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Fluorimetric Detection of Insulin in the Presence of Eu(III) - {Pyridine - 2,6 - Dicarboxylate} Tris Complex

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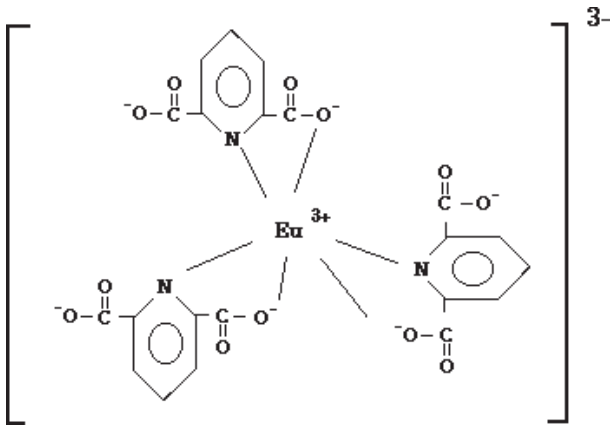
Bovine insulin solutions (pH=9.3) have maximum absorption at 278 ± 2 nm and an intrinsic emission at 305 nm when excited at 282 nm. The relative fluorescence intensities show linear dependence on its concentration: 10 μg/mL< [Ins] < 200 μg/mL. When $Eu(PDA)_3^{3-}$ tris complex is added to these solutions, it has a hyperchromic effect at 278 nm absorption band of insulin, sensitizing the emission intensities of central Eu^{3+} metal ion of the complex at 590 and 615 nm, and simultaneously quenching the emission intensity of hormone at 305 nm. Stern-Volmer plots show that a mechanism of bimolecular quenching at 305 nm and sensitization at 615 nm are valid up to a mole ratio, R = $[Eu(PDA)_3^{3-}]/[Ins]$ < 2.0. An intramolecular rather than an intermolecular energy transfer is proposed. An apparent binding constant, log K_{app}=4.70 ± 0.13, is calculated for Ins-$[Eu(PDA)_3^{3-}]_2$ type product, the presence of which may offer a new luminescence technique as a diagnostic tool and an alternative to radio-iodinated (^{131}I -) insulin.

A simple, rapid and accurate quantitation of insulin is proposed by using a fixed concentration of $Eu(PDA)_3^{3-}$, and measuring its initial F_0 at λ_{exc}/ λ_{em}=282/615 nm and the difference, ΔF after sensitization when ∼ 100 microliters of insulin sample is added. The coefficient of variation (CV), the relative error and minimum detectable amount of bovine insulin hormone are found to be 3.0%, 1.2% and 7.3 ± 0.2 μg /mL respectively.

Key Words: Insulin, Fluorescence Spectroscopy, Eu(III) - { Pyridine - 2,6 - Dicarboxylate } Tris Complex Stern-Volmer Plots, Apparent Binding Constant

Introduction

Europium aquo-ions form fluorescent chelates with many ligands such as EDTA isothiocyanotophenyl [EDTA][1], 4,7-bis (chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid [BCPDA][2], 2-naphthoyl-trifloroacetone [NTA][3] and stable mono, bis and tris complexes with pyridine-2,6- dicarboxylic acid [PDA][4].



The stability constants of $Eu(PDA)_3^{3-}$ complex, expressed as log β_1, β_2 and β_3 values are 8.83, 15.98 and 21.03 respectively[5]. The acidic dissociation constants of the pyridine-2,6-dicarboxylic acid being pK_{a1}=2.22, pK_{a2}=5.29, all ligands are in anionic dicarboxylate form (dipicolinate anion, PDA^{2-}) before complexation in neutral solutions. The importance of this europium complex is its enhanced fluorescence at 615±5 nm, the excitation radiation absorbed by the ligand near UV being transfered to the central Eu^{3+} ion by an internal energy transfer process, leading to $^5D_0 \gg {}^7F_2$ emission. This characteristic metal ion emission has a narrow half-bandwidth of ∼10 nm and is also very sensitive to the detailed nature of the ligand environment, reflecting the *hypersensitivity* of the transition[6].

In the colored proteins, light absorption takes place in the prosthetic groups, for instance, in the heme residues of hemoglobin or in the flavin moiety of the flavoproteins. In the colorless proteins and apoproteins light is absorbed predominantly by the tryptophan (Trp) and tyrosine (Tyr) residues (at 275-285 nm) and weakly by the phenylalanine (Phe) residues (transparent at 280 nm, absorb at 260 nm). Since almost all proteins contain these amino acids, absorbance at 280 nm is routinely used to estimate the concentration of protein in solutions[7]. Trp, Tyr and Phe residues reemit part of their absorbed energy as luminescence (called the protein's intrinsic fluorescence and blue phosphorescence) or transmit part of their energy to neighboring residues even if they are separated by a distance of about 20 $\AA$, for instance, to the CO-heme complex for the cleavage of CO-myglobin (or CO-hemoglobin) into free CO residue and free myglobin (or hemoglobin).

The quenching of protein's intrinsic fluorescence in the presence of lanthanide aquo ions is an interesting research topic[8]. For instance, Trp and Tyr residues of bovine serum albumine (BSA) have intrinsic fluorescence at 342 nm. When $Eu(PDA)_3^{3-}$ complex is added to neutral BSA solution, quenching of protein's fluorescence intensity at 342 nm and sensitization of the Eu^{3+} ion emission intensity at 615 nm (called protein's extrinsic fluorescence) is detected[4]. Following both intrinsic and extrinsic fluorescences of such proteins in the absence or presence of $Eu(PDA)_3^{3-}$ complex, performing simple fluorimetric titrations for analyte protein may lead to a new, sensitive method for the estimation of protein concentrations within the final sample solutions. It is the aim of this study to use this new approach for insulin hormone analysis, using Stern-Volmer equations to evaluate its concentration, a recipe that gives accurate results in the final step after performing the required separation and isolation techniques.

Insulin molecule is a polypeptide produced in the beta cells of the islets of Langerhans situated in the pancreas of all vertebrates. It is secreted directly into the bloodstream, where it regulates carbohydrate

metabolism, and influences the synthesis of protein and of RNA, and the formation and storage of neutral lipids. The insulins of various species of mammals differ from each other only by the sequence of amino acids nos. 8, 9 and 10 in the A-chain. The amino acid sequence of the bovine insulin molecule, as shown in Figure 1, has been completely clarified by a series of investigations by Sanger[9]. The A-chain has an *N*-terminal glycine residue and contains 4 residues of cysteic acid, which forms dithio bonds. The B-chain has an *N*-terminal phenylalanine residue and contains 2 residues of cysteic acid, the two chains together containing a total of 7 residues of Phe and Tyr that are fluorescent. The 8, 9, 10 sequence in cattle insulin is Ala.Ser.Val; in the sheep Ala.Gly.Val; in the horse Thr.Gly.Ileu; and in human, pig and sperm whale insulin, Thr.Ser. Ileu[10].

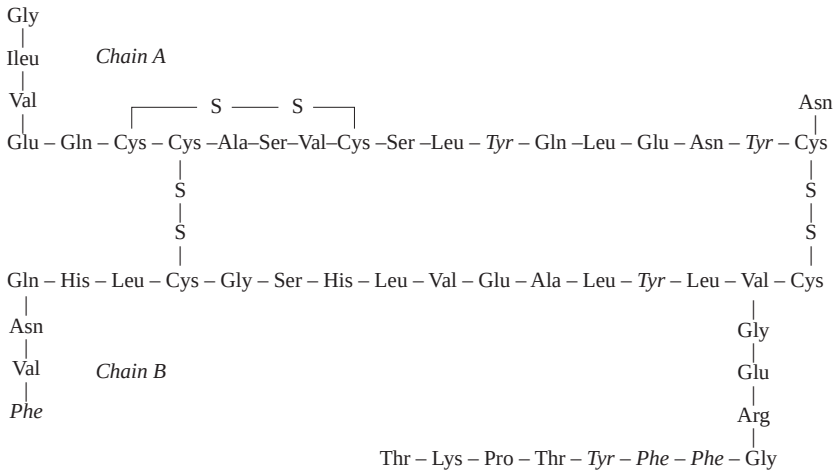


Figure 1. Bovine Insulin (the abbrevations used for amino acyl residues are: Ala = alanine, Arg = arginine, Asp = aspartic acid, Asn = asparagine, Cys = cysteine, Glu = glutamic acid, Gln = glutamine, Gly = glycine, His = histidine, Ileu = isoleucine, Met = methionine, Ser = serine. Phe = phenylalanine, Pro=proline, Thr = threonine, Tyr = tyrosine).

Insulin molecules aggregate and form polymers whose sizes increase with its concentration in aqueous solutions. Sedimentation, osmometric and light scattering techniques have yielded insulin molecular weights up to 47,800 in solutions, but extrapolation to zero concentration gives a minimum molecular weight of 6,000. The unit cell of crystalline insulin, according to X-ray measurements, contains 8 subunits of the molecular weight 6,000 and small amounts of heavy metals such as zinc, nickel, cobalt and cadmium as components of the molecule. Insulin exists as a dimer in mild acid and as a monomer in 30% acetic acid solutions. It is practically insoluble from pH 4.5 to 7.0 but soluble in more acidic or basic solutions, and has an isoelectric point of 5.30 to 5.35.

Deficiency of insulin hormone causes hyperglycemia and glycosuria. Injection of insulin lowers the blood sugar, prevents the loss of glucose through the urine and also prevents dangerous acidosis in diabetic patients. Very little is known about the mechanism of these very dramatic changes in the metabolism of carbohydrates and lipids. Radio-iodinated (^{131}I -) insulin is usually preferred for studying its hormonal activity, and insulin binding factors from insulin resistant sera. If there is an associative binding reaction between the insulin molecule and the $Eu(PDA)_3^{3-}$, then fluorescent labeling of insulin molecule may also be used as an alternative to radio-isotope labeling for following its activity and behavior in many biological systems.

Experimental

***Reagents*:** Pyridine-2,6-dicarboxylic acid (Aldrich Chem.) was used without further purification. The purity was checked potentiometrically. A stock solution of the disodium salt was prepared by dissolving an accurately weighed amount of the acid in a solution containing 99% equivalence of sodium hydroxide. The solution was filtered through Millipore filter paper and stored in a polyethylene bottle.

0.2 mol.dm^{-3} europium perchlorate stock solution was prepared from Eu_2O_3(99.9%, BDH). A weighed amount of the oxide was suspended in ∼20 cm^3 hot water. The mixture was added slowly with stirring to a dilute solution (∼30 cm^3) containing slightly less than the equivalent amount of perchloric acid (BDH) to dissolve ca. 98% of the oxide and kept near boiling point. The mixture was left boiling until the reaction was complete (pH>6). The solution was then filtered to remove unchanged oxide and evaporated to ∼25 cm^3. The solution was acidified with dilute perchloric acid to pH=2.8±0.1, filtered and boiled for 20 min. If the pH showed any increase, more acid was added and the procedure repeated. The final solution, ∼25 cm^3, was filtered again and transfered directly to a volumetric flask. Stock solutions showed no precipitation for a period of more than a year. Europium perchlorate stock solutions were standardized prior to use, by titrating against 0.01 mol.dm^{-3} EDTA solution using xylenol orange as the indicator with the reaction buffered (by acetate) at pH 5.8[4].

$Eu(PDA)_3^{3-}$ complex solutions were prepared by mixing the appropriate volumes of metal perchlorate and pyridine-2,6-dicarboxylate solutions, carefully adjusting pH to 5.8 with dilute NaOH and total ionic strength, and μ to 0.5 mol.dm^{-3} with 2.0 mol.dm^{-3} $NaClO_4$. Final formal concentrations of the metal ion varied from 2.5x10^{-5} to 3.5x10^{-4} mol.dm^{-3} in the series studied.

Bovine insulin (27.3 USP unit/mg, contained 0.4% Zn, Sigma) was used without further purification to prepare 0.4 mg/mL stock solution of the hormone. To prevent dimerization of the hormone, an accurately weighed amount of crystal was dissolved in an appropriate volume of dilute NaOH solution (pH = 9.3).

Measurements: All measurements were made on a Jasco Model FP-550 Spectrofluorometer using 10 mm quartz cell or flow cell. The instrument was connected to an IBM (PS 1) computer using a model PCL-818, high performance data acquisition card (programmable gain, maximum of 10 μs/data, PC-LabCard) and excel macro programs to plot all synchronized spectra or to average all monochromatic readings (∼1000 points) with 3 nm bandwidth on the excitation side, 5 nm on the emission side.

The pH of stock solutions, buffer solutions and solutions investigated spectroscopically were measured on the Micro-Processor pH-meter (Hanna Instr. Model HI-8521) with an accuracy ±0.05 pH unit. UV-visible absorption spectra were recorded by Hitachi Model 100-GD Spectrophotometer.

Results and Discussion

It is known that the Eu^{3+} aquo-ions form $[Eu(PDA)_3]^{3-}$, $[Eu(PDA)_2(H_2O)_3]$ and $[Eu(PDA)(H_2O)_6]^+$ species, showing a total coordination number of nine, with each dipicolinate anion acting as a tridentate and water molecules bonded to the central metal ion[5,6,11]. For the $Eu(PDA)_3^{3-}$ ion essentially all the emission emanates from the nondegenerate $^5D_0$ level of the metal ion when the excitation is at λ_{ex} <578 nm. The fluorescence excitation and emission spectra of Eu^{3+} in the presence of PDA^{2-} are given in Figure 2. Stepwise formation of mono-bis-tris complexes with this ligand lead to significant enhancement

in $^5D_0 \gg ^7F_1$ (∼590nm) and $^5D_0 \gg ^7F_2$ (∼ 615 nm) band intensities. This typical behavior is explained by Eu^{3+} energy acceptor levels being suitable for energy transfer throughout the near ultraviolet and visible regions (absorption bands like $^5F_1 \gg ^5I_6$. $^5H_6$ at 285.4 nm and $^7F_1 \gg ^7I_7$ at 285nm) and the overlap of these excitation bands with the broad absorption band of PDA ligands, which act as energy donors transfering the absorbed energy via a ligands-to-metal ion.

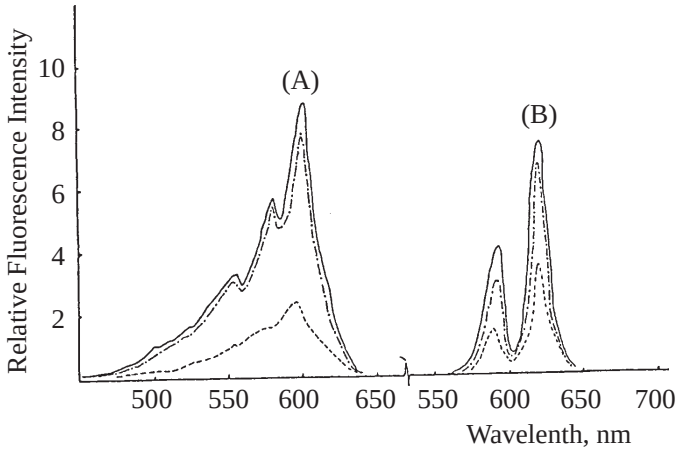


Figure 2. (A) The excitation spectra at constant λ_{em} = 615 nm, **(B)** The emission spectra at constant λ_{ex} = 566.8 nm of Eu^{3+} - PDA complexes. The final metal ion concentration is 5.0x10^{-5} mol.dm^{-3} in its
(i) mono (. . . . .), (ii) bis (-. -. -. - .), and (iii) tris (———) complex solutions with PDA at pH = 5.8 and = 0.5 mol.dm^{-3}.

Insulin solutions have maximum absorption at 278±2 nm with the molar absorptivity of ∼3.6x10^3 $molar^{-1}.cm^{-1}$ and an intrinsic fluorescence at 305 nm when excited at 282 nm (Figure 3-A and 4). Using fixed $\lambda_{exc}/\lambda_{em}$ of 282/305 nm, the computed (average of 1000 values) relative flourescence intensities show a linear dependence on the insulin concentrations (10.0 μg/mL < [In] < 200.0 μg/mL):

$$\mathbf{F}_{\mathrm{In}}, 305 = 111.64 + 1.159[\mathrm{Ins}] \quad r^2 = 0.9729.$$

When equal volumes of 0.4 mg/mL insulin and 3.2x10^{-4} mol.dm^{-3} $Eu(PDA)_3^{3-}$ solutions are mixed, there is a hyperchromic effect (the molar absorptivity increases to ∼2.2x10^4 $molar^{-1}.cm^{-1}$) at 278 nm (Figure 3-B) and sensitization of the emission intensities at 590 and 615 nm, with simultaneous quenching of the emission intensity at 305 nm (Figure 5). In order to clarify the results, microtitrations are performed at fixed $\lambda_{exc}/\lambda_{em}$ in a normal quartz cell (or in a flow cell, by peristaltic pumping of the same solution in a closed system). Each time 2.00 to 5.00 cm^3 of analyte solution is titrated with microliter volumes of titrant solution. The titration curves (average of 5 similar additions, 100 data/addition) are computed as the relative emission intensities against the mole ratio $\mathbf{R} = [Eu(PDA)_3^{3-}]/[Ins]$ at constant wavelengths; 282/305 nm and 282/615 nm as given in Figures 6 and 7 respectively. In Table 1, an excel sheet is given that shows the titration data at 282/305 nm: **the sample** being a bovine insulin solution titrated with $Eu(PDA)_3^{3-}$, **the control** being the same analyte solution that is diluted to same extent and **the difference** being the changes in emission intensities, $\Delta F = F_{sample}$ - $F_{control}$.

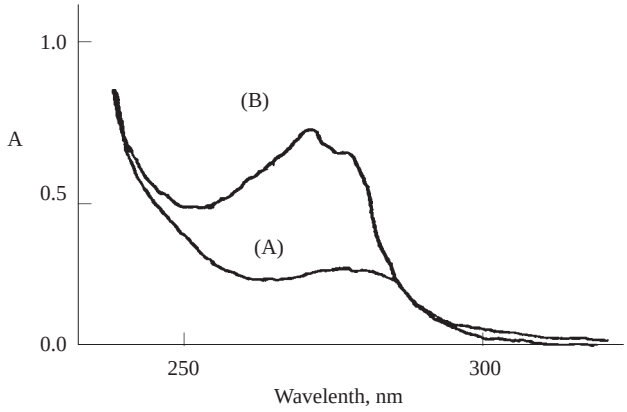


Figure 3. The absorption spectrum of

(A) 0.4 mg/mL bovine insulin,

(B) 1 : 1 (v/v) mixture of (A) and 3.2x10^{-4} mol.dm^{-3} Eu(PDA)$_3^{3-}$ solutions both at pH = 9.3.

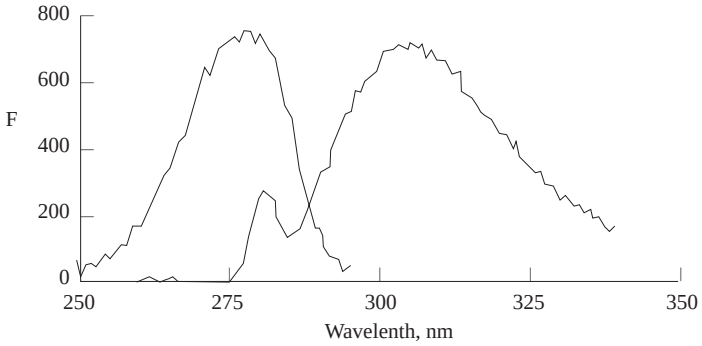


Figure 4. The excitation and emission spectra of 0.4 mg/mL bovine insulin (pH=9.3) solution at constant λ_{em} = 305 nm, λ_{ex} = 282 nm respectively.

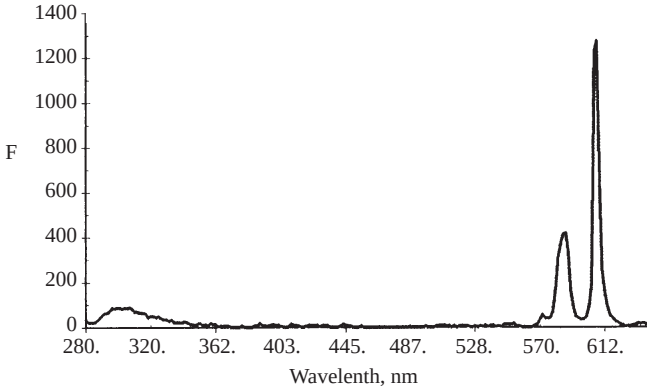


Figure 5. The emission spectrum at constant λ_{ex} = 282 nm of 1 : 1 (v/v) mixture of 0.4 mg/mL bovine insulin and 3.2 x 10^{-4} mol.dm^{-3} Eu(PDA)$_3^{3-}$ solution at pH =9.3.

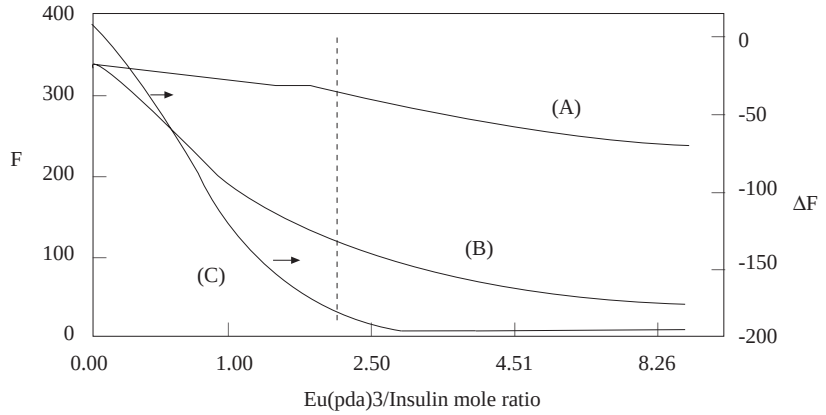


Figure 6. Fluorimetric titration curves plotted as the relative emission intensity (or the intensity difference) against the mole ratio of $Eu(PDA)_3^{3-}$/Ins. at constant λ_{ex} = 282 nm, λ_{em} = 305 nm, pH = 9.3
(A) 2.0 mL of 3.23x10^{-5} mol.dm^{-3} bovine insulin hormone solution's control titrations, simple dilution, F_A,
(B) 2.0 mL of 3.23x10^{-5} mol.dm^{-3} bovine insulin hormone solution titrated with 3.23x10^{-4} mol.dm^{-3} $Eu(PDA)_3^{3-}$ solution, F_B,
(C) Fluorimetric intensity difference, $\Delta F = F_B - F_A$.

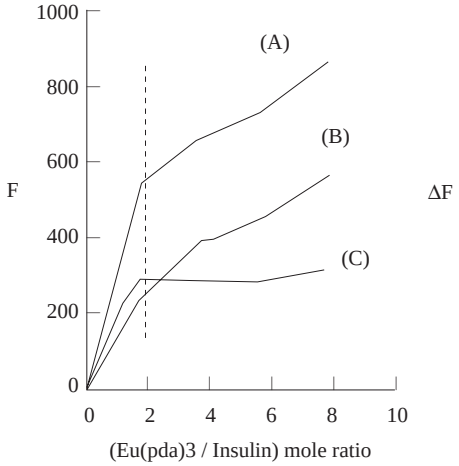


Figure 7. Fluorimetric titration curves plotted as the relative emission intensity (or the intensity difference) against the mole ratio of $Eu(PDA)_3^{3-}$/Ins.at constant λ_{ex} = 282 nm, λ_{em} = 615 nm, pH=9.3
(A) 2.0 mL of 3.23x10^{-5} mol.dm^{-3} bovine insulin hormone solution titrated with 3.23x10^{-4} mol.dm^{-3} $Eu(PDA)_3^{3-}$ solution, F_A,
(B) 2.0 mL of blank solution titrated with 3.23x10^{-4} mol.dm^{-3} $Eu(PDA)_3^{3-}$ solution, F_B.
(C) Fluorimetric intensity difference, $\Delta F = F_A - F_B$.

Table 1 and Figure 6-C show that the quenching of insulin fluorescence at 305 nm is significantly dependent on the $Eu(PDA)_3^{3-}$ concentration up to a mole ratio of R = ∼2.0 and no change is observed over this value. Thus, it is more evident when 615 nm extrinsic fluorescence of insulin is followed (Figure 7-C) and the sensitization of this band is up to R=2, a critical $[Eu(PDA)_3^{3-}]$ /[Ins] value.

Table 1. Titration of 2.000 mL 3.23x10^{-5} mol.dm^{-3} Bovine Insulin solution (pH = 9.3) with 3.23x10^{-4} mol.dm^{-3} $Eu(PDA)_3^{3-}$ solution.

Total Volume, mL	Insulin μg/mL	M	Eu(pda)^3 μg/mL	M	Eu(pda)^3/Ins μg/μg	Ratio, R mole/mole	Exc. at 282 nm Sample	Fluo.at 305 nm. Control	Difference
2.000	200.0	3.23E-05	0.00	0.00E+00	0.000	0.000	356	356	0
2.050	195.1	3.15E-05	0.00	0.00E+00	0.000	0.000	342	342	0
2.100	190.5	3.07E-05	0.00	0.00E+00	0.000	0.000	328	328	0
2.150	186.0	3.00E-05	2.35	7.51E-06	0.013	0.250	300	325	-25
2.200	181.8	2.93E-05	4.60	1.47E-05	0.025	0.501	262	322	-60
2.250	177.8	2.87E-05	6.74	2.15E-05	0.038	0.751	223	318	-95
2.300	173.9	2.81E-05	8.79	2.81E-05	0.051	1.001	185	313	-128
2.350	170.2	2.75E-05	10.76	3.44E-05	0.063	1.252	155	308	-153
2.400	166.7	2.69E-05	12.64	4.04E-05	0.076	1.502	136	308	-172
2.500	160.0	2.58E-05	16.18	5.17E-05	0.101	2.003	108	298	-190
2.600	153.8	2.48E-05	19.45	6.21E-05	0.126	2.503	94	292	-198
2.700	148.1	2.39E-05	22.48	7.18E-05	0.152	3.004	86	283	-197
2.800	142.9	2.30E-05	25.28	8.08E-05	0.177	3.505	77	274	-197
2.900	137.9	2.22E-05	27.90	8.91E-05	0.202	4.005	70	266	-196
3.000	133.3	2.15E-05	30.34	9.69E-05	0.228	4.506	62	258	-196
3.100	129.0	2.08E-05	32.63	1.04E-04	0.253	5.007	58	254	-196
3.200	125.0	2.02E-05	34.77	1.11E-04	0.278	5.507	54	250	-196
3.500	114.3	184E-05	40.46	1.29E-04	0.354	7.009	50	246	-196
3.750	106.7	1.72E-05	44.50	1.42E-04	0.417	8.261	47	243	-196
4.000	100.0	1.61E-05	48.04	1.53E-04	0.480	9.512	44	240	-196

Under steady illumination and no irreversible photochemical reactions, energy transfer from an excited donor (insulin) molecule, **Ins*** to an acceptor (**A** = $Eu(PDA)_3^{3-}$) molecule can be expressed by the **Stern-Volmer equation** and experimental *prima facie* evidence can be found by the Stern-Volmer plot[12]. The Stern-Volmer expression (1) is the ratio of equation (2) to (3) where the experimentally measured relative intensities can be used instead of quantum yields:

$$\frac{F_0}{F_A} = \frac{\Phi_0}{\Phi_{\mathbf{A}}} = \frac{k_1 + k_3 + k_2[\mathbf{A}]}{k_1 + k_3} = 1 + k_2\,\tau[\mathbf{A}] \tag{1}$$

where the quantum yield of emission at 305 nm from Ins* in the absence of **A** is

$$\Phi_0 = \frac{k_1[\mathrm{Ins}^*]}{I_a} = \frac{k_1}{k_1 + k_3} \tag{2}$$

and when a concentration of **A** equal to [A] is added, the quantum yield of emission from Ins* is

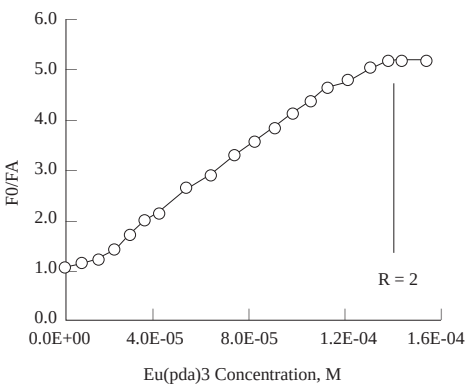
$$\Phi_A = \frac{k_1[\mathrm{Ins}^*]}{I_a} = \frac{k_1}{k_1 + k_3 + k_2[\mathbf{A}]} \tag{3}$$

I_a is the rate of light absorption = rate of Ins* formation (which may be a singlet or triplet) and k_1, k_2 and k_3 are the specific rate constants for emission, for energy transfer from Ins* and for the thermal deactivation of Ins* respectively. τ is the measured lifetime of Ins* in the absence of **A**, defined as $1/(k_1 + k_3)$.

As shown in Figure 8, a plot of (F_0/F_A) vs [$Eu(PDA)_3^{3-}$] gives a straight line with a slope of $k_2\tau$ = 3.055 x10^4 (which obeys Stern-Volmer equation 1) with a regression coefficient, r^2 = 0.9982. From equation (4), $[A]_{1/2} = [Eu(PDA)_3^{3-}]_{1/2}$ = 3.27 x10^{-5} mol.dm^{-3} is found to be a concentration which reduces Φ_0 to one-half of its original value.

$$k_2\tau[\mathbf{A}]_{1/2} = 1 \tag{4}$$

Figure 8 also shows that the linear increase in F_0/F_A values continues up to R=2 ratio and is constant over this critical value, indicating (i) that a bimolecular quenching mechanism is competing with unimolecular Ins* deactivation processes (e.g. its emission at 305) or (ii) that there might be an association of $Eu(PDA)_3^{3-}$ and insulin molecules (in the mole ratio of 2: 1) as given in reaction (1).



Eu(pda)3; M	F0/FA
0.00E+00	1.000
7.51E-06	1.083
1.47E-05	1.179
2.15E-05	1.389
2.81E-05	1.692
3.44E-05	1.987
4.04E-05	2.128
5.17E-05	2.591
6.21E-05	2.863
7.18E-05	3.291
8.08E-05	3.558
8.91E-05	3.840
9.69E-05	4.161
1.04E-04	4.379
1.11E-04	4.630
1.20E-04	4.825
1.29E-04	5.030
1.37E-04	5.175
1.42E-04	5.200
1.53E-04	5.220

Figure 8. Stern-Volmer plot of bovine insulin intrinsic fluorescence at 305 nm. Quenching of hormone emission (F_0/F) as a function of $Eu(PDA)_3^{3-}$ molar concentration.

$$\mathrm{Ins} + 2Eu(PDA)_3^{3-} - - - - - - > \mathrm{Ins} - [Eu(PDA)_3^{3-}]_2 Rea. \quad (1)$$

Such a binding reaction will cause an *intramolecular energy transfer* rather than an intermolecular energy transfer, the distinction of the former being that its

- k_2 rate constant must be bigger than the diffusion-controlled quenching rates, kq (which is of the order 10^9-10^{10} liters. $mol^{-1}.sec^{-1}$ for typical solvents at 25°C),

- k_2 rate constant must not depend on the rate of encounters or on the rate of collisions of the two partners.

One can assign t $<\sim 10^{-8}$ sec for Ins^* intrinsic fluorescence (as a fast emission), and finds k_2 of the order $\sim 3 \times 10^{12}$ from equations (1) or (4). This value means that an associative reaction has more validity than a simple bimolecular quenching mechanism.

Using the same experimental approach above but this time measuring the sensitized fluorescence of $Eu(PDA)_3^{3-}$ at $\lambda_{exc}/\lambda_{em}$= 282/615 nm after each addition of insulin solution encouraged us to offer an alternative method for hormone analysis. If we assign F_0 as the initial emission intensity at 615 nm from $Eu(PDA)_3^{3-}$ in the absence of sensitizer **A** = insulin (F_0 = 375, constant for $[Eu(PDA)_3^{3-}]$ = 1.00 x10^{-4} mol.dm^{-3} in this work), F_A as the emission intensity of $Eu(PDA)_3^{3-}$ when a concentration of **A** equal to [Ins] is added and k_{sen} as the rate of energy transfer from sensitizer to europium complex, we can rewrite the Stern-Volmer equation (1):

$$\frac{F_A}{F_0} = \frac{\Phi_A}{\Phi_0} = 1 + k_{sen}[\mathbf{Ins}] \quad (5)$$

when [**Ins**] = 0, $F_A = F_0$. A plot of (F_A/F_0) vs [Ins] gives a straight line with a slope of K_{sen} = 2.255 x10^4 and a regression coefficient, r^2 = 0.9879. From equation (5), [Ins] = 4.43 x10^{-5} mol.dm^{-3} is found to be a concentration which increases F_0 to twice its original value. The increase in emission intensity is linear up to R = 2 ratio ([Ins] = 5.0 x10^{-5} mol.dm^{-3} in this work and selectively depends on the formal concentration of $Eu(PDA)_3^{3-}$). Its bandwidth is very narrow. It is in a characteristic visible region, easy to work compared to UV emission where many proteins and biomolecules luminesce. The precision and accuracy of quantitation

of the insulin hormone between 10.0 μg/mL and 200.0 μg/mL is much better than the former method and needs only ∼100 microliter additions of sample solution to 2.000 mL of 1.0 x 10^{-4} mol.dm^{-3} $Eu(PDA)_3^{3-}$ solution (pH=9.3).

Applying this new method of analysis, the coefficient of variation (CV), the relative error and the minimum detectable quantity of bovine insulin hormone are found to be 3.0 %, 1.2 % and 7.3±0.2 μg/mL (difference = $\Delta F = F_A - F_0 = 10$, N=∼50 experiments) respectively. All experiments being carried out with the standard solutions, selectivity to insulin in a real sample must be tested. It seems that this method is applicable to some other proteins or biomolecules too. Our recent work on thyroxine hormone has shown a similar trend, as good as that of insulin.

Our recent research interest is to isolate an Ins-$[Eu(PDA)_3^{3-}]_2$ type product thinking that the associative reaction (1) is valid and to show that europium dipico-linate complex may be used for fluorescent labeling of some biomolecules, at least for the insulin hormone. If one assigns K_{app} as the apparent binding constant of insulin with $Eu(PDA)_3^{3-}$, ΔF as the differences in emission intensity at 305 nm (refering to Table 1), $[Ins]_0$ as the initial formal concentration, which is an experimental constant, and as the proportionality constant that contains all other experimental and instrumental parameters, one can write equation (6), an expression described previously[13] and recently used by us to compute the inclusion complexation of warfarin with β-cyclodextrins[14].

Table 2. Quenching of 2.000 mL 3.23x10^{-5} mol.dm^{-3} Insulin Fluorescence at 305 nm with additions of 100 μL $Eu(PDA)_3^{3-}$ complex solutions, pH = 9.3. Last column gives the Kapp values computed from Equation (6).

Ins +	2Eu(pda)3	≪ − − − − ≫	Ins-Eu(pda)3	
	R	Exc. at 282 nm	Fluo. at 305 nm	
Eu(pda)3, M	mole ratio	F	1/ΔF	K_{app}
0.00 E+00	0.00	328		
3.81 E-06	0.13	294	0.0294	3.89E+04
4.76 E-06	0.16	286	0.0238	3.99E+04
7.14 E-06	0.24	269	0.0169	4.05E+04
9.52 E-06	0.32	254	0.0135	4.11E+04
1.19 E-05	0.40	241	0.0115	4.15E+04
1.43 E-05	0.48	228	0.0100	4.29E+04
1.67 E-05	0.56	217	0.0090	4.37E+04
1.91 E-05	0.64	207	0.0083	4.46E+04
2.14 E-05	0.71	198	0.0077	4.56E+04
2.38 E-05	0.79	190	0.0072	4.63E+04
2.86 E-05	0.95	175	0.0065	4.86E+04
3.81 E-05	1.27	152	0.0057	5.30E+04
4.29 E-05	1.43	142	0.0054	5.62E+04
4.43 E-05	1.48	139	0.0053	5.75E+04
4.76 E-05	1.59	134	0.0052	5.89E+04
5.00 E-05	1.67	130	0.0051	6.08E+04
5.50 E-05	1.83	122	0.0049	6.55E+04
6.00 E-05	2.00	116	0.0047	6.91E+04
			0<R<1.0	4.30E+04
			1.0 < R < 2.0	6.01E+04
			Average	4.97E+04

$$\mathbf{1/\Delta F} = 4.0 \text{ x } 10^{-8}[\mathbf{Eu(PDA)_3^{3-}}] + 0.0038 \qquad R^2 = 0.9658$$

For the quenching of insulin fluorescence intensity at 305 nm:

$$(\Delta F)^{-1} = (\alpha K_{app}[\mathrm{Ins}]_0[Eu(PDA)_3^{3-}])^{-1} + (\alpha[\mathrm{Ins}]_0)^{-1} \quad (6)$$

The linear relationship in equation (6) between $[\mathrm{Ins}]_0/\Delta\mathrm{F}$ and $1/[\mathrm{Eu(PDA)}_3^{3-}]$ and the ratio of intersect/slope gives the apparent binding constant, K_{app}. The K_{app} values, linear regression with correlation constant (R^2), are given in Table 2. For $0 < \mathrm{R} < 2.0$, $\mathbf{K_{app} = (5.00 \pm 1.25)x10^4}$ is found to be an average value which is statistically the same (no significant difference at the 95 % probability level) when its exstrinsic fluorescence at 615 nm is followed.

Stern-Volmer plots and the apparent binding constant ($\log \mathrm{K}_{app} = 4.70 \pm 0.13$) found above are evidence of Ins-$[\mathrm{Eu(PDA)}_3^{3-}]_2$ type product formation as given in reaction (1). They show that such a reaction is more favorable than a simple bimolecular quenching of hormone in solution. More work must be done on the separation and isolation of this new product. The labeled insulin hormone may open the doors to a new in vivo luminescence methodology as a diagnostic tool and as an alternative to radio-iodinated (^{131}I-) insulin.

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