CHAPTER 6

STEADY STATE AND TIME RESOLVED FLUORESCENCE SPECTROSCOPIC CHARACTERIZATION OF FLAVINS IN URINE OF NORMAL AND ORAL CANCER SUBJECTS

6.1 INTRODUCTION

In the preceding chapters, the steady state fluorescence characteristics of urine of normal subjects and cancer patients of different stages and of different origins were discussed. Although steady-state approaches have been tested as a diagnostic tool for cancer diagnosis, the potential of time-resolved techniques to improve the diagnostic ability has been scarcely investigated. Time-resolved measurements resolve fluorescence intensity decay in terms of lifetimes, thus providing additional information with regard to the underlying fluorescence dynamics. Time-resolved fluorescence measurements capture the transient decay, which depends on the relative concentrations and lifetimes of the fluorophores contributing to the fluorescence signal. It is also extremely sensitive to the local biochemical environment which may differ in healthy and diseased conditions. In addition, the fluorophore lifetimes are independent of absolute intensity and so do not change with variations in excitation intensity. The lifetime measurements enable the discrimination of fluorophores with overlapping emission spectra but different lifetimes.

Also, it is observed from the results of the steady state fluorescence measurements, that the contribution of flavin plays an important role in the disease diagnosis. However, the steady state fluorescence characteristics of
oral cancer prove low sensitivity and specificity in the disease diagnosis when compared to the cervical cancer where the sensitivity and specificity are high. In this context, attempt was made to study the excited state kinetics of flavins present in urine samples of normal subjects and oral cancer patients in order to check whether the diagnostic accuracy can further be improved. Urine samples of fifty oral cancer patients and fifty normal subjects were collected. The fluorescence emission characteristics were measured for 450 nm excitation. The decay characteristics of the same samples were measured at 520 nm emission for excitation at 460 nm. The goodness of decay fit was judged by chi-square value.

6.2 RESULTS

6.2.1 Fluorescence Emission Spectroscopic Characterization of Urine Samples of Normal Subjects and Patients with Oral Cancer at 450 nm Excitation

The averaged and normalised fluorescence emission spectrum of normal subjects and oral cancer patients at 450 nm excitation are shown in Figure 6.1 (a&b). Figure 6.1 (c) shows the difference between the fluorescence emission spectrum of cancer subjects from that of normal subjects. From Figure 6.1 (a&b), it is observed that the normal and oral cancer subjects exhibits a broad spectrum in the wavelength region 500 nm to 600 nm and the fluorescence emission intensity of oral cancer patients is greater than normal subjects. From Figure 6.1 (b), it is observed that the emission maximum occurred at 525 nm for both normal subjects and oral cancer patients. The full width at half maximum (FWHM) of normal subjects is 89 nm and for cancer subjects it is 88 nm. Further, from the difference spectrum 6.1 (c), it is observed that the normal subjects shows positive difference around 480 nm and negative difference in the region 530 nm to 650 nm.
Figure 6.1  (a) Averaged (b) Normalized fluorescence emission spectra of urine samples of normal subjects and oral cancer patients and (c) Difference spectrum between normal and oral cancer subjects at 450 nm excitation
6.2.2 Time Resolved Fluorescence Characteristics of Urine Samples of Normal Subjects and Oral Cancer Patients for Emission at 520 nm

As there was a considerable spectral difference between the fluorescence emission intensity of normal subjects and oral cancer patients, the time resolved fluorescence spectroscopic characterisation of urine was also carried out. For decay at 520 nm emission, out of fifty normal subjects, forty subjects exhibit tri-exponential decay and ten samples with bi-exponential decay. Whereas, out of fifty oral cancer subjects, thirty samples show tri-exponential decay and twenty samples with bi-exponential decay. Figure 6.2 (a&b) shows the typical bi-exponential decay profile of urine samples of normal subjects and oral cancer patients respectively. Figure 6.3 (a&b) shows the typical tri-exponential decay profile of urine of normal subjects and oral cancer patients for emission at 520 nm. On the other hand, the standard fluorophore riboflavin which has fluorescence emission around 520 nm exhibits a bi-exponential decay and was shown in Figure 6.4.

From the Table 6.1, it is observed that bi-exponential fits well to characterise the decay profile of standard fluorophore flavin at 520 nm emission. The average lifetime for the bi-exponential decay at 520 nm for the urine samples of normal subjects is 4.6 ns and for oral cancer samples it is 3.9 ns whereas in the case of standard fluorophore riboflavin, it is 4.7 ns which is closer to the lifetime value of normal subjects. For samples with tri-exponential decay at 520 nm, the average lifetime of urine of normal subjects is 4.6 ns and for oral cancer subjects, it is 4.9 ns.
Figure 6.2  Typical bi exponential decay profile of urine samples measured at 520 nm emission for 460 nm excitation (a) normal subjects and (b) oral cancer patients along with their residuals
Figure 6.3 Typical tri exponential decay profile of urine samples measured at 520 nm emission for 460 nm excitation (a) normal subjects and (b) oral cancer patients along with their residuals.
Figure 6.4  Typical decay profile of flavin standard at 520 nm emission
Table 6.1: Shows the average lifetime components and their relative amplitudes

<table>
<thead>
<tr>
<th>Decay at 520 nm (bi-exp)</th>
<th>Group</th>
<th>( \tau_1 ) (ns)</th>
<th>( \tau_2 ) (ns)</th>
<th>( \tau_3 ) (ns)</th>
<th>Amplitude A1</th>
<th>Amplitude A2</th>
<th>Amplitude A3</th>
<th>Average Lifetime (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Riboflavin Standard</td>
<td></td>
<td>3.11±0.71</td>
<td>4.89±0.02</td>
<td>--</td>
<td>10.08±3.36</td>
<td>89.92±2.13</td>
<td>--</td>
<td>4.7</td>
</tr>
<tr>
<td>Cancer</td>
<td>Normal</td>
<td>1.41±0.6</td>
<td>4.99±1.13</td>
<td>--</td>
<td>27.27±10.90</td>
<td>72.73±10.90</td>
<td>--</td>
<td>4.6±0.7</td>
</tr>
<tr>
<td>Cancer</td>
<td>Normal</td>
<td>1.58±0.75</td>
<td>4.26±0.7</td>
<td>--</td>
<td>29.53±12.25</td>
<td>70.47±12.25</td>
<td>--</td>
<td>3.9±0.6</td>
</tr>
<tr>
<td>Cancer</td>
<td>Normal</td>
<td>0.62±0.97</td>
<td>2.62±1.18</td>
<td>5.01±1.87</td>
<td>26.86±1.87</td>
<td>33.41±15.46</td>
<td>39.73±14.90</td>
<td>4.6±0.6</td>
</tr>
<tr>
<td>Cancer</td>
<td>Normal</td>
<td>0.91±1.07</td>
<td>3.21±1.76</td>
<td>5.68±2.77</td>
<td>25.27±10.69</td>
<td>39.15±16.77</td>
<td>30.58±15.57</td>
<td>4.9±0.7</td>
</tr>
</tbody>
</table>

\( \chi^2 \) values ranges between 0.99 to 1.20
6.2.3 Stepwise Linear Discriminant Analysis for the Fluorescence Emission Spectral Data at 450 nm Excitation and for the Fluorescence Decay at 520 nm Emission

To quantify the observed spectral differences between normal and oral cancer subjects, the fluorescence spectral data were subjected to stepwise linear discriminant analysis to analyse the statistical significance in discriminating the oral cancer patients from that of normal subjects. The discriminant analysis were performed using SPSS/PC+ 17 software. The method of the statistical analysis was discussed in detail in chapter 2.

The fluorescence emission intensity values at 480 nm (V₁), 490 nm (V₂), 500 nm (V₃), 510 nm (V₄), 520 nm (V₅) and 530 nm (V₆) of the steady state fluorescence emission spectrum of urine samples of normal subjects and oral cancer patients were taken as input values for the analysis. The input values are subjected to students t-test and the significant values were included in the analysis. The mean with standard deviation and the significance (P values) of these parameters for normal and oral cancer samples studied are given in Table 6.2. The P-values of all the parameters are ≤ 0.05, indicating the statistical significance of the parameters. The stepwise linear discriminant analysis resulted in the following canonical discriminant function DF for emission spectra at 450 nm excitation.

\[ DF = V_6 - 1.224 \]

Since, there exist bi exponential fits for some cases and tri exponential fits for the other cases of normal and oral cancer subjects, the average lifetime of the urine samples were taken as input variables to find the statistical significance of the time resolved spectroscopic technique in the discrimination of oral cancer patients from that of normal subjects.
Figure 6.5 shows the distribution of average lifetime of normal subjects and oral cancer patients.

Table 6.2  Mean values (± SD) and level of significance (p) of the parameters used for statistical analysis

<table>
<thead>
<tr>
<th>Wavelength of excitation</th>
<th>Parameter</th>
<th>Normal (x10^5)</th>
<th>Cancer (x10^5)</th>
<th>Significance (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>450 nm</td>
<td>( V_1 )</td>
<td>4.9 ± 2.2</td>
<td>6.1 ± 3.4</td>
<td>0.042</td>
</tr>
<tr>
<td></td>
<td>( V_2 )</td>
<td>6.8 ± 3.3</td>
<td>9.1 ± 6.1</td>
<td>0.023</td>
</tr>
<tr>
<td></td>
<td>( V_3 )</td>
<td>9.4 ± 4.8</td>
<td>13.4 ± 11.1</td>
<td>0.019</td>
</tr>
<tr>
<td></td>
<td>( V_4 )</td>
<td>11.7 ± 6.3</td>
<td>17.4 ± 15.6</td>
<td>0.018</td>
</tr>
<tr>
<td></td>
<td>( V_5 )</td>
<td>12.9 ± 7.21</td>
<td>9.4 ± 17.9</td>
<td>0.020</td>
</tr>
<tr>
<td></td>
<td>( V_6 )</td>
<td>12.9 ± 7.3</td>
<td>13.4 ± 11.1</td>
<td>0.021</td>
</tr>
</tbody>
</table>

Figure 6.5  Scatter plot shows the distribution of average lifetime of urine of normal subjects and oral cancer patients for 520 nm emission
6.3 DISCUSSION

Riboflavin is a water soluble vitamin present in a wide variety of foods. It is well established that the cofactors of riboflavin, FMN and FAD which act as electron carriers participates in a diversity of redox reactions central to human metabolism. These enzymes play a fundamental role in the mitochondrial electron transport chain (Powers 2003). Some reactions involve transfer of a single hydrogen to a flavin, forming flavin-H⁺, which is then recycled in a separate reaction. Sometimes two molecules of flavin each accept one hydrogen atom from the substrate to be oxidized. Other reactions involve the sequential transfer of two hydrogen on to the flavin, forming first the flavin-H⁺ radical, then fully reduced flavin-H2. The reoxidation of reduced flavins in enzymes that react with oxygen is a major source of potentially damaging oxygen radicals (Tavares 2005, Bender et al 1997). Inadequate intake of riboflavin would therefore be expected to lead to disturbances in intermediary metabolism, with functional implications.

The literature relating riboflavin with cancer is complex. To the best of our knowledge, Morris and Robertson (1943) was the first to report the effect of riboflavin upon tumor growth and they reported that the deficiency of riboflavin slow down the growth of tumours. Some studies indicate that riboflavin deficiency increases the risk of cancer at certain sites, whereas others point to a possible attenuating effect of riboflavin in the presence of some carcinogens and a protective effect of deficiency. Some carcinogens are metabolized by flavin-dependent enzymes, and in these instances riboflavin may enhance or ameliorate the effects of the carcinogen (Powers 2003).

It has been reported that deficiency of riboflavin plays a prominent role in progression of various cancers as well as increased vulnerability of cells to cancer and may increase the carcinogenesis (Webster et al 1996). In
addition, it has been implicated in the enhancement of antitumor activity of many anticancer drugs, as well as in activation of the immune system to kill tumor cells. Poor riboflavin status has also been reported as a risk factor for cervical dysplasia, a precursor condition for invasive cervical cancer (Liu et al 1993). It has been reported that, riboflavin captures reactive metabolites like tamoxifen and carcinogens to form a complex, thereby preventing formations of DNA adduct, preventing DNA methylation, and maintaining genomic stability (Wojcieszynska et al 2012).

De Vogel et al (2008) studied the relationship between riboflavin intake and colorectal cancer risk and showed that riboflavin level was associated with decreased proximal colon cancer risk among women. Recently, Aili et al (2013) reported that riboflavin levels not only decreased in plasma from CIN and CSCC patients compared with control subjects, but also decreased in CSCC tissue compared with normal tissue. Association between dietary intakes of riboflavin and colorectal, cervical, breast, and prostate cancer incidence and mortality was also found (Basset et al 2012, Powers et al 2005). It is well documented that, the fluorophore flavins that is not bound to proteins in the plasma is filtered by glomerulus and excreted in urine (Grooper et al 2009).

Several epidemiological studies have reported that, riboflavin deficiency is linked to an increased risk of gastric cancer (GC) because riboflavin is involved in essential oxidation-reduction reactions and its deficiency leads to skin and mucosal disorders (Manthey et al 2005, Eussen et al 2010). Although riboflavin supplements can significantly reduce the risk of GC, different individual intervention effects were observed after dietary supplementation with riboflavin (Qiao et al 2009). He et al (2009) investigated the influence of riboflavin-fortified salt nutrition on the risk of
esophageal cancer and reported that riboflavin was a protective factor for this type of cancer incidence.

Due to the energy metabolism pertaining to various pathological conditions, the protein may be disrupted and interactions with flavin may be altered, usually leading to the increase of flavin in blood plasma. The fluorophores that is not bound to proteins in the plasma is filtered by glomerulus and excreted in urine (Grooper et al. 2009). It has been reported that Riboflavin is the primary flavin excreted in human urine with smaller amounts of other catabolites (Chastain & McCormick 1987).

Several techniques have been developed, including microbiological and liquid chromatographic methods for the determination of vitamin and protein concentrations in body fluids. Mostly, high performance liquid chromatographic (HPLC) methods have been designed for the detection of the concentrations of riboflavin in food, pharmaceutical preparations, and urine. Only a few have been used to measure riboflavin in whole blood, serum, or plasma (Hustad et al. 1999).

Also, in spite of the evident importance of riboflavin in cancer diagnosis, relatively few reports are available on the use of fluorescence spectroscopy and to the best of our knowledge no reports are available on the decay kinetics of riboflavin in urine samples of oral cancer patients when compared to tedious and time consuming methods like high performance liquid chromatography, capillary electrophoresis, etc. Though, reports are available on the fluorescence characteristics of urine, more studies are required to understand the decay kinetics of riboflavin in urine samples towards cancer diagnosis.
In this context, the potential use of steady state and time resolved native fluorescence spectroscopic technique in the diagnosis and characterization of flavin levels in the urine samples of normal subjects and oral cancer patients were explored and the spectral data were subjected to statistical analysis to validate the potentiality of the present technique in the diagnosis of cancer. Figure 6.1 (a&b) compares the averaged and normalised fluorescence emission spectrum of urine of normal subjects and oral cancer patients.

From the Figure 6.1 (a), it is observed that the normal and cancer subjects exhibited a broad emission spectrum with maxima intensity at 520 nm. The emission around 520 nm may be attributed to the contribution of riboflavin and its derivatives. Earlier reports also confirm the origin of the peak around 520 nm is mainly due to riboflavin (Alfano et al 1984, Dawson et al 1986, Engel 1996, Masilamani et al 2010). The difference spectrum Figure 6.1 c shows a positive difference in the wavelength region 470 nm to 500 nm which may be due to increased contribution of pteridines/NADH for normal subjects than cancer patients. The negative difference observed in the region above 525 nm may be due to the increased contribution of flavins for oral cancer patients than that of normal subjects.

While discussing the lifetime of flavins in urine samples and standard fluorophore at 520 nm emission, the flavin standard exhibits a bi exponential decay with an average lifetime of 4.7 ns. Whereas, the average lifetime of both the bi and tri exponential decay of urine samples of normal subjects is 4.6 ns. The cancer samples with bi and tri exponential decay shows an average lifetime of 3.9 ns and 4.9 ns respectively. Not much difference was observed between the lifetime of normal subjects and riboflavin standard. However, the average lifetime of oral cancer patients with bi exponential decay is less than that of normal subjects and cancer patients with tri
exponential decay. Since urine is a multicomponent fluid, it is not possible to measure the lifetime of individual component as there exists the possibility of influence from other fluorophores and the varied microenvironment and has to be probed further. To our knowledge, no reports are available on the time resolved spectroscopic characterization of urine of oral cancer patients in order to validate the reason for the variation in the lifetime values.

In this study, stepwise linear discriminant analysis was performed to find the sensitivity and specificity of the present technique in the diagnosis of oral cancer. Different intensity parameters were taken from the steady state fluorescence emission spectra at 450 nm excitation and were used as input values in the discriminant analysis. The emission wavelength used as input parameters represents the contribution of riboflavin and its metabolites.

At 450 nm excitation, the intensity parameter at 530 nm which is mainly due to the contribution of riboflavin is significant in discriminating the oral cancer subjects from the normal subjects. In the original and leave one out cross validated classification, out of 50 oral cancer subjects, 22 cases are correctly classified as cancer with a sensitivity of 44% and 28 cases are misclassified as normal subjects out of 50 normal subjects, 41 cases are correctly classified with a specificity of 82 % and 9 cases are misclassified as cancer subjects with an overall accuracy of 63%. Whereas, for the lifetime data, no statistical difference was observed between the lifetime values of normal subjects and oral cancer patients.

6.4 CONCLUSION

Based on the spectral information and results of statistical analysis, it was concluded that the steady state fluorescence emission and lifetime characterization of riboflavin was not much significant in the discrimination
of urine of oral cancer patients from healthy subjects. Further studies are to be carried out to collect more data and to analyse the possibility of discriminating oral cancer from normal subjects. In order to elucidate the possible reason for the altered spectral signatures due to the native fluorescence from flavin and its derivatives and the relation connecting to riboflavin metabolism, further combined studies by the biochemist, oncologist and physicians are required.