An investigation into the altered binding mode of green tea polyphenols with human serum albumin on complexation with copper

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Green tea is rich in several polyphenols, such as (−)-epicatechin-3-gallate (ECG), (−)-epigallocatechin (EGC), and (−)-epigallocatechin-3-gallate (EGGC). The biological importance of these polyphenols led us to study the major polyphenol EGC with human serum albumin (HSA) in an earlier study. In this report, we have compared the binding of ECG, EGC, and EGCG and the Cu(II) complexes of EGCG and ECG with HSA. We observe that the gallate moiety of the polyphenols plays a crucial role in determining the mode of interaction with HSA. The binding constants obtained for the different systems are \(5.86 \pm 0.72 \times 10^4\) M\(^{-1}\) \((K_{\text{ECG-HSA}})\), \(4.22 \pm 0.15 \times 10^4\) M\(^{-1}\) \((K_{\text{ECG-Cu(II)-HSA}})\), and \(9.51 \pm 0.31 \times 10^4\) M\(^{-1}\) \((K_{\text{EGCG-Cu(II)-HSA}})\) at 293 K. Thermodynamic parameters thus obtained suggest that apart from an initial hydrophobic association, van der Waals interactions and hydrogen bonding are the major interactions which held together the polyphenols and HSA. However, thermodynamic parameters obtained from the interactions of the copper complexes with HSA are indicative of the involvement of the hydrophobic forces. Circular dichroism and the Fourier transform infrared spectroscopic measurements reveal changes in \(\alpha\)-helical content of HSA after binding with the ligands. Data obtained by fluorescence spectroscopy, displacement experiments along with the docking studies suggested that the ligands bind to the residues located in site 1 (subdomains IIA), whereas EGC, that lacks the gallate moiety, binds to the other hydrophobic site 2 (subdomain IIIA) of the protein.

Keywords: (−)-epicatechin-3-gallate; (−)-epigallocatechin-3-gallate; copper complex; altered mode of binding; fluorescence; docking

1. Introduction

The presence of polyphenols, such as (−)-epicatechin (EC), (−)-epicatechin-3-gallate (ECG), (−)-epigallocatechin (EGC), and (−)-epigallocatechin-3-gallate (EGCG) (Figure 1), in green tea with antioxidant and anticancer potency has made them emerge as an important chemopreventive natural products (Cai et al., 2002; Dreostic, Wargovich, & Yang, 1997). EGCG, the major polyphenol of green tea, has a wide range of biological and biochemical activities such as inhibition of cell proliferation, induction of cell cycle arrest or apoptosis, inhibition of vascular tumor growth and tumor angiogenesis as well as evidence for osteo-protective evidence on the bone mass (Ahmad, Cheng, & Mukhtar, 2000; Fassina et al., 2004; Ferruzzi, 2010; Hastak et al., 2003; Yang, Wang, Lu, & Picinich, 2009). The daily consumption of green tea and coffee has been shown to reduce the possibility of severe chronic and degenerative disease including cardiovascular disorders, obesity, diabetes, and certain neurodegenerative disorders (Shen, Yeh, Cao, Chyu, & Wang, 2011; Wang & Ho, 2009). Green tea polyphenols have been investigated earlier from the laboratory, and reports have revealed the inhibitory effect of green tea crude extract on angiogenin-induced angiogenesis (Maiti, Chatterjee, & Dasgupta, 2003). The ribonucleolytic activity exhibited by the protein (Shapiro, Riordan, & Vallee, 1986) and its structural homology to Ribonuclease A (RNase A) (Russo, Acharya, Vallee, & Shapiro, 1996) prompted us to further investigate the action of the four green tea polyphenols on RNase A (Ghosh, Maiti, & Dasgupta, 2004; Ghosh, Maiti, Debnath, & Dasgupta, 2007). Comparatively better inhibition was achieved from the gallate containing polyphenols (ECG and EGCG) than the nongallate containing polyphenols (EC and EGC). The presence of the gallate moiety has also shown better inhibitory effects on several other enzymes (Chung, Huang, Meng, Dong, & Yang, 1999; Kumamoto, Sonda, Nagayama, & Tabata, 2001; Lee, Shim, & Zhu, 2005; Zhang & Rock, 2004). The role of
the gallate moiety in ECG and EGCG is also important in metal chelation and has been shown to enhance their metal (copper and iron) chelation ability (Hu, 1998). Recent studies with EGCG have shown that it is responsible for the increased biliary cholesterol secretion and phospholipids and its presence reduced the total plasma cholesterol (copper and iron) chelation ability (Hu, 1998). Recent metal chelation and has been shown to enhance their metal (copper and iron) chelation ability (Hu, 1998). Recent metal chelation and has been shown to enhance their metal (copper and iron) chelation ability (Hu, 1998). Recent metal chelation and has been shown to enhance their metal (copper and iron) chelation ability (Hu, 1998). Recent metal chelation and has been shown to enhance their metal (copper and iron) chelation ability (Hu, 1998). Recent metal chelation and has been shown to enhance their metal (copper and iron) chelation ability (Hu, 1998). Recent metal chelation and has been shown to enhance their metal (copper and iron) chelation ability (Hu, 1998).
plotting mole fraction of the metal ion against the absorbance of each solution at 323 and 325 nm for EGCG and ECG, respectively. The breakpoint in the plot corresponding to the copper mole fraction gives the binding stoichiometry of the Cu(II) with EGCG (2:1) and ECG (1:2), respectively (Figure S1) (Harris, 1995).

2.3. Determination of the association constant from the UV spectroscopy

The EGCG–Cu(II) and ECG–Cu(II) complexes were prepared in methanol and diluted with 20 mM phosphate buffer of pH 7 permitting a maximum alcohol content of 5%. A 25 μM of the copper complex was titrated with successive addition of HSA (24 and 22 μM for EGCG–Cu(II) and ECG–Cu(II) complexes, respectively). To obtain the ground state association constant \( K_a \) for a 1:1 complexation, the Benesi–Hildebrand equation was used (Benesi & Hildebrand, 1949).

\[
\frac{1}{\Delta A} = \frac{1}{(c_b - c_i)L_T} + \frac{1}{(c_b - c_i)L_T K_a M}
\]

where \( c \) is the extinction coefficient, the subscripts b, f, and T denote bound, free, and total ligand, \( L_T \) is the polyphenol–Cu(II) complex concentration, and \( \Delta A \) is the change in absorbance at a particular wavelength. The association constant, \( (K_a) \), for complex formation is obtained from the ratio of the intercept to the slope of the double-reciprocal plot of absorbance change vs. HSA concentration.

2.4. Fluorescence spectroscopy

Fluorescence spectra were recorded on Hitachi-850 and Horiba Jobin Yvon (Fluoromax-4) spectrofluorometers using an excitation wavelength of 295 nm. The excitation and emission bandwidths were 5 nm. Quantitative analysis of the interaction between protein and ligands was performed using a fluorimetric titration method by successive addition of ligands.

Three mL of 2 μM HSA was titrated with successive addition of the copper complexes (0–16 μM) at four different temperatures (20, 26, 32, and 38 °C) under experimental conditions. Blank spectra were collected under the same experimental conditions and subtracted from the HSA bound copper complex spectra in each case. The type of fluorescence quenching can be explained using the Stern–Volmer equation.

\[
\frac{F_0}{F} = 1 + K_q r_0 [Q] = 1 + K_{SV} [Q]
\]

where \( K_q \) is the bimolecular quenching constant, \( r_0 \) (5 ns) is the lifetime of the fluorescence in the absence of quencher, \([Q]\) is the quencher concentration, and \( K_{SV} \) is the Stern–Volmer quenching constant. From the quenching parameters the mode of quenching can be easily assigned (Lakowicz, 2006).

The binding constant \( (K_b) \) and the binding stoichiometry \( (n) \) for the interaction of ECG have been calculated at four different temperatures from the fluorescence-quenching data using Scatchard plots (Scatchard, 1949) according to Equation (3).

\[
\frac{r}{D_i} = n K_b - r K_b
\]

where \( r \) is the number of moles of ligand bound per mole of protein and \( D_i \) is the molar concentration of free ligand.

The following double-logarithm Equation (4) was used to calculate the binding constant \( (K_b) \) of interactions in the binding of copper complexes with HSA (Hu, Liu, Wang, Xiao, & Qu, 2006).

\[
\log \frac{\Delta F}{F} = n \log [Q] + \log K_b
\]

where \( \Delta F = F_0 - F; F_0 \) and \( F \) are the fluorescence intensities of HSA in the absence and presence of the complexes, respectively, \( n \) is the number of binding sites, and \( K_b \) is the equilibrium-binding constant. The values of \( K_b \) are estimated by plotting \( \log \frac{\Delta F}{F} \) vs. \( \log [Q] \).

The modified Stern–Volmer quenching constant \( (K_A) \) and the fraction of fluorophore accessible to the quencher \( (f_k) \) were also obtained from the fluorescence-quenching data by applying the modified Stern–Volmer equation (Lakowicz, 2006) according to Equation (5).

\[
\frac{F_0}{\Delta F} = \frac{1}{f_k} + \frac{1}{[Q] f_k K_A}
\]

where \( \Delta F = F_0 - F; F_0 \) and \( F \) are the relative fluorescence intensities in the absence and presence of the quencher, respectively, and \([Q]\) is the concentration of quencher. If it is assumed that the enthalpy change \( (\Delta H^o) \) does not vary significantly over the experimental temperature range, then \( \Delta H^o \) and \( \Delta S^o \) can be calculated using the van’t Hoff Equation (6).

\[
\log K_b = \frac{\Delta H^o}{2.303RT} + \frac{\Delta S^o}{2.303R}
\]

where \( K_b \) is the binding constant at corresponding temperature and \( R \) is the universal gas constant. The free energy of binding is thus estimated from \( \Delta H^o \) and \( \Delta S^o \) using the following relationship:

\[
\Delta G^o = \Delta H^o - T \Delta S^o
\]
2.5. **Fluorescence resonance energy transfer (FRET)**

The Förster nonradiative energy transfer theory is useful in many biological systems to determine the distance ($d$) between the donor and the acceptor (Hong, Lei, Kong, Chen, & Hu, 2004). The energy transfer depends on a multitude of factors, such as the essential fluorescence of the donor and the acceptor less than 7 nm and an overlap of the fluorescence emission spectrum of the donor with the absorption spectrum of the acceptor. The extent of energy transfer also depends on the critical distance between the donor and the acceptor. The efficiency of energy transfer can be obtained from the following equation:

$$ E = 1 - \frac{F}{F_0} = \frac{R_0^6}{R_0^6 + d^6} $$  \hspace{1cm} (8)

where $R_0$ is the Förster distance at which 50% of the excitation energy is transferred to the acceptor and $d$ is the separation distance between the donor and the acceptor.

$$ R_0^6 = 9.78 \times 10^{10} \left[ (\kappa^2 n^{-4} Q_D J(\lambda)) \right] $$  \hspace{1cm} (9)

where $Q_D$ is the quantum yield of the donor in the absence of acceptor, $n$ is the refractive index of the medium, and $J(\lambda)$ is the overlap integral of the emission spectra of donor and absorption spectra of the acceptor. $\kappa^2$ is the relative orientations in space of the transition dipoles of the donor and the acceptor. The overlap integral, $J(\lambda)$, is presented by the following equation:

$$ J(\lambda) = \frac{\int F(\lambda) \varepsilon(\lambda) \lambda^2 d\lambda}{\int F(\lambda) d\lambda} $$  \hspace{1cm} (10)

where $F(\lambda)$ is the corrected fluorescence intensity of the donor in the range of $\lambda$ to $\lambda + \Delta\lambda$ with the total intensity normalized to one, with the quantity $F(\lambda)$ dimensionless and $\varepsilon(\lambda)$ is the molar extinction coefficient of the acceptor at $\lambda$ nm.

2.6. **Site marker displacement studies**

HSA (2 $\mu$M) was saturated with ANS (6 $\mu$M) as described by Mahesha et al. (Mahesha, Singh, Srinivasan, & Rao, 2006) in 20 mM phosphate buffer pH 7.0 and the experiment was conducted with the addition of each of the ligands, namely, ECG, EGC, EGCG, ECG-Cu(II), and EGCG-Cu(II). The excitation wavelength for ANS-bound HSA was set at 375 nm. For warfarin displacement studies, excitation wavelength for warfarin-bound HSA was set at 308 nm. To determine the binding affinity of the ECG–Cu(II) and EGCG–Cu(II) complexes with the protein in the presence of the site markers, warfarin, and ibuprofen, we have conducted the fluorescence-quenching study at 26 °C as described earlier. Blank titrations with only ligands were carried out for the displacement experiments and corrected for the dilution.

2.7. **Circular dichroism (CD) measurements**

All far-UV CD experiments were carried out on a Jasco 810 automatic spectrophotometer at room temperature using a 0.1 cm cell path length under the experimental conditions. The spectra were recorded in the range of 190–240 nm with a scan speed of 50 nm/min and a response time of 4 s. Three sets of solutions containing HSA:ligand in 1:0, 1:1, and 1:2 molar concentrations ratios were prepared at pH 7. The concentration of HSA was 2 $\mu$M. The secondary structure was analyzed with the help of DICHROWEB (Whitmore & Wallace, 2004), an online server for protein secondary structural analyses from CD measurements. To determine the effect of the copper complexes on the secondary structure of HSA, near-UV CD measurements at pH 7 were carried out at room temperature using a 1.0 cm cell path length in the wavelength range of 250–320 nm with a scan speed of 100 nm/min. The concentrations of HSA and the copper complexes were kept at 100 $\mu$M.

2.8. **Fourier transform infrared (FTIR) spectroscopy**

FTIR spectral measurements for the interaction of the copper complexes with HSA were carried out on a Nexus 870 FTIR spectrometer (Thermo Nicolet Corporation) equipped with a zinc selenide attenuated total reflectance accessory, a deuterated triglycine sulfate detector, and a KBr beam splitter at room temperature. HSA (15 mg/mL) was dissolved in 20 mM phosphate buffer of pH 7 giving a final concentration of 0.23 mM. Three sets of solutions were prepared containing HSA: ligand molar ratios corresponding to 1:0, 1:1, and 1:2. A 256-scan interferogram with 4 cm$^{-1}$ resolution was used for the collection of the spectra after an incubation period of 2 h. Blank spectra were collected under the same experimental conditions and subtracted to get the difference spectra for each set.

The method of Byler and Susi (1986) were used to determine the secondary structure content of HSA and HSA–ligand complexes from FTIR studies. The secondary structural components were estimated using the position of the amide I band (1700–1600 cm$^{-1}$). To minimize noise, the corrected spectra were smoothed by a 13-point Savitsky–Golay smooth function (Byler & Susi, 1986). The Fourier self-deconvolution and the second derivative calculation of the smoothed spectra were used to resolve the major peaks. The Gaussian curve-fitting method was used in the region 1700–1600 cm$^{-1}$ to calculate the total area and the area corresponding to each secondary structural component.
2.9. 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The antioxidant capacity of the catechins and their copper complexes were determined in the absence and presence of HSA based on their DPPH radical scavenging activity. HSA was incubated with each ligand prior to the DPPH assay maintaining the HSA and ligand concentrations at 30 and 15 \(\mu\)M, respectively. The absorbance of DPPH (0.1 mM) was measured at 517 nm in the absence and presence of different ligands and protein–ligand systems after incubation in the dark for 10 min. The DPPH radical scavenging activity was estimated using the following Equation (11).

\[
\%\text{-DPPH scavenged} = \left[1 - \frac{A(\text{test})}{A(\text{control})}\right] \times 100
\]

where \(A(\text{test})\) is the absorbance of the individual sample set and \(A(\text{control})\) is the absorbance in the absence of any reagent.

2.10. Docking studies

The crystal structures of HSA (PDB ID: 1AO6) were downloaded from the Protein Data Bank (Berman et al., 2000). The 3D structures of ECG and EGC were generated in Sybyl 6.92 (Tripos Inc., St. Louis, USA) and their energy-minimized conformations were obtained with the help of the MMFF94 force field using MMFF94 charges with a gradient of 0.005 kcal/mol. The iteration number was taken to 1000 with all other default parameters. The FlexX software as part of the Sybyl suite was used for docking of the ligands to HSA. PyMol (DeLano, 2004) was used for visualization of the docked conformations.

The accessible surface area (ASA) of all the amino acid residues in uncomplexed HSA and those in the HSA-polyphenol docked complexes were calculated using NACCESS (Hubbard & Thornton, 1993). The structure of the ligands corresponding to the final docked conformation was chosen and composite coordinates were generated to form the docked complex. The change in ASA of the \(i\)th residue was calculated using the expression, \(\Delta\text{ASA}_i = \text{ASA}_i^{\text{HSA}} - \text{ASA}_i^{\text{HSA-ligand}}\). If a residue lost more than 10 Å² ASA on going from the uncomplexed to the complexed state, it was considered as being involved in interaction.

3. Results and discussion

3.1. Ground state association constant by UV–vis spectroscopy

The absorption spectra of the EGCG–Cu(II) and ECG–Cu(II) complex with an increase in concentration of HSA are shown in Figure 2(a) and (b), respectively. Addition of HSA to the copper complexes results in a decrease in the absorbance value and the isobestic points are at 295 and 291 nm for EGCG–Cu(II) and ECG–Cu(II) complexes, respectively. These observations point toward a ground state complexation between the HSA and the copper complexes. No such spectral shift was observed in the absorption maxima of the copper complexes after the addition of HSA. The ground state association constants \((K_a)\) were calculated from the Benesi–Hildebrand equation as given in the inset of Figure 2(a) and (b). The values were found to be \((6.44 \pm 1.41) \times 10^5 \text{M}^{-1} \left(R^2 = 0.987\right)\) and \((5.61 \pm 0.29) \times 10^4 \text{M}^{-1} \left(R^2 = 0.971\right)\) for the interactions of the EGCG–Cu(II) and ECG–Cu(II) complexes with HSA, respectively. The possible reason of higher association constant observed for the EGCG–Cu(II) complex has been discussed later.
3.2. Fluorescence-quenching mechanism

The fluorescence emission spectra of HSA in the absence and presence of polyphenols and their copper complexes are shown in Figure 3. The strong fluorescence emission of Trp 214 gradually decreased on addition of ECG [similar to our previous study with EGCG reported in this journal (Maiti et al., 2006)]. This gives a primary indication of interaction of ECG with HSA. A distinct red shift of the wavelength maxima of the emission spectra of HSA was also observed on addition of ECG [same as EGCG (Maiti et al., 2006)]. The red shift is likely due to the presence of the polar moiety, which may shift the fluorophore to a more polar environment. Therefore, it may be possible that the polar phenolic OH groups of ECG and EGCG are in the proximity of Trp 214. The almost unaltered fluorescence intensity of HSA on addition of the nongallate-containing polyphenol, EGC, indicates that the gallate moiety primarily takes part in the tryptophan quenching process. In the case of the copper complexes of ECG and EGCG, due to the involvement of the gallate moiety in the chelation of copper, the mode of their interactions with HSA is expected to be altered. Representative fluorescence emission spectra of HSA in the absence and presence of ECG-Cu(II) and EGCG-Cu(II) at 299 K are shown in Figure 3(c) and (d). The gradual decrease in fluorescence intensity of HSA on addition of the copper complexes is indicative of specific interactions with HSA. A similar observation was noted in the interaction of bilirubin with HSA in the presence of aspirin (Hosainzadeh, Gharanfoli, Saberi, & Chamani, 2012). The blue shift in emission maxima ($\lambda_{em}$) of HSA upon addition of both the copper complexes indicates the presence of hydrophobic forces in the binding processes (Hosainzadeh et al., 2012; Lakowicz, 2006). Therefore, it is likely that for free ECG and EGCG, the red shift of the HSA emission maxima is attributed to the polyhydroxy gallate part, which in the case of the complex is less available due to its involvement in metal chelation.

Fluorescence quenching occurs through different possibilities, such as: (i) ground or excited state complexation between donor and acceptor, (ii) molecular rearrangements, (iii) energy transfer, and (iv) collisional quenching (Lakowicz, 2006). Static quenching refers to the complex formation between the quencher and the fluorophore in the ground state. On the other hand, dynamical quenching happens when a transient contact between the quencher and the fluorophore occurs in the excited state. Dynamical quenching is differentiated from a static one with the help of temperature and viscosity measurements (Lakowicz, 2006). If an increase in temperature causes an enhancement of $K_S$, this indicates the involvement of dynamical quenching as it depends on diffusion.

![Figure 3](image-url)
To analyze the quenching mechanism (static or dynamic) of the interactions of the copper complexes with HSA, the Stern–Volmer plot is used. The nonlinear nature of the Stern–Volmer plot in both the cases (Figure S2) is either the result of a combination of static plus dynamical quenching or due to the higher concentrations of the ligands around the fluorophore (Lakowicz, 2006). The rise in temperature causes a decrease in the Stern–Volmer constants (Tables S1 and S2) indicating a static quenching mode in the binding of the copper complexes with HSA (Chi & Liu, 2011; Kabiri, Amiri-Tehrani-zadeh, Baratian, Saberi, & Chama-ni, 2012; Sandhya, Hedge, Kalanur, Katrahalli, & Seetharamappa, 2011; Teng, Liu, Li, Xia, & Zhang 2011). Again the quenching constants ($K_q$) were found to be in the order of $10^{13}$ M$^{-1}$s$^{-1}$, which is greater than $2 \times 10^{10}$ M$^{-1}$s$^{-1}$, the largest possible value of $K_q$ for dynamical quenching reported in the literature, clearly indicating the key role of the static quenching mechanism (Naveenraj, Raj, & Anandan, 2012). The UV–vis study reveals the ground state complexation between the HSA and the copper complexes (Figure S3), which supports the presence of static quenching mode in the binding processes (Chen, Huang, Xu, Zheng, & Wang, 1990; Hu, Yu, Dong, Yang, & Liu, 2006; Zhang, Chen, Zhou, Shi, & Wang, 2011).

Considering a model which involves both modes of quenching, a modified Stern–Volmer equation is obtained (Lakowicz, 2006):

$$F_0/F = (1 + K_D[Q])(1 + K_s[Q])$$

$$\left[\frac{F_0}{F} - 1\right]/[Q] = (K_D + K_s) + K_D K_s [Q]$$

where $K_s$ and $K_D$ are the static and dynamic quenching constants, respectively, and $[Q]$ is the quencher concentration. The values of $K_S$ and $K_D$ were obtained by plotting $[F_0/F - 1]/[Q]$ vs. $[Q]$ (Figure S4). The $K_S$ (static quenching constant) and $K_D$ (dynamic quenching constant) can be estimated for the interaction from this equation. The $K_D$ values calculated are found to be imaginary implying that the assumption was incorrect in this context. This, therefore, ruled out the possibility of a dynamic quenching mechanism. Thus, the nonlinearity and upward curvature of the Stern–Volmer plot is likely to be due to the presence of either more than one binding site or a high quencher concentration surrounding the fluorophore present in the system (Ghosh et al., 2008; Lakowicz, 2006). Recently we have published a paper containing the interactions of quercetin–Cu(II) complex with serum albumins, where a similar type of result was obtained (Singha Roy, Tripathy, Ghosh, & Dasgupta, 2012).

### 3.3. Binding affinity

To estimate the binding affinity of the polyphenols and their copper complexes to HSA by a static quenching mechanism, the Modified Stern–Volmer equation was considered. The plots for the interactions of the copper complexes with the protein at different temperatures are presented in Figure S5. The increase in temperature causes a decrease in binding affinity of the polyphenols [EGCG and EGCG (Maiti et al., 2006)], whereas the copper complexes show opposite trends. Results from both the modified Stern–Volmer equation and the binding analysis using Equations (3) and (4) are summarized in Table 1. The decrease in binding constants with an increase in temperature suggests the involvement of weak interacting forces during the complexation process taking place between the green tea polyphenols and the HSA, which become even weak on increase in temperature. The linear nature of the binding plots indicates that the ligands bind to a single class of sites in HSA (Figure S6). For EGCG, as there is negligible quenching with almost overlapping spectra at different ligand concentrations (Figure 3), the calculation of binding parameters was not possible. The number of binding sites for the interactions of the copper complexes with the proteins obtained are all greater than one (Table 1), suggesting a nonlinear nature of the Stern–Volmer plots at different temperatures that has been obtained. The gallate containing tea catechins are very good metal ion chelators. The bound metal is coordinated with the polyphenol through the –OH moieties with an acetate and water occupying the other coordination sites. This makes available further possibilities for the interactions with proteins most likely by additional hydrogen bonds. This accounts for the greater stability of the EGCG–Cu complex compared to EGCG alone. The binding affinity of EGCG–Cu(II) complex to HSA was found to be greater than other ligands (Table 1). The larger and bulkier size of the complexes formed between the HSA and the Cu(II) complexes (HSA–EGCG–Cu(II) or HSA–EGCG–Cu(II)) also accounts for the higher stability as seen for the green tea polyphenols with β-casein and β-lactoglobulin (Hasni et. al., 2011; Kanakis et al., 2011). Apart from this, an increase in the helical content of HSA, as observed from CD and FTIR studies, on the interaction with the complexes accounts for a structural stabilization. This may be due to the presence of hydrophobic forces since site displacement studies reveal that site 1, a hydrophobic binding site, is involved.

### 3.4. Binding modes

The changes in enthalpy ($\Delta H^o$) and entropy ($\Delta S^o$) were estimated to investigate the binding pattern of the ligands with HSA with the help of the van’t Hoff equation (Figure 4(a)). The values of thermodynamic parameters for the interaction of ECG and the copper complexes are
summarized in Table 2. According to Ross and Subramanian (1981), the negative sign of $\Delta H^\circ$ and positive sign of $\Delta S^\circ$ are indicative of an electrostatic interaction with partial immobilization of the protein and the ligand. This occurs in an initial step involving hydrophobic association that results in a positive $\Delta S^\circ$ value. Again both the positive values of $\Delta H^\circ$ and $\Delta S^\circ$ indicate the involvement of hydrophobic interaction in the binding process. The polyphenols, ECG and EGCG (Maiti et al., 2006), bind to HSA via an initial hydrophobic association. The involvement of the phenolic –OH groups in the chelation of copper renders the complex relatively more hydrophobic in nature, thus resulting in positive values for $\Delta H^\circ$ and $\Delta S^\circ$. This hydrophobic nature (decrease in polarity around Trp and Tyr residues) has also been reflected in the blue shift that occurs during Trp fluorescence quenching in the case of the copper complexes. This kind of blue shift due to hydrophobicity around the fluorophore was also observed in the case of the interaction of fluoxymesterone with HSA (Zohoorian-Abbootorabi, Sanee, Iranfar, Saberi, & Chamani, 2012). In the interacting complex with the polyphenols, the negative $\Delta H^\circ$ contribution comes from van der Waals interactions and hydrogen bonding between the protein and the ligands.

Table 1. Binding parameters for binding of HSA with ECG, ECG–Cu(II), and EGCG–Cu(II).

| Temp (K) | $K_a$ (10$^4$ M$^{-1}$) | $K_b$ (10$^4$ M$^{-1}$) | $n$ | $K_s$ (10$^4$ M$^{-1}$) (modified Stern-Volmer) |
|---------|----------------|----------------|---|----------------|
| 293     | 6.56 ± 0.19  | 2.34 ± 0.18 | 3.24 ± 0.21 | 6.86 ± 0.72 |
| 299     | 4.84 ± 0.32 | 11.45 ± 0.54 | 4.84 ± 0.54 | 8.57 ± 0.21 |
| 303     | 4.81 ± 0.32 | 6.45 ± 0.21 | 4.01 ± 0.07 | 5.86 ± 0.21 |
| 305     | 4.81 ± 0.32 | 6.45 ± 0.21 | 4.01 ± 0.07 | 5.86 ± 0.21 |
| 308     | 4.01 ± 0.07 | 8.09 ± 0.47 | 4.01 ± 0.07 | 8.09 ± 0.47 |
| 311     | 3.25 ± 0.15 | 13.81 ± 1.82 | 3.25 ± 0.15 | 13.81 ± 1.82 |
| 313     | 3.25 ± 0.15 | 13.81 ± 1.82 | 3.25 ± 0.15 | 13.81 ± 1.82 |

Figure 4. (a) Temperature dependence of binding constants (Van’t Hoff plot) for the interactions of ECG, ECG–Cu(II), and EGCG–Cu(II) with HSA. (b) The bar diagram presenting the contributions from the different thermodynamic parameters.
The decrease in $\Delta G^\circ$ values in all the cases with the increase in temperature implies the spontaneity of the enthalpy and entropy driven binding of ECG (or EGCG) and the copper complexes with HSA as represented in the bar diagram (Figure 4(b)). The interaction of the copper complex of quercetin with the serum albumins also provided such kind of hydrophobic forces as reported from our group recently (Singha Roy et al., 2012).

3.5. Energy transfer

The spectral overlap between the fluorescence emission spectrum of the protein with the absorption spectrum of all the ligands is given in the supplementary section (Figure S7) and the corresponding energy transfer parameters have been summarized in Table 3. Using the values of $\kappa^2$, $n$, and $Q_D$ as 2/3, 1.336, and 0.118, respectively, the energy transfer parameters were calculated (Lakowicz, 2006). It was noticed that all the distances were less than 7 nm and they were in the range $0.5R_0 < d < 2R_0$, indicating the higher possibility of energy transfer from the Trp 214 to the ligands (Lakowicz, 2006). As expected, there exists a direct correlation between the binding constant ($K_b$) values and the distance ($d$) between the donor and the acceptor. These observations are in good correlation with the binding affinity of the individual systems as observed in the case of the interaction of ropinirole hydrochloride with HSA (Kabiri et al., 2012).

3.6. Site of interaction of ligands

To determine the site of interaction of the ligands within the protein molecule, displacement studies of some site-specific markers were performed. 8-Anilino-1-naphthale sulfonic acid ANS binds to the hydrophobic subdomains (IIA and IIIA) of HSA. The displacement of ANS by ECG and EGCG (Figure 5(a) and (b)) indicates that ANS and the ligands bind to one common site that is a hydrophobic pocket (IIA and/or IIIA) of HSA. The displacement of ANS by copper complexes (Figure 5(c) and (d)) also indicates that ANS and the copper complexes bind to the same site in the protein. However, for EGC, the displacement of ANS is negligible from the HSA–ANS complex that corroborates the previous observation of no quenching of the fluorescence intensity of Trp 214 of native HSA (Figure 5(e)). Hence, it is likely that it occupies the other hydrophobic pocket (site 2 of subdomain IIIA).

Displacement of ANS by ECG, EGCG, and their copper complexes also indicates the role of hydrophobic interactions in the complexation process, which has been substantiated by the thermodynamic parameters. To confirm which hydrophobic site (IIA or IIIA) the ligands occupy, warfarin displacement studies were also done. Warfarin itself has a weak fluorescence, which is enhanced on binding to HSA. The fluorescence intensity

Table 2. Thermodynamic parameters for binding of HSA with ECG, ECG–Cu(II), and EGCG–Cu(II).

| Temp (K) | $\Delta G^\circ$ (kJ/mol) | $\Delta H^\circ$ (kJ/mol) | $\Delta S^\circ$ (J/mole K) |
|---------|---------------------------|--------------------------|---------------------------|
| 293     | $(-26.76 \pm 0.19)$       | $(-27.91 \pm 0.39)$      | $-22.79 \pm 1.53$         |
| 299     | $(-24.85 \pm 0.18)$       | $(-26.87 \pm 0.19)$      | $-22.42 \pm 1.58$         |
| 303     | $(-27.22 \pm 0.11)$       | $(-27.48 \pm 0.24)$      | $-27.55 \pm 0.21$         |
| 305     | $(-27.04 \pm 0.11)$       | $(-27.53 \pm 0.21)$      | $-27.74 \pm 0.19$         |
| 308     | $(-27.13 \pm 0.16)$       | $(-27.48 \pm 0.24)$      | $-28.71 \pm 0.13$         |
| 311     | $(-27.13 \pm 0.16)$       | $(-27.74 \pm 0.24)$      | $-28.71 \pm 0.13$         |
| 313     | $(-27.13 \pm 0.16)$       | $(-27.74 \pm 0.24)$      | $-28.71 \pm 0.13$         |
Table 3. FRET parameters of HSA with ligands.

| Ligand       | $J \times 10^{-15}$ M$^{-1}$ cm$^3$ | $R_0$ (nm) | $d$ (nm)  | $E$   |
|--------------|----------------------------------|------------|-----------|-------|
| ECG          | 16.94 ± 3.16                     | 2.67 ± 0.08| 3.36 ± 0.16| 0.21 ± 0.02 |
| EGCG         | 4.32 ± 0.45                      | 2.13 ± 0.03| 2.71 ± 0.08| 0.19 ± 0.01 |
| ECG–Cu(II)   | 10.47 ± 0.08                     | 2.47 ± 0.02| 3.58 ± 0.03| 0.11 ± 0.04 |
| EGCG–Cu(II)  | 7.35 ± 0.21                      | 2.33 ± 0.01| 2.26 ± 0.29| 0.16 ± 0.05 |

Figure 5. Displacement of bound ANS from HSA–ANS complex by (a) ECG, (b) EGCG, (c) ECG–Cu(II), (d) EGCG–Cu(II), and (e) EGC. [HSA]: 2 μM; [ANS]: 6 μM; [ligand]: 0–16 μM; and $\lambda_{ex}$: 375 nm. Arrows indicate the increase in ligand concentration.
of bound warfarin to HSA is reduced if a second ligand competes for the same site in the protein. In Figures S8a and S8c, the decrease in fluorescence intensity of bound warfarin on addition of ECG and EGCG confirms that they specifically bind to site 1 of HSA. There is no quenching of HSA-bound warfarin on addition of EGC as depicted in Figure S8b. This ruled out the possibility of EGC to interact with site 1. Therefore, the small ANS quenching observed can be explained by its affinity toward site 2. In Figures S7d and S7e, the decrease in fluorescence intensity of HSA-bound warfarin on addition of the copper complexes confirms that they also bind to site 1 of the protein. Ibuprofen, another site-specific marker, is known to bind specifically to site 2 (subdomain IIIA) of HSA. The quenching of the fluorescence intensity of bound ibuprofen on addition of EGC (Figure S9) confirms its proximity to site 2 of HSA.

The effect of site markers on the binding of the copper complexes has also been monitored. The corresponding fluorescence emission spectra are given in Figure 6 (a) and (b). Fluorescence-quenching techniques were followed to measure the binding constants ($K_b$) of the copper complexes with the protein in the presence of the site markers. In both the cases, the binding constants decreased in the presence of warfarin with no such changes observed in the presence of ibuprofen for the binding constants of the interactions (Table S3). These results clearly revealed that the copper complexes bind to the hydrophobic site 1 (subdomain IIA) near Trp 214. Warfarin and ibuprofen bind to different hydrophobic pockets of HSA (subdomains IIA and IIIA, respectively). In the site selectivity studies, thermodynamic parameters in correlation with FTIR analyses (discussed later) suggest that the polyphenol–Cu(II) complexes interact with HSA mainly via hydrophobic forces. This is evident from the blue shift in the emission spectra of HSA after the addition of the polyphenol–Cu(II) complexes, whereas the addition of ECG and EGCG (Maiti et al., 2006) causes a red shift. The binding of the copper complexes near Trp 214 (subdomain IIA) thus increase the hydrophobicity around the chromophore as in the case of the interaction of bilirubin with HSA, where a similar observation has been made (Hosainzadeh et al., 2012).

HSA possesses two specific Cu(II) binding sites: (i) the N-terminal sites (NTS) and (ii) the multimetal binding sites (Bal, Christodoulou, Sadler, & Tucker, 1998). The NTS of HSA comprised of the first three amino acids (Asp–Ala–His) has a higher affinity for Cu(II) than the multimetal site. Cu(II) mainly binds at the NTS site of HSA (or BSA) via a square planar geometry and the mode of interaction is hydrophilic in nature. The EGCG–Cu(II) and ECG–Cu(II) complexes due to their larger sizes stabilize the protein structure by increasing the helical content similar to the effect of tea polyphenols on milk β-lactoglobulin (Kanakis et al., 2011).

![Figure 6](image_url)

**Figure 6.** Displacement of bound warfarin from HSA–warfarin complex by (a) EGCG–Cu(II) and (b) ECG–Cu(II). [HSA]: 2 μM; [warfarin]: 2 μM; [ligand]: 0–16 μM; and $\lambda_{ex}$: 295 nm. Arrows indicate the increase in ligand concentration.
3.7. CD results

The CD spectra of HSA exhibit two negative bands at 208 nm (π→π* transition) and 222 nm (n→π* transition). The far-UV CD of HSA in the presence of the polyphenols and their copper complexes is shown in Figures 7 and S10. Green tea polyphenols cause a decrease in the α-helical content of HSA from 54 to 49% and 52% at 1:2 M ratios after binding with ECG and EGC, respectively. A similar result was observed in the interaction of EGCG with HSA as published earlier (Maiti et al., 2006). However, the % α-helix increases from 52 (free HSA) to 54 and 56 on 1:2 binding with the EGCG–Cu(II) and ECG–Cu(II) complexes, respectively. In the ECG–Cu(II) complex, two ECG moieties are involved; hence, there is a chance of better stabilization of the helix compared to EGCG–Cu(II) complex. Hence, we conclude that the binding of the polyphenols and their copper complexes induces conformational changes in the secondary structure of HSA. This indicates that binding of the copper complexes to HSA could cause a stabilization of the helical content similar to the effect of 3'-azido-3'-deoxythymidine (AZT) on HSA (Gaudreau, Neault, & Tajmir-Riahi, 2002). A recent study from this laboratory showed that the copper complex of quercetin was also found to stabilize the helical content of the serum albumins (Singha Roy et al., 2012).

The near-UV CD spectrum (Figure 7(c)) of HSA showed two consecutive minima positions at 263 and 267 nm and 279 and 289 nm, characteristics of the disulfide and aromatic chromophores, and asymmetric environment in the protein (Uversky, Narizhneva, Ivanova, Tomashevski, & Omashevski, 1997). Binding with the copper complexes resulted in peak shifts indicating that the microenvironment around aromatic residues and disulfide bonds were affected.

3.8. FTIR measurements

The conformational resistivity of amide bands in the protein structures has been governed by the coupling between the transition dipoles along with H-bonding. The amide I band (1700–1600 cm⁻¹) in the protein structure arises due to C=O stretching of the polypeptide chains. The amide II band (1575–1480 cm⁻¹) generated from the coupling of C–N stretching with N–H bending modes possesses less conformational sensitivity than the amide I region (Elliott & Ambrose, 1950; Krimm & Bandekar, 1986). The shift in the peaks of amide I and II bands of HSA after binding with the ECG and EGC (spectra not shown) and the copper complexes (Figure 8) is indicative of the complex formation between the HSA and the ligands.

A quantitative estimation of the secondary structure of HSA at room temperature before and after complexation with different ligands was performed. After binding with ECG and EGC the % α-helix decreases from 58 to
53 and 54 upon 1:2 binding with ECG and EGC, respectively (Figure S11). On the other hand, the value increases after binding with the copper complexes that shows a corresponding decrease in the β-sheet content. The % α-helix of free HSA at pH 7 was estimated to be 53 which increase to 62 and 60 and the corresponding % β-sheet decreases from 32 to 21 and 27 upon 1:2 binding with EGCG-Cu(II) and ECG-Cu(II) complexes, respectively (Figure 9), which is consistent with the results obtained from the CD measurements. The increase in alpha-helix content from 53 to 62% upon interaction of the Cu complexes with HSA indicates that stabilization of the secondary structure of HSA occurs on complexation. The larger sizes of the polyphenol–Cu(II) complexes are responsible for the stabilization of the protein structure. Kanakis et al. (2011) reported that green tea polyphenols (specifically EGCG and ECG) stabilize the milk β-lactoglobulin by increasing its helical content upon binding due to their larger and bulkier sizes.

The presence of strong hydrophobic interactions in the case of the copper complexes interacting with HSA was also investigated using FTIR analyses. The binding of the copper complexes are entropy-driven processes, whereas the binding of the polyphenols are enthalpy-driven pathways. The symmetric and asymmetric bending vibrational frequencies of CH₂ and CH₃ in the region 1500–1100 cm⁻¹ were used (Krimm & Bandekar, 1986). The positions of stretching frequencies of free HSA and complexed HSA with the copper complexes at pH 7 are presented in Figure 8. It was found that each position of free HSA was shifted to a certain degree after binding with the ligands at different molar ratios. The shift of stretching vibrations is indicative of the involvement of strong hydrophobic interactions of the copper complexes with HSA.

3.9. Radical-scavenging activity

Smith, Halliwell, & Aruoma (1992) found that the binding of polyphenols to albumin reduced their prooxidant activity. Protein–ligand binding can thus modulate the bioavailability of the ligand. The binding of EGCG, ECG, and their copper complexes to HSA reduced their corresponding DPPH-scavenging activity (Figure S12a). It has been found that greater the affinity for HSA, higher is the % decrease of the scavenging activity of the ligands (Arts, Haenen, Voss, & Bast, 2001; Buxton, 2005). This is because a molecule once bound to the protein with a high affinity is unlikely to dissociate itself from the protein molecule (Arts et al., 2001; Buxton, 2005). This in turn results in a reduction of their radical-scavenging properties when compared to the free form. In this case, EGCG–Cu(II) complex has the highest binding constant with HSA, which thus shows a greater decrease in the scavenging activity in the presence of HSA (Figure S12b).

3.10. Molecular docking

To substantiate the experimental results, preferred docking positions of ECG and EGC with HSA have been studied. The stereoview of the docking poses of ECG and EGC with HSA is shown in Figure S13. The docked structure shows that ECG is located within the binding pocket of site 1 (subdomain IIA) as expected from displacement studies. The inside wall of the pocket is comprised of hydrophobic side chains, whereas the entrance to the pocket is enclosed by positively charged residues, such as Lys 195, Lys 199, Arg 218, Arg 222, His 242, and Arg 257. We find that in the best docked conformation obtained, the hydroxy groups of ring D (gallate moiety) of ECG is within hydrogen-bonding distance of Trp 214 (Table S4), which can explain the observed fluorescence quenching of HSA. The docking result resembles our previous observation for EGCG (Maiti et al., 2006), for which the gallate ring is also within hydrogen-bonding distance to Trp 214. Therefore, docking of ECG and EGCG creates a more polar environment near Trp 214, which causes the large red shift in the fluorescence spectra of HSA after pol-

Figure 8. FTIR spectra of HSA and HSA bound with (a) ECG–Cu(II) and (b) EGCG–Cu(II) in the region 1700–1100 cm⁻¹ in 20 mM phosphate buffer of pH 7.0. The presence of hydrophobicity in the binding of fisetin with HSA was studied in the region of 1400–1100 cm⁻¹.
phenol binding. EGC, however, does dock to site 2 (sub-domain IIIA), where it interacts with Glu 383, Gln 390, Arg 410, Lys 414, and Glu 492. This can explain the negligible quenching of Trp fluorescence of HSA by EGC and the ibuprofen displacement observed.

To further identify the residues taking part in the interaction, we have calculated the ASA of the interacting residues of HSA and that in HSA–ligand complexes. Residues where the absolute ASAs have decreased by more than 10 Å² on complex formation are given in Table S5. We find that most of the residues involved in the interaction with ECG and EGCG belong to subdomains IIA as expected from our experimental results. However, for EGC, all the residues which lose substantial ASA are in subdomain IIIA. The decrease in solvent ASA of Trp 214 on complexation with ECG and EGCG indicates increased ligand accessibility to the fluorophore. For EGC there is no change in ASA of Trp 214, which indicates no possible interaction of EGC with HSA.

4. Conclusions
Considering the antiribonucleolytic and/or antiangiogenic activities of green tea polyphenols and their copper complexes and their potential therapeutic importance, interactions have been studied with HSA, the most abundant carrier protein. Gallate containing green tea polyphenols and their copper complexes quench the fluorescence intensity of HSA. The compounds bind to HSA with association constants of the order of $10^4$ M$^{-1}$ as obtained from Scatchard, modified Stern–Volmer and double-logarithm plots. The nongallate polyphenol, EGC does not quench the fluorescence of HSA, further suggests the role of the gallate moiety in the interaction process. The displacement of warfarin by these ligands confirms that they occupy site 1 (subdomain IIA) of HSA and thereby perturb the environment near Trp 214 causing fluorescence quenching. The binding of the polyphenols with HSA is enthalpy-driven processes and the copper complexes interacting via entropy-driven pathways. The strong hydrophobicity present in the binding of the copper complexes with the protein is also explained on the basis of the symmetric and asymmetric bending modes of the CH$_2$ and CH$_3$ groups of the protein. Docking studies corroborate the experimentally determined sites of interaction.

Supplementary material
The supplementary material for this paper is available online at http://dx.doi.org/10.1080/07391102.2012.729158.

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