Chapter 6

Spectroscopic Investigation of De- and Re-mineralization of Tooth Enamel \textit{in vitro}
6.1 INTRODUCTION

Cyclic demineralization and remineralization of the tooth replicate the natural process of demineralization and remineralization that takes place in the oral cavity environment. Characterization of the behaviour of tooth enamel lesions in a simulated natural environment by optical methods leads to improvement in sensitivities for detection of early demineralization and helps to introduce appropriate treatment modalities that avoid the risk of aesthetic damage or restorative intervention. Towards this, nitrogen laser-induced fluorescence and tungsten halogen lamp-induced DR spectra were recorded on a miniature fiber-optic spectrometer from tooth samples subjected to cyclic de- and re-mineralization (CDR) for 10 days, followed by continuous remineralization (CR) for 14 days. The LIF and DR spectral intensities were found to decrease with CDR, but get reversed during CR. This chapter presents the applicability of LIFRS to detect de- and re-mineralization changes in tooth in vitro and correlates the results of curve-fitting with those obtained from raw spectral data and DR measurements.

6.2 STUDY MATERIAL AND PROTOCOL

Twenty five sound teeth were collected from a nearby dental clinic following extraction, for various reasons including periodontal problems and transported to the laboratory in isotonic saline. Prior to measurements, the samples were washed in deionized water, cleared of food particles or blood clotting and dried with tissue paper. A square window of dimensions 3.5 mm × 3.5 mm was made on buccal side of each tooth by painting the remaining portions with acid resistant nail polish. After recording the baseline spectra, tooth samples were exposed to demineralization and remineralization by a cyclic process to stimulate a near natural in vivo environment (Jones et al, 2006). The daily schedule for cyclic treatment consisted of 6 hr demineralization and 17 hr remineralization. During demineralization, each tooth sample was immersed in 40 mL aliquot buffer solution containing 2.0 mmol/L calcium, 2.0mmol/L phosphate and 0.075mol/L acetate, maintained at pH 4.3 and 37°C temperature. Afterwards, each sample was remineralized in a 20mL solution of 1.5mmol/L calcium, 0.9 mmol/L phosphate and 150mmol/L potassium chloride maintained at pH 7.0 and 37°C temperature. Tooth samples were subjected to 10
days of cyclic demineralization and remineralization (CDR) regime, which represents an early caries lesion. The tooth samples were then subjected to continuous remineralization (CR) for 14 days in the above mineralizing solution, with the addition of 2-ppm F in the form of NaF to enhance the remineralization effect.

The LIF and diffuse reflectance spectra were then recorded in the 350-700 nm spectral range using the OOI Base32 software (Ocean Optics Inc., USA) after each phase of de-mineralization/ remineralization treatment of tooth lesions. The OOI Base32 software was configured to record the spectra, averaged for 40 scans, with a boxcar width of 10 nm and an integration time of 350 ms. Ten sets of autofluorescence and diffuse reflectance spectra were recorded sequentially from the exposed region of tooth samples after each treatment of CDR and CR. The mean spectra from each treatment is then determined and used in data analysis. The mean LIF spectra from the sound, demineralized and remineralized regions were curve-fitted using Gaussian spectral functions. The deconvoluted peak intensities and Gaussian curve areas were then utilized to identify de- and re-mineralization changes in tooth enamel.

6.2.1 Visual assessment of lesions

An experienced clinician, who was blinded to the spectral findings examined each tooth after completion of fluorescence and diffuse reflectance measurements. Any signs of demineralization, normally a white spot or surface roughness along with the
time at which it occurred, were recorded. An analysis of variance (ANOVA) was performed to detect whether the average LIF and DR spectral intensities differed between the sound, demineralized and remineralized enamel groups.

6.3 RESULTS

6.3.1 LIF spectral features

The fluorescence spectrum of sound tooth consists of a broad peak at 490 nm with a less intense shoulder around 440 nm. Although the spectral shape remains the same during de-mineralization and re-mineralization, the fluorescence intensity follows a decreasing trend in 10 days of CDR and an increasing trend in 14 days of CR, as shown in Fig. 6.1a. In order to ascertain changes in the emission peak intensity, the LIF spectra were normalized with respect to the intensity of the 490 nm peak as shown in Fig. 6.1b. Fig.6.2 shows the extent of change in the 440 nm peak intensity after CDR and CR. A gradual decrease in intensity was observed for the 440 nm band during CDR, which was followed by an intensity enhancement in CR. Further, beyond 500 nm, we noticed a corresponding increment in intensity followed by a decline in the long wavelength tail of the 490 nm peak.

The mean LIF spectra during various stages of CDR and CR were curve-fitted using Gaussian spectral functions. It can be seen that peak fitting of LIF spectra using four Gaussian spectral peaks gives the best fit, with an $r^2$ value of 0.998. Table 6.1 shows the peak position of the various bands, their Gaussian curve areas, full width at half intensity maximum (FWHM) and the $\chi^2$ and $r^2$ values of fitting during different stages of CDR and CR. In sound tooth, the four bands are located at 411.32, 440.08, 484.37 and 521.98 nm. These
bands will henceforth be designated as 410, 440, 485 and 525 nm peaks, for simplicity. It is seen that the 525 nm peak shifts towards the red region by 35.3 nm during CDR and these shifts gets completely nullified in 14 days of CR (Table 6.1). In addition, it was observed that the FWHM width of the 525 nm peak reduced by 27.7 nm in 10 days of CDR, which gets reversed within 14 days of CR whereas an opposite trend was observed in the case of the 485 nm peak, as shown in Fig.6.3. Furthermore, a decreasing trend followed by an enhancement is observed in the curve-fitted peak amplitude and Gaussian curve area of all the four peaks at 410, 440, 485 and 525 nm during CDR and CR. However, these variations were pronounced for the 410 and 525 nm peaks, as shown in Fig. 6.4(a, b).

6.3.2 Diffuse reflectance spectral features

Fig. 6.5 a, b shows the mean diffuse reflectance spectra during various stages of CDR and CR, and the same normalized to the intensity at 675 nm. It can be seen that CDR produces a gradual decline in spectral intensity, whereas CR produces an increase in intensity (Fig. 6.5a). However, in the normalized DR spectra there are no noticeable changes in the spectral shape, except for a slight decrease in spectral intensity in the 450 to 600 nm region.

6.3.3 Spectral intensity and curve area plots

The temporal profile of raw LIF intensity at 440 and 490 nm and the DR spectral intensity at 500 nm is plotted in Fig. 6.6a along with changes in the Gaussian curve area of the deconvoluted peaks at 410 and 525 nm in Fig. 6.6b during CDR, whereas
the corresponding changes during CR is shown in Fig. 6.7(a, b). The overall fluorescence and diffuse reflectance spectral intensities and Gaussian curve areas of the constituent bands were found to decrease gradually with CDR (Fig. 6.6) owing to mineral losses in tooth, whereas the intensity increases with CR owing to mineral restoration (Fig. 6.7). As compared to the variations seen in raw spectral intensity at 440, 490 and 500 nm, the Gaussian curve area of 410 and 525 nm peaks show a pronounced variation. This can also be seen from the slope of the corresponding lines in Fig. 6.6 and 6.7.

**Table 6.1. Results of curve-fitting on the mean LIF spectrum from sound and exposed areas of tooth during CDR and CR.**

<table>
<thead>
<tr>
<th>Tooth</th>
<th>Peak Centre (nm)</th>
<th>FWHM (nm)</th>
<th>Area</th>
<th>Amplitude</th>
<th>$\chi^2$</th>
<th>$r^2$</th>
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<tr>
<td>Sound</td>
<td>411.32</td>
<td>33.722</td>
<td>6064.6</td>
<td>143.49</td>
<td>32.79</td>
<td>0.998</td>
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<td></td>
<td>440.08</td>
<td>30.699</td>
<td>7637.9</td>
<td>198.52</td>
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<td></td>
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<tr>
<td></td>
<td>484.37</td>
<td>50.489</td>
<td>17575</td>
<td>277.74</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>521.98</td>
<td>107.23</td>
<td>27189</td>
<td>202.31</td>
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<td></td>
</tr>
<tr>
<td>CDR(3 day)</td>
<td>406.83</td>
<td>23.869</td>
<td>1891</td>
<td>63.21</td>
<td>12.68</td>
<td>0.999</td>
</tr>
<tr>
<td></td>
<td>436.13</td>
<td>33.589</td>
<td>6685.5</td>
<td>158.81</td>
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<tr>
<td></td>
<td>485.23</td>
<td>55.147</td>
<td>16718</td>
<td>241.88</td>
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<td></td>
<td>534.02</td>
<td>81.969</td>
<td>10450</td>
<td>101.72</td>
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<td>CDR(6 day)</td>
<td>406.79</td>
<td>22.62</td>
<td>967</td>
<td>34.109</td>
<td>5.56</td>
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<td>435.74</td>
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<td>486.74</td>
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<td>6177.6</td>
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<td>CDR(10 day)</td>
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<td>544.96</td>
<td>18.297</td>
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<td>436.12</td>
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<td>557.37</td>
<td>79.485</td>
<td>2902.1</td>
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<td>CR(7 day)</td>
<td>408.1</td>
<td>23.232</td>
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<td>CR(14 day)</td>
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Abbreviations: FWHM- full width at half maximum; $\chi^2$- chi-square; $r^2$- correlation coefficient; CDR- cyclic demineralization and remineralization; CR- continuous remineralization

Visual inspection of the teeth failed to detect any signs of demineralization until 6 days. At the 10th day, visual evidence of demineralization was noted in all experimental samples. Spectral data were analyzed by ANOVA and statistically significant differences in mean spectral intensities ($p<0.05$) were noticed between sound, demineralized and remineralized enamel in LIF and DR spectral measurements,
demonstrating a significant decrease in spectral intensity during demineralization and an increase during remineralization.

6.4 DISCUSSION

Tooth enamel is a very highly mineralized tissue consisting of approximately 96% of dry weight as inorganic material while the rest is water and organic material (Hunt, 1959; Dawes, 2003). Demineralization and remineralization have a vital impact on the strength and hardness of dental enamel. Usually, a tooth is in a constant state of cyclic or back-and-forth demineralization and remineralization due to interactions with fermentable carbohydrates and surrounding saliva. Initial enamel caries develop when the pH level at the tooth surface is lower than which could be counterbalanced by remineralization. It has been found that at lower pH, millions of calcium and other mineral ions are released from the hydroxyapatite latticework, which eventually leads to loss in structural integrity of tooth enamel. Caries advance may be arrested at this stage. In the present CDR study, the pH was maintained at a lower value of 4.3 by periodic monitoring using a pH meter. Monitoring demineralization and remineralization of dental hard tissues is essential for the prevention and minimally invasive treatment of dental caries. Optical spectroscopic techniques provide an approach to identify these incipient changes in enamel so that a proper restorative procedure can be formulated to prevent caries progression.

We have used the in vitro cyclic demineralization and remineralization which is the best possible way to simulate an in vivo environment for studying the dynamics of tooth caries development (ten Cate,
This is because in the oral cavity, the demineralization process involves cycles of acid attack following dietary carbohydrate intake, which results in an increase in acid production by the oral microflora, leading to the dissolution of minerals. As time goes on, there is a reversal of the acidic environment to the neutral status, due to various physiological and chemical factors. This return of acidic pH to neutral status results in deposition of lost minerals, thus retaining the tooth integrity (Loesche WJ, 1986).

In our CDR study, it was observed (Fig. 6.1a) that impairment of enamel due to demineralization leads to a decrease in the fluorescence signal intensity whereas remineralization produces an increase in intensity during CR treatment. By normalizing the spectrum with respect to the intensity of the 490 nm band (Fig. 6.1b); we were able to observe changes in fluorescence spectral intensity of the 440 nm band that appears as a shoulder to the 490 nm peak. Borisova et al (2004) also observed a significant reduction in the intensity of fluorescence signal of demineralized and carious tooth.

Normally, tooth enamel is composed of millions of prisms or rods with waveguide properties that facilitate deep penetration when illuminated with UV-visible light. During tooth demineralization, the prism structure is damaged and the waveguide properties are lost so that the irradiated light does not penetrate deeply (Ando et al, 2001). This leads to a reduction in the fluorescence intensity (Fig. 6.1 and Fig. 6.2). It was also reported that changes in fluorescence might be due to the changes in the calcium and phosphate mineral content of tooth enamel, which makes them soft (Al-khateeb et al, 1997a). Demineralization of tooth enamel is not a simple chemical interaction between enamel crystals and surrounding fluid, because enamel is a microporous solid and these inter-crystalline spaces are filled with tissue fluid from the pulpo-
dentinal organ. The fine mesh work of protein that covers enamel crystals also influences the chemical behaviour of the enamel.

The LIF spectra of sound tooth was resolved into four constituent peaks centered at 411.32, 440.08, 484.37 and 521.98 nm by curve-fitting using Gaussian spectral functions, which falls close to the values reported in an earlier study for detection of in vitro dental erosion (Shiny et al, 2008). The emission peak around 410 nm could be attributed to the presence of hydroxypyridinium chromophore, a collagen crosslink and dityrosine, as reported earlier (Fujimoto et al, 1977; Walters and Eyre, 1983; Booij and ten Bosch, 1982). There are noticeable variations not only in the peak emission but also in the peak amplitude, FWHM width, and the Gaussian curve area during CDR and CR. The variations in the curve-fitted peak amplitude and Gaussian curve area of the 410 and 525 nm peaks (Table 6.1, Fig. 6.4) and the shift in peak position and variation in the FWHM width of the 525 nm peak during CDR and CR (Fig. 6.3) has the potential to be utilized to understand the de- and re-mineralization status of tooth in an in vivo environment. This variation observed in peak position and FWHM width of the 525 nm could be due to the changes in chemical composition during the disintegration process as observed during caries development (Subhash et al, 2005). The red-shift of the 525 nm peak during CDR is similar to the shift seen in dentin and pulp level caries. Therefore, these changes in spectral characteristics could be useful in the identifying the extent of demineralization and stage of tooth caries (Subhash et al, 2005).

Further, the concurrent increase and decrease in spectral intensity noticed beyond 500 nm during CDR and CR could be due to an energy transfer between the levels.
involved in the 440-nm emission and the long wavelength tail of the 490 nm band. The red shift of the 525 nm peak seen in the curve-fitted spectra during CDR and its complete retrace to initial values (Table 6.1) during CR supports this hypothesis.

The diffuse reflectance intensity in sound tooth is higher owing to increased reflection from the enamel surface. This leads to a lower amount of radiation entering beyond the enamel to generate sufficient back scattering from the underlying enamel and dentine layers. Further, during demineralization, changes in light scattering properties associated with the destruction of the waveguide structure of the enamel diminish the diffuse reflectance spectra (Ando et al, 2001).

However, the nucleic acids, urocanic acid and proteins that are present in tooth have strong absorption in the ultraviolet and blue-green spectral regions and this dominates over signal in the shorter wavelength region and results in diminished diffuse reflectance spectral intensity in the 400-600 nm range (Fig. 6.5). It is also seen that an increase in mineral volume from fluoride-enhanced remineralization significantly decreases optical reflectivity of artificial lesions within an enlarged surface zone, whereas reflectivity does not necessarily decrease in the body of underlying lesion after remineralization (Jones and Fried, 2006).

As carious lesion undergoes remineralization, the deep porosities accumulate more mineral than lesion surface zone. It is also reported that successful remineralization of the surface zone limits the continued diffusion of ions into the deeper regions of the lesion, which was observed during in vivo remineralization studies of early caries (Arends and Gelhard, 1983). As lesion progresses, the mineral
content at specific depth does not vary significantly after a certain volume; instead, diffusion of ions through this area doesn’t affect the mineral volume (Arends et al, 1997), whereas in remineralization, mineral volume is increased mainly by growth of the remaining crystals, restoration of partially demineralized crystals and the deposition of newly formed crystals (Yanagisawa and Miake, 2003), which might be the reason for the gradual variations in fluorescence and DR spectral features during remineralization (Fig. 6.1 and Fig. 6.5). Also, remineralization process is slower as compared to demineralization, when mineral uptake occurs in very small quantities.

6.5 CONCLUSIONS

The study shows that both LIF with excitation by a UV laser (337 nm) and DR spectroscopy with white light illumination have the potential to identify de- and re-mineralization changes in tooth from spectral intensity variations. Among the various parameters studied, variation in the FWHM width and peak position of the 525 nm peak deconvoluted by curve-fitting, and the changes in the Gaussian amplitude and curve area of the 410 and 525 nm peaks appear to be effective in identifying incipient changes in tooth. Although the diffuse reflectance intensity variation during CDR and CR are marked, the information contained in the LIF spectral intensity and constituent peak contributions achieved through curve fitting are more suited for monitoring of early transformations in tooth enamel. Nevertheless, these modalities need to be further explored in a clinical environment to test its efficacy for monitoring changes in mineral content.