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Native Fluorescence Spectroscopic Evaluation of Chemotherapeutic Effects on Malignant Cells using Nonnegative Matrix Factorization Analysis

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The native fluorescence spectra of retinoic acid (RA)-treated and untreated human breast cancerous cells excited with the selective wavelengths of 300 nm and 340 nm were measured and analyzed using a blind source separation method namely Nonnegative Matrix Factorization (NMF). The results show that the fluorophores of human malignant breast cells change their compositions when they are treated with RA. The reduced contribution from tryptophan, NADH and flavin to the fluorescence of the treated breast cancerous cells was observed in comparison with that of the untreated cells. The results indicate that the decrease of adenosine triphosphate (ATP) in the RA-treated cells. The possible clinical applications of this native fluorescence study are discussed.

Key words: Breast cancer cells; Selective excitation wavelength; Native fluorescence; Tryptophan; Reduced nicotinamide adenine dinucleotide (NADH); Flavin; Nonnegative Matrix Factorization (NMF); Chemo-therapeutic effect; and Retinoic acid.

Introduction

Optical spectroscopy has been considered as an alternative technique for cancer detection over other conventional diagnostic methods for more than two decades. The optical method has many advantages such as minimal invasiveness, no tissue removal, less time consumption, fast and reproducibility (1). Alfano and coworkers were the first to measure native fluorescence spectra of malignant and non-malignant breast and lung tissues (1, 2). Breast tissue is mainly composed of extracellular matrix of collagen fiber, proteins, fat, water, and epithelial cells. Epithelial cells, where cancer primarily happens, contain a number of endogenous fluorophores: tryptophan, reduced nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD) *etc.* (3). The primary fluorophore in the breast tissue extracellular matrix is type I collagen (4). Tryptophan accounts for the majority of protein fluorescence. NADH and FAD are involved in the oxidation of fuel molecules and can be used to probe changes in cellular metabolism (4). Chance *et al* exploited this phenomenon and show that direct monitoring of NADH fluorescence dynamically interprets the metabolic activity within the cell (5). A novel application of this technique is to study chemopreventive effects by therapeutic agents such as retinoic acid (RA) in cancer therapy in a variety of organ sites (6). Recently, the differences between normal and malignant human breast tissue fluorescence spectra have been reported to be attributed in part by differences in the intrinsic cellular fluorescence of normal and malignant breast epithelial cells, fibers and the interstitial fluid (7, 8). There are few works concerning how fluorescence spectroscopy can be used to evaluate the biological activity arising from the therapeutic agents on malignant cell and tissue samples.

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In this work, we report a native fluorescence spectroscopy study to evaluate the chemotherapeutic effects on breast malignant cells using Nonnegative Matrix Factorization (NMF) analysis. In the native fluorescence spectral evaluation of therapeutic effects on cancer, there are two main factors hampering the reliability of extracting the difference of the biochemical and/or morphological information between treated and un-treated cancerous samples. One is that the individual difference exists among same type samples from different sources. Another is the presence of significant distortions induced by endogenous absorber and scatterer (7). The first pronounces unreliability to compare the emission intensities of certain biomarker (*e.g.* NADH) in different kinds of samples (7). The second results the imperfection of reconstructing the emission spectra from the measured individual fluorophores in solution (8).

Non-negative Matrix Factorization method may offer a way for extracting the intrinsic fluorescence spectrum of each principal biochemical component (fluorophore) from the mixed spectra of many active ones (9). NMF is a maximum likelihood approach for blind source separation (BSS) using non-negativity constraints to extract individual information of the mixed observation (9). Blind source separation, also known as blind signal separation, is the separation of a set of signals from a set of mixed signals without the aid of information (or with very little information) about the source signals or the mixing process (7, 9). NMF learns parts-based representation and finds the hidden components. In spectral analysis, NMF is superior to other BSS methods such as Principal Component Analysis (PCA), Independent Component Analysis (ICA), and Factor Analysis (FA) since the spectra and contents recovered by NMF are non-negative (9). This makes the calculated results represent more physically and/or biologically meaning because the spectra and contents of the fluorephores should have non-negative values. The advantages of NMF relies on: (1) spectral data and concentrations of constituents are positive values, therefore it is natural to use non-negative constraints; and (2) the unknown constituents in the mixed compounds of complicated environment such as biological cells and tissues may show different spectra other than pure individual biochemical because of the complex surroundings, and the spectra decomposed by NMF may find the "real" spectra in mixed environment (7, 9).

The aim of the present research is to address a question on whether the native fluorescence spectroscopy measurements are effective enough to detect changes of fluorophore compositions related to the treatment of chemotherapy on malignant cell lines (10). Retinoic acid (RA) had been evaluated as a chemotherapeutic agent for oral cancer (11). Fluorescence of the key fluorophores in human breast cells was measured and analyzed using NMF. This paper reports our experimental results and show how native fluorescence spectroscopy is used to monitor the changes in underlying fluorophores due to the treatment with the chemotherapeutic agents. This approach may provide an alternative tool in evaluation of the cancer therapy for breast cancer.

Samples and Methods

There are eight main fluorophores and/or chromophores (tryptophan, collagen, elastin, NAD+, NAD, NADH, flavin and tyrosine) which have been reported to exist in breast cells and tissue (6, 7, 10). In our study, these molecules were obtained commercially from Mallinckrodt Baker, Inc. The cultured human breast cells were purchased from American Type of Culture Collection (ATCC), Rockville, MD. The cell lines were further sub-cultured to increase the cell population. The cell lines used in the study were: malignant human breast cell lines ATCC HTB22 (Adenocarinoma Pleural Effusion) and ATCC HTB126 (Dutal Carcinoma). The cells were cultured with 90% modified Dulbeco's minimal essential medium (Eagle) (GIBO) and 10% of the fetal bovine serum (GIBCO) in a humidified atmosphere of 4% CO_2 at 37°C. Half of the cells were treated with ~10^{-6} M retinoic acid and the remainder served as controls while the cell lines are cultured. On day 10, the cells were harvest by the treatment with trypsin EDTA (5% trypsin and 5.3×10^{-6} M EDTA: GIBCO) for 5-10 minutes, diluted in the medium, then isolated by centrifugation. They were re-suspended in phosphate buffered saline (SIGMA) to be washed; the washing procedure was repeated three times. The cells were finally packed in $3 \times 3 \times 24$ mm quartz cuvette using a centrifuge. The cell concentration was estimated as ~$105 - 110 \times 10^6$ cells/ml.

The fluorescence emission spectra of the fluorophores and cells were measured using the CDScan (trade name) provided by Mediscience Technology Corp. based on Perkin-Elmer LS 50 spectrometer. The excitation light with 5nm spectral-width was focused on samples with spatial size of ~3×1 mm. The power of incident light was ~0.5 μW. The scan speed was 240 nm per minute. The fluorescence was collected with a resolution of ~2.5 nm. The excitation wavelengths of 300nm and 340 nm were selected for our study based on the absorption spectra of the main fluorophores/chromophores in breast cells, and the previous study, (4, 7, 8, 10-15). To eliminate the possible background noise from cell sample preparation, the emission of the quartz, Trypsin-EDTA and the culture medium were measured, and the results show that there is no measurable fluorescence contributed from them. These fluorescence measurements were used to exploit differences between RA-treated and untreated breast cancer cells using the NMF method.

Nonnegative Matrix Factorization (NMF) is used to recover the corresponding spectra and contents of the individual fluorophores. NMF method is also known as Non-Negative

Matrix Approximation (NNMA). Unlike other data analysis tools, NMF uses non-negativity constraints with the optimal method depending on the specific conditions to decompose constituent in a blind source. The NMF is briefly described as: $\boldsymbol{X = AS + N}$, where $\boldsymbol{X}$ is data matrix containing the spectral or other measuremental results of multiplex components (fluorophore); $\boldsymbol{S}$ denotes constituent spectra. Each row of S is a constituent spectrum under specific condition. $\boldsymbol{A}$ is a weight matrix. Each row of $\boldsymbol{A}$ denotes the fractional abundance/concentration of a chemical constituent material; $\boldsymbol{N}$ is noise or residual error matrix between $\boldsymbol{X}$ and model $\boldsymbol{AS}$. NMF constrain $\boldsymbol{A} \geq 0$ and $\boldsymbol{S} \geq 0$ to obtain a physically and/or biologically meaning for the calculated results. $\boldsymbol{A}$ and $\boldsymbol{S}$ can be found by using a maximum likelihood approach such as minimizing the square of Euclidean distance.

Experimental Results and Discussion

To investigate which biochemical components mainly contribute to the cell fluorescence, the absorption and emission spectra of tryptophan, collagen, elastin, NADH, flavin, and other key fluorophores (or chromophores) (such as NAD+, NAD, and tyrosine) were measured individually with the excitation at 300 nm and 340 nm, and the results has been reported in our previous work (15). The absorption and emission peaks of tryptophan, NADH, flavin and other fluorophores of interest are concluded in Table 1.

The emission intensities of tryptophan obtained with 300 nm excitation are much stronger than that of other fluorophores such as NADH, flavin and collagen because the absorption peaks of other fluorophores are far from 300 nm. The emission intensities obtained with 340 nm excitation for other fluorophores such as tryptophan are much weaker than NADH because the absorption peak of NADH is at ~340 nm, and the absorption peak of tryptophan locates at ~280 nm, far from 340 nm (15).

Fourteen pairs of the RA-treated and untreated breast cancer cell samples were investigated using fluorescence spectroscopy with excitation wavelength of 300 nm. The average fluorescence spectral profiles with standard deviation error bars at key wavelengths for RA-untreated and treated cancerous breast cells are shown in Figure 1 (A-B), respectively. Each spectral profile was normalized to unit value of 1 (*i.e.,* the sum of squares of the elements in each emission spectra data was set as 1) before taken averaging and calculation.

Table I
The absorption and emission peaks of the fluorophores of interest.

Molecules	Tyrosine	Tryptophan	Collagen	Elastin	NADH	Flavin
Absorption (nm)	275	287	339	351	340	375
Emission (nm)	303	342	380	410	460	525

The main emission peaks of both untreated and treated cancerous breast cells were found at 337 nm, which is mainly contributed by the emission of tryptophan. The major difference of the fluorescence profiles between the untreated and treated cells is that there exists a much lower local peak at ~460 nm for untreated cells while no such a peak for treated cells. The 460 nm peak is mainly contributed from NADH (15). A tiny local peak at 529 nm may be observed in the emission of untreated cells, which is known as the characteristic peak of flavin. This can be more clearly seen by enlarging the fluorescence profile at the spectral range of 700 nm to 900 nm as shown in insert of Figure 1 (A). Compared with the fluorescence spectra of tissue (15), no other shoulder peaks at ~380 nm and 410 nm were observed, which are contributed from collagen and elastin, respectively. This can be understood because collagen (4) and elastin (16) are construction components of extracellular matrix fiber, and mainly exists in tissues (4, 16). Therefore, it is reasonable to choose tryptophan, NADH and flavin as three leading principal components (PCs) when NMF is applied to analyze the fluorescence profiles of breast cells excited at 300 nm.

The extracted spectra of PCs (fluorophores) of the 1st component-tryptophan, the 2nd - NADH and the 3rd - flavin for the treated and untreated cells are shown as dash, dot and dash-dot lines in Figure 2 (A), respectively. The measured spectra of individual tryptophan, NADH and flavin in solution are plotted as thicker, thick and thin solid lines, respectively, as references. The two groups of spectra (the extracted and the measured) for each fluorophore show reasonable agreement, which demonstrates that NMF model accounts for the major spectroscopic feature observed, and describes that the measurements are reasonable (7). The difference between the extracted and measured spectra of each component was mainly arisen from different measuremental environment: such as viscosity, PH value of solvent medium, concentration of the fluorophore (17) and distortions caused by cell scattering and absorption (7, 8).

To investigate the relative content changes of PCs, the contents of tryptophan, NADH and flavin in two types of cells were extracted from the measured total fluorescence spectra using NMF anaysis. Figure 2 (B) shows the fractional content of the 1st PC - tryptophan vs. the 2nd PC - NADH; 2(c) displays the 3rd PC - flavin vs. the 1st PC - tryptophan and 2(d) exhibits the 3rd PC - flavin vs. the 2nd PC - NADH of the untreated (solid circles) and treated (solid squares) cancerous breast cells. The most salient feature of Figure 2(B) is that all data points for the untreated cells are located on the upper side over the data points for the treated cells, indicating that the relative contribution of tryptophan is higher in the untreated cancerous cells in comparison with the treated. Figure 2 (C) shows that all data points for the untreated cells are located on the right side compared with the data points

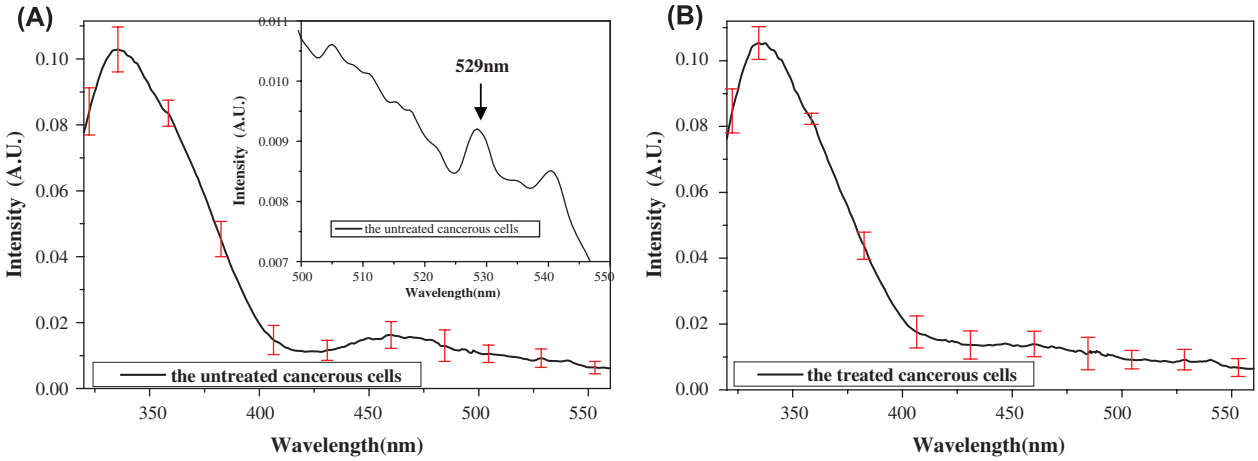

Figure 1: Average fluorescence spectra of (**A**) untreated and (**B**) Retinoic Acid (RA)-treated cancerous breast cells obtained with the excitation of 300 nm.

for the treated cells, indicating again that the relative fluorescence contribution of tryptophan is higher in the untreated cancerous cells in comparison with the treated. Figure 2 (D) shows that most data points for the untreated cells are located in the up-right side in comparison with the data points for the treated cells, indicating that the relative contents of NADH and flavin are higher in the untreated cancerous cells in comparison with the treated. Solid lines were added in Figure 2 (B-D) to guide eyes for separating the data points of the untreated and treated cells.

Spectral analysis using NMF on breast cancer cell lines show distinct differences between RA-treated and untreated samples. The following biomedical studies may help to understand these changes. Brown *et al* studied chemotherapy effects for a variety of cancer patient types including two breast, three lung, and one each of colon, rectal, pancreatic cancer *etc* (18). After chemotherapy, the decreased plasma amounts of tryptophan in plasma were observed (18). Tryptophan is an amino acid required by all forms of life for protein synthesis and other important metabolic functions (18). It was also reported that increased tryptophan levels are somehow related to increased tumor cell proliferation (19). NADH is one of the most important coenzyme catalyzing more than a thousand of metabolic reactions in the human body, the most important of which is the production of Adenosine-Tri-Phosphate (ATP) (20). When cells are incubated with NADH, a 30% increase in ATP production is observed (20). Given the fact that NADH is used primarily for generation of ATP in cell, our results of decrease of NADH may reflect a reduction in the production of ATP in RA-treated cancerous cells. The big ratios of tryptophan vs. NADH and flavin shown in Figure 2 (B and C) can be understood because the absorption peak of tryptophan is close to 300 nm, but those of NADH and flavin are far from 300 nm. The contribution from emission of tryptophan to the total fluorescence spectroscopy is much higher than those of NADH and flavin. Since tryptophan is related to tumor cell proliferation (19) and NADH is important for production of ATP, the decrease of tryptophan and NADH somehow reflects the less activity of cells. Therefore, it can be hypothesized that the RA-treatment results in cancerous cell death or reduction of the activity of cancer cells. The reduced contents of tryptophan and NADH in the RA-treated breast cancerous cells indicate that retinoic acid may be used as an effective chemotherapeutical agent for breast cancer treatment.

In the NMF analysis, the residual fluorescence spectra are generated by subtracting the contributions of the three leading PCs from the corresponding experimental fluorescence spectra. The residual profiles of all measurements for both untreated and treated cancerous breast cells lost the patterns and show random properties. This indicates that the three leading PCs: tryptophan, NADH and flavin were accounted most variance. It does not change much in the variance if using one more extra component (the fourth PC) than using the three PCs.

Emission spectra of nine pairs of the RA-treated and untreated breast cancer cell samples were measured with the excitation of 340 nm. The average fluorescence spectral profiles of the untreated and treated cells with standard deviation error bars at key wavelengths are displayed in Figure 3 (A-B), respectively. The fluorescence data obtained with the excitation of 340 nm were acquired, processed and normalized using the same way as those excited by 300 nm.

Figure 3 shows that the emission profiles of the untreated and treated cells were mainly contributed from NADH corresponding to a peak at ~459 nm, and flavin corresponding to a peak of ~530 nm. The major difference of the profiles between the untreated and treated cells is that the shoulder peak at ~530 nm for the treated cells is a little stronger than that for the untreated cells. Since our measurements show

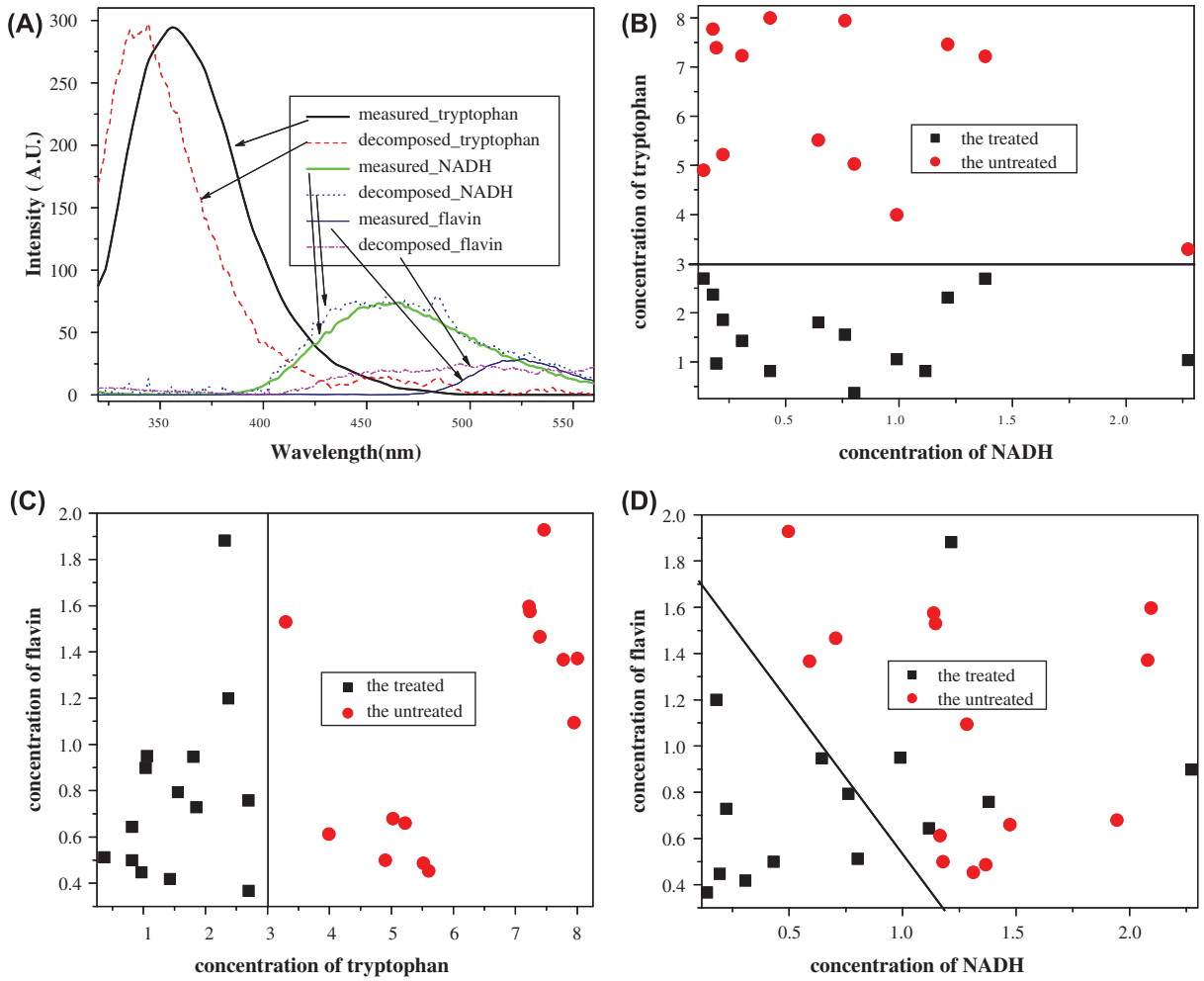

Figure 2: (**A**) Comparison of the extracted spectra of the three principal components (PCs) of the 1st PC - tryptophan (dash line), 2nd PC - NADH (dot line) and 3rd PC - flavin (dash-dot line) for the untreated and treated breast cancerous cells, and the measured spectra of individual tryptophan (thicker line), NADH (thick line) and flavin (thin line) in solution; (**B**) Fractional content of the 1st PC - tryptophan vs. that of the 2nd PC - NADH; (**C**) Fractional content of the 3rd PC - flavin vs. that of the 1st PC - tryptophan; (**D**) Fractional content of the 3rd PC - flavin vs. that of the 2nd PC - NADH by analyzing emission spectra excited at 300 nm using NMF method. The data for the untreated and treated cells are displayed with the solid circles and solid squares in 2 (**B**) – 2 (**D**), respectively.

that the emission intensity of tryptophan excited by 340 nm is very low, only two PCs (NADH and flavin) were used for the NMF analysis of the fluorescence of the untreated and treated breast cells obtained with an excitation of 340 nm.

Figure 4 (A) displays the extracted spectra of the 1st PC - NADH (dash line), and the 2nd PC - flavin (dot line). The measured spectra of NADH and flavin were plotted as thick and thin solid lines, respectively, as references. Figure 4(B) shows the relative content of the 2nd PC (flavin) vs. the 1st PC (NADH) of the untreated (solid circle) and treated (solid square) cancerous breast cells. The obvious feature of Figure 4 (B) is that most data points for the untreated cells are located in the right side over the data points for the treated cells indicating that the relative contribution of NADH is higher in the untreated cancerous cells in comparison with the treated, which reflects the decrease of the relative content of NADH in the treated cancerous breast cells in comparison with the untreated cells. The residual fluorescence spectra at the excitation of 340 nm are calculated using the same way as that with excitation of 300 nm. The residual spectra also lost the patterns and show random properties. This indicates that the two PCs: NADH and flavin were accounted most variance. It does not change much in the variance if using one more extra component (the third PC) than using the two PCs.

In the evaluation of chemo-therapeutic effect on a malignant breast cells, the analyzed outcome will be determined by the rationale of the pair of PCs to be either treated or untreated, while the actual results may be different. The sensitivity and specificity are used to determine whether a drug or a treatment has been given. The following definitions are used for analyzing the sensitivity and specificity: the true positive - the given treatment correctly determined as given;

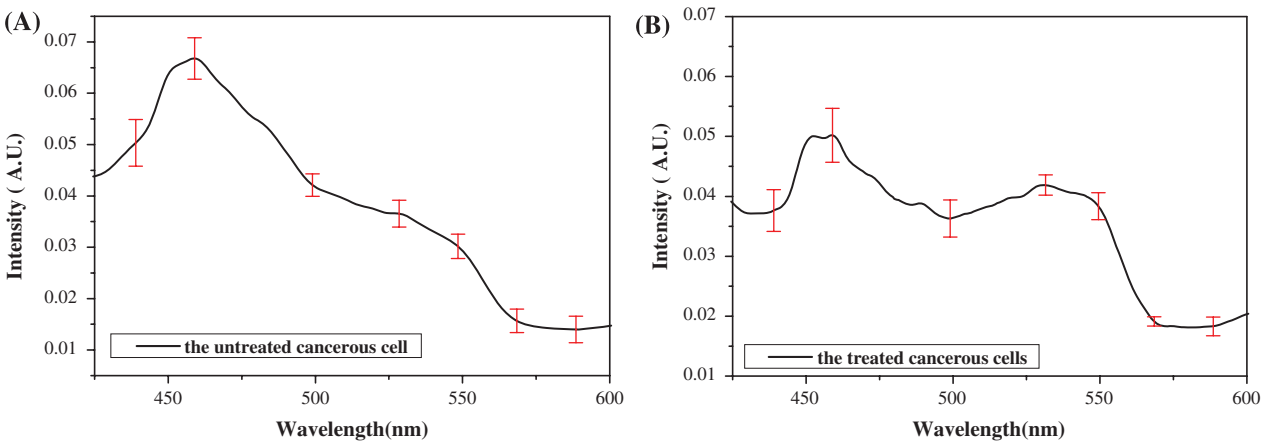

Figure 3: Average fluorescence spectra of the (**A**) untreated and (**B**) RA-treated cancerous breast cells obtained with the excitation wavelength of 340 nm.

false positive - the ungiven treatment incorrectly identified as given; the true negative - the ungiven treatment correctly determined as untreated; false negative - the given treatment incorrectly identified as ungiven. The sensitivity and specificity in our fluorescence spectral analysis for evaluation of RA-treatment can be calculated as

$$sensitivity = \frac{\#\ of\ True\ Positives}{\#\ of\ True\ Positives + \#\ of\ False\ Negatives}$$

$$specificity = \frac{\#\ of\ True\ Negatives}{\#\ of\ True\ Negativess + \#\ of\ False\ Positives}$$

To evaluate NMF analysis of native fluorescence spectroscopy as a criterion for evaluation of chemo-therapeutic effect on malignant breast cells, the sensitivity and specificity for our case were calculated and are summarized in Table II.

Conclusions

This study reports on detecting the chemotherapeutic effects on malignant breast cells by measuring the nature fluorescence spectra of cells and extracting the fluorescence spectral features of principal components using an NMF method. The presence and contents of the detectable fluorophores in breast malignant cells such as tryptophan, NADH and flavin were analyzed as the Principal Components for the NMF. We demonstrated that fluorescence intensities of tryptophan, NADH and flavin decreased in the RA-treated cells in comparison with those in the untreated cells. This work shows the change of relative contents of tryptophan, NADH and flavin studied using native fluorescence spectroscopy with NMF analysis may present potential criteria for evaluation of chemotherapeutic effects on malignant breast cells.

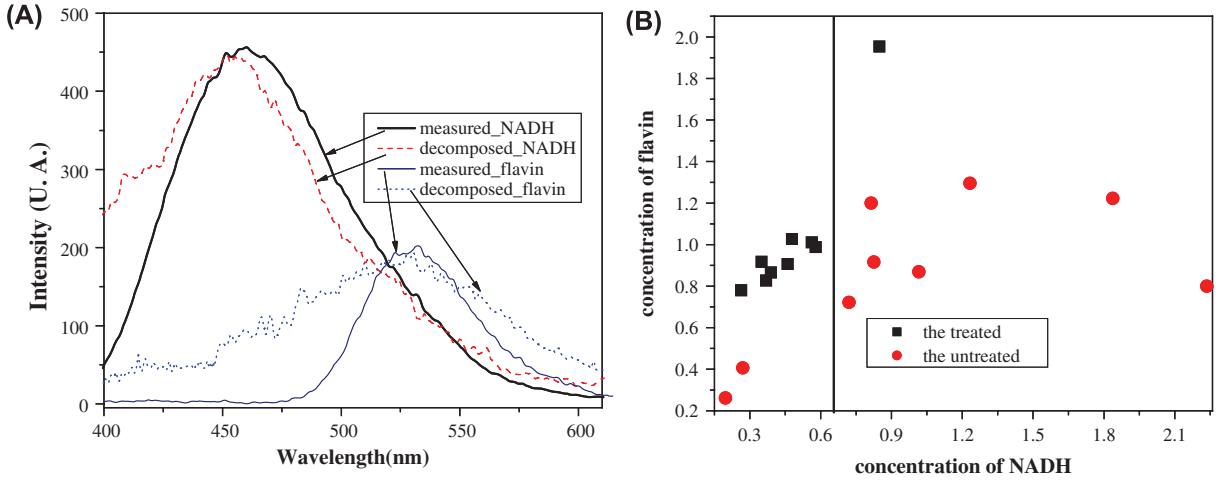

Figure 4: (**A**) The comparison of extracted spectra of two principal components of 1st PC - NADH (dash line) and 2nd PC - flavin (dot line), and the measured spectra of individual NADH (thick line) and flavin (thin line) in solution; (**B**) content of the 1st PC - NADH vs. that of the 2nd PC - flavin, obtained from the measured fluorescence spectroscopy of the breast cells with 340 nm excitation using the NMF analysis.

Table II
The native fluorescence spectroscopy and NMF analysis as a criterion for evaluation of chemo-therapeutic effect on malignant breast cells.

Excitation (λ)	Evaluated Components	Sensitivity	Specificity
300 nm	tryptophan vs. NADH	100%	100%
300 nm	tryptophan vs. flavin	100%	100%
300 nm	flavin vs. NADH	64.3%	100%
340 nm	flavin vs. NADH	88.9%	77.8%

Future Work

Retinoids, a group of metabolites and synthetic analogs of vitamin A, have been shown to have chemopreventive effect to modulate the growth and differentiation of preneoplastic and malignant cells in both laboratory and clinical studies (6, 21). Although the exact mechanism for explaining how retinoids affect breast cancer cells is unknown, it is hypothesized that they inhibit the promotion stage of carcinogenesis and suppress the progression of preneoplasms to carcinomas (20). Our primary study shows that retinoids can be used as a chemopreventive agent in breast cancer treatment. Further investigation of retinoid acid as a chemotherapeutic agent is needed to study the chemopreventive effects of RA as a function of RA concentration and treating time in breast cancer cells and animal models.

This study also suggests that native fluorescence spectroscopy may hold a promise as an alterative tool for diagnosing breast cell carcinomas since the observation of changes of fluorophore contents in the RA-untreated and treated cancerous cells extracted from fluorescence spectra using NMF analysis is in good agreement with changes observed in cancerous and normal cells and tissues using other approaches (3, 4, 7, 8, 15). Once the fluorescence fingerprints of normal and benign breast tissues were recognized, alterations in these patterns progressing to the malignant state can be reflected by the fluorescence spectra and highlighted by the NMF analysis. It is believed that the biochemical or morphologic changes that cause the spectra changes would appear earlier than the histological aberration. Animal studies combining the measured nature fluorescence spectra and the extracted fluorescence spectral features of PCs need to be achieved for the purpose of diagnosing dysplasia and malignancy *in vivo*. Furthermore, laser-induced fluorescence spectroscopy holds a promise as a useful intra-operative tool for determining adequate surgical margins of resection.

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