



Terbium Bipyridyl Complex as a Photo Probe for the Determination of Carbohydrate Antigen CA15.3 in Different Breast Cancer Patient Samples

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Abstract

A novel, simple, sensitive and selective spectrofluorimetric method has been developed for the assessment of Carbohydrate Antigen (CA 15.3) in serum samples of breast cancer patients by using photo probe Tb-Bipyridyl (Tb-Bipy) complex. The method Based on the quenching of the luminescence intensity of Tb-Bipy complex by different concentrations of CA 15-3 in acetonitrile at pH 8.0 and $\lambda_{ex} = 340$ nm. The quenching of luminescence intensity Tb-Bipy complex especially, the electrical band at $\lambda_{em} = 545$ nm is typically used for determining the Carbohydrate Antigen (CA 15.3) in **different serum samples**. **The dynamic range is 0.1-15.4 U mL⁻¹, and the limit of detection (LOD) and quantitation limit of detection (LOQ) are (0.109 and 0.57) U mL⁻¹**, respectively. This method was practical, simple and relatively free from interference effect. It was successfully applied to measure Carbohydrate Antigen (CA 15.3) in samples of human serum and from this method we can assess some biomarkers of cancer related diseases in human body.

Keywords: Tb-Bipy, Carbohydrate Antigen CA 15-3, Photo Probe, Quenching, Luminescence.

Introduction :

Breast cancer is the most common life-threatening malignancy among women and the second leading cause of death from cancer in women today [1]. The earlier this cancer is discovered, the earlier the likelihood of treatment and thus the lower the risk of death. Numerous serum tumor markers, including members of the mucin glycoprotein family MUC1, have been identified for breast cancer Carcino Embryonic Antigen (CEA), on coproteins (e.g. HER-2/c-erbB-2) and cytokines (e.g., CA 15-3, BR 27.29 and MCA, CA 549) (e.g., tissue polypeptide antigen and tissue polypeptide-specific antigen). It is widely accepted that tumor markers are not a method for primary diagnosis in breast cancer patients because of their poor sensitivity and specificity [2]. In the present situation, the diagnosis of breast cancer is by Mammogram, MRI and its examination for spectroscopy and breast biopsy. All these methods of detection are very expensive, requiring surgical procedures, time-consuming and often unfavorable outcomes. Therefore, a cost-effective technique needs to be developed which can detect breast cancer qualitatively and/or quantitatively at an early stage.

Therefore, it is important to develop a highly sensitive sensor system for breast cancer monitoring so that the patient can remain alert about the risk of breast cancer and the extent of reoccurrence [3]. For Carcinoma Antigen 15-3, CA 15-3 is a tumor marker for many cancer types, most notably breast cancer [4]. It is derived from MUC1[5]. The most frequently used serum marker in breast cancer patients is CA 15-3, which detects soluble forms of the MUC-1 protein. Its primary application is in patients with metastatic disease for tracking therapy. CA 15-3 should not be used alone but assessed in combination with medical imaging, clinical history and physical examination in tracking therapy in this setting. For postoperative monitoring of asymptomatic women who have undergone surgery for invasive breast cancer, CA 15-3 can also be used [6]. A range of techniques, such as chemiluminescence immunoassays [7], enzyme immunoassays [8], radioimmunoassay (RIA) [9], Electrochemical Immunoassay [10], electrochemiluminescence immunosensor [11], and enzyme-linked immunosorbent assays, have been developed for the determination of CA15-3 [12]. The above strategies, however, suffer from the drawbacks

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of time-consuming separations, complex label collection, and difficulty meeting the growing clinical requirements for the rapid determination of CA15-3. In addition, using these techniques, ultra-low biogenic concentrations of tumor markers cannot be desirably detected. Therefore, new techniques need to be created [10]. In this paper the photo probe Tb-Bipy is used to determine CA 15-3 in different serum samples of the breast cancer patients.

2. Experimental:

2.1. Materials:

Carbohydrate antigen 15-3 (CA15-3), Ethanol, Acetonitrile, DMSO and DMF were purchased from Sigma Aldrich.

2.2. Reagents and solutions:

Solution (10^{-2} mol L⁻¹) was prepared by dissolving 0.0869 g of Tb(NO₃)₃ (delivered from Aldrich- 99.99%) in small amount of ethanol in 25 mL measuring flask, then dilute to the mark with ethanol. In another 25 mL measuring flask (10^{-2} mol L⁻¹) was prepared by dissolving 0.0394 g of Bipy (delivered from Aldrich- 99.99%) in small amount of ethanol, then dilute to the mark with ethanol. The working solution of Tb-Bipy of 1.0×10^{-4} mol L⁻¹ was obtained by appropriate dilution with acetonitrile.

Stock solution of CA15-3 were prepared (0.5 gm of CA15-3 (delivered from Aldrich- 99.99%) dissolved in 2ml distilled Water) then prepare more concentration by dilution with distilled water. Stock and working solutions are stored at 2-8°C when are not in use.

2.3. Apparatus :

The absorption spectra were recorded with a PerkinElmer Lambda 25 UV-Visible double beam spectrophotometer equipped with a tungsten halogen lamp for visible range operation and a deuterium lamp for UV range operation. With a Meslo-PN (222-263000) Thermo Science Lumina fluorescence Spectrometer in the range (190-900 nm), all luminescence measurements were registered. Using the pHs-JAN-WAY 3330 study pH meter, the pH was calculated.

2.4. General procedure:

2.4.1. Preparation of lanthanide complex Tb-Bipy solution:

To 10 mL measuring flask, solutions were added in the following order: 0.1 mL of 1.0×10^{-2} mol L⁻¹ Tb(NO₃)₃ solution and 0.3 mL of 1.0×10^{-2} mol L⁻¹ Bipy **Figure (1)** to give 1.0×10^{-4} mol L⁻¹ of Tb(NO₃)₃ and 3.0×10^{-4} mol L⁻¹ of Bipy, then the mixture was diluted to the mark with acetonitrile. The above procedure was used for the subsequent measurements of absorption, emission spectra and

effect of pH and solvents. The luminescence intensity was measured at $\lambda_{ex}/\lambda_{em}=395/545$ nm.

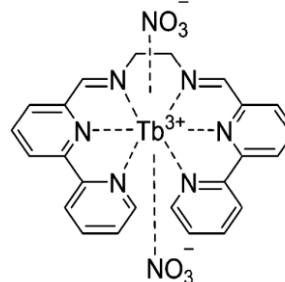


Fig.1. Structure of Tb-Bipy

2.4.2. Calibration curve:

After the preparation of the different standard solutions [0.05-75 U/mL] of CA15-3 as described above, the optical sensor Tb-Bipy was mixed with each standard solution of CA15-3 in the cell of the spectrofluorimetric device, then the luminescence spectrum was measured at the selected excitation wavelength $\lambda_{ex} = 340$ nm.

2.4.3. Determination of CA15-3 in serum samples:

A 1.0 mL of sample of blood collected from healthy volunteer was centrifuged for 15 min at 4000 r/min to remove proteins. The concentration of the CA15-3 was determined by using different concentrations from the corresponding calibration graph.

3. Result and discussion:

3.1. Absorption and emission spectra :

The absorption spectra of Tb-Bipy complex is shown in Figure (2). the absorption spectrum of bipy (spectrum 1) shows a peak at 292 nm due to $\pi-\pi^*$ transition in Bipy rings, after complexation with Tb³⁺ the red shift was obtained by 2 nm (spectrum 2).

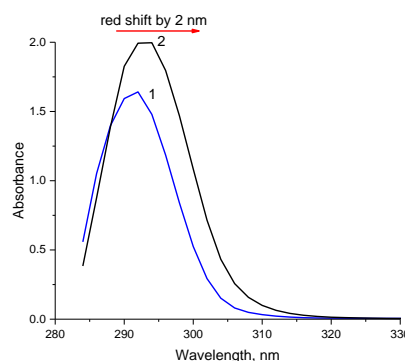


Fig. 2. the UV-vis absorption spectra of 1) 1×10^{-4} mol/L Bipy and 2) (1×10^{-4} mol/L Tb- 3×10^{-4} mol/L Bipy) in acetonitrile.

The emission spectra of Tb-Bipy complex in different concentrations of CA15-3 are shown in **Figure (3)**. After the addition of different concentrations of CA15-

3 (0.05 -75 U/mL) into the Tb-Bipy complex, the intensity of the characteristic peaks ($^5D_4 \rightarrow ^7F_3= 480$ nm, $^5D_4 \rightarrow ^7F_2=545$ nm, $^5D_4 \rightarrow ^7F_1= 620$ nm, $^5D_4 \rightarrow ^7F_0= 680$ nm), especially the peak at $\lambda_{em} = 545$ nm of Tb^{3+} was quenched due to energy transfer from the photo probe to CA15-3 [13-20].

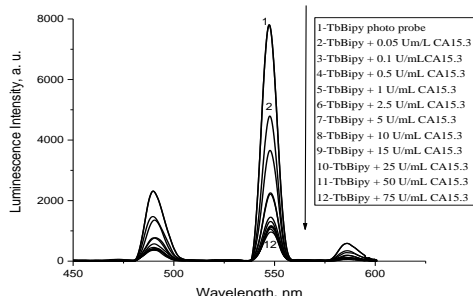


Fig. 3. Luminescence emission spectra of Tb-Bipy complex in the presence of different concentrations of CA15-3 in acetonitrile at $\lambda_{ex}=340$ nm and pH 8.

3.2. Effect of experimental variables:

3.2.1. Effect of solvent:

The influence of the solvents on the luminescence intensities of the solutions containing 1.0×10^{-4} mol L^{-1} of Tb- 3.0×10^{-4} mol L^{-1} Bipy was studied under the conditions established above. The results show Tb-Bipy has a high luminescence intensity in the acetonitrile, **Figure (4)**. The luminescence intensity of the Tb-Bipy is very weak in water, ethanol, DMF and DMSO solvents. This is because the acetonitrile has a moderate polarity between the water and ethanol (protic solvents) and dimethylformamide and dimethyl sulfoxide (aprotic solvents) [21-30].

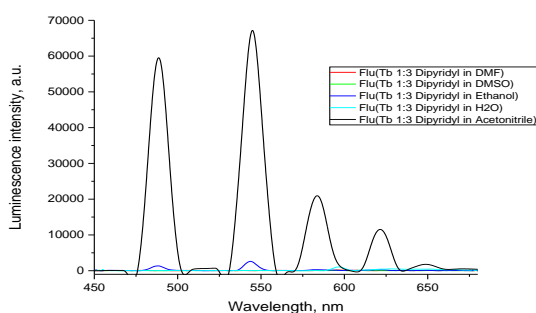


Fig. 4. Luminescence emission spectra of the photo probe Tb-Bipy in different solvents.

3.2.3. Effect of pH:

The pH of the medium has a great effect on the luminescence intensity of Tb-Bipy complex. The pH has been adjusted using 0.1 mol L^{-1} of NH_4OH and HCl solutions. The optimum pH value where the peak at $\lambda_{em} = 545$ nm has the highest intensity was obtained at pH = 8, **Figure (5)** [31-34].

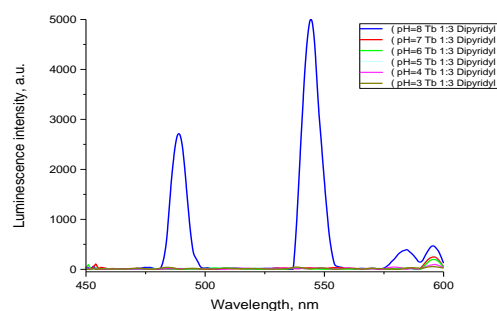


Fig. 5. Luminescence emission spectra of Tb-Bipy photo probe in acetonitrile at $\lambda_{ex}=340$ nm and different pH.

4. Analytical performance:

Method validation :

4.1. Analytical parameters of photo probe method:

A linear correlation was found between luminescence emission intensity of photo probe at $\lambda_{em} = 545$ nm and concentration of CA15-3 in the range given in $0.1 - 15.4$ $U mL^{-1}$ **Figure (6)**.

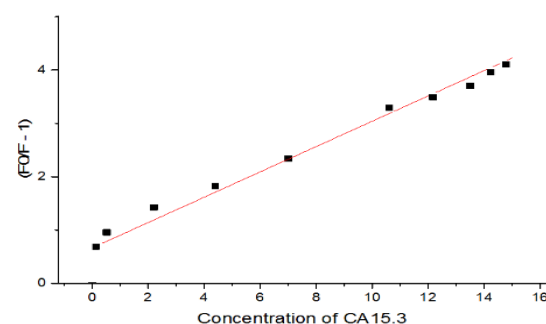


Fig. 6. Stern volmer plot $(F_0/F)-1$ Vs CA 15-3 concentration

Figure (6), represent the calibration curve, which was obtained by applying the **Stern-Völmer** plot at $\lambda_{em} = 545$ nm:

$$\left[\left(\frac{F_0}{F} \right) - 1 \right] = K_{sv} [Q]$$

Where, F_0 and F are the luminescence intensities of photo probe in absence and in presence of the quencher (CA15-3, respectively, Q is the concentration of the CA15-3 and K_{sv} is called Stern-Völmer constant. From the plot of $[F_0/F]-1$ against $[CA15-3]$, K_{sv} and the critical concentration of CA15-3 values are 0.1 U/mL and 15.4 U/mL respectively, and the distance between CA15-3 and the ionophore, R_0 is 3.36 \AA . This means that the quenching mechanism is electron transfer mechanism ($R_0 < 10 \text{ \AA}$). The quenching process is taken through an electron transfer from ionophore to CA15-3 and this very comparable with $R_0 = 3.36 \text{ \AA}$ value obtained from stern-Völmer equation [35] The regression analysis of luminescence intensity data using the Stern-Völmer equation was made to

evaluate the slope (b), intercept (a) and correlation coefficient (r^2) and the values were given in **Table (1)**. The limit of detection (LOD) and quantitation (LOQ) calculated according to ICH guidelines using the formula: $LOD = 3.3S/b$ and $LOQ = 10S/b$ U/mL (where S is the standard deviation of blank luminescence intensity value, and b is the slope of the calibration plot) are also given in **Table (1)**.

Table (1) Sensitivity and regression parameters for photo probe:

Parameter	Value
λ em (nm)	545
Linear range, (U mL ⁻¹)	0.1 – 15.4
Limit of detection (LOD), (U mL ⁻¹)	0.109
Limit of quantification (LOQ), (U mL ⁻¹)	0.57
Regression equation, Y*	Y=a+bX
Intercept (a)	0.45
Slope (b)	0.49
Standard deviation	0.04
Variance (Sa ²)x 10 ⁻⁵	1.2
Regression coefficient (r)	0.99

*Where Y= fluorescence intensity, X= concentration in n UmL⁻¹, a= intercept, b= slope.

4.2. Selectivity:

The method selectivity and validity was investigated by studying the effect of possible interfering substances on the luminescence spectra of Tb-bipy photo probe after addition of CA 15-3 (31 U/ mL). The interfering substances included (2.0 x 10⁻³ mol L⁻¹) for both potassium and sodium chloride, (0.06 g L⁻¹) for both urea and triglycerides, (0.08 g L⁻¹) for glucose and uric acid, (0.01 g L⁻¹) total protein and (0.7 g L⁻¹) albumin. The influence of CEA, CA 15-3 and CA 19-9 was also studied in concentration equivalent to (130 U mL⁻¹) each. All of the results obtained implied insignificant influence on the photo probe luminescence intensity.

4.3. Application to formulations:

The proposed methods were applied to the determination of CA15-3 in serum samples of the health state human. The results in **Table (2)** show that the method is successful for the determination CA15-3 in serum samples.

The average recovery and R.S.D for the serum samples in our method found to be (0.42-45.09) and R.S.D (0.011-1.167) % were also presented for comparison and show a good correlation with those obtained by the proposed methods. The results obtained by the proposed methods agreed well with those of reference method [36] and with the label claimed.

4.4. Recovery, Accuracy and precision study:

Recovery tests were conducted by applying the standard-addition technique to further determine the accuracy of the methods. The recovery was tested by evaluating the agreement between the normal measured concentration and the known concentration applied to the sample. In order to measure the precision and accuracy, the experiments were repeated three times a day to determine the repeatability (intra-day accuracy) and three times a day to determine the intermediate accuracy (inter-day accuracy) of the process. For three levels of the analyte, these assays were performed. The results of this study are summarized in **Table (2)**. In all cases, with relative standard deviation in the range (0.011-1.256) percent for serum samples, the recovery percentage values ranged from (0.39-45.4). The reasonably good precision of the system was shown by the closeness of the findings to 100 percent.

5. Conclusion:

Tb-Bipy complex has high sensitive and characteristic peaks in the presence of CA15-3. The luminescence intensities of these peaks are quenched by increasing the concentration of CA15-3, due to energy transfer from Tb-Bipy complex. This method can be used for determination of CA15-3 in serum with high accuracy.

Table (2). Evaluation of Intra-day and Inter-day Accuracy and Precision:

Sample	Standard method Average U/mL [36]	Propose Method									
		Intra-day accuracy and precision (n=3)					Inter-day accuracy and precision (n=3)				
		Average found			% RE	%RSD	Average found			% RE	% RSD
		U/mL	±	CL			U/mL	±	CL		
patient (1)	32.1	32.0	±	0.004	0.44	0.015	31.8	±	0.004	0.78	0.02
patient (2)	9.91	9.72	±	0.007	1.92	0.050	9.81	±	0.007	1.01	0.05
patient (3)	20.3	20.3	±	0.005	0.34	0.024	20.3	±	0.005	0.15	0.02
patient (3)	10.5	10.7	±	0.006	1.42	0.046	10.6	±	0.007	1.14	0.05
patient (5)	0.95	1.00	±	0.021	5.26	0.490	1.02	±	0.020	7.37	0.48
patient (6)	45.9	45.0	±	0.003	1.83	0.011	45.4	±	0.001	1.15	0.01
patient (7)	1.54	1.75	±	0.016	13.6	0.280	1.69	±	0.016	9.74	0.29
patient (8)	0.40	0.42	±	0.032	5.00	1.167	0.39	±	0.033	2.50	1.26
patient (9)	4.97	5.20	±	0.009	4.63	0.094	4.91	±	0.009	1.21	0.10
patient (10)	30.2	30.1	±	0.004	0.46	0.016	30.0	±	0.003	0.79	0.02

RE. Percent relative error, %RSD.relative standard deviation and CL. Confidence limits were calculated from: $CL = \pm tS/\sqrt{n}$. (The tabulated value of t is 4.303, at the 95% confidence level; S = standard deviation and n = number of measurements). Theoretical values of t- and f-tests at 95% confidence limits are 4.303 and 19.0, respectively

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