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Protein-Mediated Efficient Synergistic "Antenna Effect" in a Ternary System in D_2O Medium

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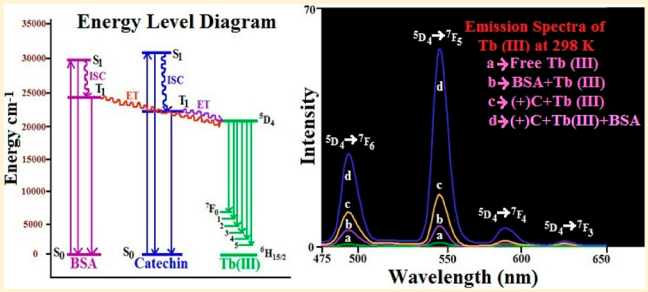
Supporting Information

ABSTRACT: A ternary system consisting of a protein, catechin (either + or − epimer), and Tb(III) in suitable aqueous buffer medium at physiological pH (= 6.8) has been shown to exhibit highly efficient "antenna effect". Steady state and time-resolved emission studies of each component in the binary complexes (protein with Tb(III) and (+)- or (−)-catechin with Tb(III)) and the ternary systems along with the molecular docking studies reveal that the efficient sensitization could be ascribed to the effective shielding of microenvironment of Tb(III) from O−H oscillator and increased Tb−C (+/−) interaction in the ternary systems in aqueous medium. The ternary system exhibits protein-mediated efficient antenna effect in D_2O medium due to synergistic ET from both the lowest $\pi\pi^*$ triplet state of Trp residue in protein and that of catechin apart from protection of the Tb(III) environment from matrix vibration. The simple system consisting of (+)- or (−)-catechin and Tb(III) in D_2O buffer at pH 6.8 has been prescribed to be a useful biosensor.



1. INTRODUCTION

The "antenna effect" sensitizing lanthanide(III) emission in aqueous medium has attracted many researchers[1−41] to develop imaging techniques in the visible and NIR region, biosensors, and biomedical assay.[42−45] The main challenge in this field is to reduce nonradiative decay of Ln(III) emission through vibronic coupling of high-frequency O−H oscillator in water. The complexes of Ln(III) have been designed to shield the Ln(III) ion from the O−H group in the microenvironment of Ln(III) using suitable ligands.[46−48] One also needs the complex to be kinetically stable in water. In these systems energy transfer usually takes place from the lowest $\pi\pi^*$ triplet state of the ligand following Dexter's exchange mechanism.[49] Thus the ligands must have high $\pi\pi^*$ absorption with suitably placed lowest $\pi\pi^*$ triplet state (T_1) in order to have efficient energy transfer (ET). An energy gap of at least 2000−3000 cm^{-1} between T_1 and the emissive state of Ln(III) has been prescribed[50] to prevent back ET. Choice of two different ligands attached to Ln(III) puts another restriction of $T_1 \leftrightarrow T_1$ ET between the ligands.[51] In recent times, some d−f hybrid complexes using Ru(III), Os(III), Ir(III), Re(III), and Pt(III) have been developed where ET takes place from the MLCT state. All these d−f hybrid complexes exhibit ET effect in nonaqueous medium.[52−66]

Some ternary systems where protein is one component of the system have been reported for biomedical assay.[67−69] One such system is the terbium−gadolinium−protein−cetylpyridine bromide system where the enhancement of Tb(III) emission is explained due to shielding of Tb(III) from O−H oscillator.[69] However, the protein used, bovine serum albumin (BSA), in this system gets denatured due to the high ionic strength of the medium.[69] We recently carried out a detailed investigation of binary complexes of serum albumins (bovine serum albumin, BSA, and human serum albumin, HSA) with stereoisomeric catechin (C) [both (+) and (−) forms] using steady state and time-resolved emission as well as CD and FTIR at room temperature and 77 K.[70] Molecular docking was employed to find the location of the ligand in these protein−ligand complexes and to determine the amino acid residues involved in the binding.[70] In a recent study we also found that the both the epimers (+) and (−) of catechin (Figure 1) bind Tb(III). The ET from catechin moiety to Tb(III) is observed to be efficient.[71]

In this paper we report very efficient antenna effect in a ternary system consisting of BSA/HSA, (+)- or (−)-catechin, and Tb(III) in aqueous medium and D_2O using suitable buffer

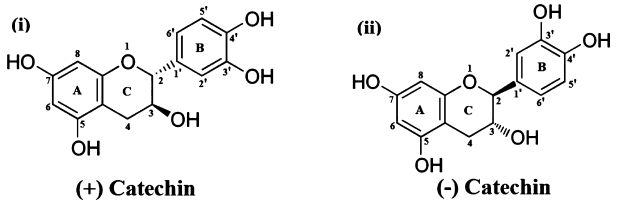


Figure 1. Structure of (i) (+)-catechin and (ii) (−)-catechin.

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at physiological pH maintaining the native structure of the protein in the system. The NO_3^- ion is used as counteranion.

The BSA molecule is made up of 3 homologous domains which are divided into 9 loops by 17 disulfide bonds. BSA has two tryptophan residues, Trp 134 in the first domain and Trp 213 in the second domain.[72] However, the exact crystal structure of BSA is unknown to date. HSA, on the other hand, consists of 585 amino acids and is cross-linked by 17 disulfide bonds. HSA contains only a single Trp residue at 214. It is considered to have three specific domains, I, II, and III, each of which consists of two subdomains a and b possessing common structural motifs.[73] A comparative study of the amino acid sequences of BSA and HSA by Brown shows that they have similar general structural features, the difference in sequence being generally conservative.[74]

In this report, we presented the steady state and time-resolved emission studies monitoring each species in the binary complexes consisting of BSA/HSA and Tb(III), C (+ or −), and Tb(III) and the ternary systems consisting of BSA/HSA, C (+ or −), and Tb(III) to characterize the binary and ternary systems. The ET efficiency observed in the ternary systems has been compared with that observed in each of the binary systems. Time-resolved studies of Tb(III) emission in all the binary and the ternary systems in H_2O and D_2O are also used to find the number of water coordination of Tb(III) in the ternary systems and in the binary systems. Molecular docking studies are employed to find the location of (+)- or (−)-catechin in the complexes of catechin with proteins as well as to calculate the accessible surface area (ASA) of the amino acid residues of BSA/HSA involved in the binding and the ASA of the bound ligand catechin. Docking studies are also utilized to detect whether Trp 134 or Trp 213 of BSA is involved in the ET process in the complexes. The highly efficient sensitization of Tb(III) emission in the ternary system observed in aqueous medium at room temperature has been ascribed to (i) the enhancement of Tb−C (+/−) interaction in the ternary system containing protein compared to that in the binary system of Tb−C (+/−) in aqueous medium and (ii) efficient shielding of Tb(III) in the ternary complexes from O−H oscillators capable of vibronic coupling. Protein-mediated efficient antenna effect is observed in D_2O medium due to synergistic effect of ET from the lowest triplet state (T_1) of Trp residues in BSA/HSA and the T_1 of catechin in addition to the shielding effect of Tb(III) environment from matrix vibration. Finally, we have shown how the simple system of (+)- or (−)-catechin complex with Tb(III) in D_2O buffer at pH 6.8 could be very effective for biosensor development.

2. EXPERIMENTAL SECTION

2.1. Materials and Methods. All chemicals were of reagent grade, which were used without further purification unless otherwise mentioned. (+)-Catechin ((+)-C), (−)-catechin ((−)-C), terbium nitrate ($Tb(NO_3)_3$), serum albumin from bovine (BSA), serum albumin from human (HSA), and *α,α,α*-tris(hydroxymethyl)methylamine were purchased from Sigma-Aldrich. The 0.1 M tris-HCl buffer of pH 6.8 was prepared in triply distilled water and used for making all experimental solutions. The stock solutions of (+)-C, (−)-C were prepared using extinction coefficient in water (ε = 10 233 M^{-1} cm^{-1} at 276 nm). Binary complexes of catechin and Tb(III) were prepared by mixing 100 μM catechin ((+)-C or (−)-C) and 25 μM Tb(III) in the tris buffer of pH 6.8. The composition used (4:1 mole ratio of catechin and Tb(III)) for the preparation of the binary complexes of (+)- or (−)-C with Tb(III) is discussed in section 3.3. The ternary systems were prepared by using 25 μM Tb(III), 100 μM catechin ((+)-C or (−)-C), and 10 μM BSA/HSA in the buffer of pH 6.8. The binary systems of BSA/HSA with Tb(III) were prepared by mixing 10 μM BSA/HSA and 25 μM $Tb(NO_3)_3$ in the tris buffer of pH 6.8. A slight increase in pH from 7 resulted in precipitation of Tb(III). We found that pH 6.8 is the optimum pH for the preparation of the binary and ternary systems.

2.2. Instrumentation. UV−vis absorption spectra were recorded on a Hitachi U-4010 spectrophotometer at 298 K. The steady state emission measurements were carried out using a Hitachi Model F-7000 spectrofluorimeter equipped with a 150 W xenon lamp, at 298 K using a stoppered cell of 1 cm path length.

The lifetime of the singlet state was measured by using TCSPC from PTI, U.S., using the subnanosecond pulsed LED source (280 and 290 nm having pulse width 600 ps [full width at half-maximum]) (from PicoQuant, Germany) operating at high repetition rate of 10 MHz driven by PDL 800-B driver, PicoQuant, Germany. LED profiles were measured at the respective excitation 280 or 290 nm with a band pass of 3 nm using Ludox as the scatterer.

The decay parameters were recovered using a nonlinear iterative fitting procedure based on the Marquardt algorithm.[75] The deconvolution technique used can determine the lifetime up to 150−200 ps. The quality of fit has been assessed over the entire decay, including the rising edge, and tested with a plot of weighted residuals and other statistical parameters, e.g., the reduced χ^2 ratio.[76]

The decay times of the Tb(III) complexes in the microseconds region were also acquired by phosphorescence decay mode in a QM-30 fluorimeter from PTI, U.S., using a gated detection system having start and end window time 0 and 6000 μs, respectively. The decay times in the milliseconds or longer range were measured by phosphorescence time-based acquisition mode of the QM-30 fluorimeter in which emission intensity is measured as a function of time. The decay parameters were recovered using a nonlinear iterative fitting procedure based on the Marquardt algorithm.[75]

2.3. Docking Studies. The crystal structure of HSA (PDB entry 1AO6) is obtained from the Protein Data Bank.[77] Since the structure of BSA is unavailable in the PDB, a homology model was used for the docking studies.[78−82] The 3D structures of catechin are generated by Sybyl 6.92 (Tripos Inc., St. Louis, MO, U.S.), and their energy-minimized conformations are obtained with the help of the TRIPOS force field using Gasteiger−Hückel charges with a gradient of 0.005 kcal/mol. Details of docking studies and calculation of accessible surface area (ASA) in the free protein and the complex have been described in detail in our earlier work.[70]

The change in accessible surface area for residue i is calculated using the following equation

$$\Delta ASA^i = ASA^i_{HSA/BSA} - ASA^i_{HSA/BSA\text{-}complex} \quad (1)$$

If a residue loses more than 5 Å^2 ASA on going from the uncomplexed to the complexed state, then it is considered to be involved in the interaction.

3. RESULTS AND DISCUSSION

3.1. Absorption Spectra at 298 K in Aqueous Buffer. All the absorption spectra of various species are recorded in tris

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buffer of pH 6.8 at 298 K. The absorption spectra of free BSA and the binary complex of BSA with Tb(III) (NO_3^- as counteranion) are shown in Figure 2A as a and a′, respectively.

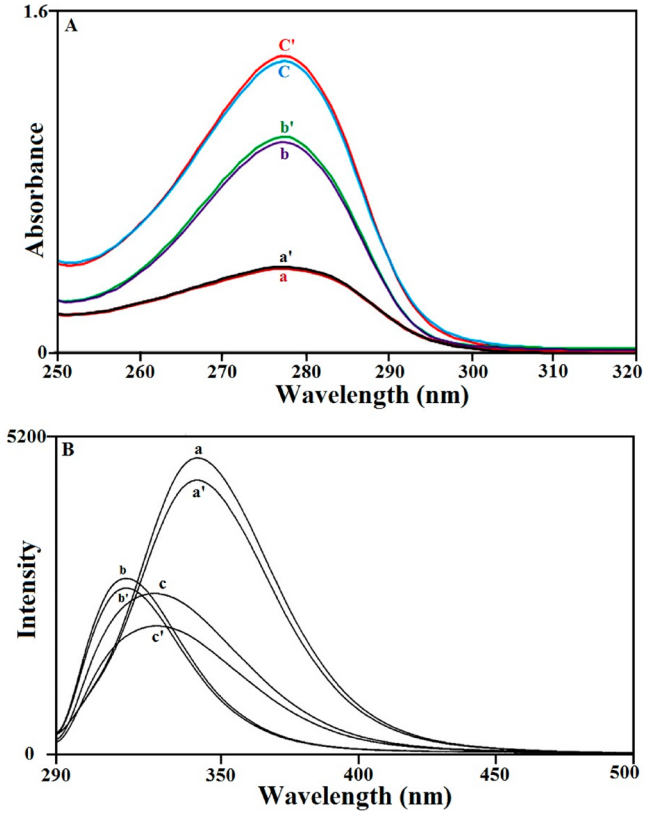

Figure 2. (A) Absorption spectra of (a) BSA, (a′) BSA + Tb(III), (b) (+)-C, (b′) (+)-C + Tb(III), (c) BSA + (+)-C, and (c′) (+)-C + Tb(III) + BSA in aqueous buffer at 298 K. (B) Steady state fluorescence spectra of (a) BSA, (a′) BSA + Tb(III), (b) (+)-C, (b′) (+)-C + Tb(III), (c) BSA + (+)-C, (c′) (+)-C + Tb(III) + BSA in aqueous tris-HCl buffer at pH 6.8 at 298 K. λ_{exc} = 280 nm, excitation band pass = 10 nm and emission band pass = 5 nm. [BSA] = 10 μM, [(+)-C] = 100 μM, [Tb(III)] = 25 μM.

In Figure 2A, b and b′ represent the absorption spectra of free (+)-C and the binary complex of (+)-C and Tb(III).The absorption spectra of the binary complex of BSA and (+)-C and the ternary system of BSA, (+)-C with Tb(III) are shown in Figure 2A (c and c′, respectively). The λ_{max} and the absorbance remain the same in the free (+)-C and in the binary complex of (+)-C with Tb(III). The complex of (+)-C with BSA shows λ_{max} at 276 nm, and the absorbance is observed as the sum of the individual absorbances of (+)-C and BSA. The λ_{max} and the absorbance remain same in the ternary system as that observed in the binary complex of (+)-C with BSA. The same trend is observed in the complexes with (−)-C. The absorbance pattern is also found to be the same when the binary and the ternary systems are prepared using HSA. The pH 6.8 was chosen to prevent the oxidation of catechin to quinone[83] at higher pH and taking into consideration the physiological pH.

3.2. Steady State and Time-Resolved Emission Studies at 298 K in Aqueous Buffer. The emission spectra of (+)-C, free BSA, and the binary complexes of (+)-C with Tb(III), of BSA with Tb(III), and of BSA with (+)-C are shown in Figure 2B with λ_{exc} = 280 nm. The emission spectra of the ternary system consisting of BSA, (+)-C, and Tb(III) are also shown in Figure 2B. The λ_{max} of the emission in the different systems is given in Table 1. In the binary complexes of BSA with Tb(III) and of (+)-C with Tb(III), the λ_{max} remains the same as that of free BSA and free (+)-C, respectively. The λ_{max} of the emission of the complex of BSA with (+)-C is observed at 325.6 nm (with λ_{exc} = 280 nm) due to overlap of the emission spectra of BSA and (+)-C. In all the binary complexes of Tb(III) and in the ternary systems, the emission of BSA/HSA or of (+)/(−)-C are quenched (Table 1) with concomitant increase of the Tb(III) emission ($^5D_4 \rightarrow {}^7F_6$, $^5D_4 \rightarrow {}^7F_5$, $^5D_4 \rightarrow {}^7F_4$, $^5D_4 \rightarrow {}^7F_3$) as compared to that of free Tb(III) in the same buffer under similar experimental conditions (Figure 3A). The maximum enhancement of Tb(III) emission is observed in the ternary system. In order to compare the intensity of Tb(III) emission in the different systems, λ_{exc} is kept at 295 nm to avoid inner filter effect (o.d. is less than 0.2 in each case) and to prevent excitation of tyrosine residues (Tyr) in protein.

In order to find the nature of quenching in the binary systems with Tb(III), we measured the decay of (+)-C in the free state and in the complex with Tb(III) in tris HCl buffer monitored at 315 nm (Figure 4A). It was observed that the lifetime of (+)-C fluorescence remains same in the free state and in the complex (Table 2). It is noted that the decay fits nicely with one-component analysis. The lifetime of Trp emission of BSA is also found to remain the same in the free protein and in the complex with Tb(III) (Figure 4B, Table 2). In the protein complexes the decay was fitted with two components. The value of $\langle\tau\rangle$ for free protein matches very well with the reported data.[84,85] The results clearly indicate that static quenching is taking place with the formation of ground state complex in each case. Similar results were observed in the complexes of (−)-C with Tb(III) and in the complex of HSA with Tb(III).

3.3. Binding Constant of Catechin and Tb(III). In order to determine the composition of the complex of (+)-C and Tb(III) in tris-HCl buffer, we followed the enhancement of Tb(III) emission ($^5D_4 \rightarrow {}^7F_5$) due to the addition of different concentration of (+)-C (shown in Figure 5A). It was observed that 1:4 ratio of metal−ligand composition gives the maximum luminescence intensity of Tb(III) ($^5D_4 \rightarrow {}^7F_5$ peak) (Figure 5B). The binding interaction between catechin ((+)-C or (−)-C) and Tb(III) was examined by determining the binding constant from the luminescence intensity of the $^5D_4 \rightarrow {}^7F_5$ peak of Tb(III) as a function of added catechin concentration employing modified Benesi−Hilderbrand equation[86−88]

$$[F_\infty - F_0]/[F_x - F_0] = 1 + 1/K[Q] \quad (2)$$

where F_0, F_x, and F_∞ are the lumenescence intensity of Tb(III) in the absence of catechin, at an intermediate catechin concentration, and at a concentration of complete interaction, respectively; K being the binding constant and [Q] the concentration of added catechin ((+)-C or (−)-C).

The slope of the linear plot of $[F_\infty - F_0]/[F_x - F_0]$ against the $[Q]^{-1}$ provides the binding constant (K). Such a plot of the complex of (+)-C with Tb(III) is shown in Figure 5C. The binding constants using both the forms of catechin ((+)-C and (−)-C) are given in Table 3.

3.4. Enhancement Factor at 298 K and Lifetime of Tb(III) in Various Systems. It is noted that the enhanced ratio of luminescence emission of Tb(III) in the ternary system of (+)-C, BSA with Tb(III) is more than that of the sum of the enhanced ratio of Tb(III) in the individual binary complexes

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Table 1. Quenching of Emission of the (+)-C, BSA, and HSA and Enhancement of Luminescence Emission of Tb(III) Ion in the Binary Complexes and Ternary Systems in Aqueous Tris Buffer and D_2O Buffer at pH 6.8 at 298 K[a]

medium	system	λ_{max} of fluorescence with λ_{exc} = 280 nm[b] (nm)	quenching of the fluorescence at (0, 0) band ($S_1 \rightarrow S_0$) (I_F/I^0_F)[d] (λ_{exc} = 280 nm)	enhancement of Tb(III) emission at 547 nm[c] ($^5D_4 \rightarrow {}^7F_5$) transition ($I_M/I^0_M$)[e] ($\lambda_{exc}$ = 295 nm)	enhancement of Tb(III) emission at 491 nm[c] ($^5D_4 \rightarrow {}^7F_4$) transition ($I_M/I^0_M$)[f] ($\lambda_{exc}$ = 295 nm)
aqueous buffer	(+)-C + Tb(III)	315.8	0.94	15.7	14.8
	BSA + Tb(III)	342.0	0.93	5.1	4.8
	(+)-C + Tb(III) + BSA	325.6	0.80	53.5	47.0
	HSA + Tb(III)	339.0	0.91	7.0	6.4
	(+)-C + Tb(III) + HSA	325.8	0.79	44.8	39.3
D_2O buffer	(+)-C + Tb(III)	315.2	0.92	15.0	14.5
	BSA + Tb(III)	341.4	0.93	7.0	6.8
	(+)-C + Tb(III) + BSA	325.0	0.78	90.1	86.0
	HSA + Tb(III)	339.2	0.92	6.8	6.2
	(+)-C + Tb(III) + HSA	325.2	0.80	70.0	65.1

[a]The intensities are calculated in each case using the total area under the respective band. [b]λ_{max} does not change with λ_{exc} = 295 nm. [c]Error in the measurement of area under the Tb(III) emission = ± 2% [d]I_F and I^0_F are the intensities of total fluorescence spectra of the catechin in the complex and in the pure state, respectively. [e]I_M and I^0_M are the intensities of emission for the $^5D_4 \rightarrow {}^7F_5$ transitions of the Tb(III) ion at 547 nm in the complex and in the pure $Tb(NO_3)_3$, respectively. [f]I_M and I^0_M are the intensities of emission for the $^5D_4 \rightarrow {}^7F_4$ transitions of the Tb(III) ion at 491 nm in the complex and in the pure $Tb(NO_3)_3$, respectively.

with two donors ((+)-C and BSA) (Figure 3A). The enhanced intensity ratio of the band ($^5D_4 \rightarrow {}^7F_5$) at 547 nm of Tb(III) in different complexes compared to that of free $Tb(NO_3)_3$ is shown in Table 1. The enhancement could be due to shielding of Tb(III) environments from the potential quencher viz. OH-oscillator or intramolecular energy transfer from both the donors (protein and catechin ligand) to the Tb(III) in the ternary system or both.

The efficiency of energy transfer was calculated using the equation[89]

$$\text{ET efficiency} = (A_{acc}/A_{don})[I_M/I^0_M - 1] \quad (3)$$

where A_{acc} and A_{don} are the absorbances of the acceptor (Tb(III)) and donor, respectively, at the same excitation wavelength. I_M and I^0_M are the luminescence intensities of the Tb(III) in the presence and in the absence of energy transfer. The calculation of the ET efficiency ratio using the eq 3 includes the effect of both the shielding factor and the ET effect.

The ET efficiency ratios calculated for the $^5D_4 \rightarrow {}^7F_5$ peak and $^5D_4 \rightarrow {}^7F_4$ peak of Tb(III) in the ternary systems compared to that in the binary systems are recorded in Table 4. It is noted that although the ratio (E_T/E_{B1}) for the transition $^5D_4 \rightarrow {}^7F_5$ is ≈2 when compared to that of the binary complex of catechin and Tb(III), the ratio (E_T/E_{B2}) is enhanced to 6 when compared to that of the binary complexes of BSA and Tb(III) (Table 4). It is also found that the ET efficiency ratio is somewhat less in the complexes containing HSA compared to that in the complexes containing BSA (Table 4) in H_2O buffer used.

To find the mechanism of enhanced emission in the ternary systems, the decay of Tb(III) luminescence monitoring the band at 547 nm of Tb(III) in the free state ($Tb(NO_3)_3$ in tris buffer at pH 6.8) and in all the different binary and ternary systems [(+)-C with Tb(III); BSA with (+)-C and Tb(III)] has been measured (Figure 4C). The significant change in lifetime of Tb(III) in different complexes with BSA and HSA is shown in Table 5. The experiments described above were also carried out in the ternary systems with (−)-C. A similar result was observed with (−)-C as that of (+)-C (see the data in the Supporting Information).

3.5. Emission of Tb(III) in Different Complexes in D_2O Buffer at 298 K. The luminescence emission of Tb(III) in different binary and ternary systems ((+)-C with Tb(III); BSA with Tb(III); (+)-C with BSA and Tb(III)) was also carried out in D_2O buffer with λ_{exc} = 295 nm. It was observed that the intensity of luminescence emission of different peaks and luminescence lifetime of Tb(III) in D_2O buffer are greatly enhanced compared to that in the different binary and ternary complexes in aqueous buffer (Figure 3B). The luminescence lifetime of the $^5D_4 \rightarrow {}^7F_5$ transition is also enhanced as given in Table 5. The enhancement ratios of luminescence emission of Tb(III) in the ternary systems and in the binary complexes in D_2O buffer are presented in Table 1.

3.6. ET Mechanism. Recently, we have investigated the interaction of both (+) or (−) forms of catechin with BSA/HSA using steady state and time-resolved fluorescence, phosphorescence, circular dichroism (CD), FTIR, and protein ligand docking studies.[70] Binding constants are found to be 5.1 × 10^3 and 1.0 × 10^4 for BSA and HSA, respectively, at 298 K with catechin using emission studies. The values agree well with

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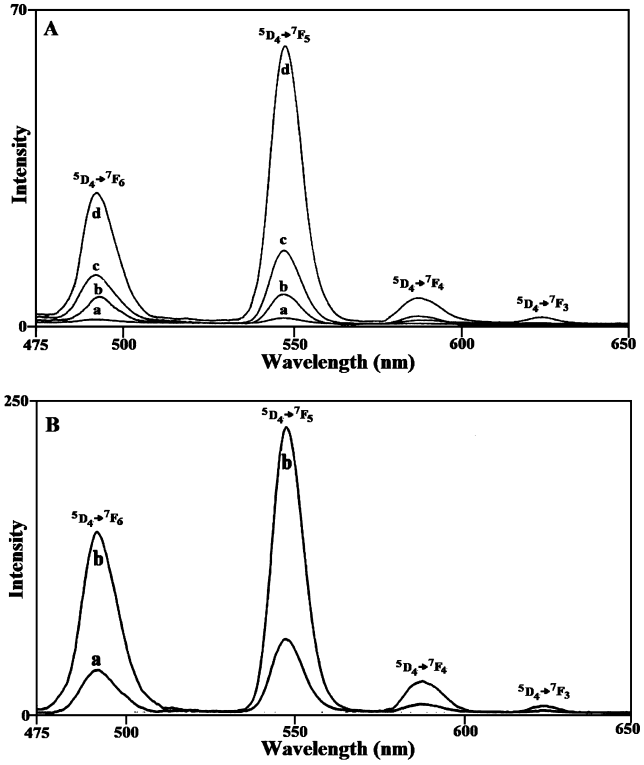

Figure 3. (A) Luminescence spectra of Tb(III) in (a) free Tb(III), (b) BSA + Tb(III), (c) (+)-C + Tb(III), (d) (+)-C + Tb(III) + BSA in aqueous tris-HCl buffer at 298 K; λ_{exc} = 295 nm, excitation band pass = 10 nm and emission band pass = 5 nm; [BSA] = 10 μM, [(+)-C] = 100 μM, [Tb(III)] = 25 μM. (B) Luminescence spectra of Tb(III) in the ternary system consisting of (+)-C + Tb(III) + BSA (a) in aqueous buffer, (b) in D_2O buffer at 298 K; λ_{exc} = 295 nm, excitation band pass = 10 nm and emission band pass = 5 nm; [BSA] = 10 μM, [(+)-C] = 100 μM, [Tb(III)] = 25 μM.

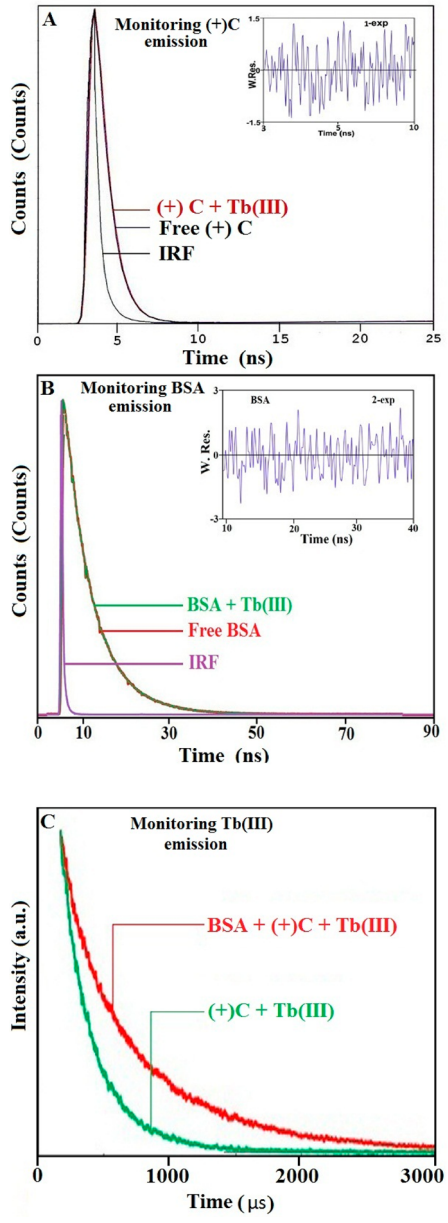

Figure 4. (A) Fluorescence decay of free (+)-C and the complex with Tb(III) at 298 K in aqueous buffer; λ_{exc} = 280 nm, $\lambda_{monitor}$ = 315 nm (excitation band pass = 10 nm, emission band pass = 5 nm); [(+)-C] = 100 μM, [Tb(III)] = 25 μM. (B) Fluorescence decay of free BSA and the complex with Tb(III) in aqueous buffer at 298 K; λ_{exc} = 280 nm, $\lambda_{monitor}$ = 340 nm (excitation band pass = 10 nm, emission band pass = 5 nm); [BSA] = 10 μM, [Tb(III)] = 25 μM. (C) Decay of Tb(III) monitored at 547 nm in tris-HCl buffer at 298 K in different systems; λ_{exc} = 295 nm, excitation band pass = 10 nm, emission band pass = 5 nm. [BSA] = 10 μM, [(+)-C] = 100 μM, [Tb(III)] = 25 μM.

those obtained from CD measurements. The number of binding sites for catechin are found to be one using both emission and CD measurements. The low-temperature phosphorescence and molecular docking with both (+) and (−) forms of C indicated that catechin binds in the proximity of Trp134 of BSA and Trp 214 of HSA. The major residues involved in the binding in all the cases are shown in Tables 6 and 7. We included only those residues which lose ≥10 Å^2 ASA due to interaction. The distances of Trp 134 and Trp 213 of BSA and Trp 214 of HSA from the ring A, B, and C of (+)- or (−)-catechin are provided in Table 8. This clearly indicates that the Trp 134 residue is involved in the binding of (+)- or (−)-C in the complex of BSA while the Trp 214 is near the binding site in the complex of HSA. The distances calculated are within 10 Å in each case. This suggests the exchange mechanism involving the lowest triplet state of respective Trp residues and $^5D_4$ state of Tb(III) operating in the ET process.[90−93] Furthermore, the energy levels of the T_1 state of Trps of proteins and of catechin[70] and the emissive state of Tb(III) are depicted in Figure 6. The position of the energy levels of the T_1 states is an indication of synergistic energy transfer in the ternary system.

The docked pose of (+)- or (−)-C and the nearby residues of BSA and HSA involved in the binding are shown in Figure 7. The total loss of ASA due to binding for BSA and HSA is provided in Table 9. ASA of ligands (+)- and (−)-C in the complex are also calculated and shown in Table 9.

If the Tb(III) complexes of (+) and (−)-C are located in the same region of protein as that of pure (+)- and (−)-C, then the ASA values calculated for (+)- or (−)-C in the binary complexes (Table 9) clearly imply that Tb(III) is shielded from H_2O in the ternary complexes. The loss of ASA of various residues near the binding site of (+)- or (−)-C in HSA and BSA (Table 9) is also a clear indication of shielding of Tb(III) in the protein environment.

The lifetime (τ) of Tb(III) can be expressed as[94]

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Table 2. Singlet State Lifetime of (+)-C (Monitoring at 315 nm) and Serum Albumins (Monitoring Trp Emission) at 298 K in the Free State and in the Complexes with Tb(III); λ_{exc} =280 nm

system	τ_1 (ns)	τ_2 (ns)	$\langle\tau\rangle$ (ns)	$\chi 2$
(+)-C	0.52(100%)	–	0.82	0.98
Tb(III) + (+)-C	0.53(100%)	–	0.83	1.10
BSA	6.49(87%)	3.27(13%)	6.07	0.91
Tb(III) + BSA	6.53(83%)	3.91(17%)	6.10	0.99
HSA	6.74(57%)	2.92(43%)	5.09	1.13
Tb(III) + HSA	6.91(48%)	3.25(52%)	5.00	1.06

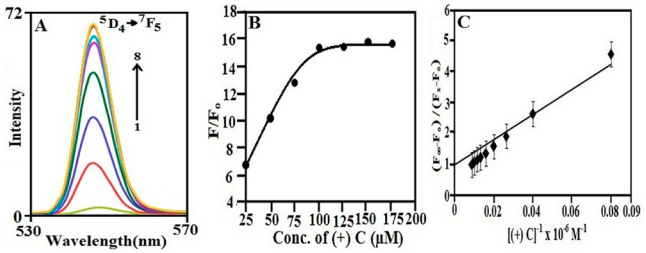

Figure 5. (A) Luminescence spectra of Tb(III) (peak 547 nm) with varying concentration of (+)-C: (1) 0, (2) 25, (3) 50, (4) 75, (5) 100, (6) 125, (7) 150, and (8) 175 μM, respectively, in aqueous buffer at 298 K, and in each case the concentration of Tb(III) is kept at 25 μM; λ_{exc} = 295 nm; excitation band pass = 10 nm and emission band pass = 5 nm. (B) Plot of F/F_0 against [(+)-C]. (C) Plot of $[F_\infty - F_0]/[F_x - F_0]$ vs [(+)-C] obtained from modified Benesi–Hilderbrand equation at 298 K monitoring the peak at 547 nm of Tb(III) emission.

Table 3. Binding Constants of the Catechin and Tb(III) Complexes at 298 K Using the Modified Benesi–Hilderbrand Equation (Monitoring the Peak of Tb(III) at 547 nm in Aqueous Buffer of pH = 6.8)

system	binding constant (K)
(+)-C + Tb(III)	6.2×10^4 M^{-1}
(−)-C + Tb(III)	6.0×10^4 M^{-1}

$$1/\tau = k_r + k_{nr} + k_q \quad (4)$$

where k_r, k_{nr}, and k_q are the rate constants of radiative, nonradiative, and quenching deactivation (due to presence of any quencher or the microenvironment of Tb(III) in the systems), respectively. Assuming that rate constants k_r and k_{nr} are relatively stable,[69,88] the rate constant k_q controls the lifetime. In order to find the difference of the microenvironment of Tb(III), one can utilize the lifetime of Tb(III) in all the complexes in H_2O and D_2O. The number of coordinated "H_2O" in all the binary and ternary systems using the equation developed by Horrocks et al.[95] is given in Table 5.

Table 5. Luminescence Lifetime of Tb(III) ($^5D_4 \rightarrow {}^7F_5$ Transition) (Monitored at 547 nm) at 298 K in the Different Systems in Aqueous Buffer and D_2O Buffer of pH = 6.8; λ_{exc} = 295 nm

	aqueous buffer[a]		D_2O buffer[a]		
system	τ (μs)	$\chi 2$	τ (μs)	$\chi 2$	q^b
Tb(III) [Tb$(NO_3)_3$]	410	1.10	946	1.15	–
Tb(III) + (+)-C	240	1.18	398	1.09	6.9
Tb(III) + BSA	274	1.08	510	1.03	7.1
Tb(III) + HSA	260	1.10	447	1.15	6.8
Tb(III) + (+)-C + BSA	569	1.10	1069	1.20	3.5
Tb(III) + (+)-C + HSA	526	1.18	1019	1.20	3.9

[a]Error in the analysis of τ = ±5 μs. [b]q is the number of coordinated water molecules calculated using eq 5

$$q = 4.2[\tau_{H2O}^{-1} - \tau_{D2O}^{-1}] \quad (5)$$

where q is the number of H_2O molecules coordinated in the complexes and τ_{H2O} and τ_{D2O} are the lifetimes of Tb(III) in various complexes in H_2O and D_2O buffer, respectively. The values of q calculated thus clearly demonstrate that Tb(III) is shielded from H_2O in the ternary complexes compared to that in the binary complexes. This shielding effect which reduces the vibronic coupling with O–H oscillator could decrease the quenching rate (k_q) of Tb(III) emission in the ternary complexes leading to enhanced emission of Tb(III). It is noted that although (+)-C or (−)-C acts as bidentate ligand for Tb(III) in the binary complex, the value of q obtained in the ternary system suggests that catechin bound to protein acts as monodentate ligand.

In order to find the contribution of synergistic energy transfer, one should modify eq 3 for ET efficiency as

$$\text{ET efficiency} = (A_{acc}/A_{don})[(I_M/I^0{}_M)(q^0{}_M/q_M) - 1] \quad (6)$$

where a correction factor for the enhancement ratio has been incorporated taking quantum efficiency into account (where q_M and $q^0{}_M$ are the quantum yields of the Tb(III) in the presence and in the absence of energy transfer, respectively). The correction factor $q^0{}_M/q_M$ could be replaced by $\tau^0{}_M/\tau_M$ assuming that k_r and k_{nr} are relatively stable in all the systems, where τ_M and $\tau^0{}_M$ are the lifetimes in the presence and the absence of energy transfer. The corrected ET efficiency ratios thus obtained for all the systems for $^5D_4 \rightarrow {}^7F_5$ transition using (+)-C are provided in Table 4. It is noted that although the energy transfer efficiency ratios of the ternary systems compared to the binary complex of (+)-C and Tb(III) (E_T/E_{B1} and $E_{T'}/E_{B1}$ in Table 4) are somewhat less than 1, the ratio compared to that of the binary complex of BSA and Tb(III), i.e., E_T/E_{B2}, is 2.8 (Table 4). The ratio $E_{T'}/E_{B'2}$ is 1.8 for the

Table 4. Energy Transfer Efficiency (E) Ratio of Tb(III) in the Ternary Systems[a]

		E_T/E_{B1}		E_T/E_{B2}		$E_{T'}/E_{B1}$		$E_{T'}/E_{B'2}$	
medium	system (emission band monitored)	from eq 3	from eq 6	from eq 3	from eq 6	from eq 3	from eq 6	from eq 3	from eq 6
aqueous buffer	complex with (+)-C ($^5D_4 \rightarrow {}^7F_5$, 547 nm)	2.0	0.8	6.3	2.8	1.5	0.7	3.9	1.8
	complex with (+)-C ($^5D_4 \rightarrow {}^7F_4$, 491 nm)	1.8	0.8	4.8	2.6	1.3	0.6	3.8	1.7
D_2O buffer	complex with (+)-C ($^5D_4 \rightarrow {}^7F_5$, 547 nm)	3.6	1.3	7.3	3.2	2.5	0.9	6.4	2.6
	complex with (+)-C ($^5D_4 \rightarrow {}^7F_4$, 491 nm)	3.6	1.3	7.2	3.2	2.4	0.9	6.6	2.6

[a]$E_T = E_{[(+)\text{-C+Tb(III)+BSA}]}$, $E_{T'} = E_{[(+)\text{-C+Tb(III)+HSA}]}$, $E_{B1} = E_{[(+)\text{-C+Tb(III)}]}$, $E_{B2} = E_{[BSA+Tb(III)]}$. $E_{B'2} = E_{[HSA+Tb(III)]}$. E in each case is the energy transfer efficiency; the subscript describes the system. Error in the energy transfer efficiency ratio = ±2%

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Table 6. Accessible Surface Area (ASA) (in Å^2) for the Docked Complexes of BSA with (+)/(−)-Catechin[a]

BSA + (+)-catechin[b]			BSA + (−)-catechin[b]		
residues[c]	ASA(protein) (Å^2)	ASA(complex) (Å^2)	residues	ASA(protein) (Å^2)	ASA(complex) (Å^2)
GLU-17	147.8	114.5(33.3)	GLU-17	147.8	94.0(53.8)
LYS-20	58.0	38.1(19.9)	GLU-130	75.7	51.8(23.9)
LEU-24	15.0	0.0(15.0)	LYS-131	165.6	133.4(32.2)
GLU-130	75.7	59.0(16.7)	TRP-134	53.3	13.5(39.8)
LYS-131	165.6	102.8(62.8)	ASN-158	87.7	66.9(20.8)
TRP-134	53.3	19.1(34.2)	ASN-161	40.4	24.4(16.0)
ASN-161	40.4	23.7(16.7)			

[a]Underlined residues are polar residues. [b]The changes in accessible surface area (ΔASA) (in Å^2) are given in parentheses. [c]Residues showing ΔASA ≥ 10 Å^2

Table 7. Accessible Surface Area (ASA) (in Å^2) for the Docked Complexes of HSA with (+)/(−)-Catechin[a]

HSA + (+)-catechin[b]			HSA + (−)-catechin[b]		
residues[c]	ASA(protein) (Å^2)	ASA(complex) (Å^2)	residues	ASA(protein) (Å^2)	ASA(complex) (Å^2)
TYR-150	17.3	3.5(13.8)	LYS-195	91.4	61.7(29.7)
SER-192	28.8	16.7(12.1)	LEU-198	29.0	1.6(27.4)
LYS-195	91.4	45.3(46.1)	TRP-214	61.7	10.7(51.0)
LYS-199	32.0	8.2(23.8)	ARG-218	42.0	19.6(22.4)
TRP-214	61.7	36.6(25.1)	VAL-344	13.2	1.6(11.6)
ARG-218	42.0	11.1(30.9)	ASP-451	30.2	2.2(28.0)
ARG-222	37.1	9.0(28.1)	LEU-481	35.6	14.5(21.1)
ALA-291	37.0	10.9(26.1)			
ASP-451	30.2	14.9(15.3)			

[a]Underlined residues are polar residues. [b]The changes in accessible surface area (ΔASA) (in Å^2) are given in parentheses. [c]Residues showing ΔASA ≥ 10 Å^2

Table 8. Distances of the Different Rings of Isomeric Catechin from Indole Nitrogen of Trp of Serum Albumins

system	residues	5-OH of A ring (Å)	7-OH of A ring (Å)	3′-OH of B ring (Å)	4′-OH of B ring (Å)	3-OH of C ring (Å)
BSA + (+)-C	Trp 134	9.93	9.73	4.99	7.29	7.12
	Trp 213	34.81	35.70	34.26	35.66	35.66
BSA + (−)-C	Trp 134	4.96	6.64	8.92	6.59	9.80
	Trp 213	31.54	27.41	37.69	35.80	37.19
HSA + (+)-C	Trp 214	11.13	10.33	8.06	6.18	8.58
HSA + (−)-C	Trp 214	9.48	11.59	5.64	7.74	9.72

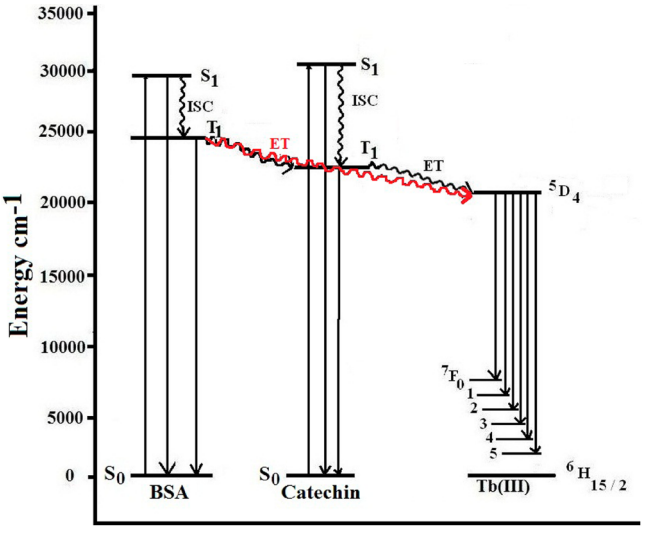

Figure 6. Energy level diagram of different systems.

ternary system containing HSA. This clearly indicates that Tb−C (+/−) interaction is more dominant in the ternary system containing protein in aqueous medium.

Furthermore, it is interesting to note that the value of E_T/E_{B1} becomes 1.3 and E_T/E_{B2} is 3.3 for systems containing BSA in D_2O buffer (Table 4). A similar trend is observed for systems containing HSA (Table 4). These results definitely imply that synergistic ET takes place in the ternary systems in D_2O buffer. Thus, the synergistic effect of ET (Figure 6) as well as an effective protection of Tb(III) from quenching by matrix vibration are responsible for protein-mediated efficient antenna effect in the ternary systems in D_2O medium.

3.7. Analytical Assay of Serum Albumins. The detection limits were determined by calculating the minimum amount of serum albumins that could be significantly determined using the reagent complex ((+)-C with Tb(III)) fluorimetrically in D_2O buffer at physiological pH 6.8 at 298 K. Under optimum condition the calibration curve of luminescence intensity of Tb(III) against concentration of serum albumins added is shown in Figure 8. From the curve it is observed that the limit of detection (LOD) for HSA is about 10×10^{-9} mol L^{-1} (670 ng/mL). The sensitivity is better for BSA, and the LOD is about 6×10^{-9} mol L^{-1} (398 ng/mL). The regression coefficients were always ≥0.98.

3.8. Conclusion. The paper reports the efficient enhancement of Tb(III) emission in a ternary system consisting of two donors (a protein and a ligand, (+)- or (−)-catechin) and an acceptor Tb(III) in aqueous buffer at physiological pH 6.8. The

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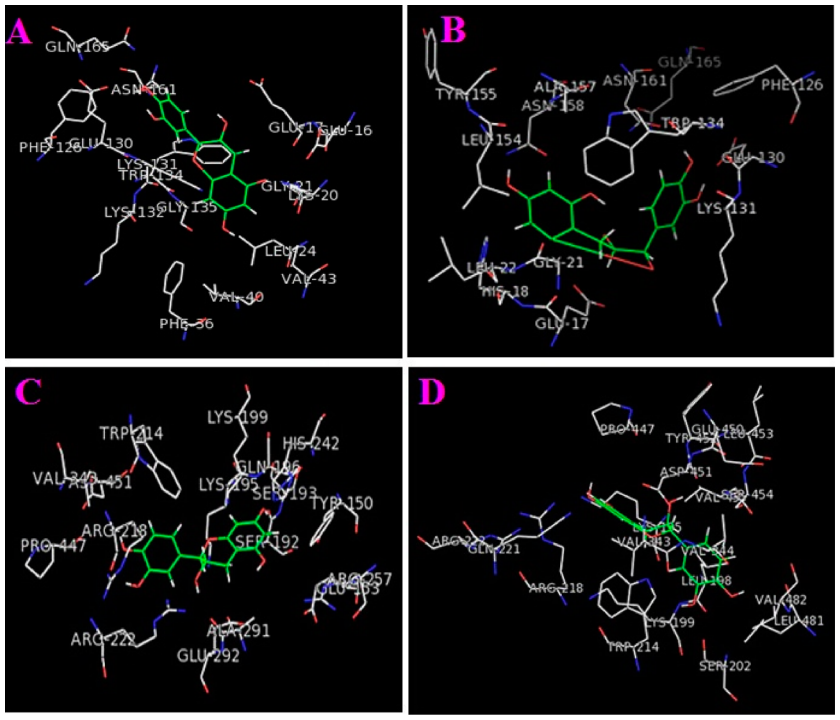

Figure 7. Stereoview of residues of (A) BSA + (+)-C, (B) BSA + (−)-C, (C) HSA + (+)-C, and (D) HSA + (−)-C complexes, the various residues present within 5 Å of the probe.

Table 9. Accessible Surface Area (ASA) (in Å^2) of the (+)/(−)-Catechin and Changes in Accessible Surface Area (ΔASA) (in Å^2) of the Serum Albumins after Complexation

system	ASA of ligand (Å^2)	ΔASA for serum albumins[a] (Å^2)
HSA + (+)-C	76.00	250.2
HSA + (−)-C	95.86	237.3
BSA + (+)-C	99.07	236.7
BSA + (−)-C	108.98	238.6

[a]Total decrease of ASA of the residues involved in the binding.

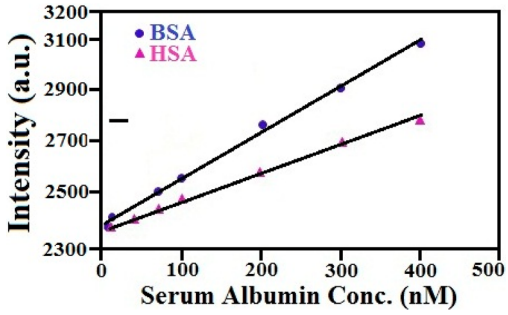

Figure 8. Callibration graphs of BSA and HSA in D_2O buffer at pH 6.8 at 298 K; λ_{exc} = 295 nm, excitation band pass = 10 nm and emission band pass = 5 nm; [(+)-C] = 100 μM, [Tb(III)] = 25 μM. Intensities are calculated in each case using the total area under the band at 547 nm.

steady state and the time-resolved emission studies of each component in the ternary as well as in the binary systems consisting of one donor (either the protein or the ligand) and the acceptor Tb(III) revealed that the enhancement of Tb(III) emission in the ternary system could be due to (i) efficient protection of Tb(III) microenvironment from OH oscillator and (ii) synergistic energy transfer involving both the lowest triplet state of Trp residues of protein and that of catechin. Molecular docking studies of the systems suggest that the Trp 134 of BSA and Trp 214 of HSA act as donor in the ternary systems involving exchange mechanism. Calculation of the accessible surface area of the ligands and the loss of ASA of the residues of proteins involved in the binding together with experimental determination of the number of coordinated H_2O of Tb(III) in the binary and the ternary systems imply that the Tb(III) microenvironment is effectively shielded from O−H oscillator. The calculation of ET efficiency ratio in the ternary system compared to that in the binary systems eliminating the shielding effect by using a modified equation of ET efficiency suggests that Tb−C (+/−) interaction is more dominant in the ternary complex in aqueous buffer medium. However, the results observed in D_2O medium directly prove the synergistic energy transfer from both the lowest triplet states of the Trps in protein and that of catechin. The system provides a unique situation where both the donors absorb in the same region and the lowest $\pi\pi^*$ triplet states of both the donors are suitably placed compared to the $^5D_4$ emissive state of Tb(III) minimizing the possibility of back ET. The simple system consisting of the ligand catechin and Tb(III) in suitable buffer in D_2O at pH 6.8 could be used for efficient bioassay of the albumins.

■ ASSOCIATED CONTENT

Supporting Information

Quenching of emission and enhancement of luminescence emission (Table 1), energy transfer efficiency ratio of Tb(III) (Table 4), and luminescence lifetime of Tb(III) (Table 5). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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