CHAPTER 7

SYNCHRONOUS LUMINESCENCE
SPECTROSCOPIC CHARACTERIZATION
OF BLOOD PLASMA

7.1 INTRODUCTION

The use of native fluorescence emission and excitation spectroscopy in the characterization of blood plasma and acetone-extracted blood formed elements of normal subjects and cervical cancer patients have been already discussed in the chapters 3, 4, 5 and 6. In conventional fluorescence spectroscopy, fluorescence emission spectra at one or more excitation wavelengths and/or excitation spectra corresponding to one or more emission wavelengths are being used in diagnostic purpose. As tissues and cells are highly heterogeneous with native intrinsic fluorophores, Excitation and Emission Matrix (EEM) can be used to identify the excitation wavelengths at which tissues /cell classification is optimized and to identify the origin of the measured fluorescence signals in a more reliable manner (Richards-Kortum et al 1991, Mahadevan et al 1993). However, EEM measurement also requires a series of fluorescence emission scans at sequential excitation wavelengths at small wavelength interval. This is a time-consuming process and there may be tissue/cell deterioration and/or photo bleaching of native fluorophores during EEM measurement.

Further, the conventional luminescence methods have the following limitations: First the emission spectra of complex mixtures often cannot be
resolved satisfactorily. Second, in highly heterogeneous media with many native fluorophores, severe overlapping of individual peaks occurs, particularly if the bands are broad. Vo Dinh (1978) suggested the idea of Synchronous Luminescence Spectroscopy (SLS) in the analysis of multi-components. In this technique, the fluorescence signals are measured by simultaneously varying both the excitation and emission wavelengths with a fixed wavelength ($\Delta \lambda$) between them. Using this technique many researchers have reported the use of SLS in the analysis of multi components present in environmental pollutant, contaminations in food and oil (Jone and Soutar 1976). However, not much of data are available on the characterization of tissues in particular biotic fluids viz. blood using SLS. Based on these an attempt was also made to characterize the blood plasma and acetone extracted blood formed elements using SLS technique.

In this context, the study is carried out under the following three categories of analysis:

i) blood plasma of 29 normal subjects verses 68 cervical cancer patients were studied

ii) blood plasma of 29 normal subjects verses 33 early and 31 advanced cervical cancer patients

iii) blood plasma of 29 normal subjects verses 40 MDSCC, 10 WDSCC and 8 PDSCC of cervical cancer patients.

The synchronous luminescence spectra of blood plasma of normal and cervical cancer patients were recorded by simultaneously scanning both the excitation and emission wavelengths with a fixed interval of $\Delta \lambda \approx 20$ nm between the excitation and emission wavelengths. The measured SL spectra, in the region 250-750 nm, reveals a more resolved structure from a complex
system in contrast to the generally featureless and broadband appearance of the conventional fluorescence spectra.

### 7.2 SYNCHRONOUS LUMINESCENCE SPECTRAL CHARACTERISTIC OF BLOOD PLASMA OF NORMAL SUBJECTS AND CERVICAL CANCER PATIENTS.

The averaged SL spectra of blood plasma of normal subjects and cervical patients are shown in the Figure 7.1(a). From the Figure 7.1(a) it is observed that both the normal subjects and cervical cancer patients show similar spectral profile however, it is clear that the intensity of the peaks for blood plasma of cervical cancer patients exhibits higher intensity than that of normal subjects.

The measured SL spectra were normalized and the normalized spectra for the blood plasma of normal subjects and cervical cancer patients are shown in the Figure 7.1(b). Blood plasma of normal subjects showed peaks at 305, 353, 450, 482, 491 and 594 nm two valleys at 327 and 408 nm between the peaks at 305 and 353 nm, 353 and 450 nm respectively. From these peaks it is observed that there will be some contribution of amino acid tryptophan, enzymes NADH/NADPH, FAD, bilirubin some vitamins, riboflavin and porphyrines in the blood plasma of normal subjects. Although the blood plasma of cervical cancer patients show more or less similar spectral profile, with respect to normal subjects however, the peaks at 305nm and 353 nm are red shifted to 3 and 7 nm respectively. Further, it is also observed that the concentration of FAD, NADH / NADPH and porphyrines are more in blood plasma of cervical cancer patients than the normal subjects.

To clearly understand the spectral signatures of blood plasma of normal and cervical cancer patients, the difference spectrum was computed by
Figure 7.1  SL spectra of (a) Averaged, (b) Normalized spectra and (c) difference spectrum of normal subjects and cervical cancer patients blood plasma
subtracting the spectral signatures of blood plasma of cervical cancer patients from normal subjects and it shown in the Figure 7.1(c). From the difference spectrum it is observed that there is a sharp positive peak at 297 nm and two broad positive peaks centered at 332 and 414 nm. From the positive peaks, we can understand that the contribution of amino acids tryptophan and enzymes NADH / NADPH and structural proteins such as elastin are more in the blood plasma of normal subjects than the blood plasma cervical cancer patients. Further, it is also observed that the spectrum shows a major sharp negative peak at 311 nm and six negative peaks at 369, 453, 472, 484, 493 and 592 nm. From the negative peaks, it is attributed that the blood plasma of cervical cancer patients exhibiting higher intensity of luminescence above 420 nm and this may be due to higher concentration of amino acid 4-pyridoxic acid, lipids such as phospholipids, lipofuscin, ceroid and vitamins like riboflavin in the blood plasma of cervical cancer patients than the normal subjects. However, it must be further studied to know the exact reason for altered spectral characteristics.

7.3 SYNCHRONOUS LUMINESCENCE SPECTROSCOPIC CHARACTERIZATION OF BLOOD PLASMA OF NORMAL SUBJECTS AND EARLY, ADVANCED CERVICAL CANCER PATIENTS

The averaged SL spectra of blood plasma of normal subjects, early and advanced cervical patients are shown in the Figure 7.2(a). From the Figure 7.2(a) it is observed that both the normal subjects, early and advanced cervical cancer patients show similar spectral profile. Blood plasma of normal subjects as well as early and advanced cervical cancer patients showed peaks at 305, 350, 450, 469, 482, 491 and 594 nm, two valleys at 327 and 408 nm between the peaks at 305 and 353 nm, 353 and 450 nm respectively. From these peaks, it is observed that there will be some contribution of aminoacid
tryptophan, enzymes NADH / NADPH, FAD, bilirubin, some vitamins, riboflavin and porphyrines in the blood plasma of normal subjects, at the same time it is clear that the intensity of the peaks for blood plasma of advanced cervical cancer patients exhibits higher intensity than that of normal subjects and the order of intensity is advanced > early > normal.

The measured SL spectra were normalized and the normalized spectra for the blood plasma of normal subjects, early and advanced cervical cancer patients are shown in the Figure 7.1(b). Blood plasma of normal subjects showed peaks at 305, 350, 450, 469, 482, 491 and 594 nm, two valleys at 327 and 408 between the peaks at 305 and 353, 353 and 450 nm respectively. It is observed that, the normalized spectrum of the normal subjects, early and advanced cervical cancer patients shows the similar spectral profile as that of normal subjects except the following observation. The sharp peaks at 305 and 350 nm are red shifted to 3 nm and 7 nm respectively, for both early and advanced cervical cancers. Further, it is also observed that up to 320 nm all the three spectra showed same intensity, beyond 320 nm, the intensities are in the order of advanced > early > normal.

To clearly understand the spectral signatures of blood plasma of normal, different stages of cervical cancer patients the difference spectrum was computed by subtracting the spectral signatures of blood plasma of early cervical cancer patients from normal subjects, advanced cervical cancer patients from normal subjects and shown in Figure 7.2(c). From the difference spectrum between early and normal subjects, it is observed that there is a sharp positive peak at 297 nm and two broad positive peaks centered at 332 and 414 nm. From the positive peaks, we can understand that the contribution of amino acids tryptophan and enzymes NADH / NADPH and structural proteins such as elastin are more in the blood plasma of normal
Figure 7.2  Synchronized spectra of (a) Averaged, (b) Normalized and (c) Difference spectra of normal subjects, early and advanced cervical cancer patients blood plasma
subjects than the blood plasma cervical cancer patients. Further it is also observed that the spectrum shows a sharp negative peak at 311 and six negative peaks at 364, 453, 472, 484, 493 and 592 nm. From the negative peaks, it is attributed that the blood plasma of early cervical cancer patients may have some higher concentration of amino acid 4-Pyridoxic acid, lipids, phospholipids, lipofuscin, ceroid and vitamins, riboflavin in their blood plasma than that of normal subjects. The difference spectrum between the advanced cervical cancer patients and normal subjects also shows similar spectral profile, at the same time up to 320 nm the intensities of normal minus early and normal minus advanced are same beyond 320 nm, the intensity of normal minus early is more than normal minus advanced. This clearly shows that the concentration of enzymes NADH / NADPH, FAD, Bilirubin some vitamins, riboflavin and porphyrines in the blood plasma of normal subjects than that of early and advanced cervical cancer (Yang et al 1997).

7.4 SYNCHRONOUS LUMINESCENCE SPECTRAL CHARACTERISTIC OF BLOOD PLASMA OF NORMAL SUBJECTS MDSCC, WDSCC AND PDSCC OF CERVICAL CANCER PATIENTS

The average fluorescence emission spectra of blood plasma of normal subjects and cervical cancer patients under MDSCC, WDSCC and PDSCC conditions are shown in Figure 7.3(a). The averaged SL emission spectra show considerable differences between the normal and different histopathological conditions of cervical cancer patients. From the Figure 7.3(a), it is observed that the MDSCC blood plasma exhibits overall higher SL emission intensity than the normal subjects as well as the other histo pathologically cervical cancer patients. The order of emission intensity is MDSCC > normal >PDSCC > WDSCC.
The normalized SL emission spectra of blood plasma of normal subjects, MDSCC, WDSCC and PDSCC of cervical cancer patients is shown in Figure 7.3 (b). From the normalized spectra, it is observed that the spectra of normal as WDSCC cervical cancer patients showed a primary predominant peak at 306 nm, a secondary peak with lesser intensity than the primary peak at 358 nm, a broad peak centered at 480 nm and a hump at 595 and 640 nm. However, the primary peak of normal subjects is centered at 306 nm and MDSCC cervical cancer patients have red shift of 4 nm with respect to normal subjects. The red shift is more for MDSCC, whereas other conditions of cervical cancer patients showed minimal red shift of the order of 1-2 nm with respect to normal subjects. The primary and secondary peaks at 306 and 358 may be due to the presence of aromatic amino acids, tryptophan lipids such as lipofuscin ceroid etc. From the Figure 7.3(b), it is also observed that the contribution of NADH and vitamins are more of the order of WDSCC > PDSCC > MDSCC > normal.

Further to understand the spectral difference between the blood plasma of normal subjects and different histopathologically classified cervical cancer patients such as MDSCC, WDSCC and PDSCC, the difference spectra were computed by subtracting the spectral signatures of MDSCC, WDSCC and PDSCC of cervical cancer patients from the normal subjects. From the Figure 7.3(c) it is found that the difference spectrum of normal subjects and MDSCC cervical cancer patients has a positive sharp peak at 299 nm, with comparatively two small broad positive peaks at 331 and 415 nm and a broad negative peak centered at 482 nm.

The difference spectrum between normal and WDSCC patients shows very high intensity differences both in the positive as well as negative side of the spectrum with respect to the other two spectra. It is observed the positive peak at 299 nm and 331 nm are red shifted to 6 nm and blue shifted to
Figure 7.3  Synchronized spectra of (a) Averaged, (b) Normalized and (c) difference spectra of normal subjects, MDSCC, WDSCC and PDSCC of cervical cancer patients blood plasma.
3 nm respectively with respect to MDSCC. On the other hand it is having a broad negative peak centered at 363 nm. It is further noted that the broad higher intensity negative peak centered at 482 nm is having very high intensity on the negative side compared to the MDSCC.

The difference spectrum between normal subjects and PDSCC cervical cancer patients shows moderate intensity differences both in the positive as well as negative side of the spectrum with respect to the other two spectra. The positive peak at 299 nm in the MDSCC and WDSCC is completely absent in this. Further it is also observed that the peaks at 331 and 415 nm are blue shifted to 2 and 15 nm respectively with respect to moderately. The intensity of the order of negative broad peak centered at 482 nm is WDSCC > PDSCC > MDSCC on the negative side.

7.5 **STEPWISE DISCRIMINANT ANALYSIS OF SYNCHRONOUS LUMINESCENCE SPECTRAL CHARACTERISTIC OF BLOOD PLASMA OF NORMAL SUBJECTS AND CERVICAL CANCER PATIENTS.**

In the statistical discrimination of blood plasma of normal subjects from cervical cancer patients, 36 ratio variables were calculated from the SL spectra intensities at 216, 252, 272, 285, 294, 324, 361, 395 and 431 nm. The linear discriminant analysis performed across the normal and cervical cancerous groups, using these 36 ratio variables as input, resulted in the following expression for a canonical discriminant function (7.1).

\[
DF_{20} = -39.217*I_{216/285} + 61.783*I_{272/285} - 49.283*I_{272/294} \\
- 0.442*I_{285/395} + 4.164*I_{324/361} - 6.859
\]  

(7.1)

It is found that out of the only 36 input variables, five ratio variable viz. \(I_{216/285}\), \(I_{272/285}\), \(I_{272/294}\), \(I_{285/395}\) and \(I_{324/361}\) resulted in the significant in
discriminating the blood plasma of normal from cervical cancerous subjects, Figure 7.4 shows the scatter plot of the discriminant score $DF_{20}$ of normal and cervical cancerous subjects. It is found that $DF_{20}$ classified 24 normal subjects and 58 cervical cancerous subjects correctly, resulting in a specificity and sensitivity of 82.5% and 85.3% respectively. Five normal were misclassified as cervical cancer and 10 cervical cancers were misclassified as normal, 84.5% of original groups classified correctly.

Figure 7.4 Scatter plot showing the distribution of the discriminate score of the blood plasma of normal and cervical cancer for SLS

7.6 DISCUSSION

SL spectroscopic techniques, it is observed that the SL spectra also provide significant spectral variation not only between normal and cancerous subjects, but also with respect to stage and hisopathological classifications. However, from the statistical classification, it is observed that good discrimination is obtained only between normal subjects and cervical cancer,
but not with respect to stage of histopathological classification. This may be
due to very small sample size or other possible statistical methods are to be
adapted.

The synchronous luminescence spectra of the blood plasma of normal subjects and cervical cancer patients were subjected to statistical analysis. From the Figure 7.4 it is observed that, the synchronous luminescence spectroscopy provides only 82.8% sensitivity and specificity of 85.3%.