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Characterization of the Binding of the Finland Trityl Radical with Bovine Serum Albumin

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Abstract

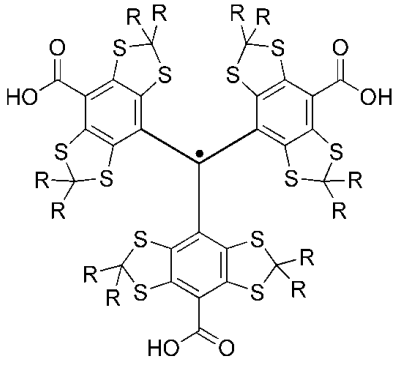
Understanding the interactions of trityl radicals with proteins is required to expand their biomedical applications. In this work, we demonstrate that the Finland trityl radical CT-03 binds to bovine serum albumin (BSA) in aqueous solution. Upon binding with BSA, CT-03 exhibits a much broader electron paramagnetic resonance (EPR) signal and this line broadening can be reversed by proteolysis of the BSA. The binding induces a red-shift of the maximal UV-Vis absorbance wavelength of CT-03 around 470 nm likely due to localization of CT-03 in the relatively hydrophobic region of the protein. The interaction between CT-03 and BSA is driven by hydrophobic interaction with an estimated binding constant of 2.18×10^5 M^{-1} at 298 K. Furthermore, only one CT-03 is bound to each molecule of BSA and the binding site is determined to be the sub-domain IIA (Sudlow's site I). This protein binding of the trityl probe to albumin can be used to study the structure and function of albumin and also must be considered for its use as *in vivo* imaging agent or spin label.

1. Introduction

Trityl radicals have a long history in radical chemistry ever since the initial report of the triphenylmethyl radical by Gomberg in 1900.[1] By the late 1990s, a series of fully substituted tetrathiatriarylmethyl (trityl) radicals such as OX063 and CT-03 (Chart 1) were developed by Nycommed Innovations (now a subsidiary of GE Healthcare) and other research groups for use as contrast agents in Overhauser-enhanced magnetic resonance imaging.[2-6] These sterically crowded carbon-centered radicals show high biostability with a single and sharp electron paramagnetic resonance (EPR) signal, and therefore are well suited for EPR imaging applications.[7,8] Besides the major application in EPR oximetry,[9-12] much interest has been involved in the functionality of these trityl radicals as superoxide radical,[13,14] pH,[15-18] redox[19,20] and GSH probes.[21] Recently, distance measurements in proteins in the liquid state have been achieved using trityl radicals as spin labels along with pulsed EPR techniques.[22] This approach provides an exciting new way to investigate the structure and dynamic of proteins at physiological conditions. Moreover, these trityl radicals can also find applications in the study of protein-protein interactions. Thus, understanding the interactions of trityl radicals with proteins is required for their applications either in magnetic resonance-related imaging or in structural biology. While intensive efforts have focused on the functionality of these trityl radicals (as mentioned above) as well as the synthesis of their analogues,[23-25] few studies have been performed to characterize the interaction of trityl radicals with biological macromolecules.[26,27]

In the present study, bovine serum albumin (BSA) and CT-03 (also called as Finland trityl radical) were chosen as a model protein and compound, respectively, to investigate the interactions of proteins with trityl radicals. CT-03 is one of the most commonly used trityl radicals.

Serum albumins are the major soluble protein constituents of the circulatory system and have many physiological functions.[28] BSA has been widely studied due to its strong ligand binding capacity and structural homology with human serum albumin.[29,30] Herein, the interaction between BSA and CT-03 were systematically investigated by EPR, UV-Vis and fluorescence spectroscopies. The interaction mechanism, binding constant and binding stoichiometry of CT-03 with BSA were determined. The binding site of CT-03 on BSA was assigned using the fluorescence competition experiments and the nature of their interaction was analyzed based on thermodynamic parameters. Moreover, energy transfer from BSA to CT-03 was also explored.



OX063, R = CH_2CH_2OH
CT-03, R = CH_3

Chart 1. Molecular structure of trityl radicals OX063 and CT-03

2. Experimental section

2.1 Materials

Bovine serum albumin (fatty acid-free, BSA), site markers (walfarin and ibuprofen) and pronase were purchased from Sigma-Aldrich (USA). In order to remove trace metals, BSA was dissolved in citric acid solution (10 mM) and dialyzed against water and phosphate buffer solution which were pretreated with Chelex. All other chemicals used throughout the experiments were of

analytical grade.

2.2 Spectroscopic measurements

All EPR spectra were recorded at room temperature using a Bruker X-band EPR spectrometer with the following parameters: microwave power, 0.2-2 mW; modulation frequency, 100 kHz; sweep time, 41.94 s; modulation amplitude, 0.01-0.2 G. For the variable temperature EPR experiments, the following EPR parameters were used: modulation amplitude, 0.04 G; microwave power, 0.2 mW; number of scans, 5.

All fluorescence spectra were recorded on a fluorophotometer (SPEX FluoroMax) equipped with a 1.0 cm quartz cell and a thermostat bath using a xenon lamp excitation source. Fluorescence quenching spectra were recorded at 298, 303 and 308K in the range of 300-450nm. The width of the emission slit was set at 1.0 nm. An excitation wavelength of 286 nm was chosen for BSA and its very dilute solutions (< 25 μM) were used in the experiments to avoid inner filter effect.

The UV-Vis absorption spectra were recorded at room temperature on a Cary 50 UV-Vis spectrophotometer equipped with a 1cm quartz cell and a thermostat bath.

3. Results and Discussion

3.1 Interaction between CT-03 and BSA studied by EPR and UV-Vis spectroscopies

EPR spectroscopy is the most direct and specific technique to detect paramagnetic species and has been widely used in the field of spin labeling to investigate the microenvironment of biological systems. Here, we initially used EPR spectroscopy to study the interaction of CT-03 with BSA since this interaction may change the motion and local microenvironment of CT-03 and

thus its EPR properties. As show in Figure 1, CT-03 (20 μM) has a sharp single EPR signal with a linewidth of 176 mG in air-equilibrated PBS. Upon addition of BSA, the singlet signal was gradually broadened with the linewidth of 288 mG in the presence of 100 μM of BSA (Figure 1B). Accordingly, the signal intensity of CT-03 in the presence of 100 μM of BSA was more than 5 times weaker than that in PBS. The near identical double integration values of the EPR signals in the absence and presence of BSA indicate that no chemical reaction(s) occur between CT-03 and BSA (see Figure S1 in Supplementary Information). These results were consistent with our previous observation from the deuterated analogue of CT-03[31] and also supported by the high stability of CT-03.[7]

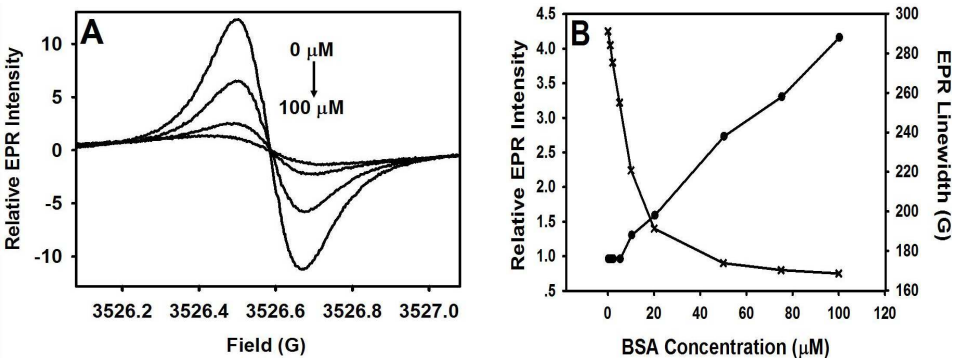


Figure 1. (A) EPR spectra of CT-03 (20 μM) in the presence of various concentrations of BSA (0, 5, 20 and 100 μM) at room temperature at pH 7.4. (B) EPR linewidth and relative intensity of CT-03 in the presence of various concentrations of BSA (0, 1.25, 2.5, 5, 10, 20, 50, 75 and 100 μM).

The EPR line broadening of CT-03 in the presence of BSA may be due to the shortening of its relaxation time by binding with BSA or due to its spin-spin interaction with residual paramagnetic metals (copper or iron ions) from the commercial source of BSA in solution. To verify if any potential paramagnetic metal ion in the commercial BSA was involved, steps were

taken to remove any bound metal ions by extensive dialysis with citric acid solution and PBS in chelex-treated water. No difference in the linewidths of CT-03 was observed in the untreated (288 mG) and treated (285 mG) BSA solutions, indicating that paramagnetic ions did not exert significant effect on the line broadening of CT-03. Therefore, the line broadening of CT-03 is most likely due to its binding with BSA which may decrease the molecular tumbling rate of CT-03 and shorten its relaxation time. This suggestion was further verified by proteolysis of BSA bound with CT-03 (see Figure 2). Solutions of CT-03 (20 μM) in the presence of BSA (100 μM) were subjected to proteolysis using pronase (14 U mL^{-1}) for 1 hour at room temperature. After this proteolysis, the linewidth of CT-03 in the solution reverted back from 288 mG (Figure 2A) to 183 mG (Figure 2C) which is almost identical with the value of 176 mG (Figure 2D) of free CT-03 in PBS.

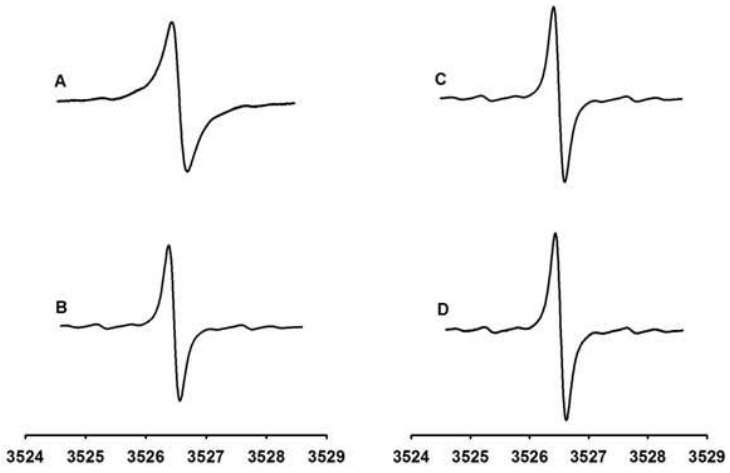


Figure 2. EPR spectra obtained by incubating the solution of CT-03 (20 μM) and BSA (100 μM) with pronase (14U mL^{-1}) at the different time points at pH 7.4. (A) 0 min; (B) 30 min; (C) 1 h; (D) free CT-03.

In order to further explore the interaction of CT-03 with BSA, the

concentration-dependent effect of BSA on the UV-Vis spectra of CT-03 was also investigated. As shown in Figure 3, the maximal UV-Vis absorbance wavelength (λ_{max}) of CT-03 (20 μM) was red-shifted at 467 nm to 472 nm when the concentration of BSA increased from 0 to 60 μM. No further obvious shift was observed with further increase in the concentration of BSA, indicating that CT-03 (20 μM) is almost completely bound to BSA at concentrations > 60 μM, consistent with our EPR results (see Figure 1B). The red-shift of λ_{max} for CT-03 in the presence of BSA is most likely due to the localization of CT-03 in the relatively hydrophobic region of the protein. This is rationalized by the suggestion that the three carboxylic groups in CT-03 are easily protonated possibly due to extensive H-bonding at low polarity environment and thus the resulting acid form of CT-03 becomes hydrophobic. Similarly, due to the intermolecular interaction of BSA with CT-03, enhanced maximum absorbance intensity of CT-03 at 450-480 nm was also observed (Figure 3, insert).

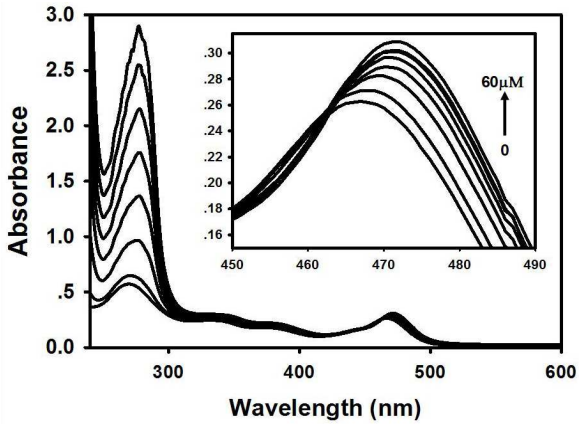


Figure 3. Absorption spectra of CT-03 (20 μM) in the presence of various concentrations of BSA (0, 2, 10, 20, 30, 40, 50 and 60 μM) at room temperature at pH 7.4.

3.2 The interaction between BSA and CT-03 studied by fluorescence spectroscopy

Fluorescence techniques have been widely utilized to investigate the interaction of

organic compounds with proteins. By measuring the variations of the intrinsic fluorescence intensity of the proteins before and after addition of compounds of interest, important information regarding their interactions such as binding constant, binding site, binding stoichiometry, type of interaction force and thermodynamic parameters can be extracted[32-34]. As shown in Figure 4, BSA has a strong fluorescence emission at 342 nm when excited at 286 nm, while the fluorescence of CT-03 is negligible under the same condition. However, the fluorescence intensity of BSA distinctly decreased upon addition of CT-03 (0-25 μM) with the slight red shift of the maximum emission. These results suggest that CT-03 interacts with BSA and quenches the intrinsic fluorescence of BSA. More than 4-fold decrease in fluorescence intensity of BSA induced by the binding of CT-03 was most likely due to the fluorescence quenching of both tryptophan residues in BSA which was also observed for other organic molecules..[35-38]

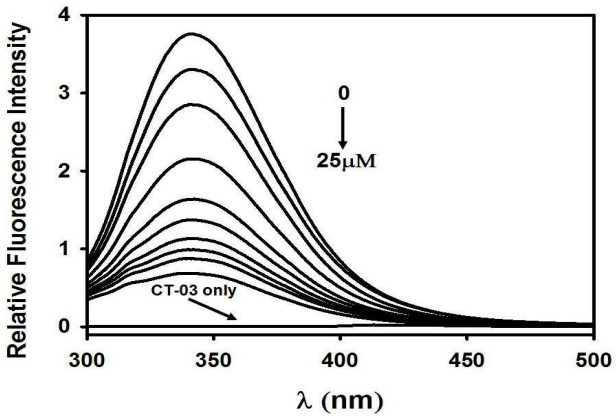


Figure 4. Emission spectra of BSA (2.5 μM) in the presence of various concentrations of CT-03 (0, 1.25, 2.5, 5, 7.5, 10, 12.5, 15, 17.5 and 25 μM) or CT-03 (25 μM) alone in PBS (pH 7.4).

3.2.1 Fluorescence quenching mechanism of BSA by CT-03

The interaction mechanism between BSA and CT-03 can be determined by investigating the concentration effect of CT-03 on the intrinsic fluorescence of BSA which can be quantitatively

described by the Stern-Volmer equation:[39]

$$\frac{F_0}{F} = 1 + K_{sv}[Q] = 1 + K_q \tau_0 [Q] \quad (1)$$

where F_0 and F represent the fluorescence intensities of BSA in the absence and presence of CT-03, respectively; K_{sv} is the Stern-Volmer quenching constant, and [Q] is the concentration of CT-03; K_q is the apparent bimolecular quenching constant and equal to K_{sv}/τ_0; τ_0 is the average fluorescence lifetime of BSA without any quencher and taken as 10^{-8} s.[40] As shown in Figure 5, the regression curves of F_0/F versus [CT-03] were plotted with a good linearity and the values of K_{sv} and K_q were determined from the slopes of the curves. The calculated value of K_q ($(1.55 \pm 0.02) \times 10^{13}$ M^{-1} s^{-1}) at 298 K is about 10^3 times higher than the maximum scatter collision quenching constant (2.0×10^{10} $M^{-1}s^{-1}$),[41] indicating that a static quenching mechanism dominates in the interaction and that a complex may be formed between CT-03 and BSA.

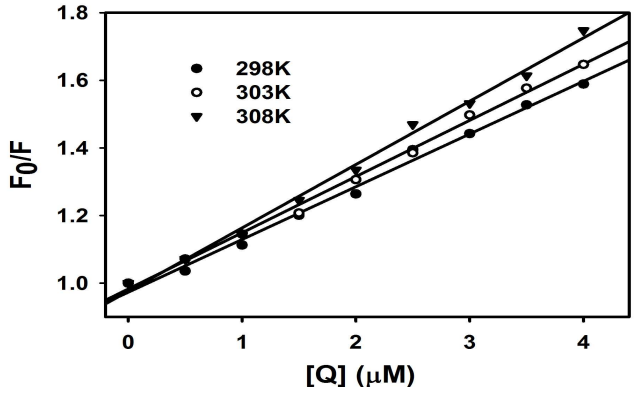


Figure 5. Stern-Volmer plots of the quenching of BSA fluorescence by CT-03 at different temperatures at pH 7.4. According to the Stern-Volmer equation, K_q is calculated to be $(1.55 \pm 0.02) \times 10^{13}$ M^{-1} s^{-1} at 298 K, $(1.66 \pm 0.01) \times 10^{13}$ M^{-1} s^{-1} at 303 K, and $(1.87 \pm 0.02) \times 10^{13}$ M^{-1} s^{-1} at 308 K. Here Q is specifically designated as CT-03.

According to the static quenching mechanism, K_q will decrease with the temperature.[39] However, increased K_q with the temperature was observed in our experiment (Figure 5). This phenomenon may be explained by the Arrhenius theory.[42]

$$\ln\frac{K_{sv}}{\tau_0} = \ln K_q = -\frac{E_a}{RT} + \ln A \qquad (2)$$

K_q, which equals K_{sv}/τ_0, is the apparent bimolecular quenching rate constant; E_a is the activation energy of the quenching reaction of CT-03 with BSA; and A is the pre-exponential factor. From the slopes of the $\ln K_q$ – 1000/T plots, E_a was estimated to be 14.7 kJ mol^{-1} (see Figure S2 in Supplementary Information). According to the Arrhenius theory, increasing temperature is feasible for the interaction of BSA and CT-03 and leads to a high rate constant (K_{sv} or K_q), consistent with our above observation.

3.2.2 Effect of a diamagnetic derivative of CT-03 (CT-03H) on the fluorescent quenching of BSA

Since CT-03 is a stable paramagnetic compound, the paramagnetic feature of CT-03 may enhance the fluorescence quenching of BSA. In order to clarify this, a reduced and diamagnetic derivative of CT-03, CT-03H, was synthsized[26] and concentration effect of CT-03H on the fluorescence of BSA was also studied at 298K. As shown in Figure 6, almost the same K_q's were obtained for CT-03 ((1.55 ± 0.02) × 10^{13} M^{-1} s^{-1}) and CT-03H ((1.56 ± 0.04) × 10^{13} M^{-1} s^{-1}). This result implies that the paramagnetic character of CT-03 does not exert any effect on the fluorescent quenching of BSA.

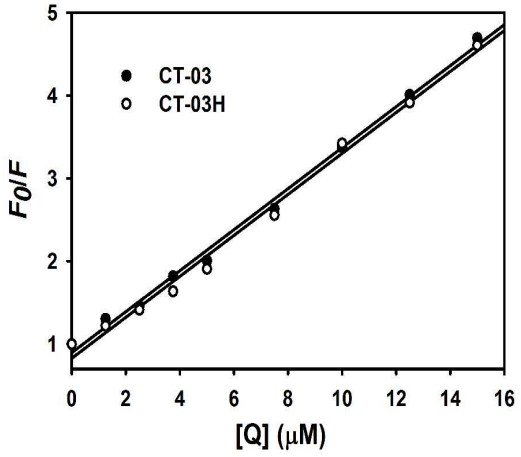


Figure 6. Stern-Volmer plots for the fluorescence quenching of BSA by CT-03 and CT-03H at 298K. C_{BSA} = 2.5μM; C_{CT-03} or C_{CT-03H} = 0, 1.25, 2.5, 3.75, 5.0, 7.5, 10, 12.5 and 15.0 μM;λ_{ex}= 286 nm; pH = 7.4. Here Q is CT-03 or CT-03H.

3.2.3 Binding constant and stoichiometry

In order to determine the binding constant (K_b) and stoichiometry (n) for the interaction of CT-03 with BSA, the relationship between the intrinsic fluorescence intensity of BSA and the concentration of CT-03 was further fitted using equation (3):[43]

$$\log\frac{(F_0-F)}{F} = n\log[Q] + \log K_b \quad (3)$$

where [Q] is the concentration of CT-03. Table 1 summarizes the values of K_b and n at different temperatures. High value of K_b ((2.18 ± 0.02) $\times 10^5$ M^{-1} at 298 K) indicates a strong binding between CT-03 and BSA. The binding capacity of BSA to CT-03 was further enhanced at higher temperatures with a K_b value of (1.11 ± 0.02) $\times 10^6$ M^{-1} at 308 K. The obtained n value (~ 1) indicated that there is only one binding site in BSA for CT-03 on average.

Table 1. Binding constants and stoichiometries for the interaction of CT-03 with BSA at different temperatures at pH 7.4.

T (K)	K_b (M^{-1})	n	R
298	2.18×10^5	1.03	0.9942
303	4.38×10^5	1.08	0.9973
308	1.11×10^6	1.14	0.9972

Note： K_b and n are the binding constant and stoichiometry, respectively. R is the correlation coefficient for the K_b values.

3.2.4 Thermodynamic parameters and nature of the binding interaction

In principle, there are four types of forces of interaction between small organic molecules and

biological macromolecules: hydrophobic force, hydrogen bond, van der Waals force, and electrostatic interactions, which can be distinguished by the sign and magnitude of thermodynamic parameters: (a) enthalpy change (ΔH) > 0 and entropy change (ΔS) > 0, hydrophobic force; (b) $\Delta H < 0$ and $\Delta S < 0$, van der Waals force and hydrogen bond; (c) $\Delta H < 0$ and $\Delta S > 0$, electrostatic interactions.[44] Assuming that both ΔH and ΔS are temperature-independent constants under the investigated temperatures, their values can be estimated by the van's Hoff equation:[45]

$$\ln K = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \tag{4}$$

where K and R are the binding constant K_b and gas constant, respectively. Then, free energy change (ΔG) can be further estimated according to the following equation:

$$\Delta G = \Delta H - T\Delta S = -RT \ln K_b \tag{5}$$

The values of ΔH, ΔS and ΔG were summarized in Table 2. Negative values of ΔG at different temperatures reveal that the binding process between CT-03 and BSA is spontaneous. As described above, the positive values of ΔH (123.8 kJ mol^{-1}) and ΔS (517.3 J mol^{-1} K^{-1}) indicates that the hydrophobic force plays a major role in their binding process and contributes to the stability of the resulting complex, consistent with the above UV-Vis spectral analysis. Furthermore, the positive values of ΔH indicate that the interaction of CT-03 with BSA is endothermic and easier to occur at the elevated temperature, consistent with the above results that the apparent bimolecular quenching rate constant K_q increases with the temperature.

Table 2. Thermodynamic parameters of the binding of CT-03 with BSA at the different temperatures.

T (K)	ΔH (kJ mol^{-1})	ΔG (kJ mol^{-1})	ΔS (J mol^{-1} K^{-1})	R
298		-30.4		
303	123.8	-32.9	517.3	0.9957
308		-35.5		

Note: R is the correlation coefficient for the van's Hoff plot (see Figure S3 in Supplementary Information).

3.2.5 Binding sites of CT-03 on BSA

Based on the distribution of disulfide bridges and amino acid sequence, BSA consists of three homologous domains (I-III) with two sub-domains (A and B) on each domain. The intrinsic fluorescence in BSA mainly comes from Trp-212 and Trp-134 which are located on sub-domain IIA (Sudlow's site I) and sub-domain IIIA (Sudlow's site II), respectively.[46] The binding affinity offered by site I is mainly through hydrophobic interaction, whereas site II involves a combination of hydrophobic, hydrogen bonding, and electrostatic interaction.[47,48] Warfarin (an anticoagulant drug) and ibuprofen (a nonsteroidal anti-inflammatory agent) have been considered as stereotypical ligands for Sudlow's site I and II, respectively.[49] To identify the binding site of CT-03 on BSA, competitive binding experiments using warfarin or ibuprofen for CT-03 were performed by EPR spectroscopy. As shown in Figure 7, addition of warfarin into the BSA-CT-03 system gradually induced the enhancement of the EPR signal due to free CT-03, while ibuprofen did not have any significant effect on the EPR signal. Thus, warfarin may release CT-03 from the binding site on BSA and restore its sharp EPR signal. This result implies that CT-03 binds to the Sudlow's site I of BSA, consistent with our above result that the binding between CT-03 and BSA is driven by the hydrophobic interaction.

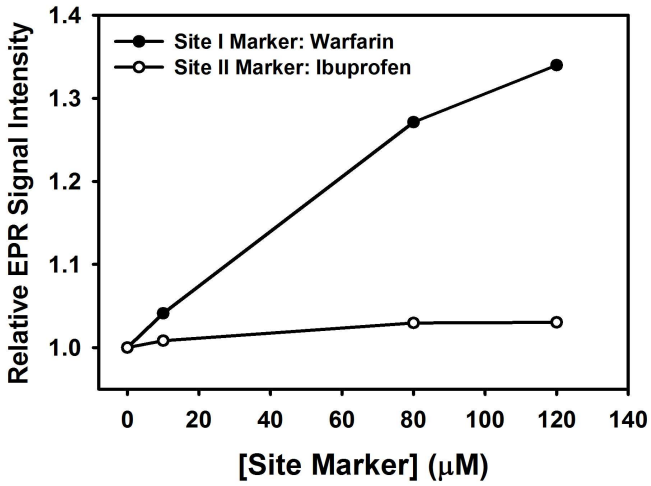


Figure 7. EPR competition displacement experiments with measurements performed at room temperature. $C_{BSA} = C_{CT-03} = 10\mu M$, $C_{warfarin} = C_{ibuprofen} = 0$, 10, 80, 100 and 120 μM. pH = 7.4.

To further confirm the binding site of CT-03 on BSA, fluorescence replacement experiments

were also carried out (see Figure 8). The system consisting of BSA and CT-03 was titrated by the site marker (warfarin or ibuprofen). Both concentrations of BSA and CT-03 were 2.0 μM, while the concentration of each site marker increased from 1.0 to 7.0 μM. Upon excitation at 286 nm, neither CT-03 or the site markers showed significant fluorescence (data not shown). Addition of warfarin to the solution containing BSA and CT-03 resulted in a slight red shift (from 342 nm to 345 nm) of the maximum emission wavelength of BSA and attenuated the fluorescence intensity (data not shown). The Stern-Volmer quenching constant of the BSA-warfarin-CT-03 system was determined to be (1.34 ± 0.01) × 10^5 M^{-1} (R = 0.9964) according to equation (1). Similarly, the addition of ibuprofen into the BSA-CT-03 system also reduced the fluorescence intensity. The calculated quenching constant ((1.49 ± 0.03) × 10^5 M^{-1}) of the BSA-CT-03-ibuprofen system was much higher than that in the BSA-CT-03-warfarin system ((1.34 ± 0.01) × 10^5 M^{-1}) but similar to the value observed in the BSA-CT-03 system ((1.55 ± 0.02) × $10^5 M^{-1}$). This result reveals that CT-03 competes with warfarin for the Sudlow's site I in BSA and this site is the main binding site for CT-03, well consistent with the result from the above EPR experiment.

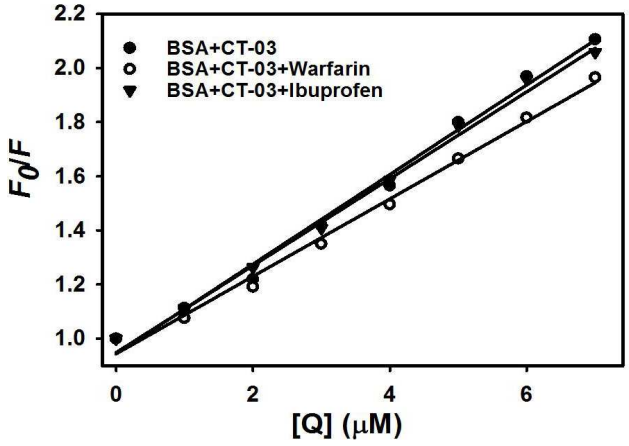


Figure 8. Fluorescence competition displacement experiments at 298K. C_{BSA} = C_{CT-03} = 2.0 μM, $C_{warfarin}$ = $C_{ibuprofen}$ = 0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 and 7.0 μM. pH = 7.4.

3.3.6 Energy transfer from BSA to CT-03

In biochemistry, the efficiency of energy transfer can be used to estimate the distance between the donor and acceptor in protein. According to the Förster's resonance energy transfer theory, the

efficiency of energy transfer depends on (i) the relative orientation of the donor and acceptor dipoles, (ii) the overlapping extent of the fluorescence emission spectrum of the donor with the absorption spectrum of the acceptor and (iii) the distance between the donor and acceptor.[50,51] The efficiency of energy transfer between the donor and acceptor, *E*, could be calculated by the following equation:

$$E = 1 - \frac{F}{F_0} = \frac{R_0^6}{R_0^6 + r^6} \quad (6)$$

where *r* is the distance between the donor and acceptor (*Note*: the *r* calculated for BSA–CT-03 complex is actually the average value between the bound CT-03 and the two tryptophan residues);[52,53] R_0 is the critical distance when the efficiency of energy transfer is 50% and can be calculated by the following equation:

$$R_0^6 = 8.79 \times 10^{-25} k^2 N^{-4} \varphi J \quad (7)$$

where *N* is the refracted index of medium; φ is the quantum yield of the donor in the absence of acceptor; k^2 is the orientation factor between the emission dipole of the donor and the absorption dipole of the acceptor and can range from 0 to 4. If both the donor and acceptor are tumbling rapidly and free for any orientation, k^2 equals 2/3. In most cases, however, even modest reorientation of the donor and acceptor can still result in enough orientational averaging such that $\kappa^2 = 2/3$ does not lead to a large error in the estimated energy transfer distance due to the sixth power dependence of R_0 on κ^2.[54,55] In the present case, CT-03 has a relatively free mobilization upon binding to BSA since the EPR signal of CT-03 bound to BSA only showed a slightly immobilization with the linewidth of 288 mG as compared to that of the strongly immobile CT-03 in frozen solution (linewidth = 710 mG, see Figure S4 in Supplementary Information). Thus, the value of k^2 can be still set to 2/3. *J* expresses the degree of spectral overlap between the emission spectrum of BSA and the absorption spectrum of CT-03 (Figure 9), which could be calculated by the following equation:

$$J = \frac{\sum F(\lambda)\varepsilon(\lambda)\lambda^4 \Delta\lambda}{\sum F(\lambda)\Delta\lambda} \quad (8)$$

where $F(\lambda)$ is the fluorescence intensity of the donor in the wavelength range λ to $\lambda+$, $\Delta\lambda$; $\varepsilon(\lambda)$ is the molar absorption coefficient of the acceptor at λ.

In the present case, N = 1.36 and φ = 0.15.[56] From Eqs. (6) - (8), J = 1.96 ×10^{-14} cm^3 L mol^{-1}, E = 0.209, R_0 = 2.64 nm, and r = 3.29 nm were estimated. Although two donors (i.e., two tryptophan residues) interact with an acceptor (i.e., CT-03) in this study, this system can be still regarded as a single donor system as suggested in the previous study.[57] Thus, the estimated average energy transfer distance (r = 3.29 nm) is valid. For the more accurate calculation of the distance, the Monte Carlo simulation method may be used.[58,59] The fact that the average distance was on the 2-8 nm scale and $0.5R_0 < r < 1.5R_0$ indicates that the energy transfer from BSA to CT-03 occurred with high probability.[60]

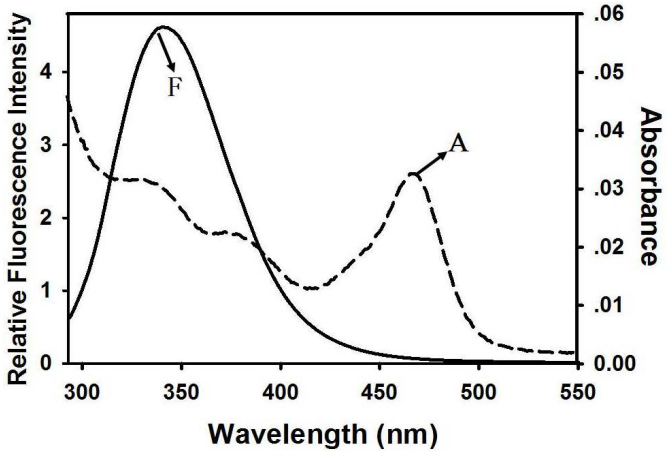


Figure 9. Spectral overlap of CT-03 absorption (A) and BSA fluorescence (F). C_{BSA} = C_{CT-03} = 2.0μM.

4. Conclusions

In this study, the binding of the Finland trityl radical CT-03 with BSA was systematically investigated by the fluorescence spectroscopy in combination with EPR and UV-Vis spectroscopies. While the binding with BSA significantly broadened the EPR signal of CT-03 primarily due to the relatively slower molecular tumbling, the binding also quenched the intrinsic fluorescence of BSA. The fluorescent quenching experiments demonstrated that the binding of CT-03 with BSA was mainly through the static mechanism with an estimated E_a of 14.7 kJ mol^{-1}. The binding was spontaneous (ΔG < 0) and characteristic of hydrophobic interaction (ΔH > 0 and ΔS > 0) with a binding constant of 2.18

$\times 10^5$ M^{-1} at 298 K and binding stoichiometry of one on average. Competitive binding experiments performed by both EPR and fluorescent techniques showed that the binding site of CT-03 on BSA was in sub-domain IIA (Sudlow's site I) with the average energy transfer distance of 3.29 nm away from the two tryptophan residues in BSA.

Since high concentration of serum albumin (~ 5%) is present in blood, the binding of trityl radicals such as CT-03 with the albumin also occurs which may be mainly responsible for transportation of CT-03 to different sites. The site-specific binding of CT-03 provides a new and convenient approach to label proteins without preparation of mutants and will be very useful to study the structure and dynamic of the proteins. However, the albumin binding-induced line broadening of trityl radicals may complicate their uses as *in vivo* imaging agents, especially for EPR oximetry. Thus additional calibration curves for linewidths of trityl radicals as a function of oxygen levels are required. Recently, in order to avoid the albumin binding, a PEGylated trityl-based EPR oximetry probe has been developed which shows the concentration-independent effect of albumin on the linewidth of the probe.[31] In summary, the present study provides critical information and enables a deeper understanding of the *in vivo* behavior of trityl radicals and will be very helpful to develop new trityl radicals for biomedical applications.

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References and Notes

(1) Gomberg, M. *J. Am. Chem. Soc.* **1900**, *22*, 757.
(2) Ardenkjaer-Larsen, J. H.; Leunbach, I. PCT Int. Appl. wo/9709633, 1997.
(3) Andersson, S.; Radner, F.; Rydbeek, A.; Servin, R.; Wistrand, L. G. U.S. Patent 5530140, 1996.
(4) Reddy, T. J.; Iwama, T.; Halpern, H. J.; Rawal, V. H. *J. Org. Chem.* **2002**, *67*, 4635.
(5) Dhimitruka, I.; Velayutham, M.; Bobko, A. A.; Khramtsov, V. V.; Villamena, F. A.; Hadad, C. M.; Zweier, J. L. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 6801.
(6) Liu, Y. P.; Villamena, F. A.; Sun, J.; Xu, Y.; Dhimitruka, I.; Zweier, J. L. *J. Org. Chem.* **2008**, *73*, 1490.

(7) Ardenkjaer-Larsen, J. H.; Laursen, I.; Leunbach, I.; Ehnholm, G.; Wistrand, L. G.; Petersson, J. S.; Golman, K. *J. Magn. Reson.* **1998**, *133*, 1.

(8) Kuppusamy, P.; Wang, P. H.; Chzhan, M.; Zweier, J. L. *Magn. Reson. Med.* **1997**, *37*, 479.

(9) Liu, Y. P.; Villamena, F. A.; Sun, J.; Wang, T. Y.; Zweier, J. L. *Free Radic. Biol. Med.* **2009**, *46*, 876.

(10) Bobko, A. A.; Dhimitruka, I.; Eubank, T. D.; Marsh, C. B.; Zweier, J. L.; Khramtsov, V. V. *Free Radic. Biol. Med.* **2009**, *47*, 654.

(11) Driesschaert, B.; Charlier, N.; Gallez, B.; Marchand-Brynaert, J. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 4291.

(12) Elas, M.; Ahn, K. H.; Parasca, A.; Barth, E. D.; Lee, D.; Haney, C.; Halpern, H. J. *Clin. Cancer Res.* **2006**, *12*, 4209.

(13) Rizzi, C.; Samouilov, A.; Kutala, V. K.; Parinandi, N. L.; Zweier, J. L.; Kuppusamy, P. *Free Radic. Biol. Med.* **2003**, *35*, 1608.

(14) Liu, Y. P.; Song, Y. G.; De Pascali, F.; Liu, X. P.; Villamena, F. A.; Zweier, J. L. *Free Radic. Biol. Med.* **2012**, *53*, 2081.

(15) Bobko, A. A.; Dhimitruka, I.; Zweier, J. L.; Khramtsov, V. V. *J. Am. Chem. Soc.* **2007**, *129*, 7240.

(16) Driesschaert, B.; Marchand, V.; Leveque, P.; Gallez, B.; Marchand-Brynaert, J. *Chem. Commun.* **2012**, *48*, 4049.

(17)Dhimitruka, I.; Bobko, A. A.; Eubank, T. D.; Komarov, D. A.; Khramtsov, V. V. *J. Am. Chem. Soc.* **2013**, *135*, 5904.

(18) Liu, Y. P.; Villamena, F. A.; Zweier, J. L. *Chem. Commun.* **2008**, 4336.

(19) Liu, Y. P.; Villamena, F. A.; Rockenbauer, A.; Zweier, J. L. *Chem. Commun.* **2010**, *46*, 628.

(20) Liu, Y. P.; Villamena, F. A.; Song, Y. G.; Sun, J.; Rockenbauer, A.; Zweier, J. L. *J. Org. Chem.* **2010**, *75*, 7796.

(21) Liu, Y. P.; Song, Y. G.; Rockenbauer, A.; Sun, J.; Hemann, C.; Villamena, F. A.; Zweier, J. L. *J. Org. Chem.* **2011**, *76*, 3853.

(22) Yang, Z. Y.; Liu, Y. P.; Borbat, P.; Zweier, J. L.; Freed, J. H.; Hubbell, W. L. *J. Am. Chem. Soc.* **2012**, *134*, 9950.

(23) Decroos, C.; Prange, T.; Mansuy, D.; Boucher, J. L.; Li, Y. *Chem. Commun.* **2011**, *47*, 4805.

(24) Tormyshev, V. M.; Rogozhnikova, O. Y.; Bowman, M. K.; Trukhin, D. V.; Troitskaya, T. I.; Vasiliev, V. G.; Shundrin, L. A.; Halpern, H. J. *Eur. J. Org. Chem.* **2014**, 371.

(25) Rogozhnikova, O. Y.; Vasiliev, V. G.; Troitskaya, T. I.; Trukhin, D. V.; Mikhalina, T. V.; Halpern, H. J.; Tormyshev, V. M. *Eur. J. Org. Chem.* **2013**, 3347.

(26) Decroos, C.; Li, Y.; Bertho, G.; Frapart, Y.; Mansuy, D.; Boucher, J. L. *Chem. Res. Toxicol.* **2009**, *22*, 1342.

(27) Decroos, C.; Li, Y.; Soltani, A.; Frapart, Y.; Mansuy, D.; Boucher, J. L. *Arch. Biochem. Biophys.* **2010**, *502*, 74.

(28) Peters, T., Jr. *Adv. Protein Chem.* **1985**, *37*, 161.

(29) Carter, D. C.; Ho, J. X. *Adv. Protein Chem.* **1994**, *45*, 153.

(30) Peters, T., Jr. *All about Albumin: Biochemistry, Genetics and Medical Applications*; Academic Press: San Diego, CA, 1996.

(31) Song, Y. G.; Liu, Y. P.; Hemann, C.; Villamena, F. A.; Zweier, J. L. *J. Org. Chem.* **2013**, *78*, 1371.

(32)Tian, F. F.; Jiang, F. L.; Han, X. L.; Xiang, C.; Ge, Y. S.; Li, J. H.; Zhang, Y.; Li, R.; Ding, X. L.; Liu, Y. *J. Phys. Chem. B* **2010**, *114*, 14842.

(33) Zhao, X.; Liu, R.; Chi, Z.; Teng, Y.; Qin, P. *J. Phys. Chem. B* **2010**, *114*, 5625.

(34) Shi, Y.; Liu, H.; Xu, M.; Li, Z.; Xie, G.; Huang, L.; Zeng, Z. *Spectrochim. Acta A Mol. Biomol. Spectrosc.***2012**, *87*, 251.

(35) Paul, B. K.; Samanta, A.; Guchhait, N. *J. Phys. Chem. B* **2010**, *114*, 6183.

(36) Bourassa, P.; Kanakis, C. D.; Tarantilis, P.; Pollissiou, M. G.; Tajmir-Riahi, H. A. *J. Phys. Chem. B* **2010**, *114*, 3348.

(37) Mote, U. S.; Han, S. H.; Patil, S. R.; Kolekar, G. B. *J. Lumin.* **2010**, *130*, 2059.

(38) Bourassa, P.; Dubeau, S.; Maharvi, G. M.; Fauq, A. H.; Thomas, T. J.; Tajmir-Riahi, H. A. *Eur. J. Med. Chem.* **2011**, *46*, 4344.
(39) Sherwin, L. S. *Biochemistry* **1971**, *50*, 3254.
(40) Lakowicz, J. R.; Weber, G. *Biochemistry* **1973**, *12*, 4161.
(41) Eftink, M. R.; Ghiron, C. A. *Anal. Biochem.* **1981**, *114*, 199.
(42) Shahabadi, N.; Nemati, L. *Appl. Biochem. Biotechnol*. **2014**, *172*, 2800.
(43) Xie, M. X.; Xu, X. Y.; Wang, Y. D. *Biochim. Biophys. Acta* **2005**, *1724*, 215.
(44) Ross, P. D.; Subramanian, S. *Biochemistry* **1981**, *20*, 3096.
(45) Klotz, I. M.; Urquhart, J. M. *J. Am..Chem. Soc.* **1949**, *71*, 847.
(46) Carter, D. C.; Ho, J. X. *Adv. Protein Chem.* **1994**, *45*, 153.
(47) Jisha, V. S.; Arun, K. T.; Hariharan, M.; Ramaiah, D. *J. Am. Chem. Soc.* **2006**, *128*, 6024.
(48) Sudlow, G.; Birkett, D. J.; Wade, D. N. *Mol. Pharmacol.* **1976**, *12*, 1052.
(49) He, X. M.; Carter, D. C. *Nature* **1992**, *358*, 209.
(50) *Modern Quantum Chemistry*; Förster, T.; Sinanoglu, O., Eds.; Academic Press: New York, 1966.
(51) Lakowicz, J. R. *Principles of Fluorescence Spectroscopy*, 3rd ed.; Springer: New York, 2006.
(52) Liu, J. Q.; Tian, J. N.; Zhang, J. Y.; Hu, Z. D.; Chen, X. G. *Anal. Bioanal. Chem.* **2003**, *376*, 864.
(53)Kandagal, P. B.; Ashoka, S.; Seetharamappa, J.; Shaikh, S. M. T.; Jadegoud, Y.; Ijare, O. B. *J. Pharm. Biomed. Anal.* **2006**, *41*, 393.
(54) Demchenko, A. P. *Introduction to Fluorescence Sensing*; Springer: Dordrecht, 2008.
(55) Lakowicz, J. R. *Principles of fluorescence spectroscopy*; Plenum: New York, 1999.
(56) Mahammed, A.; Gray, H. B.; Weaver, J. J.; Sorasaenee, K.; Gross, Z. *Bioconjug. Chem.* **2004**, *15*, 738.
(57) Fabian, A. I.; Rente, T.; Szollosi, J.; Matyus, L.; Jenei, A. *Chemphyschem* **2010**, *11*, 3713.
(58) Berney, C.; Danuser, G. *Biophys. J.* **2003**, *84*, 3992.
(59) Corry, B.; Jayatilaka, D.; Rigby, P. *Biophy. J.* **2005**, *89*, 3822.
(60) Valeur, B. *Molecular Fluorescence: Principles and Application*; Wiley: New York, 2001.