CHAPTER 9

RESULTS AND DISCUSSION

9.1 INTRODUCTION

Cervical cancer is one of the most common cancers among women in India, with approximately 71,600 new cases occurring each year. Over 80% of the women were not aware of cancer and more than 99% had never heard of a test for cancer. However, 70% expressed their interest in undergoing such a test. Among the global community, cervical cancer ranked the second most common malignancy. Women less than 35 years of age account for up to 24.5% of patients with invasive cervical cancer and the incidence continues to increase for women in this age group (Wright et al 1994). Cervical cancer is 98 percent curable if detected early, yet one third of the women diagnosed with the disease will die from it and the remaining two third will undergo radial treatment to avert terminal illness because the cancer wasn’t detected early enough.

The conventional diagnostic techniques that are adopted for cervical cancer diagnosis are Pap smear, colposcopy, endocervical curettage, punch or target biopsy and cone biopsy. Though these techniques are being used as the current diagnostic modality to identify cervical abnormalities, they are tedious, time consuming and more dependent on the expertise of the person who is examining resulting in subjective errors. The accuracy of the pap smear is limited by both sampling and reading errors. Approximately 60% of false-negative smears are attributed to insufficient sampling; the remaining...
40% are due to reading errors. In this context, an efficient diagnostic modality to identify the normal from abnormal cervical tissues is mandatory for early detection of cancer, in order to control the alarmingly increasing mortality rate in India.

Based on the need for an effective diagnosis tool, have explored the potentiality of utilizing the native fluorescence spectroscopy in discriminating the normal from abnormal tissues. Several studies have been reported on the autofluorescence characterization of different pathological conditions of tissues like breast, lung, oral cavity and colon (Alfano et al 1987, Majumdar et al 1999, Richards Kortum et al 1991 and Yang et al 1994). In particular, several studies have been reported on the discrimination of normal from abnormal cervical tissues (Lohmann et al 1989, Glassman et al 1992, Mahadevan et al 1993, Ramanujam et al 1996 and Brookner et al 2000).

Recently, many reported on Excitation Emission Matrix and analyzed the multicomponents (Fluorophores) present in the tissues. It was reported multiwavelength excitations and probing more than one fluorophores has provided better statistical significance than single wavelength of excitation,

Although, fluorescence spectroscopy of tissues provides better statistical significance, oncologists and biomedical scientists are looking for early cancer detection method and to develop a routine screening technique .Since, at early stages the patient may not get symptoms or evidence of occurrence of diseases; it may not be possible to use the tissue spectroscopy for routine screening.

In this context, recently many attempted in the characterization of blood of both normal and cancer patients using native fluorescence spectroscopy. However, there is no much of studies on the characterization of
the blood of patients who are affected by various cancers. As already discussed, cervical cancer ranks number two in the world and number one in India, it is assumed to study the native fluorescence spectroscopy of blood of both normal and cervical cancer patients. The study was designed into three categories viz.,

i) Normal Versus Cervical Cancer Patients

ii) Normal Versus Early and advanced cervical cancer

iii) Normal versus PDSCC, MDSCC, WDSCC

The blood samples were also prepared and analyzed under two different conditions viz., blood plasma and acetone extracted blood of formed elements. These two types of samples were analyzed by native fluorescence emission, fluorescence excitation and synchronous luminescence Spectroscopy (SLS). The salient features of the thesis work are listed below:

9.2 NATIVE FLUORESCENCE SPECTROSCOPIC CHARACTERIZATION OF BLOOD PLASMA OF NORMAL SUBJECTS AND CANCER PATIENTS

- From the fluorescence emission spectra of blood plasma at 270, 275, 280 and 300 nm excitation:
  - The fluorescence emission intensity of the blood plasma normal subjects exhibits higher fluorescence around 340 nm and lesser emission around 460 nm and it is vice versa for cervical cancer patients.
  - The peak around 340nm may be attributed to the contribution of various amino acids in particular tryptophan and the secondary peak at 460nm may be due to NADH/NADPH.
The normalized spectra of both early and advanced cervical cancer patients show 5 nm red shift with respect to normal subjects. The contribution of NADH is of the order of advanced > early > normal.

It is also observed that the contribution of NADH is also higher for PDSCC than that of WDSCC, MDSCC and normal subjects.

From the Fluorescence Emission Spectra of blood plasma at 350 nm excitation:

The blood plasma of normal subjects and cervical cancer patients have similar spectral signature with a primary peak around 485 nm. The primary emission peak may be due to the contribution of some vitamins and amino acid, indoxyl peak may be due to the contribution of some vitamins and amino acid, indoxyl sulphate. These fluorophores are predominant in the blood plasma of normal subjects than that of cervical cancer patients.

The difference spectrum between blood plasma of cervical cancer patients and normal subjects indicates that many of the fluorophores viz, amino acids, NADH, FAD vitamins and lipids are available and they at elevated level in normal subjects than that of cancer patients. Further these fluorophores are elevated when early cervical cancer becomes advanced cervical cancer. The peak intensity at 536 nm is order of PDSCC > WDSCC > MDSCC.

From the fluorescence emission spectra of blood plasma of normal and cervical cancer patients at 405 and 420nm excitation:
The overall fluorescence emission intensity of the blood plasma of normal subjects exhibits higher fluorescence intensity than that of blood plasma of cervical cancer patients.

They show a primary broad peak around 506 nm and a secondary peak around 625 nm. This may be attributed to the contribution of FAD and porphyrin.

The blood plasma of advanced cervical cancer patients exhibit higher fluorescence intensity than that of normal subjects and early cervical cancer patients. The order of intensity is advanced > normal > early.

The level of NADH may be in the order of PDSCC > MDSCC > WDSCC. The blood plasma cervical cancer patients show the presence of porphyrin. The intensity of the porphyrin is of order of WDSCC > MDSCC > PDSCC.

From fluorescence excitation spectra of blood plasma of normal and cervical cancer patients for 340 nm emission:

The tryptophan emission for blood plasma of cervical cancerous is more at excitation wavelength higher than 306 nm and the peak excitation is at 311nm. This clearly indicates that the emission is due to amino acid residue which may undergo conformational changes when normal cells transform into cancer.

It is observed that for the excitation wavelengths upto 297 nm, the average fluorescence intensity for cervical cancer is lesser than that of normal subjects and beyond that it is vice versa.
- It is also noted that the normal subjects show one peak around 303 nm and the same is red shifted for advanced and early cervical cancer by 3 and 5 nm respectively.

- The amino acids present in the blood plasma of normal subjects have more absorption at excitation wavelength ranging from 250-305 nm and it is negative or excitation wavelength ranging from 305 to 320 nm with respect to MDSCC and PDSCC. Interestingly, the same is reversed for PDSCC (i.e. negative from 250 - 304 nm and positive from 304-320 nm). This clearly indicates that the position of tryptophan amino acid in some key protein may be changed depending upon pathological conditions of patients.

- From fluorescence excitation spectra of blood plasma of normal and cervical cancer patients for 390 nm emission:

  - The blood plasma of normal and cervical cancer patients show an excitation maximum at 399 nm. They also have secondary excitation maxima centered around 340 nm. This clearly indicates that the emission at 390 nm may be due to contribution of elastin and some vitamin B6 compounds viz., pyridoxine and pyridoxamine.

  - The blood plasma of normal subjects show higher fluorescence intensity than that of advanced and early cervical cancer patients at excitation wavelengths lesser than 310 nm.

  - In the case of PDSCC patients both the positive and negative peaks are different with respect to MDSCC and WDSCC patients.
• From fluorescence excitation spectra of blood plasma of normal and cervical cancer patients for 460 nm emission:
  ▪ The excitation peak at 340 nm may be due to the co-enzyme NADH/NADPH absorption. It is also observed that excitation maxima for early cancer patients shows 7 nm blue shift with respect to normal subjects.
  ▪ The order of fluorescence intensity at 460 nm emission is MDSCC > PDSCC > normal > WDSCC.
  ▪ The predominant excitation peak of normal subjects is centered at 348 nm, where as excitation peak of MDSCC and PDSCC cervical cancer patients showed a blue shift of 5 nm and the spectrum of WDSCC showed a red shift of 6 nm with respect to normal subjects.
  ▪ The predominant excitation peaks at 348 nm for 460 nm emissions may be due to the presence of aromatic amino acids 5-Hydroxyanthranilic acid as well as enzymes such as NADH/NADPH and other lipids.

• From the Synchronous luminescence spectra of blood plasma of normal and cervical cancer patients:
  ▪ The peak intensities of the blood plasma of cervical cancer patients exhibit higher intensity than that of normal subjects.
  ▪ The emission contribution of FAD, NADH / NADPH and porphyrine are more in blood plasma of cervical cancer patients than that of the normal subjects.
  ▪ The peaks at 305 and 350 nm are red shifted to 3 nm and 7 nm respectively, for both early and advanced cervical cancers with respect to normal.
The primary and secondary peaks at 306 and 358 may be due to the presence of tryptophan lipids such as lipofuscin and ceroid. The contribution of NADH and vitamins are of the order of WDSCC > PDSCC > MDSCC > normal.

9.3 NATIVE FLUORESCENCE SPECTROSCOPIC CHARACTERIZATION OF ACETONE EXTRACTED BLOOD FORMED ELEMENTS OF NORMAL SUBJECTS AND CANCER PATIENTS

- From the fluorescence emission spectra at 270, 275, 280, 290 and 300nm excitation:
  - The spectra of normal and cervical cancer subjects have two major peaks of almost equal magnitude at 380 and 440 nm. They also have a well between the two major peaks at 415 nm. The peak at 450 nm is considerably more for normal subjects than that of cervical cancer patients.
  - The peak around 380 nm may be attributed to indoxyl sulphate (Indican) and elastin. The peak centered around 440-450 nm may be considered as to 4-pyridoxic acid and NADH. The well between the two major peaks around 413-415 nm may be due to reabsorption of hemoglobin present in the blood.
  - The difference spectra show that the contribution of 4-Pyridoxic acid is more in the case of normal than the cervical cancer patients, at the same time bound tryptophan is less in the case of normal subject when compared to the cervical cancer patients. The peak
around 430 nm is lesser in the case of WDSCC and MDSCC and it is higher for PDSCC than that of normal subjects.

- From fluorescence emission spectra of formed elements of normal subjects and cancer patients at 350 nm excitation:
  - The peak emission for normal subjects is seen at 441 nm and it is blue shifted to 13 nm for cervical cancer. The peaks in the region 440-450 and 630 may be attributed to the presence of NADH/NADPH and phorphyrin respectively. The shoulder at 390 nm is may be due to the presence of elastin.
  - The peak at 630 nm, may be attributed to porphyrins and it is of the order of WDSCC>normal >MDSCC >PDSCC.

- From fluorescence emission spectra of formed elements of normal subjects and cancer patients at 405 nm excitation:
  - The spectra of normal and cervical cancer patients show two major peaks at 466 and 630nm. They are attributed to NADH and porphyrin. There is a 10 nm red shift for both early and advanced cervical cancer patients with respect to a primary peak of normal at 465nm. It also observed that the emission contribution of porphyrin is in the order of normal > advanced > early.
  - It also shows the emission peak due to FAD and riboflavin.
From fluorescence excitation spectra of acetone extracted blood formed elements for 390nm emission:

- 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. The emission at 340 and 357 nm excitations may be attributed to vitamin B$_6$ compounds viz. pyridoxin and pyridoxamine. The isosebastic point at 334 nm is demarcating the cervical cancer patients from normal subjects.

- It is also observed that the shoulder for advanced and early cervical cancer patients shows 8 nm red shift with respect to normal subjects.

- MDSCC exhibits overall higher fluorescence intensity than the normal subjects as well as the other two histopathological conditions of cervical cancer patients.

- The fluorophores which are responsible for 390 nm emission, possibly due to elastin and vitamin B complex.

From fluorescence excitation spectra of acetone extracted blood formed elements for 460nm emission:

- 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 cancer.

- Advanced cervical cancer patients exhibit higher fluorescence intensity than that of early cervical cancer patients and normal subjects. The excitation maxima for
advanced and early cervical cancer patients show 11 nm blue shift 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.

- From the SLS Characterization Of Acetone Extracted Blood Formed Elements Of Normal Subjects And Cervical Cancer Patients:
  - The fluorescence intensity of cervical cancer patients has two fold higher intensity than that of normal subjects.
  - The acetone extracted blood formed elements of cervical cancer patients show similar spectral profile. However the peak at 355 nm is red shifted to 10 nm with respect to normal subjects.
  - The primary peak of normal subjects is centered at 355 nm where as in the case of MDSCC the same peak is red shifted to 10 nm. The other conditions of cervical cancer patients showed minimal red shift of the order of 3-4 nm with respect to normal subjects. It is also observed that the contribution of NADH and vitamins is more of the order of PDSCC > MDSCC > normal > WDSCC.
9.4 OPTIMIZATION OF EXCITATION AND EMISSION WAVELENGTHS USING STATISTICAL ANALYSIS

Both the blood plasma and acetone extracted blood formed elements were characterized using fluorescence emission, excitation and synchronous luminescence spectroscopic techniques. The spectra were analyzed statistically to optimize the suitable excitation and emission wavelengths.

Figure 9.1(a) shows the percentage of sensitivity and specificity in the discrimination of cancer from normal at different excitation and emission wavelengths and also the same for SLS technique. The Figure 9.1(b) shows their respective overall accuracy. From the figures it is observed that emission spectra at 275, 290 and 300nm and excitation spectra for 340, and 460nm provide more than 95% sensitivity, specificity and overall accuracy. However, emission spectra at 300nm excitation classify the blood plasma of cervical cancer patients from normal with 100% accuracy. This clearly indicates that amino acids and NADH which are present in the blood plasma may be effectively excited at 300nm and these fluorophores may be considered as tumour markers in discriminating the cervical cancer patients from normal subjects.

Figure 9.2a and Figure 9.2b shows the percentage of sensitivity and specificity for different excitation, emission and synchronous luminescence spectra for blood formed elements. Figure 9.2b shows their response overall accuracy. From the figure it is observed that the emission spectra of formed elements in blood of normal and cervical cancer patients at 270 and 420 nm gives 100% of sensitivity, specificity and hence 100% overall accuracy. Synchronous Luminescence spectra give 91.8% of overall accuracy. From the spectral characteristics of formed elements of normal and cervical cancer
Figure 9.1(a)  The percentage of sensitivity and specificity in the discrimination of cervical cancer from normal at different excitation and emission wavelength and SLS for blood plasma

Figure 9.1(b)  The overall accuracy in the discrimination of cervical cancer from normal at different excitation and emission wavelength and SLS for blood plasma
Figure 9.2(a) The percentage of sensitivity and specificity in the discrimination of cervical cancer from normal at different excitation and emission wavelength and SLS for acetone extracted blood formed elements

Figure 9.2(b) The overall accuracy in the discrimination of cervical cancer from normal at different excitation and emission wavelength and SLS for acetone extracted blood formed elements
suspects, it is observed that amino acid, idoxyl sulphate, elastin, NADH, FAD and porphyrins are involved in the better discrimination of cervical cancer patients from normal subjects.

On comparing the spectroscopic characterization of blood plasma with acetone extracted blood formed elements, it is found that both the samples are giving 100% of accuracy. However, the wavelengths of excitations and emissions are differing with respect to sample preparation conditions. It is found that emission spectroscopy of blood plasma at 300 nm excitation and in the case of blood formed elements, the emission spectra at 270 and 420nm excitation may be considered for accurate discrimination of the samples. Although synchronous luminescence gives only 90% of accuracy, it may also be used to identify the possible fluorophores which are available in the blood.

9.5 FUTURE DIRECTIONS

The blood is highly optically heterogeneous and it consists of many fluorophores. These fluorophores may be effectively utilized as tumour markers in the discrimination of cancer patients from normal subjects, if suitable wavelength of excitation and emission are selected. Hence, the study demonstrated the following important conclusion that warranted future clinical investigation:

i) The blood plasma could be further investigated to quantify the fluorophores by other physicochemical, in particular HPLC-LIF techniques.

ii) As the spectral signature is differing with respect to stage and histopathological condition of patients, the study could also be extended to time resolved fluorescence spectroscopy to
understand the various confirmations of the fluorophores when the normal cell transformed into malignant one.

iii) The blood formed elements show difference spectral profiles with that of blood plasma, the exact components which are available in them could also be carried out.

iv) Further, studies are to be carried out with more number of samples to accurately discriminate the different stages and histopathological conditions of the cancer patients from normal subjects.

v) A portable Laser Induced Fluorescence (LIF) system with selected excited and emission wavelengths could be made possible for mass screening of the population.