Enhancement of Fluresceinyl *Cypridina* Luciferin Analog Chemiluminescence by Human Serum Albumin for Singlet Oxygen Detection

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ABSTRACT

Fluresceinyl *cypridina* luciferin analog (FCLA) is a chemiluminescence (CL) probe for detecting reactive oxygen species (ROS). Its efficiency for detecting singlet oxygen ($^1O_2$) can be significantly enhanced in the presence of human serum albumin (HSA). This phenomenon may apply to important applications for both research and clinical testing, because of the broad presence of HSA in the human system. In the current study the mechanism of the FCLA–HSA CL system is studied by means of direct CL measurement and spectroscopy techniques. Our results show that FCLA can combine with HSA *via* a single binding site to form a complex. The CL efficiency of the system is largely governed by an intersystem energy transfer between the two components upon interaction with $^1O_2$. The CL production reaches maximum in a synergetic manner when equal amounts of FCLA and HSA are present simultaneously, but production is less at other ratios. The results also show that the combination of FCLA with HSA does not significantly alter the ROS selectivity of FCLA. In conclusion, our study shows that FCLA and HSA can combine and form a complex with higher CL efficiency. This provides us a new approach in designing CL techniques for studying ROS.

INTRODUCTION

Singlet oxygen ($^1O_2$) is an excited state of molecular oxygen and one of the most important reactive oxygen species (ROS). It plays an important role in biological systems and is responsible for many types of tissue damage and an extraordinary array of diseases (1,2). Accordingly, detection of the generation and distribution of $^1O_2$ in biologic systems has great significance for both basic and clinical research. At present there are several methods for $^1O_2$ detection, including electron spin resonance, stopped flow spectrophotometry, ultra-weak chemiluminescence (CL), and direct $^1O_2$ luminescence (3–7). CL has been reported to be a sensitive and convenient method to detect the existence of $^1O_2$ in complicated biologic systems (8–10). The basic principle of this

method is that the free radical with an elevated energy level can react with a specific CL probe and initiate a series of chemical reactions that releases additional chemical energy. This results in the structurally altered probe molecule reaching an excited state. The subsequent de-excitation of the probe emits photons (CL), typically in the visible wavelength range. Compared with the other methods, the intensity and temporal profile of CL are relatively easy to record with conventional optical systems, such as a photon multiplier tube (PMT) and/or charge-coupled device (CCD).

Fluresceinyl *cypridina* luciferin analog (FCLA) is a well-established CL probe for ROS (9,10). Its significant characteristics important for biological applications include that the CL photons have the longest wavelength among the known chemiluminescent substances in the category (11) and its optimum environmental pH is within the typical physiological range (12).

Human serum albumin (HSA) is a major protein component of blood plasma. This protein contains a single intrinsic tryptophan and has internal dimensions favorable for the Förster type of energy transfer (13). HSA can easily bind to many agents and transport them in the bloodstream (14,15). It is confirmed that, in a normal physiological environment, HSA is in a favorable structural conformation for binding with a great majority of drugs (16). In addition, HSA is a known $^1O_2$ quencher and can readily react with $^1O_2$ (17–19) and generate 450 nm CL (although very weakly) (19). The CL is estimated to be emitted from a triplet state with the carbonyl group produced by the reaction between proteins and $^1O_2$ (19,20).

We have previous reported that the efficiency of FCLA CL could be enhanced in the presence of HSA (21,22). The mechanism of the enhancement and its potential applications were not discussed. The objective of the current study is to study the CL mechanism of FCLA–HSA system.

MATERIALS AND METHODS

Reagents. FCLA (free acid [3,7-dihydro-6-[4-[2-[N′-(5-fluresceinyl) thioureido]ethoxy] phenyl]-2-methylimidazo[1,2-a]pyrazin-3-one], Tokyo Kasei Kogyo Co., Tokyo, Japan) was dissolved in double-distilled water at a concentration of 100 μmol L^{-1} and stored at −80°C until needed. A stock solution of protoporphyrin IX disodium salt (PpIX) (Aldrich Chem. Co., Milwaukee, WI) was prepared according to the manufacturer's instructions to a concentration of 200 μmol L^{-1}. The stock was stored in the dark at 4°C until needed. HSA (purity, >96%; Sigma, St. Louis, MO) was prepared in double-distilled water to a concentration of 50 μmol L^{-1}.
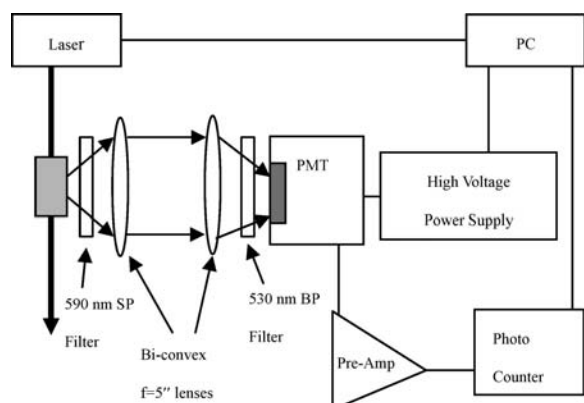
Figure 1. Schematic of the experimental setup used for CL measurements.

Figure 2. Comparison of real-time CL intensity in photosensitization reaction with 5 μmol L^{-1} FCLA, 2 μmol L^{-1} PpIX and a 635 nm, 25.5 mW cm^{-2} laser fluence rate in the presence (A) and absence (B) of 5 μmol L^{-1} HSA.

Sodium azide (NaN$_3$), hydrogen peroxide (30%) and sodium hypochlorite were purchased from the Guangzhou Chemical Reagent Company.

Singlet oxygen production. In the current study singlet oxygen was produced *via* two distinctively different methods: a photosensitization reaction and a direct chemical reaction.

In the photosensitization reaction $^1O_2$ was generated by light irradiation of photosensitizer PpIX. The irradiation light source was a custom-built 635 nm, 200 mW diode laser (diode controller: LDC2000; temperature controller: TEC2000; Thorlab, Newton, NJ).

In the direct chemical reaction $^1O_2$ was produced from the H$_2$O$_2$/NaOCl chemical reaction. The concentration of H$_2$O$_2$ and NaOCl was 200 mmol L^{-1} and 3 mol L^{-1}, respectively. The solution was titrated to pH 7.4. To compare the alter of HSA CL and FCLA CL, the concentrations of FCLA and HSA were set at 0.01 μmol L^{-1} and 5 μmol L^{-1}. To control the timing of $^1O_2$ production, NaOCl was injected into the H$_2$O$_2$ in a glass cuvette (0.5 $\times$ 1 $\times$ 4 cm^3) while CL was recorded.

Experimental setup for CL detection. The schematic for CL measurement (and that of light irradiation for the photosensitization reaction) is summarized in Figure 1. In the current study FCLA CL was measured in real-time using a PMT (model 952; Perkin Elmer Optoelectronics Instruments) operating in single-photon counting mode. The CL measurement setup was identical for both studies, although there was no laser irradiation in the direct chemical reaction study. All CL measurements were made at room temperature and the data were sampled every 0.2 s (sampling rate, 5 Hz s^{-1}).

For the photosensitization study a 590 nm short-pass (SP) and a 530 nm band-pass (BP) filter were used in front of the PMT to minimize the scattered excitation light (635 nm) but allow the 530 nm CL photons to reach the detector. The optical axis of the irradiation light was set at a 90° angle to the detector, to further improve the signal-to-noise ratio. For all photosensitization experiments the samples were irradiated uniformly with a light fluence rate of 25.5 mW cm^{-2}. For comparison of the CL from HSA and FCLA in the H$_2$O$_2$/NaOCl chemical reaction, CL was measured before and after FCLA and HSA were mixed, using either a 455 nm (for HSA) or a 527 nm (for FCLA) BP filter set between the sample and the PMT for signal discrimination.

Experimental procedures. To simplify the investigation the study was performed in a cell-free environment. After HSA and FCLA were mixed at different ratios according to the experimental protocols ([HSA]/[FCLA]:0.25 to 3 with [FCLA] = 1 or 5 μmol L^{-1} was added to the solution. In the current study the order of chemical addition was very important to avoid HSA interaction with the photosensitizer. The pH of the samples was adjusted to a standard level of 7.4. The prepared sample was immediately irradiated according to the protocol for the photosensitization reaction.

The $^1O_2$ production rate was varied by changing the concentration of PpIX (0.2–2 μmol L^{-1}). CL from 5 μmol L^{-1} FCLA and 5 μmol L^{-1} FCLA mixed with 5 μmol L^{-1} HSA were measured from different concentrations of $^1O_2$.

To identify the nature of the enhanced CL when FCLA is combined with HSA, total CL production from FCLA alone (5 μmol L^{-1}) and the FCLA–HSA mixture (5 μmol L^{-1} each) was measured by continuously irradiating the samples until the signal diminished to the background level.
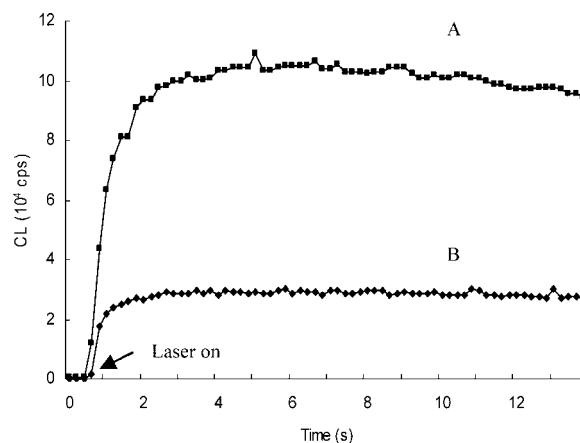
To verify the ROS selectivity of FCLA and FCLA–HSA a known $^1O_2$ quencher, NaN$_3$ (1 mmol L^{-1}) was added to the solution before the light measurement. The concentrations of FCLA and HSA were both 5 μmol L^{-1}.

Spectroscopic measurements. To identify potential structural change and/or binding of the probe and HSA, absorption spectra of FCLA and HSA alone and of the FCLA–HSA mixture were recorded with a Lambda 35 UV/VIS spectrometer (Perkin Elmer). The fluorescence and CL spectra of the samples were recorded with a LS 55 luminescence spectrometer (Perkin Elmer). For the FCLA-quenching of HSA fluorescence, various concentrations of FCLA and a fixed concentration of HSA were mixed to make a total volume of 600 μL in a standard quartz cuvette. The final protein concentration was 2.5 μmol L^{-1}, and the molar ratios of FCLA to protein varied from 0 to 4. The fluorescence spectra were recorded with 279 nm excitation. All experiments were performed at room temperature.

RESULTS

CL measurements of FCLA with and without HSA during the photosensitization reaction

Photosensitization reaction, similar to photodynamic therapy, provides a very well-controlled $^1O_2$ source. With light irradiation there was a noticeable luminescence (*ca* 400 cps at laser fluence rate of 25.5 mW cm^{-2}) due to scattered irradiation light reaching the detector. This background luminescence was nearly identical for samples containing water only, water with HSA, water with PpIX, water with HSA and PpIX only. For samples containing FCLA but no light irradiation we observed a small but consistent luminescence signal above the dark-current counting of the optical system (Fig. 2). The background luminescence without light irradiation was likely due to the FCLA self-oxidation.

After subtraction of the background luminescence and scattered irradiation light FCLA CL was observed from all samples containing FCLA and PpIX, regardless of the presence or absence of HSA. The CL intensity was consistently stronger in samples from the FCLA–HSA mixture, compared with samples from FCLA alone. Figure 2 shows a representative comparison of the real-time CL intensities.

Figure 3 shows the CL intensities for various HSA/FCLA ratios at two fixed FCLA concentrations. For each FCLA concentration the largest CL intensity was observed in samples with equal molar
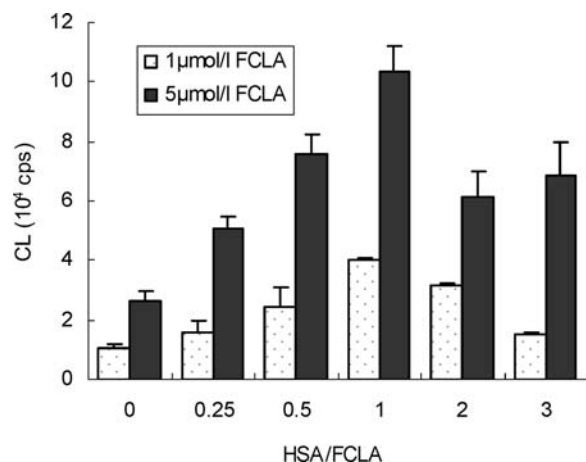
Figure 3. Comparison of CL intensities from various HSA/FCLA concentration ratios with 5 μmol L^{-1} or 1 μmol L^{-1} FCLA, 2 μmol L^{-1} PpIX and 25.5 mW cm^{-2} fluence rate. The largest CL intensity was observed in samples with equal molar concentrations of FCLA and HSA. Data are presented as mean ± SD of at least three separate experiments.

concentrations of FCLA and HSA and was nearly four times that in FCLA alone.

Figure 4 shows the CL intensity comparisons of FCLA and FCLA–HSA for different PpIX concentrations. The CL enhancement by HSA was consistently observed for all photosensitizer concentrations investigated.

The CL was also measured during continuous irradiation until the signal diminished to the background level. The results show that, given identical experimental protocols, the integrated luminescence signal from the FCLA–HSA mixture was seven times that from FCLA alone (Fig. 5).

Without FCLA, CL from HSA alone during the photosensitization reaction was extremely weak and practically undetectable with the current experiment setup. It is clear that the CL enhancement observed for the FCLA and HSA mixture was a synergetic effect rather than a simple summation.
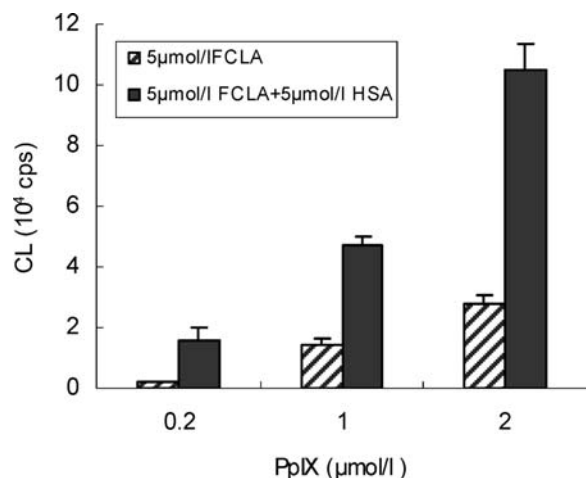

Figure 4. Comparison of CL intensities from different concentrations of PpIX with 5 μmol L^{-1} FCLA in the absence of and in the presence of 5 μmol L^{-1} HSA. Data are presented as mean ± SD of at least three separate experiments.

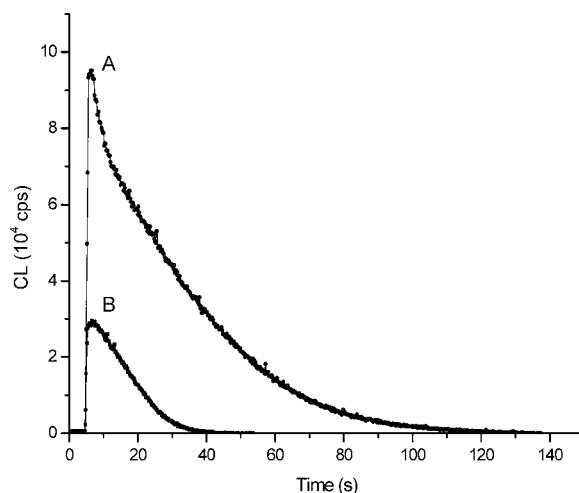**Figure 5.** CL of 5 μmol L^{-1} FCLA in the presence (A) and absence (B) of 5 μmol L^{-1} HSA with continuous irradiation until the signal diminished to the background level .

Effect of $^1O_2$ quencher NaN$_3$ on FCLA CL and FCLA–HSA CL in photosensitization reaction

To investigate the origins of FCLA CL and FCLA–HSA CL in the current study, CL from photosensitization reaction was evaluated with and without the $^1O_2$ quencher NaN$_3$. As shown in Figure 6 FCLA CL was quenched 64% and FCLA–HSA CL was quenched 79% by NaN$_3$.

Stoichiometry of FCLA–HSA interaction and binding

To analyze the characteristics of the FCLA–HSA binding, the stoichiometry of HSA fluorescence quenching by FCLA was investigated. The results show that, when mixed with different concentrations of FCLA, the fluorescence of HSA at 337 nm was quenched proportionally in comparison to its control value (Fig. 7). Aside from the self-quenching mechanism, HSA fluorescence may also be quenched by several other mechanisms, such as dynamic and static quenching. Considering that the volume of FCLA in the experiments was far smaller than that of HSA, the dilution
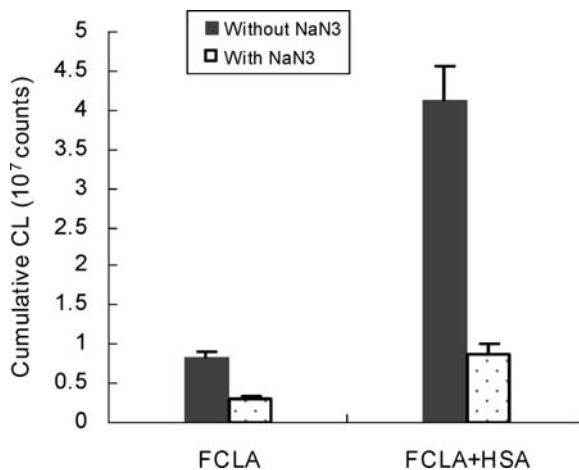

Figure 6. Effect of 1 μmol L^{-1} NaN$_3$ on FCLA CL and FCLA–HSA CL in the photosensitization reaction. Data are presented as mean ± SD of at least three separate experiments.
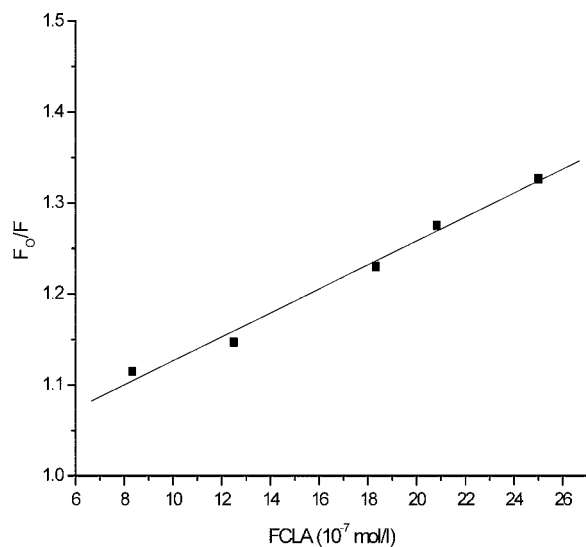
Figure 7. The curve of the relationship between F_0/F and the concentration of FCLA.

Figure 9. Absorption spectra of 5 μmol L^{-1} FCLA (dashed line), 5 μmol L^{-1} HSA (boxed line) and 5 μmol L^{-1} FCLA mixed with 5 μmol L^{-1} HSA (solid line).

effect of FCLA was thus negligible. Dynamic quenching is caused by physical collision among molecules and can be described by the Stern–Volmer equation (23)

$$\frac{F_0}{F} - 1 = k_q \cdot \tau_0[Q] = K_D[Q]$$

where F and F_0 are the relative fluorescence intensities of HSA in the presence and absence of quencher Q (FCLA), respectively. k_q is the quenching constant. τ_0 is the excited-state lifetime in the absence of Q (approximately 10^{-8} s for macromolecules) (24). K_D is the Stern–Volmer constant. The quenching constant, k_q, for the FCLA–HSA system was calculated using the data shown in Figure 7. With the Stern–Volmer equation kq was found to be 1.31×10^{13} L mol^{-1} s^{-1}. This value is much larger than that of the limiting diffusion rate constant of typical biomolecules (2×10^{10} L mol^{-1} s^{-1}), so dynamic quenching is not likely to be a primary reason in
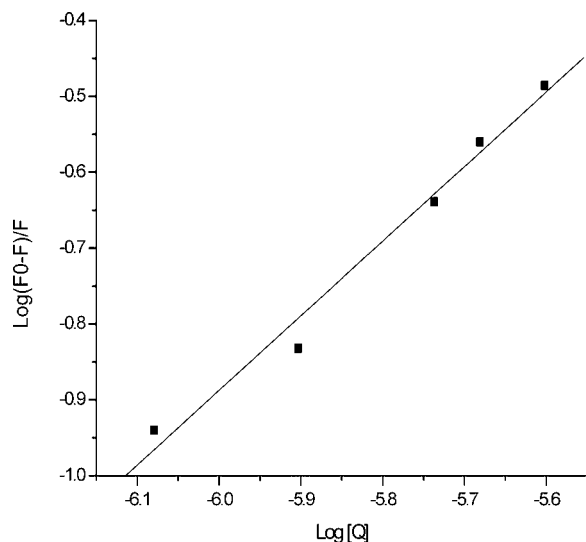
the quenching of HSA fluorescence (25,26). Static quenching can result from the formation of a ground-state complex that is non-fluorescent or weakly fluorescent. According to Jiang *et al.* (27), if molecule Q has *n* binding sites on protein HSA the relative protein fluorescence intensities F and F_0 (in the presence and absence of Q, respectively); the concentrations of Q in the reaction can be described as follows:

$$\log\frac{F_0 - F}{F} = \log Ka + n\log[Q]$$

In this study Q was regarded as probe FCLA. When the ratio of the concentration of probe to protein is less than or equal to 1 : 1, $\log\frac{F_0-F}{F}$ is highly linear to log[Q] (R = 0.99; *P* = 0.001) (Figure 8). The slope of the linear regression (the number of binding sites) was found to be 0.98. This suggests that FCLA has only one binding site on HSA.

Absorption and CL spectra of FCLA in the absence and the presence of HSA

Figure 9 shows representative absorption spectra of FCLA, HSA and their mixture at a 1 : 1 concentration ratio. With HSA alone there was a distinctive peak at 279 nm, whereas the major absorption peak of FCLA was at 496 nm. When FCLA was mixed with HSA the absorption spectrum of the compound consisted of the two corresponding absorption peaks, with one important difference. Although the absorption peak wavelength of HSA at 279 nm did not change, the one corresponding to the FCLA had shifted 5 nm (from 496 nm to 501 nm) when FCLA was mixed with HSA. The wavelength shift was also observed in the corresponding CL spectrum. As shown in Figure 10 the peak CL wavelength shifted from 522 nm of FCLA alone to 526 nm when FCLA was mixed with HSA.

Excited-state intermolecular energy transfer in FCLA–HSA complexes

To explore the mechanism of CL enhancement for the FCLA–HSA complex, the fluorescence spectra of FCLA, HSA and their complex were further examined. Figure 11 shows the excited-state

Figure 8. Logarithmic plot of fluorescence quenching of HSA treated with different FCLA concentrations. λex = 279 nm.

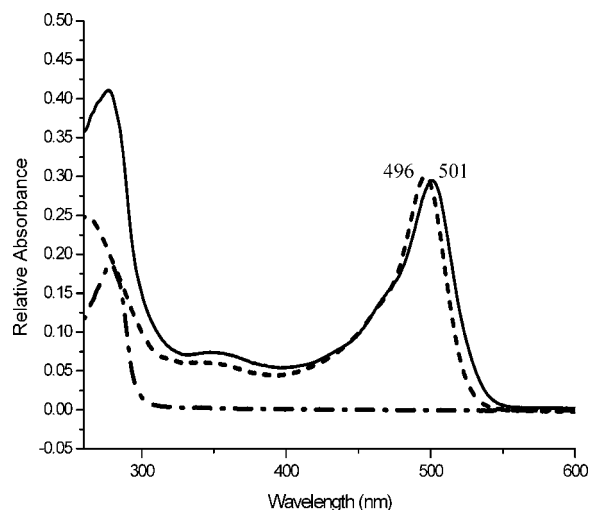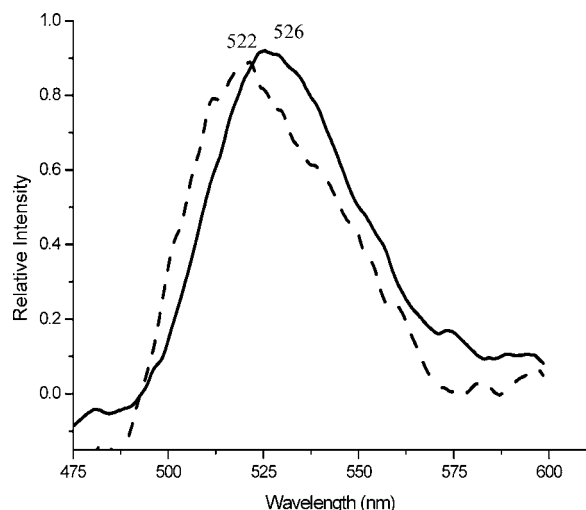**Figure 10.** CL spectra of 5 μmol L^{-1} FCLA in the absence (dashed line) and the presence (solid line) of 5 μmol L^{-1} HSA.

Figure 11. Fluorescence spectra of 2.5 μmol L^{-1} HSA in the absence (1) and the presence (3) of 2.5 μmol L^{-1} FCLA. Curve 2 represents the fluorescence spectrum of 2.5 μmol L^{-1} FCLA alone. λex = 279 nm.

intermolecular energy transfer spectra in FCLA-HSA complexes. The binding of FCLA to HSA caused an efficient quenching of the HSA fluorescence. Simultaneously, the FCLA fluorescence intensity was enhanced. As shown in Figure 12, in the H_2O_2/NaOCl reaction there was an observable HSA CL at 450 nm when HSA alone reacted with $^1O_2$ (the background luminescence of FCLA self-oxidation was close to the dark-current counting of the optical system and was ignored). When FCLA was mixed with HSA the HSA CL at 450 nm was reduced and the FCLA CL at 526 nm was enhanced. The data clearly indicate that there is an intermolecular energy transfer from HSA at its excited state to FCLA in the FCLA–HSA complex.

DISCUSSION

We have previously reported that the efficiency of FCLA CL production is highly improved in the presence of HSA (21,22). When FCLA and HSA are combined for detection of $^1O_2$ the resultant total CL is synergetic rather than a case of simple summation. Considering that HSA is a natural protein that is present in all parts of a human body and that CL has a broad range of potential applications, understanding the mechanism of this CL enhancement phenomenon becomes critical. The current study utilizes direct CL measurement and spectroscopy techniques to investigate the phenomenon and to analyze the underlying mechanism.

In the current study $^1O_2$ was produced from the following different systems: chemical reaction between H_2O_2 and NaOCl and a photosensitization reaction similar to that in photodynamic therapy. Although the $^1O_2$ source was different and the concentrations of $^1O_2$ were different (Fig. 4), the FCLA CL was enhanced by HSA in both cases, suggesting the enhancement was source independent. In a well-controlled photosensitization reaction with the $^1O_2$ quencher NaN$_3$, CL was quenched 64% from FCLA alone; the value changed to 79% when HSA was added (Fig. 6). This clearly indicates that the addition of HSA did not hamper the specificity of the ROS probe, if not improve it.

The CL was measured with a fixed concentration of FCLA mixed with various concentrations of HSA in the photosensitization reaction. When the HSA–FCLA concentration ratio was 1 : 1, the largest CL intensity (close to 4 times that from FCLA alone)
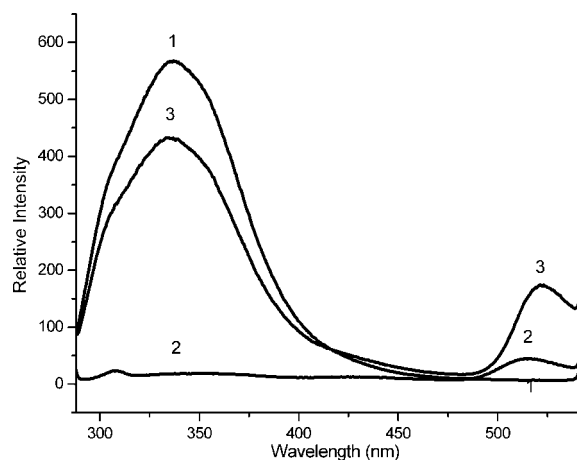
was obtained (Fig. 3). The enhancement was found to be true both in the intensity at a given moment during an irradiation and in the overall integrated CL values. With further increase of the HSA concentration, the CL enhancement was less efficient (Fig. 3). This can be explained as follows. HSA alone is a known $^1O_2$ quencher. When excess HSA is present it directly reacts with and quenches $^1O_2$ to produce a protein carbonyl group. The elevated energy state of the protein carbonyl groups typically goes through nonradiative decay with low quantum yield in photon production (18). This results in a decreased amount of $^1O_2$ available for interaction with FCLA to produce CL.

The enhancement mechanism was further investigated on the basis of spectroscopy results. Evaluation of the stoichiometry of the HSA fluorescence quenching by FCLA revealed that FCLA can combine with HSA and that FCLA has only one binding site on HSA. This finding is confirmed by the finding that maximum CL enhancement was achieved in samples with equal amounts of HSA and FCLA. When FCLA was combined with HSA the peak FCLA absorption and CL wavelengths red-shifted by 5 and 4 nm, respectively.

When combined with FCLA, it is likely that the $^1O_2$ quencher HSA can, at its elevated energy state, pass its energy *via* excited-state intermolecular energy transfer to FCLA. Comparison of the fluorescence spectra for HSA, FCLA and the FCLA–HSA complex
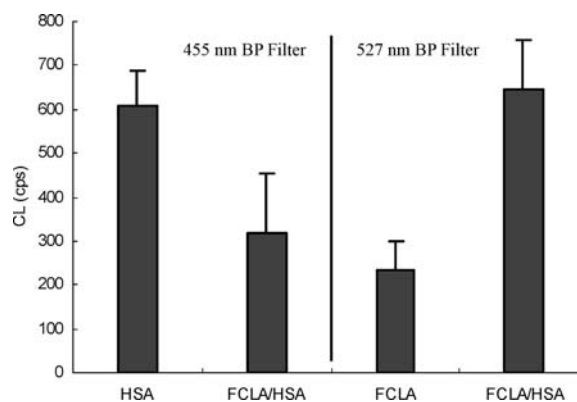

Figure 12. Comparison of HSA CL with FCLA CL before and after FCLA was mixed with HSA in the H_2O_2/NaClO solution. Data are presented as mean ± SD of at least three separate experiments.

(Fig. 11) and for HSA CL and FCLA CL (Fig. 12) clearly revealed that Förster energy transfer occurred in the FCLA–HSA complex. Because it is a very efficient $^1O_2$ quencher, HSA is easily excited by $^1O_2$. After it combines with FCLA the HSA protein carbonyl group (which is the product of reaction between HSA and $^1O_2$) transfers its elevated energy to excite FCLA. Therefore, there may be two luminescence processes by FCLA. First is the true CL process involving FCLA reaction with $^1O_2$:

$$FCLA + {}^1O_2 \rightarrow FCLA = O^* \rightarrow FCLA = O + h\nu$$
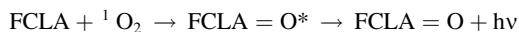
This is an irreversible process in which the chemical structure of FCLA is altered; FCLA thus cannot be involved in future CL production. Second is the luminescence from the radiative transition of the excited FCLA due to the energy transfer from HSA. This is a reversible process in which FCLA molecules can recycle in photon production.

Given same amount of photosensitizer and irradiation fluence rate, CL was measured from samples containing FCLA alone and the FCLA–HSA mixture until the signals were reduced to background level (Fig. 5). In comparison, the value from FCLA–HSA was a magnitude higher than that of FCLA alone. Fresh air/oxygen was injected into the solvent after a certain period of irradiation during the measurement and we found little or no increase in CL (data not shown). This indicated that the oxygen consumption did not affect the singlet oxygen produced with the fixed concentration of PpIX. The result clearly demonstrates that radiative transition of the excited FCLA contributes greatly to the CL collected during the measurement. This is because FCLA CL is a nonreversible process: if there was no radiative transition due to direct excitation of FCLA by energy transferred from HSA, the total integrated CL from the two experiments would be similar.

The spectra results show that the peak wavelengths of fluorescence and the CL spectra of FCLA alone are 516 nm (Fig. 11) and 522 nm, respectively. There is a 6 nm difference. When FCLA combines with HSA its fluorescence peak wavelength changes to 522 nm and the corresponding CL peak wavelength is 526 nm. This is further evidence that the spectrum of the complex is a combination of FCLA CL and excited FCLA luminescence mediated by energy transfer from HSA.

Although there is no direct proof from the current study there are other potential mechanisms by which HSA can enhance the FCLA CL. This may include but not be limited to the following. Analysis of the FCLA CL mechanism reveals that the optimum reaction environment for FCLA CL is similar to that of the typical range found in physiological reactions. During the FCLA CL process there is an important intramolecular proton transfer *via* H_2O assistance (28). HSA is a typical amphoteric molecular and is able to get and release a proton. When HSA is combined with FCLA it is likely that the presence of HSA favors the reaction between FCLA and $^1O_2$. The excitement of FCLA due to HSA energy transfer may also increase the chance of its CL reaction with $^1O_2$. Last but not least the presence of HSA may improve the quantum yield of photons via radiative de-excitation after the excited FCLA reacted with $^1O_2$, because of changes in microenvironment around the molecules.

In conclusion, the mechanism by which HSA enhances FCLA CL has been examined for the first time. The results demonstrate that the FCLA–HSA complex is a more efficient method for producing CL in $^1O_2$ detection, compared with FCLA alone. The enhancement in the photon production is due to an energy transfer process from HSA reacting with $^1O_2$ to excite FCLA.

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