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ORIGINAL ARTICLE

Monitoring Breast Cancer Response to Treatment Using Stokes Shift Spectroscopy of Blood Plasma

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Abstract
With the emerging trend of personalized cancer treatment, there is a need to develop noninvasive/minimally invasive techniques for treatment monitoring. In this regard, in this work fluorescence analysis of blood plasma of breast cancer patients has been used for the evaluation of response to treatment. This approach delivers information not only about the change in biochemical constituents but also about the altered metabolic pathway. Spectral deconvolution method is employed to compute the fluorescence intensity, peak wavelength, and full-width half maxima for different endogenous fluorophores. The fluorescence measurements were made on blood plasma collected from 10 normal subjects, 10 pre-treated cancer patients, and 10 post-treated patients. Besides, variations in relative concentration of tryptophan, collagen, NADH, and FAD, peak shifts and broadening of peaks are observed for tryptophan, NADH, and FAD, in blood plasma of pre-treated cancer patients indicating both biochemical and microenvironmental changes at cellular level. Further, the spectral profile of blood plasma of post-treated patients found to be similar to blood plasma of normal subjects. Linear discriminant analysis showed that pre-treated and post-treated breast cancer is discriminated with a sensitivity and specificity of 100% and 100% respectively.

Keywords Stokes shift spectroscopy · Blood plasma · Breast cancer · Spectral deconvolution method · Treatment monitoring

Introduction

Despite the scientific and technological advances, cancer still remains to be a social stigma in society. The American Cancer Society (ACS) has reported that breast cancer is ranking high among various cancers and is the commonest cause of cancer-related transience in women [1]. It has been established that a complete treatment of cancer is possible if it is detected at its early stage. In this context, various imaging modalities like the Thermal / X-ray / magnetic resonance mammograms and ultrasound-based elasto mammograms are being used for screening and early diagnosis of breast cancer. In addition to these techniques, biochemical assays (assessment of CA15/3, CA27/9) and sophisticated nuclear medicine imaging techniques (Positron Emission Tomography combined with Computerized Tomography or Magnetic Resonance Imaging) are being employed for the diagnosis of cancer, to know the metastatic status of the disease as well as for the assessment of treatment response in the management of cancer patients. However, except for the golden standard of histopathological evaluation (invasive), the rest of the methods pose difficulties for complete acceptance by the Oncologists. The American Society of Clinical Oncology has reported that the clinical data are insufficient to recommend the use of serum-based tumor markers CA 15–3 and CA 27–29 for cancer screening, diagnosis and staging and also for monitoring patients for recurrence after primary breast cancer therapy [2]. It is also to be noted that the accuracy of the imaging techniques like CT scan, MRI depend on the spatial resolution of the imaging systems. For example, the minimum size that could be detected by the CT scanners is around 3 mm diameter lesions, for PET–CT it is 7 mm diameter lesions and Single Photon Emission Computed Tomography (SPECT) can detect around 10 mm diameter lesions [3].

Under these circumstances, molecular diagnostics has been considered to discriminate the diseased state as well as to monitor the therapeutic response due to various

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treatment modalities. In this context, various optical spectroscopic techniques have been considered to monitor the pathological and physiological state of the cells and tissues [4–13]. Among various spectroscopic techniques, fluorescence spectroscopy of cells and tissues has been considered as an effective tool to characterize the cells and tissues [14–16]. In this regard, various complementary techniques of fluorescence spectroscopy viz., fluorescence emission spectroscopy, excitation emission matrix spectroscopy, polarized fluorescence spectroscopy, time-resolved fluorescence spectroscopy and Stokes Shift spectroscopy (SSS) have also been considered to characterize cells and tissue. SSS technique has the unique advantage of measuring multiple fluorophores in a single spectrum and with resolved peaks [17]. Furthermore, the excitation time is much lesser than other fluorescence techniques and consequently, photobleaching can be avoided. Hence, various groups used SSS for cancer diagnosis by use of biopsy tissues [18–23]. SSS of biofluids (blood plasma, urine, saliva) is gaining attention, as it yields good diagnostic accuracy and also minimally invasive [24–26].

Even though there are a number of groups working on the discrimination of cancer using fluorescence techniques, only limited data are available on the fluorescence spectroscopic characterization of bio fluids [24–30]. Among various biofluids, blood has many important fluorophores which can act as key biomarkers for characterization of various cancerous and non-cancerous conditions and considered to be one of the most prospective biofluid as it can correlate directly with the view of the disease [31]. However, only limited data are available on the use of fluorescence spectroscopy of biofluid in the treatment monitoring of breast cancer patients. In this context, the present pilot work was aimed to characterize the photophysical characteristics of blood plasma under pre-treated and post-treated condition using SSS to monitor therapeutic response in breast cancer patients.

Materials and Methods

Sample Classification and Protocol

The study consisted of 10 female breast cancer patients undergoing treatment (male Breast cancers not included in the study, considering the low prevalence) in the age group of 40–55 years with well-differentiated infiltrating ductal carcinoma mostly stage II and III (Confirmed based on fine needle aspiration cytology / trucut biopsy), with known immunohistochemistry status like ER / PR / EGFR / VEGFR / HER2neu / E-Cadherin / KI67 etc., All the patients underwent positron Emission Tomography (PET - CT) / Nuclear bone scan prior to treatment as a metastatic work up. The treatment comprised of excision of the tumor, 6/8 cycles of chemotherapy and concomitant chemo-irradiation. The spectra of plasma of 10 normal subjects with no significant history of illness were also taken for comparative study. The blood plasma samples were taken before and after treatment. Care was taken to give enough time for the chemotherapy drugs to be excreted from the body through natural detoxification process by the liver. The samples were taken prior to treatment, a month after the entire completion of treatment and once again after a period of 2 years.

Sample Preparation

Venous blood samples around 2.5 ml from all the subjects were collected in EDTA (Ethylene diamine tetra-acetic acid $C_{10}H_{16}N_2O_8$) vacutainer tubes. The samples were centrifuged for 10 min at 1500 rpm (Remi centrifuge R8C, India). The supernatant plasma was pipetted out into the vial, without disturbing the buffy coat and erythrocyte sediments, and stored at 4 °C. The study was conducted within 6 h from the time of collection of the sample and as per the Ethical Committee guidance of the Institute.

Instrumentation and SS Spectral Measurements

The SS spectra of the blood plasma samples belonging to the study group were recorded using the Fluoromax-2 Spectrofluorometer purchased from Horiba Jobin Yvon – SPEX, Edison, New Jersey, USA, with 150 W Ozone free Xenon arc lamp, acting as an excitation source, coupled to the monochromator, delivers light to the sample at the desired wavelength and the fluorescence emission from the sample is collected by an emission monochromator, connected to a photomultiplier tube (R928P, Hamamatzu, Shizuoka-ken, Japan). The gratings in the excitation and emission monochromators have a groove density of 1200 grooves/mm and were blazed at 330 and 500 nm, respectively.

SS measurements for all the samples were recorded in the wavelength regions 250–600 nm, by simultaneously scanning the excitation and emission monochromators, keeping an offset wavelength of 20 nm between them. A constant wavelength of $\Delta\lambda = 20$ nm was maintained between the excitation and emission wavelengths throughout the spectrum as expressed by $\lambda em = \lambda ex + \Delta\lambda$, in order to obtain a more resolvable spectrum based on the absorption and emission spectral characteristics of the various fluorophores and this provided maximum number of fluorophores in a single scan, with good signal to noise ratio. The collected signal was then processed by Data Max software provided by the instrument for further analysis.

Data Processing

Normalization

The spectra were initially averaged according to their respective groups and then normalized by "Divided by maximum" intensity method, i.e., each spectrum was normalized by dividing the intensity at each emission wavelength by the peak emission intensity of the spectrum. The procedure of normalization helps us in understanding the shift if any with respect to the peak positions of the various key biomolecules under the pre-treated and post-treated condition and also with respect to normal.

Deconvolution Method

The normalized average spectra of the samples belonging to the study are due to multiple endogenous fluorophores present in the samples and the fluorescence emission due to individual fluorophores were extracted using the method as discussed in our previous paper [32].

Statistical Analysis

To quantify the observed spectral results and to estimate the diagnostic potentiality of the various fluorophores, two-tailed independent student T-test analysis was performed across extracted parameters of the various key fluorophores of all the samples and also the significance (*P* value) of the selected parameters were determined. The receiver operator characteristic curve was also plotted for the spectral data of all the fluorophores obtained by the deconvolution method. Linear discrimination analysis was also performed by giving the parameters extracted using spectral deconvolution method to verify the discrimination capability of the SS technique.

Wilk's lambda method was followed for entering or removing independent variables. Wilk's lambda method is a variable selection method for stepwise discriminant analysis which chooses variables for entry into the equation on the basis of how much they lower Wilk's lambda. At each step, the variable that minimizes the overall Wilk's lambda is entered, and it is the most economical method, which selects independent variables that minimize Wilk's lambda. The discriminant analysis was performed using a partial F test (F to enter 3.84 F to remove 2.71) and a stepwise method to sequentially incorporate the set of variables into the Fisher linear discriminant function and this classification function of each group could discriminate only that group from the rest of the groups in the analysis. To verify the reliability of our analysis, leave one out cross validation method was also employed, wherein one particular sample would be eliminated and the discriminant analysis was used to form a classification algorithm using the remaining samples. The resulting algorithm was then used to classify the excluded case. This process was repeated for all the samples belonging to the group and the proportion of the classification was used to estimate the sensitivity and specificity of the analysis.

Results and Discussion

Although the rapidly growing array of chemotherapeutic agents and evolving sophisticated radiotherapy techniques have produced significant progress in the field of oncology, the choice of treatment (drug or combination of drugs / radiotherapy technique), best suited for the patient remains a challenge to the oncologists, as the "Response to treatment" varies with the individual. For effective cancer management, it is not only essential for early detection but also it is equally important to monitor the therapeutic efficacy to enhance survival. Currently, various biochemical assessment like CA125, CA15/3, CA27/9, glycogen, lipids, DNA/RNA and proteins and radiological evaluation technique (PET-CT) have been considered as a tool to diagnose and monitor treatment response in oncology. However, the accuracy of the biochemical tests is not appreciable [2] and also the accuracy of the radiological evaluation (PET-CT) depends on the spatial resolution of the imaging techniques [3]. In this regard, various optical spectroscopic techniques have been used to evaluate the molecular level changes associated with the transformation of normal cell into cancer as well as during pre-treated and post-treated conditions using biofluids. Biofluids like blood, urine, and saliva are preferred as they have many metabolites and key biomolecules and their confirmation and/ or distribution may change during the transformation of normal into cancer as well as due to treatment [24–30].

It is further noted that many metabolites are intrinsic fluorophores viz., amino acids (tryptophan, tyrosine, and phenylalanine) collagen/elastin, enzymes (NADH, FAD, etc.,) and endogenous porphyrin. Any change in the photophysical characteristics of native fluorophores have been exploited to discriminate the neoplastic condition from normal cells and tissues and also to evaluate the same due to treatment. In this study, SSS technique was used for monitoring the therapeutic response on breast cancer patients. To evaluate the photophysical characteristic changes due to treatment such as spectral shift and FWHM variations of different intrinsic fluorophores, the normalized SL spectra were also processed using spectral deconvolution method.

The SS spectra of blood plasma of 10 normal subjects, 10 pre-treated breast cancer patients (Infiltrating Ductal carcinoma confirmed based on histopathological examination of a biopsy), 10 post-treated breast cancer patients were measured in the wavelength region between 250 nm to 750 nm. The offset wavelength ($\Delta\lambda$) was fixed as 20 nm for all the groups. Figure 1 shows the average normalized SS spectra of blood

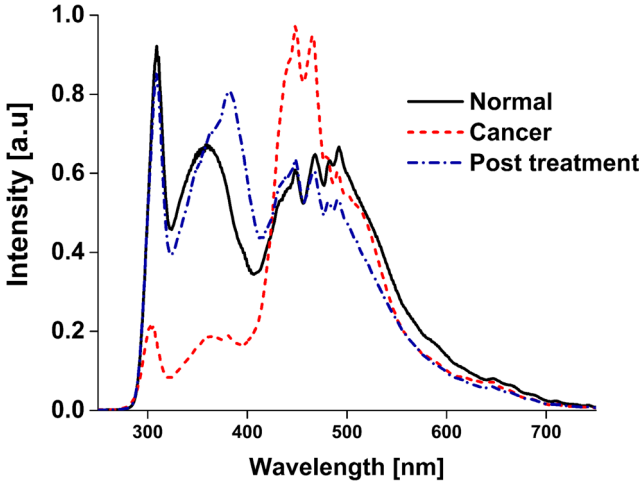

Fig. 1 Normalized mean SS spectra of blood plasma of normal subjects, pre-treated and post-treated breast cancer patients

plasma of normal subjects, patients with infiltrating ductal carcinoma, and post-treated patients. The difference spectrum between normal and pre-treated cancer blood plasma was computed by subtracting the average normalized SS spectrum of pre-treated breast cancer blood plasma from that of normal subjects and it is shown in Fig. 2. From Figs. 1 and 2, it is observed that the SS spectra exhibit different peaks around 300 nm, 350 nm, 450 nm and 500 nm with variation in their peak maxima and integrated intensity between normal subjects, pre-treated cancer, and post-treated cancer cases. From the difference spectrum (Fig. 2), it is seen that pre-treated cancer blood plasma exhibits lesser intensity between 285 nm and 417 nm and higher intensity between 417 nm and 485 nm than that of normal blood plasma. The difference spectrum has two isosbestic points at 417 and 485 nm. Subsequently, the emission intensity from 485 nm to 750 nm is considerably lower than that of normal subjects. From the difference spectrum, (Fig. 2), it is seen that spectra of post-treated blood plasma exhibit higher intensity between 286 nm and 425 nm and lesser intensity between 425 and 485 nm. The post-treated SS spectra exhibit minimal intensity on the positive scale of the difference spectrum. The difference spectrum between the blood plasma of pre-treated and post-treated SS spectra show significant variations at the wavelength of emission below 540 nm and with distinct isosbestic points at 427 nm. The variations in the SS spectra of blood plasma of breast cancer patients under pre-treatment and post-treatment condition may be attributed to the changes in the photophysical characteristics of the various native fluorophores such as tryptophan, collagen, NADH, flavin, and porphyrin.

Further, to estimate the distribution of possible fluorophores, the normalized SS spectra were processed using spectral deconvolution method. Spectral deconvolution method extracts the integrated intensity under the curve (Intensity), spectral peak wavelength (λ_{max}), and full width at half maxima (FWHM) for various fluorophores. The representation of measured and modeled spectra using deconvolution method for blood plasma of a normal subject, pre-treated, post-treated, and follow up breast cancer patients are shown in Fig. 3a–d.

The parameters for various fluorophores are computed and they are given in Table 1. The SS spectra after fit, show five major peaks due to the emission of tryptophan, collagen, NADH, FAD, and porphyrin. It is noted that the SS spectral signatures of native fluorophores with respect to λ_{max} of emission, FWHM, and integrated intensity show a considerable

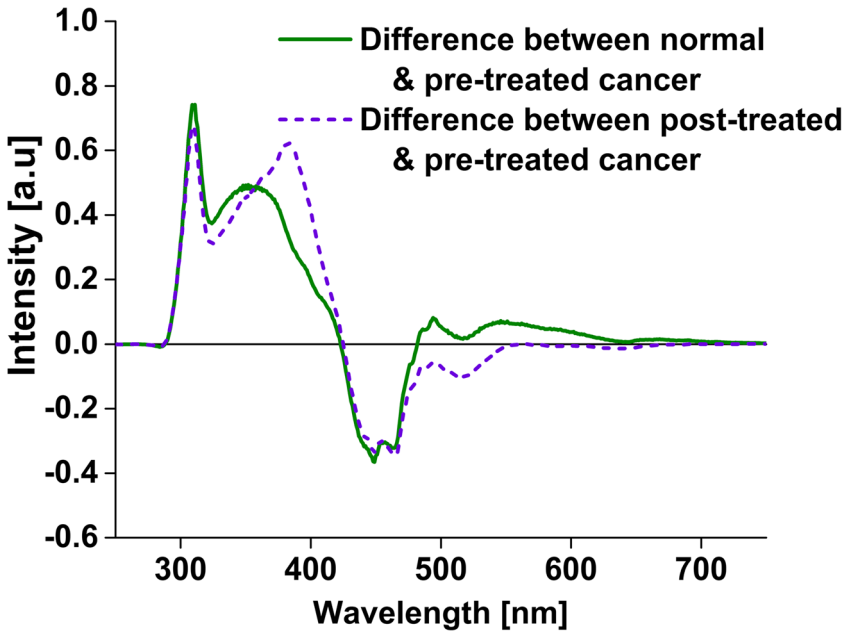

Fig. 2 Difference spectra between normalized mean SS spectra of blood plasma of normal subjects and breast cancer patients and between normal subjects and post-treated patients

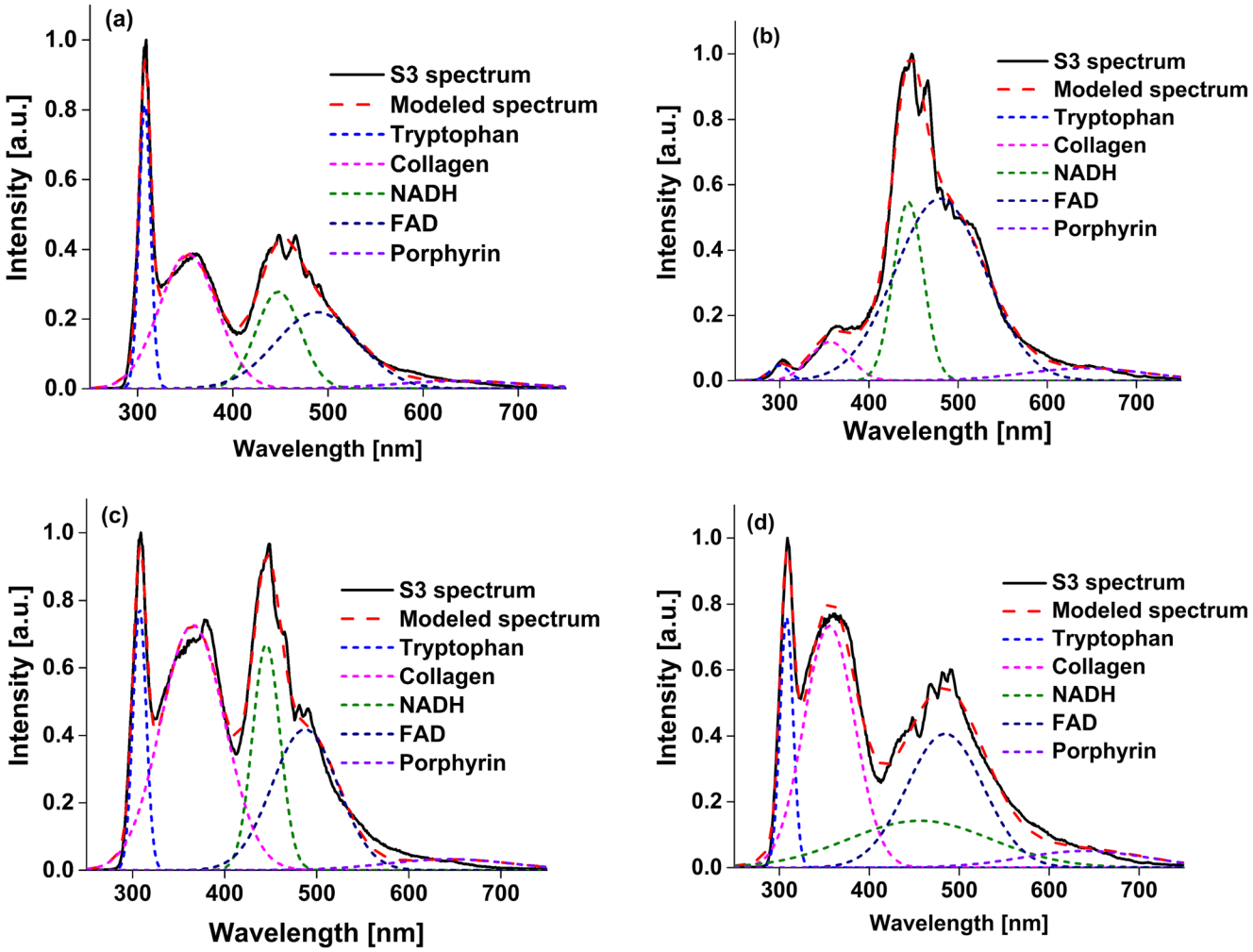

Fig. 3 Schematic representation of measured and modeled SS spectra using spectral deconvolution method (**a**) for normal subjects (**b**) for pre-treated cancer patients (**c**) for post-treated cancer patients and (**d**) for follow up patients (2 years after treatment)

difference between normal and pre-treated breast cancer blood plasma as well as between pre-treated and post-treated breast cancer blood plasma groups. It is seen that there is not much of change in the emission characteristics of endogenous porphyrin, even though there is a slight shift in the peak position and variation in the intensity. Further, it is interesting to note that there is not much variation between normal and post-treated as well as follow-up cases (2 years after treatment).

Table 1 The average, and standard deviation, of all the statistically significant variables for normal, pre-treated, post-treated, and follow up cases

Variable		Normal	Pre-treated	Post-treated	Follow up
Intensity	Tryptophan	13.18 ± 2.07	3.97 ± 3.99	12.90 ± 2.89	11.40 ± 3.27
	Collagen	41.75 ± 12.30	9.13 ± 9.90	42.08 ± 11.70	47.52 ± 15.21
	NADH	34.21 ± 20.88	30.13 ± 30.73	78.28 ± 43.44	51.97 ± 19.87
	FAD	57.16 ± 40.11	61.45 ± 24.15	10.87 ± 11.65	17.93 ± 16.24
λ_{max}	Tryptophan	307.92 ± 0.58	302.36 ± 2.20	307.66 ± 1.30	308.18 ± 1.08
	NADH	449.71 ± 4.67	447.84 ± 3.81	452.00 ± 5.76	447.35 ± 6.84
	FAD	490.78 ± 14.28	483.44 ± 10.72	493.96 ± 6.65	497.46 ± 10.34
FWHM	Tryptophan	17.38 ± 1.61	24.69 ± 5.45	17.67 ± 1.84	17.24 ± 1.91
	NADH	142.52 ± 50.27	95.99 ± 55.35	151.89 ± 43.45	170.24 ± 20.16
	FAD	107.54 ± 18.41	96.69 ± 13.47	78.86 ± 13.45	75.04 ± 16.04
Redox ratio		0.62 ± 0.31	0.72 ± 0.29	0.17 ± 0.21	0.25 ± 0.22

The peak around 300 nm may be attributed to the emission from tryptophan (Fig. 1). Varied spectral signatures between normal and breast cancer blood plasma (pre-treated) is due to change in the photophysical characteristics of tryptophan. From Table 1, it is observed that tryptophan for normal and post-treated blood plasma exhibit peaks at 307 nm, whereas for pre-treated cancer blood plasma shows a peak at 302 nm. The pre-treated cases exhibit a 5 nm blue shift with respect to normal and post-treated cases. Further, the integrated intensity under the curve exhibit variation $I_{Try}(nor) = I_{Try}(post) > I_{Try}(pre)$. The changes in the spectral signatures of tryptophan are attributed to conformational changes such as partial unfolding of protein. The change in the observed spectra may be associated with specialized protein like BRCA 1 gene which is altered when normal cells transform into cancer [33]. The low intensity of tryptophan in breast cancer patients could also be attributed due to the removal of the tumor (excision biopsy/ lumpectomy/ mastectomy) in most of the patients [34]. Zhang et al. have also reported that tryptophan can be considered as a key native biomarker in cells to determine the level of metastasis in breast cancer cells [35]. Furthermore, the study on plasma free amino acid profiling using high performance liquid chromatography (HPLC) of different types of cancers including breast cancers revealed significant low levels of tryptophan [36], which coincides with our observed result. It was also observed from Table 1 that the tryptophan intensity and their position of peak wavelength of post-treated cancer patients were almost matching with that of normal subjects.

The peak around 350 nm (Fig. 1) may be attributed to the contribution of collagen. Collagen exhibits low intensity in the beginning of treatment as seen in Table 1. But, it is also seen from the table that the intensity of the collagen peak increases during post-treatment conditions. Brisson et al. studied using murine (4 T1) and human (MDA-MB-231) breast cancer cells grown in collagen III-poor and collagen III-enriched microenvironments have noted that collagen plays a crucial role in regulating tumor progression [37]. The low intensity of collagen under pre-treatment condition may be attributed to the destruction of extra cellular matrix by cancer cells [23]. Similar results of decreased collagen in cancer were also reported by others [19, 22, 23, 38, 39]. The increase in the level of collagen under post-treated conditions could be attributed to the destruction of the vascular membrane by chemotherapy agents/increase in angiogenic activity due to cellular stress induced by chemotherapy [40]. Masouni et al. reported that collagen VI is produced by adipocytes in breast cancers and also by macrophages during inflammation. They have measured the mean serum collagen IV concentration prior to and during chemotherapy, using immunoassay and have found that mean serum collagen concentration was 167 μg ml^{-1} in 39 patients who subsequently received 6 months chemotherapy and the concentration had risen to 206 μg ml^{-1} after

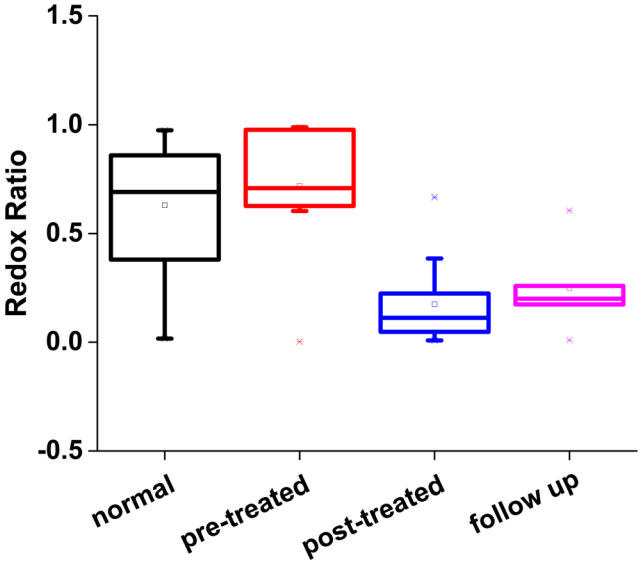

Fig. 4 Redox ratio for normal subjects, and cancer patients during pre-treated, post-treated and yearly follow up

completion of chemotherapy [40]. Miller et al. conducted metabolite profiling using a liquid chromatography mass spectrometry on early stage breast cancer patients on limonene treatment, and have noticed a significant increase of the Collagen break down products - proline, glycine, and 4-hydroxy proline in the plasma of patients after treatment [41]. This concurs with our findings that the level of Collagen of the patients increased after treatment.

In Fig. 1, the region around 450 nm and 490 nm could be attributed to the emission of NADH and FAD respectively. Decreased NADH and increased FAD is observed under pre-treatment condition. However, the NADH intensity increases during post-treated conditions as seen from Fig. 1. NADH acts as a protector against the general toxic effects of

Table 2 The p value of all the statistically significant variables for normal, pre-treated and post-treated cancer patients

Variable		*P* value (normal and pre-treated)	*P* value (pre-treated and post-treated)
Intensity	Tryptophan	$< 10^{-3}$	$< 10^{-3}$
	Collagen	$< 10^{-3}$	$< 10^{-3}$
	NADH	0.001	0.001
	FAD	$< 10^{-3}$	$< 10^{-3}$
λ_{max}	Tryptophan	0.069	0.028
	NADH	0.339	0.073
	FAD	0.065	0.022
FWHM	Tryptophan	0.210	0.017
	NADH	0.876	0.111
	FAD	0.287	0.067
Redox ratio		0.52	$<10^{-3}$

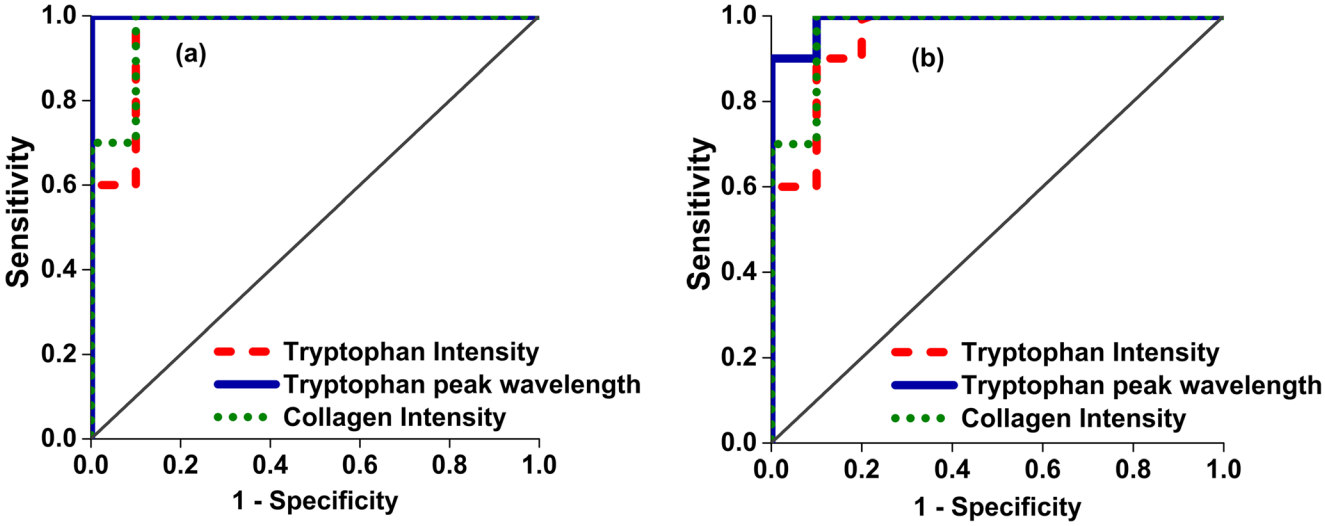

Fig. 5 ROC curves for the variables extracted using spectral deconvolution method (**a**) for normal and pre-treated cancer blood plasma (**b**) for pre-treated and post-treated cancer blood plasma

chemotherapeutic agents by stimulating the DNA repair system, promoting normal cellular bio-synthetic response after therapy [9], which could be the reason for the increase in the NADH intensity in post-treated patients. Redox ratio may provide potentially a new index which could have significance in the diagnosis and treatment of breast cancer and hence the redox ratios of the various samples were calculated as per Eq. 1.

$$Redox\ ratio = \frac{FAD}{NADH + FAD} \tag{1}$$

Increased NADH and decreased FAD molecules indicate that there is a decrease in redox ratio under post-treated condition (Fig. 4). However, the redox ratio level increases slightly during yearly follow up. The redox ratio acts as biomarkers for monitoring the oxidation – reduction state in cells. The Variation in redox ratio may be due to a shift from oxidative phosphorylation to glycolysis in cancer cells [42, 43–46].

Discrimination by Student T-Test Analysis

Two-tailed student t-test analysis was performed across 15 variables extracted using spectral deconvolution method and they are listed in Table 2 along with their p values. It was found that, out of 15 variables, only five variables turned out to be significant.

Discrimination by Receiver Operator Characteristic Curve

As there exists good statistical significance in terms of intensity and λ_{max} for tryptophan and collagen, ROC analysis was made for tryptophan intensity, tryptophan λ_{max} and collagen intensity for normal and pre-treated cancer blood plasma (Fig. 5a). The cutoff values for each variable and their respective sensitivity and specificity are given in Table 3 and it is observed that the tryptophan λ_{max} provides 100% sensitivity and 100% specificity for discriminating normal and pre-treated cancer. Tryptophan intensity and collagen intensity provide 100% sensitivity and 90% specificity. Similarly, ROC graph for tryptophan intensity, tryptophan λ_{max} and collagen intensity for pre-treated and post-treated cancer blood plasma are shown in Fig. 5b and the corresponding cut off values, the area under the curve, sensitivity, and specificity are given in Table 4. It is found that tryptophan λ_{max} and collagen provide 100% sensitivity and 90% specificity for discriminating pre-treated and post-treated cancer. While

Table 3 Cutoff value, AUC, sensitivity, and specificity of the extracted variables for normal and pre-treated cancer blood plasma

Variable	Cutoff value	AUC	Sensitivity	Specificity
Tryptophan (Intensity)	9.68	0.960	100%	90%
Tryptophan (λ_{max})	306.49	1.000	100%	100%
Collagen (Intensity)	23.39	0.970	100%	90%

Table 4 Cutoff value, AUC, sensitivity, and specificity of the extracted variables for pre-treated and post-treated cancer blood plasma

Variable	Cutoff value	AUC	Sensitivity	Specificity
Tryptophan (Intensity)	5.21	0.950	100%	80%
Tryptophan (λ_{max})	304.87	0.990	100%	90%
Collagen (Intensity)	21.72	0.970	100%	90%

Table 5 Classification results of discriminant analysis

Input variables	Sensitivity	Specificity
Normal subjects and pre-treated cancer patients	100%	100%
Pre-treated and post-treated cancer patients	100%	100%

tryptophan intensity discriminates pre-treated and post-treated cancer blood plasma with a sensitivity and specificity of 100% and 80% respectively.

Discrimination by Stepwise Multiple Linear Discriminant Analysis

The stepwise multiple linear discriminant analysis performed across the whole set of 10 normal, 10 pre-treated cancer, and 10 post-treated blood plasma by use of variables extracted using deconvolution algorithm. The discriminant analysis resulted in the following expressions for canonical discriminant functions (DF1 and DF2):

$$DF1 = -0.092 \times Tryptophan\ (Intensity) + 0.444 \times Trptophan\ (\lambda_m) + 0.071 \times Collagen\ (Intensity) - 104.606 \quad (2)$$

$$DF2 = 0.107 \times NADH\ (Intensity) + 0.018 \times FAD\ (FWHM) - 4.976 \quad (3)$$

Where,

DF1 is the discriminant function for normal and breast cancer patients prior to treatment and

DF2 is the discriminant function for breast cancer patient before and after treatment

The linear discriminant analysis provides 100% sensitivity and 100% specificity for normal and pre-treated cancer blood plasma. The second LDA was performed between the breast cancer group under pre-treated and post-treated conditions in a similar manner by giving the extracted parameter using spectral deconvolution method as input. This analysis too classified all the samples correctly namely 10 samples were correctly classified as pre-treatment group and 10 were correctly classified as post-treatment group yielding sensitivity and specificity of 100% and 100% respectively. The third PCA-LDA analysis was performed between all the three groups namely, normal subjects and breast cancer patients under pre-treated and post-treated conditions. The analysis was able to discriminate the samples based on the spectral variations observed and hence could help in monitoring the biochemical changes happening during the course of treatment to assess the therapeutic efficacy in the breast cancer patients undergoing treatment. Table 5 compares the results of the discriminant analysis for the various groups. The scatter plot of discriminant function for different combinations of normal, pre-treated cancer, and post-treated cancer blood plasma is plotted in Fig. 6.

Student t-test analysis, ROC analysis, and stepwise linear discriminant analysis all revealed that there exist significant differences between the healthy subjects and breast cancer patients as well as between the breast cancer patients under pre-treated and post-treated conditions. The variations are with respect to both peak intensity and position of the various biomolecules tryptophan, collagen, NADH, and FAD. The integrated intensities of tryptophan and collagen and tryptophan peak shift were found to have the highest discriminating capability. The LDA discriminates pre-treated cancer from the normal subjects as well as from the post-treated group.

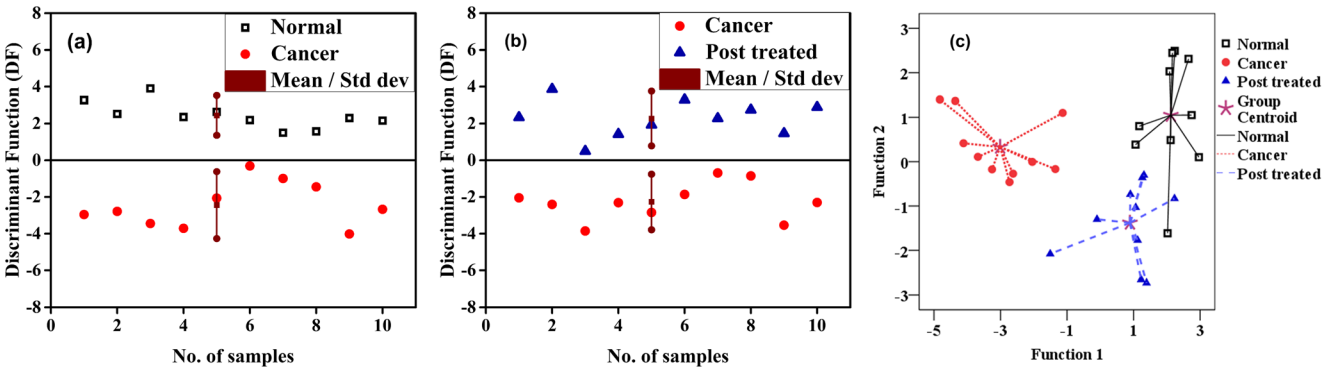

Fig. 6 Scatter plot of discriminant function (**a**) for normal subjects and cancer patients (**b**) for pre-treated and post-treated cancer patients and (**c**) for normal, pre-treated and post-treated cases

Conclusion

Synchronous luminescence spectroscopy (SLS) characterization and discrimination of blood plasma is used to monitor tumor response to therapy and for follow up care of breast cancer patients based on the expression of molecular biomarkers. Among various fluorophores, tryptophan, collagen may be considered as molecular markers as they exhibit significant spectroscopic variations as well as excellent statistical discrimination. The discriminant analysis provides 100% accuracy among all the three groups. The ultimate implications of the approach are optimization of drug dosage, testing of new chemotherapy drugs, and for development of personalized treatment. More light should be thrown in understanding the variations in the photophysical characteristics of tryptophan, collagen, NADH, and FAD due to various treatment protocols.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that there is no conflict of interest regarding the publication of this article.

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