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Targeting the heme protein hemoglobin by (-)-epigallocatechin gallate and the study of polyphenol-protein association using multi-spectroscopic and computational methods

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Supporting Information

S1. Detailed Methodologies

S1.1. UV-vis absorption spectral studies

The absorbance spectroscopic studies were performed using a PerkinElmer Lambda 35 UV-vis spectrophotometer using a quartz cuvette of 1 cm path length in the range of 200–700 nm at 20 mM Tris-HCl buffer (pH 7.4). For the ground state complexation experiment, a 1.5 μM solution of BHb was taken and for complex formation, 4.5 μM of EGCG solution was added. All the UV-vis spectral measurements were carried out at room temperature.

S1.2. Fluorescence spectroscopy

All the fluorescence emission studies were carried out in Fluoromax-4 Jobin Yvon (Horiba Scientific, Japan) except the three dimensional fluorescence studies which were carried out on a Cary Eclipse spectrofluorimeter.

A Fluoromax-4 Jobin Yvon (Horiba Scientific, Japan) spectrofluorimeter equipped with a Newport temperature controller (Model 350 B, California, USA) was utilized for carrying out the temperature dependent fluorescence emission studies using quartz cell of 1 cm path length and a slit width of 5/5 nm with an integration time of 0.3 sec. A 3 μM BHb solution was titrated successively with EGCG solution (0-32.25 μM) at different temperatures (288, 295, 303 and 310 K) by using an excitation wavelength of 295 nm (selective excitation of the Trp fluorophore).

The excited-state lifetime experiments for determining the nature of quenching mechanism involved in the complexation of BHb with EGCG was carried out on a Pico Master Time-correlated single photon counting (TCSPC) lifetime instrument (PM-3) from Photon Technology International (PTI), USA. A 295 nm excitation LED source was used to excite the samples and the corresponding emission decay profiles of the samples were recorded at a magic

angle of 54.7°. The emission wavelength was set up at 337 nm for collecting the data. A colloidal suspension of coffee whitener was used to record the IRF (instrument response function). For this experiment, a 3 μM solution of BHb and its complex with EGCG at a molar ratio of 1:0, 1:2 and 1:4 was taken. The precision of the graphical fits was accessed by (a) visual inspection of the decay profiles, (b) χ^2 values and the Durbin–Watson (DW) parameter. The Levenberg–Marquardt algorithm,[1,2] was used to express the decay profiles as the sum of exponential functions (eqn. S1).

$$F(t) = \sum_i \alpha_i \exp\left(-\frac{t}{\tau_i}\right) \quad \text{S1}$$

where α_i is the amplitude of i^{th} component corresponding to the fluorescence lifetime τ_i. The average lifetime (τ_{avg}) was estimated using the following eqn. S2.[3]

$$\tau_{avg} = \frac{\sum_i \alpha_i \tau_i^2}{\sum_i \alpha_i \tau_i} \quad \text{S2}$$

Three-dimensional (3D) fluorescence measurements was performed on a Cary Eclipse spectrofluorimeter from Agilent Technologies fitted with a PCB 1500 water Peltier system at 295 K. For this experiment, a 3 μM solution of BHb and its 1:4 complex with EGCG were scanned in the emission wavelength range of 250-500 nm with 1 nm interval. Simultaneously, the excitation wavelength was fixed at 200-380 nm with 5 nm increments and a slow scan speed was maintained. The slit width was maintained at 10/5 nm.

S1.3. Circular dichroism (CD) measurements

The secondary structural alterations in the protein on interaction with EGCG were determined through far-UV CD analysis, performed on a JASCO J-1500 instrument with a quartz cuvette

of 0.1 cm path length at room temperature. The data for each spectrum was collected at an interval of 0.1 nm having 100 nm min^{-1} scan speed with a response time of 4 s. For determining structural components of BHb using the DICHROWEB online server,[4] the spectra of 2.5 μM BHb and its complexes with EGCG prepared at different concentrations (5.0, 16.12 and 32.25 μM) were scanned in the range of 190–240 nm. In addition to the online server DICHROWEB, the α-helical content was also determined using the following equations (eqn. S3).[5,6]

$$MRE_{208} = \frac{\theta_{obs}}{C_p \times l \times n \times 10} \quad \text{S3}$$

where θ_{obs} indicates the CD in millidegrees, l is the path length of the cuvette, n is the number of amino acid residues, and C_p is the total concentration of the protein used. Following this, the percentage of the α-helical content of BHb was evaluated considering the MRE value at 208 nm using the following equation (eqn. S4).[7,8]

$$\alpha - helix = \frac{-MRE_{208} - 4000}{33000 - 4000} \times 100 \quad \text{S4}$$

where 33000 is correspond to the MRE value of pure α-helix at 208 nm and 4000 is the MRE value at 208 nm for the β-form and random coil conformation.

S1.4. Fourier transform infrared spectroscopy (FTIR)

The FTIR spectra of native BHb and its complex with EGCG were collected on a PerkinElmer Spectrum Two instrument equipped with a single reflection diamond universal attenuated total reflectance (UATR) accessory having a spectral resolution of 0.5 cm^{-1}. A solution of BHb (10 mg/ mL) was prepared in 20 mM Tris HCl buffer (pH 7.4), and its concentration determined spectrophotometrically, based on its concentration a complex of BHb-EGCG in the molar ratio of 1:2 was prepared and incubated for 2 h before recording of the spectrum. A 256 scan interferogram was used to obtain each of the spectra and then corrected by subtracting the corresponding blank.

S1.5. Preparation of samples for BHb glycation studies

BHb (5 mg/mL) samples were incubated in 20 mM Tris-HCl buffer (pH 7.4) containing 1 mM sodium azide at 37 °C for 3 days in the absence and presence of 20 mM Glyoxal (GO) solution and EGCG (100 μM). The native BHb incubated without GO or EGCG for 3 days under the same condition treated as the control. At different intervals of time, aliquots were taken out of the reaction mixtures for fluorescence studies.

S1.6. UV irradiation (UVI) mediated photo-oxidative damage studies

The photo-oxidative damage studies were carried out according to the procedure described by Pan. et al. (2012).[9] Briefly, samples of BHb (10 μM) in 20 mM Tris-HCL buffer (pH 7.4) were irradiated in a UV sterilizing chamber fitted with a light source of 254 nm having electrical power of 11 W for 60 minutes (at an interval of 10 mins) in the absence and presence of EGCG (100 μM). The fluorescence emission intensity of the samples was recorded in the range of 400-650 nm using an excitation wavelength of 365 nm. To figure out the presence of reactive oxygen species (ROS) responsible for the fluorescence enhancement, the BHb solution and its 1:10 complex with EGCG were incubated in the dark for 10 mins in the presence of 0.1 mM H_2O_2 and then were subjected to UVI treatment as mentioned above.

References

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Supporting Figures

Fig. S1

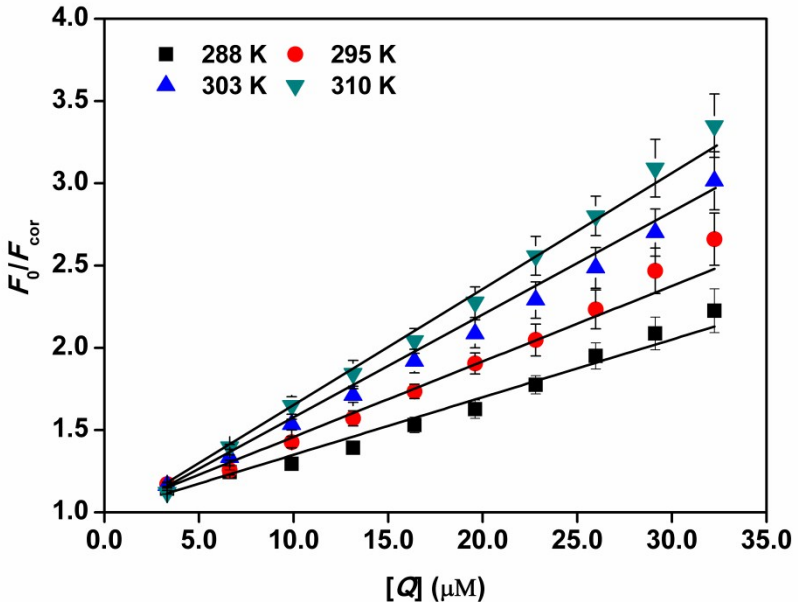

Fig. S1. Stern-Volmer plots for the interaction of EGCG with BHb at different temperatures in 20 mM Tris-HCl buffer of pH 7.4.

Fig. S2

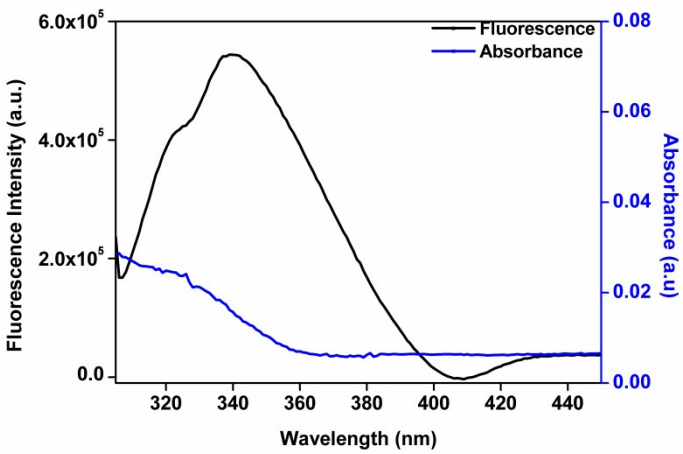

Fig. S2. Spectral overlap between the fluorescence emission of BHb and the absorbance profile of EGCG. [BHb]=[EGCG]= 3 μM.

Fig. S3

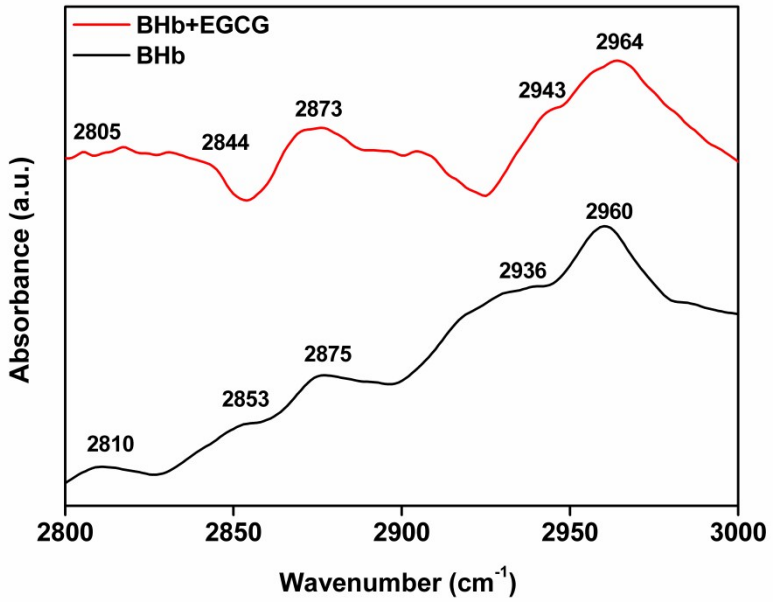

Fig. S3. FTIR spectra of CH_2 and CH_3 stretching vibrations in BHb and its complex with EGCG in the region 3000-2800 cm^{-1}.

Fig. S4

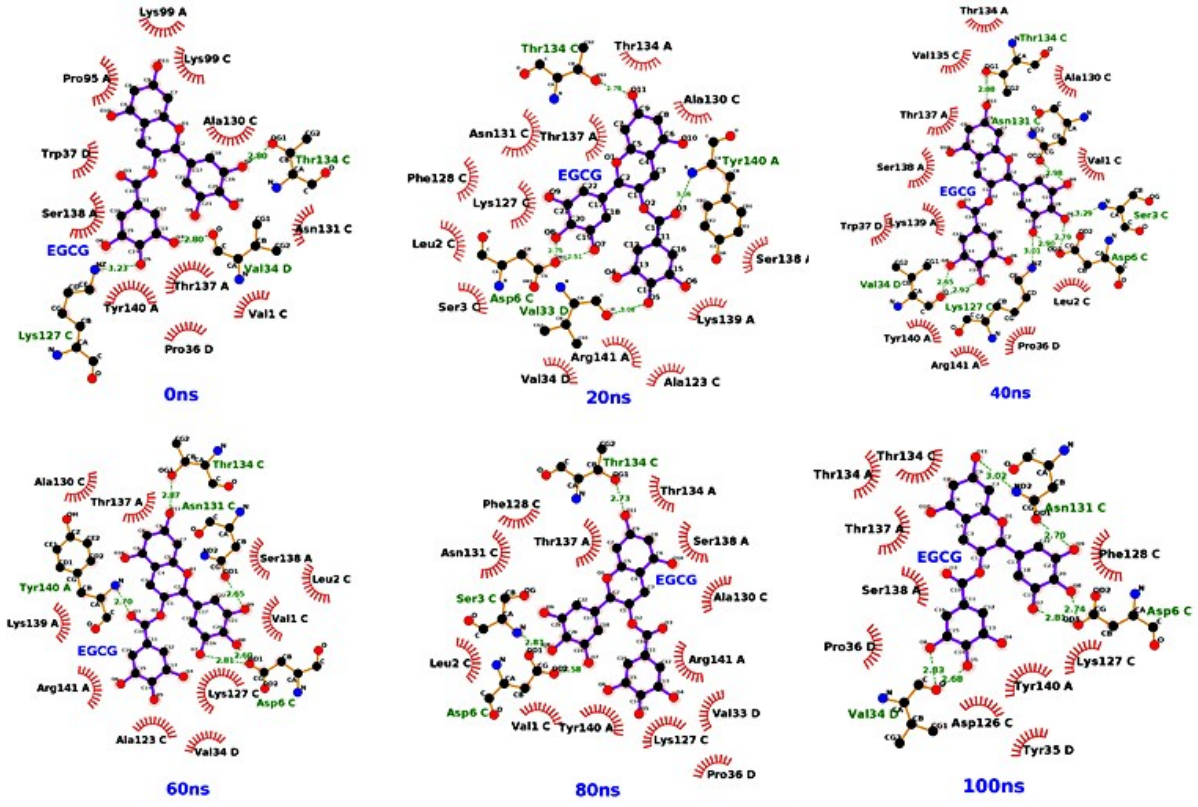

Fig. S4. The 2D interaction maps representing the interaction of EGCG with neighbouring residues of BHb at an interval of 20 ns. α_1-chain= A, α_2-chain= C, β_1-chain= B, β_2-chain= D.

Supporting Tables

Table S1. Stern Volmer parameters for the interaction of EGCG with BHb at different temperatures.

Temp. (K)	K_{SV} (10^4, M^{-1})	k_q (10^{13}, $M^{-1}s^{-1}$)
288	3.50±0.32	2.14
295	4.80±0.43	2.94
303	5.76±0.47	3.53
310	6.90±0.53	4.23

*±standard error

Table S2. Characteristics 3D fluorescence spectral parameters associated with native BHb and its complex with EGCG.

System	Peak 1 (nm)		Stokes shift $\Delta\lambda$ (nm)	Intensity (a.u.)	Peak 2 (nm)		Stokes shift $\Delta\lambda$ (nm)	Intensity (a.u.)
	λ_{ex}	λ_{em}			λ_{ex}	λ_{em}		
BHb	285	333	48	65	235	332	97	43
BHb-EGCG	285	332	47	47	235	332	97	27

Table S3. Comparison of the α-helical percentage (%) content of BHb obtained using equation (eqn. S3-S4) and DICHROWEB.

Complex	**Using eqn. (S3-S4)**	**DICHROWEB (online server)**
BHb	37.46±1.03	54.35±2.25
BHb-EGCG (5.0 μM)	33.74±0.31	49.85±0.25
BHb-EGCG (16.12 μM)	19.49±0.60	36.85±0.15
BHb-EGCG (32.25 μM)	4.63±0.29	17.10±3.40

*±standard error

Table S4. Docking summary of BHb with EGCG by the Autodock 4.2 program generating different ligand conformations with the help of Lamarckian GA.

Rank	Binding energy (kcal mol^{-1})	K_i (μM)	K_b (10^5, M^{-1})	Cluster RMS	Ref. RMS
1	-7.82	1.85	6.12	0.00	65.57
2	-7.76	2.05	5.52	0.00	63.45
3	-7.65	2.47	4.58	0.00	51.61
4	-7.54	2.95	3.80	0.00	50.62
5	-6.96	7.97	1.41	1.52	49.99
6	-7.12	6.03	1.85	0.00	51.36
7	-6.67	12.94	0.86	0.00	45.23
8	-6.66	13.22	0.84	0.00	48.63
9	-5.18	159.97	0.07	0.00	64.91
10	-5.12	178.05	0.06	0.00	74.37

*K_b calculated at 295 K

Table S5. Number of non-bonded interactions involved in BHb-EGCG complexation during the course of simulation time.

Time (ns)	**No. of non-bonded Interactions**	
	Hydrogen-Bond	Hydrophobic Interactions
0	5	17
20	6	14
40	6	12
60	5	15
80	5	16
100	5	17