusua et al., 1984). The slide is inoculated with saliva and the growth density of mutans streptococci are scored after incubation at 37 °C for 48 h. For validation purpose, this chair-side technique has been compared with conventional selective agar plate culture. The comparison

* Corresponding author. Tel.: +65-67906420; fax: +65-67912274.
E-mail address: ckishen@ntu.edu.sg (A. Kishen).

showed good correlation between the methods (Alaluu-sua et al., 1989; Jensen and Bratthall, 1989). This type of chair-side slide had a short shelf life because of the mitis salivarius-sucrose agar.

Additional methods for mutans streptococci identification are based on distinctive colonial morphology on selective and nonselective agar, Gram staining, distinctive cell shape on light microscopy, specific growth characteristics and sugar fermentation and enzymatic patterns. These techniques are time consuming and needs additional laboratory support for bacterial determination. This mainly led to the unpopularity of culture based techniques in clinical dental practice. The aim of this study is to develop a miniature, sensitive and versatile, optical fiber based biosensor to monitor in real-time the mutans streptococci mediated biochemical reaction in saliva.

A chemical sensor is a device that is used to measure the concentration or the activity of a chemical species in a sample of interest. Ideally, this device should be capable of operating in a continuous and reversible manner, directly in the sample. The ultimate strength of an ideal chemical sensor is the ability to provide real-time information on the spatial and temporal distributions of a particular molecular or ionic species. The recent availability of high quality and inexpensive optical fibers has lead to a new subclass in chemical sensor called the fiber optic chemical sensors (FOCS) (Lewis and Griffiths, 1996). The fiber optics greatly improves the chemical sensor design, since optical transduction allows a wide variety of chemical detection schemes that are not previously possible (John et al., 1999). Further, the potential of using FOCS in conjunction with spectroscopy to make rapid, selective and quantitative in-situ measurements of a specific chemical species motivates this work.

In a FOCS design an optical fiber is used to transmit electromagnetic radiation to and from the sensing region that is in direct contact with the sample. Concomitantly, a chemical recognition phase is used to generate an analyte-dependent spectroscopically detectable signal within the sensing region of the optical fiber. The chemical change that occurs because of the interaction between the analyte and the immobilized indicator are measured spectroscopically by analyzing the electromagnetic radiation that returns from the sensing region. The fiber optic biosensor (FOBS) is a modified FOCS. In the FOBS the evanescent wave absorption phenomenon at the core-cladding interface of an optical waveguide is used to monitor the chemical variables associated with mutans streptococcal activity in saliva. A sol-gel technique is utilized to immobilize the photo-sensitive indicator, while the principles of selective bacterial medium is used to render the sensor milieu, specific for mutans streptococci (Slots and Taubman, 1992). The FOBS allows rapid, chair-side and quantita-

tive measurement of mutans streptococcal activity in saliva.

2. Experiments

The experiments in this study were carried out in two stages.

2.1. Stage 1: design and development of the biosensor

In this stage experiments were conducted towards designing and development of a fiber optic evanescent wave sensor to monitor mutans streptococcal activity in the excluded saliva. The stages involved are described under the following phases.

2.1.1. Evanescent wave sensor and fiber optical transduction phase

The fiber optic sensor based on the evanescent wave is basically a spectrophotometric exploration of the variables under modified Beer–Lambert Law condition. The theoretical formalism of the design with a uniform core at the sensor region, with all bound modes launched can be seen elsewhere (John et al., 1999; Ruddy et al., 1990). There are few differences between the evanescent wave absorption spectroscopy and standard absorption measurement. The former offers numerous specific advantages compared to the latter method. In evanescent wave based sensors the interrogating light remains guided and therefore no coupling optics is required at the sensor region. Also, in-situ measurements are easily possible even if the sensing sample is remotely situated away from the measuring devices. This technique can provide enhanced sensitivity over conventional bulk optics approach, since the number of reflections per unit length is greater in the EW based sensors. Also fully or quasi-distributed sensing is highly possible if the fiber is configured to be sensitive over along its length or at discrete zones. In standard absorption measurement it is often difficult or inconvenient to perform accurate absorption measurement on highly absorbing or scattering media, whereas fiber optic evanescent wave spectroscopy is more suitable for such samples.

To fabricate the sensor, multi-mode fiber with a core diameter 100 μm was selected and the protective sheath over a 10 mm length was removed using a fiber stripper. Subsequently, the cladding of the sheath-less fiber was chemically etched in 40% hydrofluoric acid for approximately 30 min (Fig. 1). The chemically treated portion of the optical fiber was later washed and dried. Once dried, the etched portion of the fiber was treated with nitric acid. This step activates the –OH groups on the surface of the optical fiber, for the better bonding of the sol-gel thin film to the glass fiber.

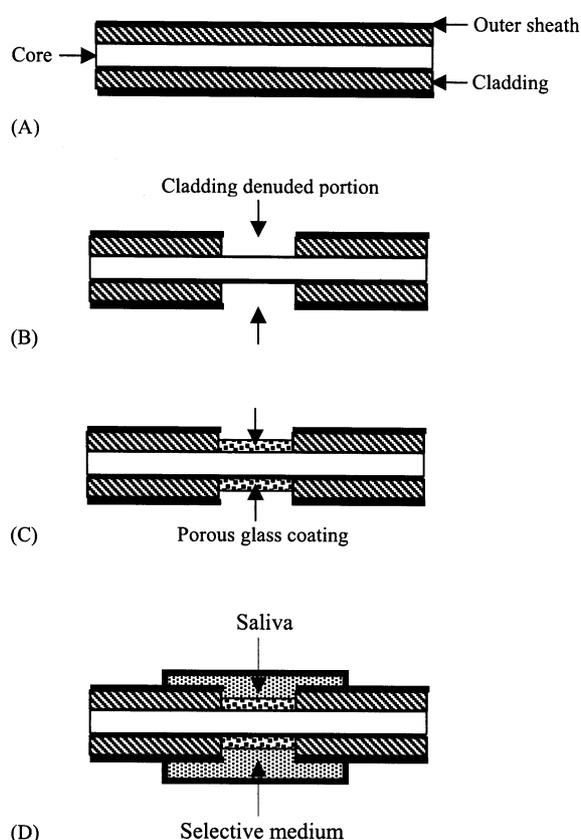


Fig. 1. Schematic diagram showing the stages of preparation of a multi-mode optical fiber (A) intact (B) cladding denuded (C) sol-gel coated (D) sensor element.

2.1.2. Biochemical recognition phase

Mutans streptococci are facultative anaerobes having their optimal growth at 37 °C (Ma and Marquis, 1997). The glucosyltransferases and fructosyltransferases in mutans streptococcus catalyzes the synthesis of water-soluble and water-insoluble glucan and fructan polymers from sucrose, leading to the formation of lactic acid, which renders the saliva acidic (Loesche, 1986). Nevertheless, the acid tolerance of the mutans streptococci enables them to continue their metabolisms even at low pH. A photosensitive pH indicator that would produce characteristic color gradients with the variations in pH is therefore utilized in the FOBS. The mutans streptococci synthesized both extracellular polysaccharides and intracellular polysaccharides from sucrose. The extracellular polysaccharide increases the adhesion of the bacteria with tooth surface, while the intracellular polysaccharide is a stored form of energy. The stored intracellular polysaccharides enable the bacteria to continue fermentation even in the absence of exogenous food supplies (Loesche, 1986).

All the chemicals used in the present study were purchased from the Sigma Aldrich (Wisconsin, USA). The precursor liquid used for the sol-gel preparation and immobilization is tetraethyl orthosilicate (TEOS).

The starting solution is prepared by partial hydrolysis of TEOS following a previously described procedure (Brinker and Scherer, 1990). The molar ratio of TEOS:water:ethanol:hydrochloric acid was 1:2.89:1.5:0.0025. Denatured anhydrous ethanol, deionized water and 12 M hydrochloric acid were used to perform the hydrolysis of TEOS. The entire mix was maintained under a constant stirring for 1 h using a magnetic stirrer and then stored at room temperature in a sealed bottle. After 24 h, a 0.01 Molar solution of bromophenol blue dissolved in ethanol was added to the precursor liquid at a ratio of 1:0.0167 (precursor solution:indicator). Following the mixing of indicator, the transparent solution of sol-gel turned orange.

The polymer at this consistency was used to coat the uncladded portion of the optical fiber. A dip coating equipment was utilized for this purpose. The precursor solution containing the indicator was placed in a 20 ml pipette. The surface prepared optical fiber, dried in a desiccator overnight was dipped into the pipette, and was drawn upwards using the dip coating equipment at a rate of 5 cm/min, at room temperature (~23 °C). The rate of fiber drawing was determined based on the viscosity of the solution at the time of drawing.

The coated fiber was then placed in a petri dish and allowed to dry for a period of 2 weeks. The petri dish was used to prevent contamination of dirt and to slow down the evaporation of the liquids in the gel. No extra solvent was added into the petri dish during the drying process. Prior to the experimentations with saliva, the sensor portion was examined to determine whether the bromophenol had been washed out of the porous glass matrix. The observed intensity of the bromophenol blue in water after repeated washing confirmed that this had not occurred. The gel formed by drying sol-gel at atmospheric pressure and room temperatures (below 100 °C) was called as xerogel. In this experiment, since the organic macromolecule (pH indicator) was immobilized in the inorganic oxide glass networks they are also known as hybrid gel (Brinker and Scherer, 1990).

A rectangular slot of 6 mm length, 4 mm breadth and 4 mm height was cut in a transparent perspex sheet of dimension (length 10 mm, breadth 6 mm and height 8 mm). The glass-coated portion of the optical fiber (sensor portion) was secured on the floor of the groove using wedges, in such a way that few drops of saliva would immerse the sensor portion completely.

2.1.3. Spectroscopic analytical phase

In developing a quantitative method for determining an unknown concentration of a given species by absorption spectrometry, the first step is the choice of the absorption band at which absorbance measurements are made. Towards this end, a UV-Visible absorption spectrum was obtained using a scanning double beam spectrophotometer for (1) pH 4 and 7 buffer solutions

with bromophenol blue at a ratio 1:0.017 (buffer solution:indicator) (2) A medium of saliva, sucrose and bromophenol blue at a ratio of 1:0.2:0.017 (saliva:sucrose:indicator), following 15 min and 24 h incubation at 37 °C. These initial experiments aided in determining the wavelength and peak characteristics for the indicator in the buffer solutions at different pH and in the saliva at different pH induced by the bacterial activity.

The UV–Visible spectroscopic analysis in the pH 7 and 4 buffer solutions with bromophenol blue showed a prominent peak at 59 nm wavelength. The intensity of the peak decreased from pH 7 to 4 (Fig. 2). In the medium of saliva, sucrose and bromophenol blue at intervals of 15 min and 24 h, it was found that the samples at 15 min interval displayed a broad absorption peak at 597 nm wavelength. The intensity of this broad absorption peak diminished in the samples 24 h after incubation (Fig. 3). Five FOBS were fabricated and tested during this study. Each sensor was experimented one with a pH 4 and 7 buffer solutions and twice with saliva (Group-1 and Group-2) as per the following Section 2.2.2.1 and Section 2.2.2.2 (Fig. 4)

2.2. Stage 2: application of the biosensor

In this stage the FOBS is connected to an optical set up and tested with human saliva. This stage is described in the following. (1) Experimental configuration (2) Testing on human saliva.

2.2.1. Experimental configuration

A fiber optic cable with the FOBS portion was secured to a fiber connector and the light from a tungsten halogen lamp was launched in it. The other end of the fiber optic cable was connected to a spectrometer. The spectrometer comprised a 1024 pixel charged couple device providing a resolution of 0.5 nm, over a

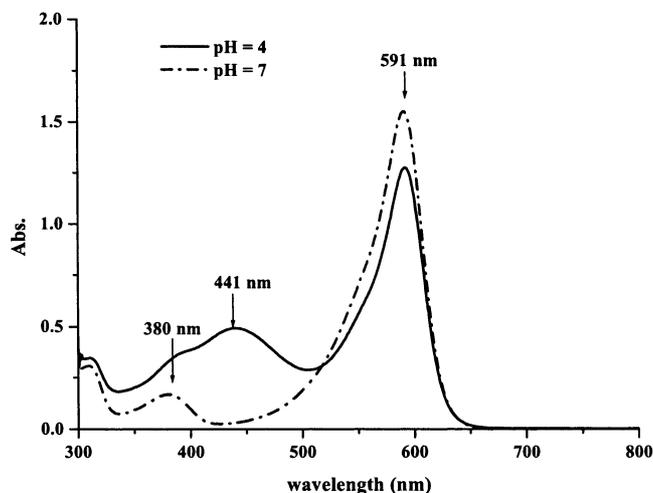


Fig. 2. UV–Visible spectrum of bromophenol blue in pH 7 and 4 buffer solutions.

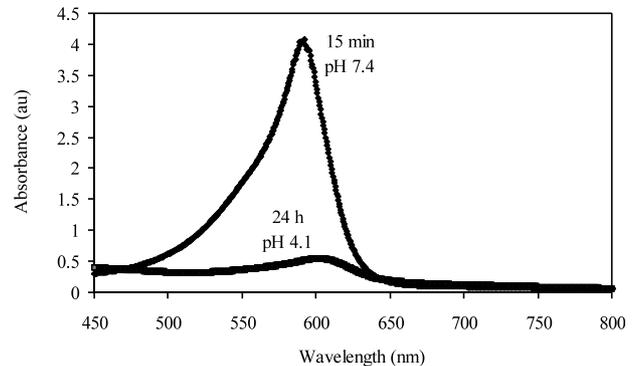


Fig. 3. UV–Visible spectrum of a medium of saliva, sucrose and bromophenol blue at an interval of 15 min and 24 h after collection and incubation.

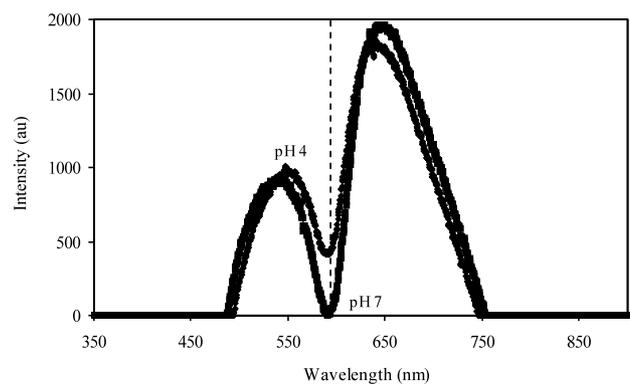


Fig. 4. Typical graph obtained from the FOBS showing the transmittance spectral variation for pH 7 and 4 solutions with bromophenol blue.

range of 400–800 nm. A personal computer with data acquisition card was linked to the spectrometer. A customized software program was used for the spectroscopic analysis.

2.2.2. Experiments

Samples from five volunteers with zero to moderate caries experience were chosen. During the analysis, each volunteer was asked to expectorate approximately 3 ml saliva samples into a test tube. The collected saliva samples were then transformed into a selective media for mutans streptococcal growth by addition of selective chemical agent and antibiotic. The selective chemical and antibiotic in a selective isolation media were designed for isolation of specific organisms, and suppress the growth of others. 1 ml of 1% solution of potassium tellurite and 2 discs of 5 µg bacitracin was added to the collected saliva samples. The potassium tellurite and the bacitracin inhibited the growth of the enterics and the lactobacilli, and rendered the saliva samples a selective medium for the growth of mutans streptococci (Emilson and Bratthall, 1976; Wade et al., 1986).

The saliva samples from each individual were then divided into two equal portions (Groups-1 and -2). 0.5 ml of 20% sucrose was added to the saliva samples in one portion and mixed well (Group-1). The saliva sample in the other portion is left as the control group (Group-2). The group-1 and group-2 saliva samples were used to monitor mutans streptococci activity using the FOBS. The testing of saliva was carried out in two ways. These experiments were conducted as follows.

2.2.2.1. Experiment-1. In this method, the saliva samples collected from the groups-1 and -2 were incubated at 37 °C. For each measurement, fresh saliva from the incubator was introduced on the FOBS and the resultant spectral response was recorded at 15 min interval over a period of 2 h.

2.2.2.2. Experiment-2. In this experiment, the saliva samples from groups-1 and -2 were separately introduced on the sensor, which was maintained in a water bath at 37 °C. In this method, the spectral response was monitored at 15 min interval over a period of 2 h.

Two approaches were used to test the saliva samples in this study. This facilitated us to examine whether the present biosensor can be used for continuous measurement of microbial activity (Section 2.2.2.2) or required additional incubation and separate measurements to be carried out at different time intervals (Section 2.2.2.1). The spectral responses at different time intervals were recorded with an integration time of 60 m s. Further, the corresponding pH value of the saliva sample at each time intervals was also recorded using a pH meter (Model 30A, Orion, USA) with a pH resolution of 0.01 and relative accuracy of ± 0.02 .

3. Results

The FOBS displayed immediate spectroscopic response on exposure to saliva. The Section 2.2.2.1 showed that the initial spectral reading obtained upon introduction of saliva became stable in less than 1 min. However, a time delay of 5 min was provided before recording the spectral change. It was also noted that there was no evident variation between the spectral response of the group-1 samples tested via Section 2.2.2.1, and those tested via Section 2.2.2.2.

It was observed from the fiber optic spectroscopic analysis of group-1 samples that there was a conspicuous variation in the transmitted intensity at 597 nm as a function of time (Fig. 5). It was found in group-1 samples that the transmitted intensity at 597 nm increased with time (Fig. 6(A)). While, the pH determined using a pH meter decreased with time (Fig. 6(B)). A negative correlation was calculated between the increase in transmitted intensity at 597 nm measured

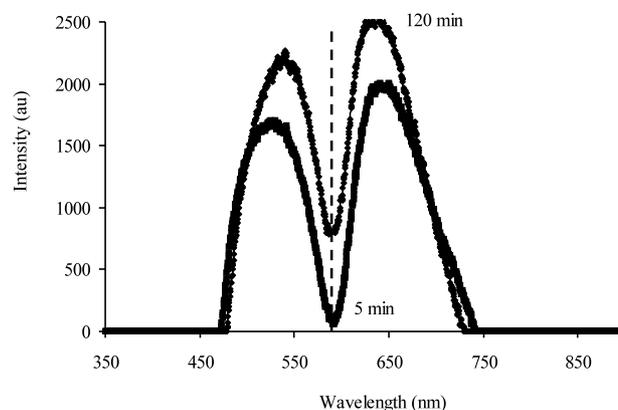


Fig. 5. Typical graph obtained from the FOBS showing the transmittance spectral variation in group-1 saliva sample from 0 to 120 min, tested via Section 2.2.2.2.

using the sensor and the decrease in pH determined using the pH meter ($\rho = -0.994$). This finding corresponded with the initial experiments conducted with the UV–Visible spectrometer, and confirmed the decrease in absorbance of saliva with bacterial activity.

The intensity at 597 nm displayed a conspicuous increase as a function of time. Two distinct regions

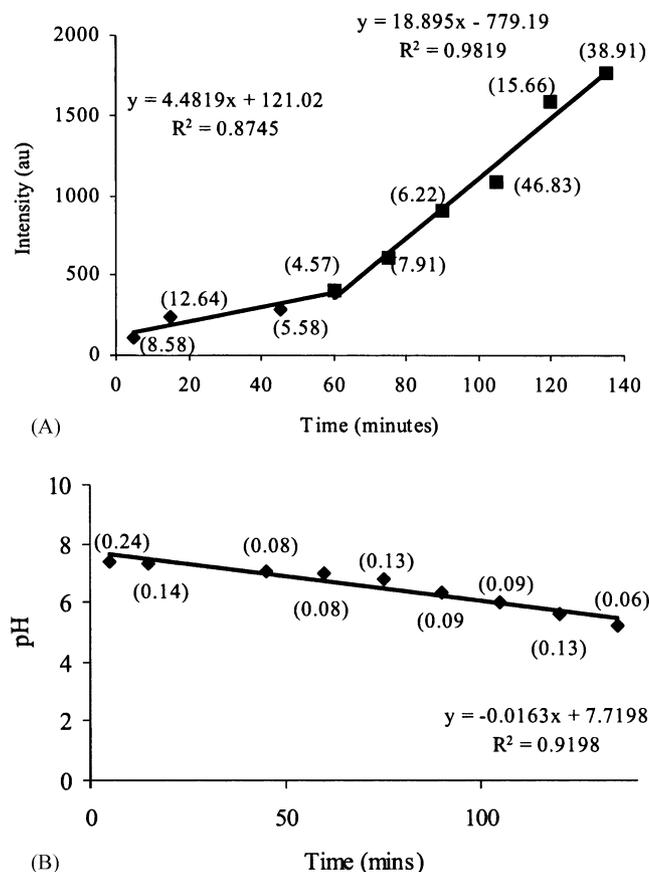


Fig. 6. The graph showing (A) the transmittance spectral intensity variation, as a function of time in FOBS and (B) the pH variation from 0 to 120 min for group-1 sample in Section 2.2.2.2 ($n = 5$; S.D. are indicated in brackets).

(phase: 1 and 2) were apparent (Fig. 6(A)). The initial phase: 1 extended from 0 to 60 min, while the phase: 2 extended from 60 to 120 min. During phase: 1, the intensity increased to 407 units in 60 min (slope 4.48), while in phase: 2, the intensity increased to 1770 units by 120 min (slope 18.89). The increase in intensity with time was much higher in phase: 2. Overall, the transmitted intensity at 597 nm increased more than 1700 units in 120 min (Fig. 6(A)). For the same duration the pH decreased linearly from 7.36 to 5.24, resulting in a decrease of 2.1 units (slope -0.016) (Fig. 6(B)).

The saliva samples in group-2 (control) however, exhibited the expected spectral pattern immediately after adding to the sensor, but did not show subsequent spectral changes with time (Fig. 7). The pH in these samples did not shift to acidic scale with time. It was noted that the entire spectral patterns in this study were repeatable and reversible in each sensor.

4. Discussion

Streptococci mutans is found commonly in man, while Streptococci sobrinus, is carried by 8–35% of people in different countries. Although Streptococci mutans and Streptococci sobrinus can be distinguished by appropriate laboratory tests, they are expensive and time-consuming. Therefore it is not always considered practical to identify down to the species level in large-scale epidemiological studies (Slots and Taubman, 1992; van Ruyven et al., 2000). Further, there has been no selective medium that would allow us to look for the presence of a single species in saliva. Consequently, most work on the relationship of bacteria to dental caries has grouped the two species together as the mutans streptococci. The selective media that are widely used for isolating caries-related streptococci uses the antibiotic Bacitracin to suppress the growth of other species but allows Streptococci mutans and Streptococci sobrinus to grow (Wade et al., 1986). Diagnostic kits designed for

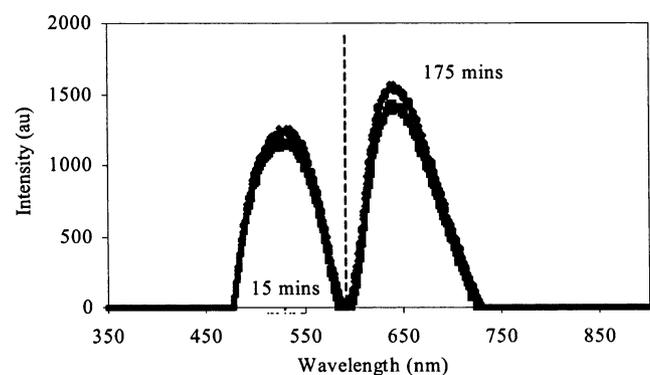


Fig. 7. Typical graph obtained from the FOBS showing the absence of spectral variation with time in the group-2 samples (saliva) in Section 2.2.2.2.

use in the dental clinic are also based on similar selective media. Therefore it should be noted that they measure the total mutans streptococci, and not just Streptococci mutans (Emilsson and Bratthall, 1976).

The FOBS developed in this study utilizes the variation in the evanescent wave absorption spectroscopy produced by the reactivity of mutans streptococci with sucrose. The mutans streptococci mediated reaction resulted in the formation of lactic acid and extracellular polysaccharide in the medium. Formation of acid would diminish the pH of the medium. Consequently, the photosensitive pH indicator immobilized in the glass thin film coating changes its color. The extracellular polysaccharide produced by the bacteria in the saliva also decreases the evanescent absorbance. The entire effect is spectroscopically observed as an increase in the transmitted intensity at 597 nm wavelength from the time of onset. It is important to realize that a selective media that restricts the growth of unwanted microbial species is particularly effective in the early hours of incubation. This would further support the advantage of using a rapid method to monitor bacterial activity (Collins et al., 1995).

During experiments it is identified that the mutans streptococci exhibited an exponential growth profile. In the initial phase from the onset to 45 min, there is a gradual increase in activity with time. While, in the second phase, from 45 min through 2 h they exhibited a conspicuous increase in activity. This biphasic activity is evident from the rate of intensity variation with time. The experiments conducted with the FOBS also displayed very short response time. It is noted that the intensity peak increased more than 1700 units for a pH change of 2.1 units towards acidic scale in a span of 2 h. The presented resolution in the spectral measurements and the capability of the FOBS to monitor spectral changes in real-time, and rapidly would facilitate further characterization of the bacterial activity.

The FOBS provides a safe means to analyze biochemical reactions. The FOBS developed and applied in this study makes use of the advantages of the optical fibers such as electrical isolation, physical flexibility and need less electrical power for driving the sensor unit (Vo-Dinh, 2000). Recently, fluorescence-based techniques have also been reported to detect bacteria from cultures. The fluorescence-based techniques are usually conducted by injecting exogenous dyes. It is crucial to discern that the biological fluids are turbid media in which the predominance of light scattering can substantially distort the measured fluorescence (Zhang et al., 2000). This signifies the advantage of a non-fluorescent optical technique to conduct real-time analysis on body fluid. The FOBS has a diameter of only 0.22 mm and a length of 10 mm. It is also flexible and portable, and the sensor geometry is more flexible to tailor different design configurations for an in-situ application. If

designed as a probe, can find potential application in detecting bacterial activity from limited accessible regions and specific regions such as root canals and periodontal pockets.

It is established that mutans streptococci population (and therefore caries activity) could vary in different regions of the mouth, and conventional methods needed larger quantities of saliva and relied on expectoration by the patient (Kneist et al., 1998). It should be noted that such samples are representative of the entire mouth, and dilution of the bacterial count is possible. It is realized that FOBS can determine the bacterial activity from few drops of saliva collected from a specific region of the mouth. Further, mutans streptococci typically produced a low pH value of less than 3.9 (van Ruyven et al., 2000), and, bromophenol blue is an effective indicator to monitor pH changes between 3 and 5 (Winans and Brown, 1975), and hence is employed in this study.

The sol–gel coating prepared from metal alkoxide such as TEOS posses a homogenous refractive index and isotropic optical property. They displays high thermal stability, chemical and environmental durability. Besides, the index of refraction of the xerogel matched with the silica core of the optical fiber (Hench et al., 1994). This is a critical factor for an evanescent wave based fiber optic spectroscopy. The sol–gel coating is also effective to immobilize photosensitive indicator. The free –OH binding sites lining the pore space and the pore size distribution within the glass matrices prevents indicator leaching. Another attractiveness of the sol–gel technology is the ability to produce glass at room temperature. Consequently, the indicator used is not exposed to high temperature and their functional activity is retained. Additionally, the surface characteristics of the sol–gel can be easily modified and they exhibit fair biocompatibility in vivo, and with certain modification the present FOBS can be used for direct in vivo applications also (Gerritsen et al., 2000).

5. Conclusions

In this study a fiber optic, evanescent wave and spectroscopy based biosensor was developed to monitor the bacterial mediated biochemical reaction in human saliva at real-time. This sensor utilized a photosensitive indicator that was immobilized within a porous glass coating on a cladding denude multi-mode optical fiber. It was observed that the intensity of the absorption valley increases conspicuously from the time of onset through the entire period of testing (120 min). A linear regression analysis demonstrated a negative correlation between the increase in absorption peak intensity and the decrease in pH of saliva with time.

Acknowledgements

The authors wish to gratefully acknowledge the School of Mechanical & Production Engineering, Nanyang Technological University, for the funds from Strategic Development Scheme (SDS) 11/2001. We also thank Prof. Hu Xiao, School of Material Engineering, Nanyang Technological University, for the facilities provided in the Polymer Laboratory.

References

- Alaluusua, S., Savolainen, J., Tuompo, H., Gronroos, L., 1984. Slide-scoring method for estimation of Streptococcus mutans levels in saliva. *Scandinavian Journal of Dental Research* 92, 127–133.
- Alaluusua, S., Kleemola-Kujala, E., Nystrom, M., Evalahti, M., Gronroos, L., 1987. Caries in the primary teeth and salivary Streptococcus mutans and lactobacillus levels as indicators of caries in permanent teeth. *Pediatric Dentistry* 9, 126–130.
- Alaluusua, S., Nystrom, M., Gronroos, L., Peck, L., 1989. Caries-related microbiological findings in a group of teenagers and their parents. *Caries Research* 23, 49–54.
- Bowen, W.H., 1996. Salivary influences on the oral micro flora. In: *Saliva and oral health*, second ed. British Dental Journal, London.
- Brinker, C.J., Scherer, G.W., 1990. *Sol–Gel Science: the Physics and Chemistry of Sol–Gel Processing*. Academic press, New York.
- Collins, C.H., Patricia, M.L., Grange, J.M., 1995. *Microbiological Methods*, seventh ed.. Butterworth-Heinemann Ltd, Great Britain.
- Duchin, S., van Houte, J., 1978. Colonization of teeth in humans by Streptococcus mutans as related to its concentration in saliva and host age. *Infection Immunity* 20, 120–125.
- Emilson, C.G., Bratthall, D., 1976. Growth of Streptococcus mutans on various selective media. *Journal of Clinical Microbiology* 4, 95–98.
- Fitzgerald, P.J., Keyes, P.H., 1960. Demonstration of the etiologic role of streptococci in experimental caries in the hamster. *Journal of American Dental Association* 61, 9–19.
- Gerritsen, M., Kros, A., Sprakel, V., Lutterman, J.A., Nolte, R.J., Jansen, J.A., 2000. Biocompatibility evaluation of sol–gel coatings for subcutaneously implantable glucose sensors. *Biomaterials* 21, 71–78.
- Gronroos, L., 2000. Quantitative and qualitative characterization of mutans Streptococcus in saliva and in dentition, Academic dissertation, University of Helsinki, Finland.
- Hench, L.L., Araujo, Fernando, Gabriel, da-Silva, West, J.K., Latorre, G.P., 1994. Gel–silica optics: theory and application. *Journal of Sol–Gel Science and Technology* 2, 647–655.
- Jensen, B., Bratthall, D., 1989. A new method for the estimation of mutans streptococci in human saliva. *Journal of Dental Research* 68, 468–471.
- John, M.S., Radhakrishnan, P., Nampoore, V.P.N., Vallabhan, C.P.G., 1999. A fiber optic evanescent wave sensor for monitoring the rate of pulsed lased deposition of metal thin films. *Measurement Science and Technology* 10, N17–N20.
- Kneist, S., Heinrich-Weltzien, R., Tietze, W., Fischer, T., Stosser, L., 1998. Salivary microbiology and dentist's ability in the prediction of caries activity. *Journal of Dental Research* 75, 1251–1251.
- Kohler, B., Andreen, I., Jonsson, B., 1984. The effect of caries-preventive measures in mothers on dental caries and the oral presence of the bacteria Streptococcus mutans and lactobacilli in their children. *Archives of Oral Biology* 29, 879–883.

- Kohler, B., Andreen, I., Jonsson, B., 1988. The earlier colonization by mutans streptococci, the higher the caries prevalence at 4 years of age. *Oral Microbiology Immunology* 3, 14–17.
- Lewis, I.R., Griffiths, P.R., 1996. Raman spectrometry with fiber optic sampling. *Applied Spectroscopy* 50, 12A–30A.
- Loesche, W.J., 1986. Role of *Streptococcus mutans* in human dental decay. *Clinical Microbiology Reviews* 50, 353–380.
- Ma, Y., Marquis, R.E., 1997. Thermophysiology of *Streptococcus mutans* and related lactic-acid bacteria. *Antonie Van Leeuwenhoek* 72, 91–100.
- Ruddy, V., MacCraith, B.D., Murphy, J.A., 1990. Evanescent absorption spectroscopy using multimode fibers. *Journal of Applied Physics* 67, 6070–6074.
- Slots, J., Taubman, M., 1992. *Contemporary Oral Microbiology and Immunology*, Mosby Yearbook.
- Tanzer, J.M., 1992. Microbiology of dental caries. In: *Contemporary Oral Microbiology and Immunology*. Mosby, St. Louis.
- van Ruyven, F.O., Lingstrom, P., van Houte, J., Kent, R., 2000. Relationship among mutans streptococci, 'low-pH' bacteria, and iodophilic polysaccharide-producing bacteria in dental plaque and early enamel caries in humans. *Journal of Dental Research* 79, 778–784.
- Vo-Dinh, T., 2000. SERS chemical sensors and biosensors: a new tools for environmental and biological analysis. *Sensors and Actuators B* 29, 183–189.
- Wade, W.G., Aldred, M.J., Walker, D.M., 1986. An improved medium for isolation of *Streptococcus mutans*. *Journal of Medical Microbiology* 22, 319–323.
- Winans, R., Brown, C.A., 1975. Fading of bromophenol blue: a combined synthesis and spectrophotometric kinetics experiment. *Journal of Chemical Education* 52, 526–527.
- Zhang, Q.G., Muller, M.G., Wu, J., Feld, M.S., 2000. Turbidity-free fluorescence spectroscopy of biological tissue. *Optics Letters* 25, 1451–1453.