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Cervical cancer detection by time-resolved spectra of blood components

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Abstract. Fluorescence spectral techniques are very sensitive, and hence they are gaining importance in cancer detection. The biomarkers indicative of cancer could be identified and quantified by spectral or time domain fluorescence spectroscopy. The results of an investigation of time-resolved spectra of cellular components of blood obtained from cervical cancer patients and normal controls are given. The cancer indicative biomarker in this paper is porphyrin; it has a fluorescence decay time of 60% more in samples of cancer patients than those of normal controls. Based on such measurements, a randomized set comprising samples from cancer patients and controls ($N = 27$ in total) could be classified with sensitivity (92%) and specificity (86%). [DOI: 10.1117/1.JBO.19.5.057011]

Keywords: time-resolved spectra; picosecond laser excitation; cervical cancer detection; blood components; porphyrin biomarker.

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1 Introduction

Optical biopsy of cancer is a novel, minimally invasive, real-time technique for cancer detection. From laboratory investigations, it has gone to clinical settings in some cases, with specific examples such as laser endoscopes.[1–3]

The concept is based on the proven principle that a certain set of fluorescence biomarkers grow out of proportion when normal tissue turns malignant. Fluorescence spectroscopy has two complementary approaches: steady-state spectral domain and transient time-resolved spectral domain.

Steady-state fluorescence spectra are not sufficient to distinguish between fluorophores that have highly overlapping spectral characteristics. Also, differences in the fluorescence intensities due to quenching by other molecules, aggregation or energy transfer can be difficult to quantify or interpret. Therefore, fluorescence lifetime data are additional parameters to distinguish the spectrally overlapping fluorophores.[4–7] Due to this advantage, there is a considerable interest in time-resolved measurements, and it is a new emerging technique to study the structure and dynamics of biological molecules. The photophysical properties of the intrinsic biomolecules and biostructures have been considered as a possible parameter that may be related to the morphofunctional state of biological substrate. It can also be used to identify cancer by using the intrinsic or extrinsic fluorophores.[1–7]

The commonly used fluorescence lifetime τ depends on the intrinsic characteristics of the fluorophore and also on local environments, such as viscosity, pH, refractive index, aggregation as well as interactions with other molecules.[4–6] The time-resolved spectral information is obtained by exciting the sample with a short pulse laser and observing the decay of the fluorescence intensity with the time-correlated single photon counting (TCSPC) technique. Employing this technique, a few reports had shown significant differentiation between normal and tumor sites of the same organ, in terms of lifetime τ.[4–6]

In this brief paper, we investigated the possibility of using time-resolved fluorescence spectroscopy to differentiate blood components drawn from normal controls and patients with cervical cancer. We reported in our previous papers[8–10] on the results of steady-state measurements of blood components for cancer diagnosis using porphyrin as a biomarker. The results showed a significant discrimination accuracy to classify the diseased blood from normal blood. Such results have motivated us to study the time-resolved measurements of porphyrin in blood components.

2 Methods and Materials

2.1 *Sample Processing*

Exactly 5 ml of venous blood from each of fourteen healthy women (age range 25 to 60 years) was collected in a violet sterile vial that contained the anticoagulant ethylenediamine tetraacetic acid (EDTA). The vial was gently rocked five times to adequately mix the EDTA with the whole blood, and the sample was centrifuged at a rotational speed (rpm) of 3000 for 15 min. Clear, pale, greenish-yellow plasma supernatant was obtained by this centrifugation. A total of 1.5 ml of supernatant plasma (containing mostly proteins and enzymes) and buffy coat (containing mostly white blood cells) were removed and discarded leaving the formed elements (FE) undisturbed.

Then, exactly 1 ml of the thick FE from the bottom layer, which contained mostly red blood cells (RBCs), was removed

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to a sterile vial and mixed with 2 ml of analytical grade acetone. Proper care was taken to ensure that the FE did not develop lumps. After thorough mixing to enable the acetone extract fluorophores within and around the cells, the sample was centrifuged again (3000 rpm for 15 min). The resulting supernatant was subjected to steady-state fluorescence emission spectra (FES) and time-resolved spectra.

The same protocol was used to process blood samples from the cervical cancer patients ($N = 13$, age range from 25 to 60) at the Government Hospital in Royapettah, Chennai. The classification of cervical cancer subjects was made by the histopathological examination as early stages (Stage I and Stage II). All of the above said patients were confirmed cases of cancer, waiting for the onset of treatment. They had been informed about the research investigation and consent from each had been obtained. Suitable permission had been given from the Internal Review Board.

2.2 *Spectral Measurements*

Steady-state FES for the acetone extract of samples (control and cervical cancer patients) were obtained by PerkinElmer Spectrofluorometer LS 55. Each sample, taken in a quartz cuvette of 1 cm × 1 cm × 4 cm, was excited at 400 nm, and the fluorescence signal from the sample was collected (transversely) and scanned from 425 to 700 nm. This was the spectral domain analysis.

The same set of samples was used to obtain transient, excited-state laser-induced fluorescence spectra, employing the TCSPC technique. This was the time domain analysis. Our attempt was to establish correspondence between these two types of spectral analyses.

The fluorescence decay measurements were carried out with a micro-channel plate photomultiplier tube (MCP-PMT) as detector and a picosecond laser as the excitation source. The laser used was mode locked Ti-Sapphire Tsunami laser (Spectra Physics, 3960) capable of generating ps/fs laser pulses at 800 nm. Such pulses were passed through the pulse picker (Spectra Physics, 3980 2S) to generate 35 ps 4 MHz pulses from the 82 MHz train of pulses. Following this, the second-harmonic laser (400 nm) output was obtained from the flexible harmonic generator (Spectra Physics, Santa Clara, California). The vertically polarized 400 nm laser beam was used to excite the sample.

The fluorescence emission at the magic angle (54.7 deg) was acquired by an MCP-PMT (Hamamatsu R 3809U, Tokyo, Japan) after passing through the monochromator and processed through a constant fraction discriminator, a time to amplitude converter, and multichannel analyzer modules. The instrument response function for this system was 50 ps. Since we are interested in studying the porphyrin lifetime of blood components at 630 nm, a band-pass filter was used before the PMT to obtain the desired native fluorescence and to exclude undesired fluorescence from other sources.

The measured fluorescence decay was the convolution of the true fluorescence decay, the excitation function, and the instrument response function. The fluorescence kinetic parameters (lifetime, amplitude, etc.) were obtained by deconvoluting the excitation function and the instrument response function from the measured fluorescence decay function. The data analysis was carried out by the software provided by the fluorocube of Horiba Jobin Yuvon (formerly IBH product) IBH (DAS-6), which was based on the reconvolution technique using iterative nonlinear least-squares methods.[11]

The fluorescence decay curves obtained from the sample were fitted to single, double, and triple exponential decay functions. The three component approaches improved the fitting, and the quality of fit was judged by the reduced χ^2 value. The three exponential functions used to fit the data were

$$I(t) = \alpha_1 \exp\left(-\frac{t}{\tau_1}\right) + \alpha_2 \exp\left(-\frac{t}{\tau_2}\right) + \alpha_3 \exp\left(-\frac{t}{\tau_3}\right),$$

where α_1, α_2, and α_3 were the amplitudes of the components at $t = 0$; τ_1, τ_2, and τ_3 were the decay times.

The fractional contribution of each component to the fluorescence intensity could be defined as

$$f_i = \frac{\alpha_i \tau_i}{\sum_j \alpha_j \tau_j}.$$

The average lifetime for the three exponential decay was

$$\tau = \frac{\alpha_1 \tau_1^2 + \alpha_2 \tau_2^2 + \alpha_3 \tau_3^2}{\alpha_1 \tau_1 + \alpha_2 \tau_2 + \alpha_3 \tau_3}.$$

2.3 *Statistical Analysis*

Statistical analyses were performed to evaluate the diagnostic potential of the collected time-resolved spectra of blood components of normal and cancer patients using ANOVA discriminant analysis commonly available in the SPSS +11 software. This technique assigns a case to group K, if its score on the function for that group is greater than its score on the function for any other group. This function called the Fisher classification function will give n classification function coefficients and $(n - 1)$ canonical discriminant functions.[11] To check the classification results, we used "leave-one-out cross-validation" method. The algorithm of "leave-one-out cross-validation" involves classifying each case into a group according to the classification functions computed from all the data except the case being classified.[12] Such an analysis gave a measure of reliability of collected time-resolved data.

3 Results

3.1 *Steady-State FES*

Figure 1(a) shows the typical FES of acetone extract of cellular components in the normal control. This spectrum has three important fluorescence peaks: one at 500 nm (due to Flavin adenine dinucleotide), next at 585 nm, and third at 630 nm (due to basic and neutral forms of porphyrin). Figure 1(b) shows the typical FES of acetone extract of cellular components of a cancer patient. This too has three peaks similar to that of Fig. 1(a), but porphyrin peaks are out of proportion. If we define ratio parameters $R_1 = I_{635}/I_{585}$, intensities at 635 and 585 nm, it is 1.1 for the control group, but 2.4 for a cancer patient. That is, the normal porphyrin is twice elevated for the patient. This is true for many other types of cancer, too.[8–12]

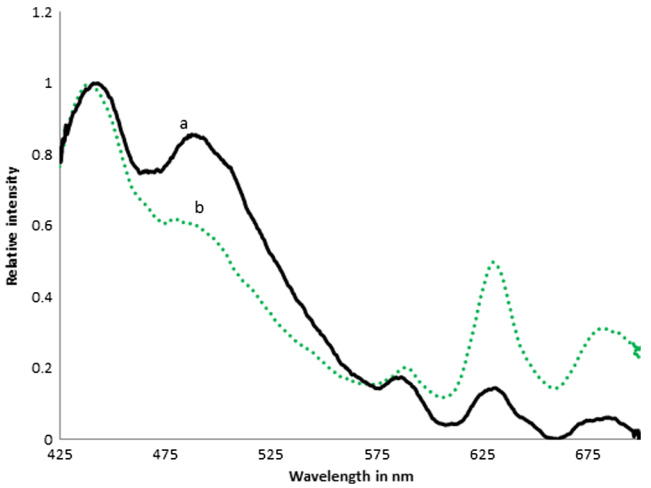

Fig. 1 (a) Steady-state fluorescence emission spectra (FES) of acetone extract of formed elements (FE) of normal control. (b) Steady-state FES of acetone extract of FE of cervical cancer patients. Both are typical spectra.

3.2 *Fluorescence Lifetime Spectra*

The native fluorescence lifetime spectra of porphyrin found in the blood samples of normal patients and cervical cancer patients were acquired at 630 nm emission under 400 nm excitation. Figure 2(a) shows the typical time-resolved spectra for normal samples. A time decay curve, such as the one shown in Fig. 2(a) may be fitted with single, double, or triple exponential decay functions. For us, it was the three component functions that gave the best fit, and the quality of such a fit could be judged by the residuals lying between −1.4 and +1.4 as shown in Fig. 2(b). A similar set of fluorescent decay curves and residuals for a typical cancer sample are shown in Figs. 3(a) and 3(b), respectively.

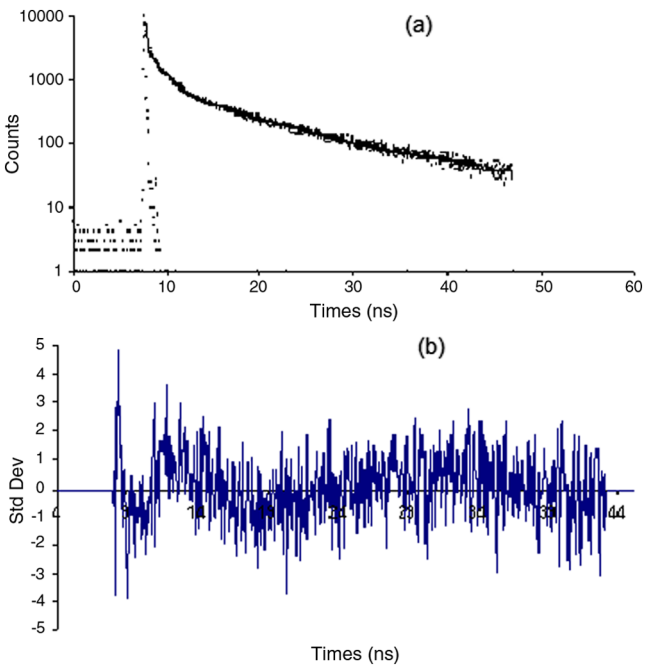

Fig. 2 (a) Fluorescence decay curve of FE of normal control and (b) with fitted function and residuals.

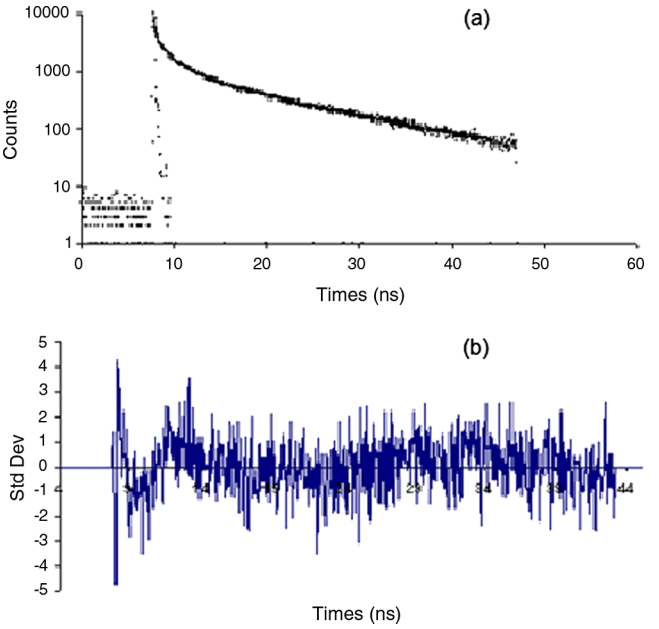

Fig. 3 (a) Fluorescence decay curve of FE of cervical cancer patients and (b) fitted functions with residuals.

The three decay times and their amplitudes for normal and cancer samples are shown in Table 1.

4 Discussion

As the steady-state fluorescence spectra for blood samples of cancer patients have been studied extensively,[8–12] the discussion will focus only on one pertinent observation. In the field of fluorescence spectroscopy, the relative intensities of different peaks are more important than the actual, absolute intensities. They reduce the errors arising out of the quantity and possible turbidity of a sample in addition to the instrumental sensitivity variation. The relative intensity between two peaks, say R_1, as given in Fig. 1, depends upon the concentration of the intrinsic nature of the fluorophores under observation and not on sample volume, or RBC concentrations.

The time-resolved spectral parameters of FE of blood samples indicated that the fluorescence at 630 nm emission originated from three molecular species with three characteristic lifetimes. For normal FE, the fast component (component 1) lifetime was 0.035 ns and had the maximum amplitude; the

Table 1 Mean values of lifetimes and their amplitudes of formed element.

Mean	Normal FE	Diseased FE
$\alpha 1$ (%)	56.53	31.69
$\tau 1$ (ns)	0.035 ± 0.006	0.091 ± 0.011
$\alpha 2$ (%)	18.58	20.41
$\tau 2$ (ns)	1.14 ± 0.09	1.6 ± 0.16
$\alpha 3$ (%)	24.88	47.89
$\tau 3$ (ns)	6.0 ± 0.6	9.31 ± 1.16

intermediate component (component 2) had the lowest amplitude and lifetime was 1.140 ns; and the third component had the longest decay time of 6.000 ns. In the cervical cancer FE, component 1 was 0.091 ns and the amplitude was low when compared to normal; the component 2 was 1.6 ns; and the component 3 was 9.31 ns and the amplitude was found higher than for the normal. One may ignore the fast component values (0.035 and 0.091 ns), as the resolution of the instrument was only 50 ps. Yet, the other two components were about 1.6 times higher for diseased FE than for normal FEs, and hence, such data did have the potential to distinguish the two sets unambiguously.

In an earlier report by Cubeddu et al., porphyrin in normal tissue had a fluorescence lifetime of 10 ns, but in tumor tissues it was 18 ns.[13,14] That is, for this tissue study, the tumor tissue had a 1.8 times longer lifetime than that of normal ones. In another similar study on oral cancer, it was again reported that the concentration of porphyrin was larger, and it had a longer lifetime.[15]

It is important to mention here that no other reports on time-resolved spectra of blood components are available for more precise comparison.

Under normal conditions, the synthesis of protoporphyrin is under a high feedback control; that is, cells produce it at a rate just sufficient to match the heme, but in excessive abnormal cell proliferation the feedback mechanism loses its control and the protoporphyrin that is produced in excess is left unutilized and released into tissue which is carried through, by the circulating fluid, blood. So the porphyrin concentration is more in the diseased blood components.[8–10] The porphyrin which gets accumulated abnormally on the tumor tissue experiences different local environment, such as aggregation, higher acidity and also bonding to the intercellular matrix. All these could lead to enhanced lifetime,[14,15] in tissue as shown by others and in blood, as shown in this paper. It is important to draw attention to two important facts:

1. There is more than one porphyrin (such as coproporphyrin I, coproporphyrin III, uroporhyrins, etc.) found in blood and, more importantly, in urine.[16] All have overlapping spectral profiles, and hence the three components in decay times could be due to three different types of porphyrins.
2. In the spectral domain also, there are three overlapping profiles for porphyrin: one at 585 nm, the next at 630 nm, and a third at 685 nm attributed to the basic, neutral, and acidic forms of porphyrins.[8,9] It might be possible that all these different porphyrins occurring in different concentrations could lead to three different lifetimes. However, an attempt has not been made to resolve them in this report, because our aim was to assess the diagnostic potential of the technique, which may have clinical value in the long run.

In conclusion, the lifetime of FE porphyrin fit well for three component models. The lifetime values of the porphyrins available in the FE showed a significant difference between the normal and cervical cancer subjects.

4.1 *Statistical Results*

The statistical analysis was performed as described in Sec. 2 to evaluate the diagnostic potential of the time-resolved spectra of blood components using porphyrin as a biomarker. One of the multivariate analyses called linear stepwise discriminant analysis was used to distinguish the diseased blood components from normal blood components. Out of twenty-seven samples, known cases of normal and cervical cancer (each four in number) were selected for preliminary measurement of lifetimes; these averaged out values are in Table 1. With these values as classification tools, three component lifetimes obtained from each sample were given as inputs for the subsequent discriminant analysis for classification into two separate sets.

4.2 *Discriminant Analysis*

Discriminant analysis gives the discriminant function, called first canonical discriminant function, that is used to classify the diseased FE from the normal control FE using discriminant scores. Figure 4 shows the scatter plot between the sample number and the discriminant score obtained from the first canonical discriminant function. It shows that normal and cervical cancer patients are well separated.

Table 2 shows the classification results of the first discriminant analysis. The classification accuracy for classifying normal subjects and cervical diseased subjects is 88.9% for the original case and 81.5% for the cross-validation case. The discriminant analysis results show that cervical malignant subjects are discriminated with a sensitivity of 92.3% and a specificity of

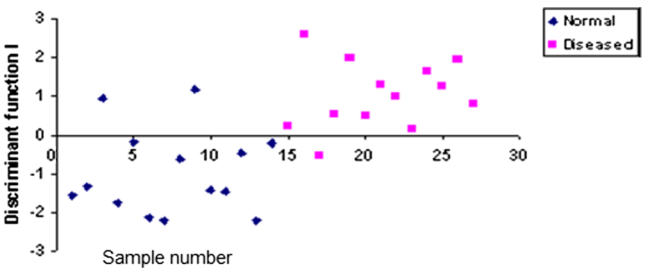

Fig. 4 Plot between the sample number and values of discriminant function for normal and cervical cancer subjects.

Table 2 Classification results of discriminant analysis.

Group		Predicted group membership		
		Normal 1	Diseased 2	Total
Original	Count normal 1	12	2	14
	Diseased 2	1	12	13
	% 1	85.7	14.3	100.0
	2	7.7	92.3	100.0
Cross-validated	Count 1	12	2	14
	2	3	10	13
	% 1	85.7	14.3	100.0
	2	23.1	76.9	100.0

85.7% for the original case and sensitivity of 76.9% and specificity of 85.7% for the cross-validated case. In other words, 88.9% of the original grouped cases were correctly classified, and 81.5% of cross-validated grouped cases were correctly classified.

5 Conclusion

In this short paper on time-resolved spectra of porphyrin found in the cellular component of blood obtained from cervical cancer patients and normal controls are reported. It was found that the fluorescence decay could be fitted very well by three exponential functions. The porphyrin of cervical cancer samples has approximately 1.6 times longer fluorescence time than that from the normal control. Based on the statistical analysis, discrimination was done for a total in 27 samples. This technique has shown a 92% sensitivity and an 86% specificity. It is important to mention that this is the only time-resolved spectral study done on blood components. Although this study was confined to cervical cancer, it may be extended to other cancers, too. Such a study is in progress in our laboratory.

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