CHAPTER 6

NATIVE FLUORESCENCE EXCITATION SPECTROSCOPIC CHARACTERIZATION OF ACETONE EXTRACTED BLOOD FORMED ELEMENTS

6.1 INTRODUCTION

As in the case of previous chapter, the fluorescence excitation spectroscopy of acetone extracted blood formed elements of both the normal subjects as well as cervical cancer patients was carried. In this chapter, the samples were analyzed for different emissions. Although the fluorescence spectroscopy for different emission was carried out, the results and discussions were given only for limited excitation spectra based on discriminant results. The results are analysed not only between normal and cervical cancer but also based on stage of the disease and histopathological conditions of the patients.
6.2 NATIVE FLUORESCENCE EXCITATION SPECTROSCOPIC CHARACTERIZATION OF ACETONE EXTRACTED BLOOD FORMED ELEMENTS AT 390 nm EMISSION

6.2.1 Native fluorescence excitation spectroscopic characterization of acetone extracted blood formed elements of normal subjects and cervical cancer patients at 390 nm emission

The averaged fluorescence excitation spectra of acetone extracted blood formed elements of normal subjects and cervical cancer patients for 390 nm emission are shown in the Figure 6.1(a). From the Figure 6.1(a), it is observed that the fluorescence excitation spectra have considerable difference between the acetone extracted blood formed elements of normal subjects and cervical cancer patients. The average fluorescence excitation intensity for cervical cancer is more than that of normal subjects.

The normalized average excitation spectra of acetone extracted blood formed elements of normal subjects and cervical cancer patients are shown in the Figure 6.1(b). The normalized excitation spectra have considerable difference in their peak emission wavelengths. The fluorescence intensity of normal subjects is more up to 334 nm excitation and at excitations higher than 334 nm the fluorescence intensity of cervical cancer predominates. From the normalized excitation spectra at 390 nm emission, it is also observed that there is a 10 nm red shift in peak emission for cervical cancer patients compared to the normal subjects. The peak at 338 nm in the normalized spectra clearly indicates the presence of elastin, other amino acids and some other vitamin B compounds present in the acetone extracted blood formed elements.
Figure 6.1  (a) Averaged (b) Normalized fluorescence excitation spectra of acetone extracted blood formed elements of normal subjects and cervical cancer patients and (c) Difference spectrum at 390 nm emission
The difference spectrum was also computed by subtracting the spectral signatures of acetone extracted blood formed elements of cervical cancer patients from normal subjects, and it is shown in the Figure 6.1(c). From the Figure 6.1(c), it is observed that there is a well defined sharp positive peak at 323 nm clearly indicating that the contribution of structural protein, elastin and co-enzyme pyridoxal phosphate and vitamin A may be more in the acetone extracted blood formed elements of normal subjects than the cervical cancer patients. On the other hand, the difference spectrum has two negative peaks at 340 and 357 nm. The emission at 340 and 357 nm excitations may be attributed to vitamin B\textsubscript{6} compounds viz. pyridoxin and pyridoxamine. The isosebestic point at 334 nm is demarcating the cervical cancer patients from normal subjects.

6.2.2 Native fluorescence excitation spectroscopic characterization of the acetone extracted blood formed elements of normal, early and advanced cervical cancer patients at 390 nm emission

The averaged fluorescence excitation spectra of acetone extracted blood formed elements of normal subjects, early and advanced cervical cancer patients are shown in Figure 6.2(a). From the Figure 6.2(a) it is observed that the acetone extracted blood formed elements of advanced cervical cancer patients exhibits higher fluorescence intensity than that of early cervical cancer patients and normal subjects. The excitation fluorescence intensity is of the order of advanced > early > normal.

The normalized excitation spectra of normal, early and advanced cervical cancer patients acetone extracted blood formed elements are shown in Figure 6.2(b). From the Figure 6.2(b), it is clearly observed that the acetone extracted blood formed elements of normal subjects, early and advanced cervical cancer patients have similar spectral signature except with some modifications in their peak emission. It is observed that the normal excitation
spectrum has a shoulder between 330 and 350 nm with a small dip at 338 nm. From the Figure 6.2(b), it is also observed that the shoulder for advanced and early cervical cancer patients shows 8 nm red shift with respect to normal subjects.

The difference spectra were computed by subtracting the spectral signatures of acetone extracted blood formed elements of advanced cervical cancer patients from normal subjects and also acetone extracted blood formed elements of early cervical cancer patients from normal subjects. From the Figure 6.2(c), it is observed that the difference spectrum between normal and early cervical cancer has a well defined positive peak at 322 nm with an isosebestic point at 344 nm. The difference spectra further confirms that the emissions at 390 nm is more for normal subjects for excitation wavelengths lesser than 344 nm and afterwards the intensity is more for early cervical cancer.

Similar positive peak and isosebestic point is observed in the difference spectrum between normal subjects and advanced cervical cancer. However, it is observed that the difference is more on positive side and less at the negative side with that of normal subjects and early cervical cancer. As there is no collagen in blood, the emission at 390 nm may be attributed to elastin and some of the vitamin B₆ compounds.

On comparison with fluorescence excitation spectrum with blood plasma (Figure 5.5 (c)), it is found that the acetone extracted blood formed elements provide considerable variation in the fluorescence excitation spectra between them. This indicates that different composition/confirmation of the fluorophores which are responsible for emission around 390 nm in the acetone extracted blood formed elements.
Figure 6.2  (a) Averaged (b) Normalized fluorescence excitation spectra of normal subjects, early and advanced cervical cancer patients acetone extracted formed elements and (c) Difference spectra at 390 nm emission.
6.2.3 Native fluorescence excitation spectroscopic characterization of acetone extracted blood formed elements of normal subjects MDSCC, WDSCC and PDSCC of cervical cancer patients at 390 nm emission

The average fluorescence excitation spectra of acetone extracted blood formed elements of normal subjects and cervical cancer patients under MDSCC, WDSCC AND PDSCC conditions at 390 nm emission are shown in Figure 6.3(a). From the Figure 6.3(a), it is observed that the acetone extracted blood formed elements from MDSCC exhibits overall higher fluorescence intensity than the normal subjects as well as the other two histopathological conditions of cervical cancer patients. The order of fluorescence excitation intensity is MDSCC > PDSCC > WDSCC > normal.

The normalized excitation spectra of acetone extracted blood formed elements of normal subjects, MDSCC, WDSCC and PDSCC of cervical cancer patients are shown in Figure 6.3(b). The spectra of normal and WDSCC and other histopathological conditions of cervical cancer patients have broad excitation maxima. Their spectra have shoulder of around 10 nm width. The shoulder of normal subjects has shifted towards blue region with respect to other pathological conditions. The predominant excitation peak at 336 nm for 390 nm emissions may be due to the elastin and some vitamin B compounds.

The difference spectra for 390 nm emissions were also computed for normal subjects minus MDSCC, normal subjects minus WDSCC and normal subjects minus PDSCC of cervical cancer patients. It is found that all the spectra have one distinct positive peak (Figure 6.3(c)). The positive peaks of normal subjects minus MDSCC, normal subjects minus WDSCC and normal subjects minus PDSCC are situated at 322, 324 and 326 nm. Similarly they
Figure 6.3  (a) Averaged (b) Normalized fluorescence excitation spectra for normal subjects and MDSCC, WDSCC and PDSCC acetone extracted blood formed elements and (c) Difference spectra at 390 nm emission
have distinct negative peak around 350 nm. The difference spectra of various histopathological conditions of patients clearly indicating that the fluorophores responsible for 390 nm emission, possibly elastin and vitamin B complex may undergo changes in their distribution and confirmations due to the transformation of normal subject into cervical cancer.

6.3 NATIVE FLUORESCENCE EXCITATION SPECTROSCOPIC CHARACTERIZATION OF ACETONE EXTRACTED BLOOD FORMED ELEMENTS AT 460 nm EMISSION

6.3.1 Native fluorescence excitation spectroscopic characterization of acetone extracted blood formed elements of normal subjects and cancer patients at 460 nm emission

The averaged fluorescence excitation spectra of acetone extracted blood formed elements of normal subjects and cervical cancer patients at 460 nm emission are shown in the Figure 6.4(a). From the Figure 6.4(a), it is observed that the fluorescence excitation spectra for 460 nm have also showed considerable difference between the acetone extracted blood formed elements of normal subjects and cervical cancer patients. The overall emission intensity at 460 nm for cervical cancer patients is more than that of normal subjects.

From the normalized spectra, it is found that the excitation maximum for normal subjects is around 365 nm and the same for cervical cancer is around 355 nm, i.e.10 nm blue shifts for cervical cancer (Figure 6.4(b)). This clearly indicates that there may be some conformational changes in the fluorophores which are present in the acetone extracted blood formed elements.
Figure 6.4  (a) Averaged (b) Normalized fluorescence excitation spectra of acetone extracted blood formed elements of normal subjects and cervical cancer patients and (c) Difference spectrum at 460 nm emission
From the fluorescence excitation difference spectrum (Figure 6.4(c)), it is observed that many fluorophores are responsible for emission at 460 nm, for example the positive and negative peaks around 323, 396 and 342 nm respectively may be attributed to NADH and/or NADPH and some of the lipids viz. lipofuscin and ceroid.

6.3.2 Native fluorescence excitation spectroscopic characterization of the acetone extracted formed elements of normal early and advanced cervical cancer patients at 460 nm emission

The averaged fluorescence excitation spectra of acetone extracted blood formed elements of normal, early and advanced cervical cancer patients are shown in Figure 6.5(a). From the Figure, it is observed that the acetone extracted blood formed elements of advanced cervical cancer patient’s exhibit higher fluorescence intensity than that of early cervical cancer patients and normal subjects. The order of fluorescence excitation intensity is advanced > early > normal.

The normalized excitation spectra of acetone extracted blood formed elements of normal subjects, early and advanced cervical cancer patients are shown in Figure 6.5(b). From the Figure 6.5(b), it is clearly observed that the formed elements of normal subjects, early and advanced cervical cancer patients have similar spectral signature with a predominant excitation peak around 355 nm and a hump at 394 nm. However, the excitation maximum for normal is observed at 366 nm. The excitation peaks for 460 nm emission may be due to the presence of enzymes viz NADH/NADPH and lipids such as lipofuscin and ceroid are present in the acetone extracted blood formed elements. From the 6.5(b), it is also observed that excitation maxima for advanced and early cervical cancer patients show 11 nm blue shift with respect to normal subjects.
The difference spectra were computed by subtracting the spectral signatures of acetone extracted blood formed elements of advanced cervical cancer patients from normal subjects, early cervical cancer patients from normal subjects. From the Figure 6.5(c), it is observed that there are two positive peaks at 323 nm, and 394 nm and a negative shoulder centered at 345 nm. The positive peaks at 394 nm is broader and having higher emission than the peak at 323 nm. The positive peaks may be attributed to some lipids such as lipofuscin and ceroid and /or NADH/NADPH.

The difference spectrum of normal and early cervical cancer patients is also showing similar spectral signature except that, the emission intensity is minimal for excitations lesser than 320 nm with respect to normal and advanced cervical cancer. It is also found from the Figure 6.5(c), that there is a 3 nm blue shift between normal and early and normal and advanced cervical cancer, show similar differences, in spite of having some difference in the spectral signature.

6.3.3 Native fluorescence excitation spectroscopic characterization of acetone extracted blood formed elements of normal subjects MDSCC, WDSCC and PDSCC of cervical cancer patients at 460 nm emission

The average fluorescence excitation spectra of acetone extracted blood formed elements of normal subjects and cervical cancer patients under MDSCC, WDSCC and PDSCC conditions for 460 nm emission are shown in Figure 6.6(a). From the Figure 6.6(a), it is observed that the MDSCC acetone extracted blood formed elements exhibit overall higher fluorescence excitation intensity than the normal subjects as well as the other histopathologically classified cervical cancer patients. The order of fluorescence excitation intensity is MDSCC > PDSCC > WDSCC > normal.
Figure 6.5  (a) Averaged (b) Normalized fluorescence excitation spectra of normal subjects, early and advanced cervical cancer patients acetone extracted formed elements and (c) Difference spectra at 460 nm emission
The normalized excitation spectra of acetone extracted blood formed elements of normal subjects, MDSCC, WDSCC and PDSCC of cervical cancer patients are shown in Figure 6.6(b). From the Figure 6.6(b), it is observed that the predominant peak of normal subjects is centered at 360 nm and the spectra of MDSCC, WDSCC and PDSCC shows a blue shift of 5 nm with respect to normal subjects. The predominant excitation peak at 360 nm and hump at 395 nm for 460 nm emissions may be due to the presence of enzymes NADH / NADPH and lipids like lipofuscin and ceroid in the acetone extracted blood formed elements of normal and other histopathologically classified cervical cancer.

The difference spectra were also computed by subtracting the spectral signatures of MDSCC, WDSCC and PDSCC conditions of cervical cancer patients from the normal subjects in the wavelength of measurement. From the Figure 6.6(c), it is found that the difference spectrum of normal subjects and MDSCC cervical cancer patients has a major positive peaks at 322 nm and a negative peak at 348 nm.

From the difference spectra of normal subjects minus WDSCC, and normal subjects minus PDSCC patients, it is observed that there are two positive peaks at 322 and 398 nm and a negative peak at 344 nm. The positive peak at 322 nm between normal subjects and WDSCC, normal subjects and PDSCC shows a red shift of 2 nm and 4 nm respectively with respect to the difference spectrum between normal subjects and MDSCC. On the other hand, the positive peak at 398 nm for normal subjects and WDSCC, normal subjects and PDSCC shows a blue shift of 4 nm with respect to normal and MDSCC. Further, it is also observed that the negative peak at 344 nm shows a 3 nm and 5 nm blue shift for normal subjects and PDSCC; normal subjects and WDSCC respectively with respect to normal subjects and MDSCC.
Figure 6.6   (a) Averaged (b) Normalized fluorescence excitation spectra for normal subjects and MDSCC, WDSCC and PDSCC acetone extracted blood formed elements and (c) Difference spectra at t 460 nm emission
From the difference spectra (Figure 6.6 (c)), it is observed that all categories of the samples have a major positive peak with a shoulder width of 10 nm. The shoulder of normal subjects minus MDSCC exhibits 4 nm red shift with respect to normal subjects minus WDSCC and normal subjects minus PDSCC. On the other hand, MDSCC has another secondary positive peak at 322 nm. The positive peak at 322 nm has almost come down to negative side. The WDSCC has completely come down to negative side and with a negative peak shift of 20 nm with respect to the difference spectrum of normal subjects subtracted from MDSCC.

6.4 STEPWISE DISCRIMINANT ANALYSIS

6.4.1 Stepwise Discriminant Analysis at 390 nm Emission

At 390 emissions 28 ratio variables were calculated from the fluorescence intensity at the excitation wavelengths, 311, 317, 323, 329, 334, 340, 350 and 357 nm in the fluorescence excitation spectra of normal and cervical cancerous subjects, at 390 nm emissions. The linear discriminant analysis performed across the normal and cervical cancerous groups, using these 28 ratio variables as input, resulted in the following expression for a canonical discriminant function:

\[ DF_{18} = 4.192 \times \frac{I_{323}}{I_{350}} - 3.541 \]  

Out of the 28 input variables, only one ratio variable \( \frac{I_{323}}{I_{350}} \) resulted significant in discriminating the acetone extracted blood formed elements of normal from cervical cancerous subjects, at 390 nm emissions. Figure 5.7 (a) shows the scatter plot of the discriminate score \( DF_{18} \) of normal and cervical cancerous subjects, it is found that \( DF_{18} \) classified 26 normal subjects and 75 cervical cancers correctly resulting the specificity and sensitivity of 81.3 and 74.3% respectively. Six normal were misclassified as cervical cancer and 26 cervical cancers were misclassified as normal, 75.9 % of original groups classified correctly.
6.4.2 Stepwise Discriminant Analysis at 460 nm Emission

At 460 emission 28 ratio variables were calculated from the fluorescence intensity at the excitation wavelengths, 313, 317, 324, 329, 344, 365, 396 and 419 nm in the fluorescence excitation spectra of the formed elements of normal and cervical cancerous subjects at 460 nm emission. The linear discriminant analysis performed across the normal and cervical cancerous groups, using these 28 ratio variables as input, resulted in the following expression for a canonical discriminant function.

\[ DF_{19} = 5.33 \cdot \frac{I_{313}}{I_{317}} - 5.7 \cdot \frac{I_{324}}{I_{329}} + 0.729 \]

(5.2)

Out of the 28 input variables, six ratio variables viz. \( I_{313}/I_{317} \) and \( I_{324}/I_{329} \) resulted in the significant in discriminating the formed elements of normal from cervical cancerous subjects, at 460 nm emission. Figure 5.8(b) shows the scatter plot of the discriminant score \( DF_{19} \) of normal and cervical cancerous subjects, It is found that \( DF_{19} \) classified 25 normal subjects and 55 cervical cancerous subjects correctly, resulting in a specificity and sensitivity of 65.6 % and 67.1 % respectively. Five normal were misclassified as cervical cancer and 15 cervical cancers were misclassified as normal, 66.7% of original groups were classified correctly.
Figure 6.7 (a) and (b) scatter plot showing the distribution of the discriminate score of acetone extracted blood formed elements normal and cervical cancer subjects at of 390 and 460 nm emission
6.5 DISCUSSION

In this chapter the fluorescence excitation spectra of the acetone extracted blood formed elements of normal subjects and abnormal cervical cancer patients were subjected to statistical analysis. From the Figures 6.7a and 6.7b it is observed that, among various excitation spectra, the excitation spectra for 390 and 460 nm emission provide 81.3% and 65.6% sensitivity and specificity of 74.3% and 67.1% respectively. From these, it is concluded that Elastin and NADH present in the acetone extracted blood formed elements may be considered as tumor marker in discriminating the abnormal from normal. However, excitation spectra of formed elements are providing only poor statistical significance.