ANALYTICAL SCIENCES JANUARY 2008, VOL. 24

A Novel Chemiluminescence Technique for Quantitative Measurement of Low Concentration Human Serum Albumin

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An efficient and highly sensitive chemiluminescence (CL) technique is proposed in the current study for detection of low levels of human serum albumin (HSA). Chemiluminescence (CL) produced during interaction between fluoresceinyl cypridina luciferin analog (FCLA)-$^1O_2$ can be modified with the presence of HSA. The conventional CL technique uses a quenching effect of HSA for its quantitative measurement. We are reporting here that the CL intensity can be enhanced, rather than quenched, by the addition of HSA. The CL signal can be linearly correlated with the HSA concentration over a clinically interesting range of 5×10^{-9} – 8×10^{-8} mol L^{-1}, with a detection limit of 2.5×10^{-9} mol L^{-1}. The determination result was consistent with that obtained from conventional methods. One possible mechanism of HSA detection technique using CL enhancement approach is discussed. Intermolecular energy transfer in chemiluminescence systems and changes of microenvironment are likely to be contributors of the CL enhancement with HSA.

(Received August 21, 2007; Accepted October 5, 2007; Published January 10, 2008)

Introduction

Human serum albumin (HSA) is the most abundant protein of human serum. It is critical for maintaining the osmotic pressure and its level in human urine is an important index for clinical assay and diagnosis. Quantitative analysis of HSA provides critical information for early diagnosis and treatment of nephrosis.[1,2]

At present, the established clinical method to determine low-level HSA is radioimmunoassay. Its principle begins with the facts that antigens with and without radioactive elements can bind competitively to specific antibodies. There is a certain functional relationship between the antigen and the impulse signal emitted by labeled antigen-antibody complexes. Under the same conditions, the impulse signal tested can provide the concentration of antigen in sample according to a functional relationship. The approach can reliably quantify HSA in urine samples up to10^{-9} mol L^{-1}, but with limitations imposed by its high cost and the long time required for the analysis. As for analysis of low level concentrations of HSA, the most commonly used methods are the Lowry,[3] CBBG-250,[4,5] electrochemiluminescence,[6] spectrophotometry,[7] fluorospectrophotometry,[8] and Rayleigh light scattering.[9,10] However, these methods are also costly, tedious or suffer from the disadvantages of low sensitivity and narrow linear range. An alternative method with low operational cost and more efficient detection speed to determine micro-amount human serum albumin is thus desired.

Among analytical techniques, chemiluminescence (CL) is considered as the most sensitive and versatile analytical technique. Current research in CL analysis is focused on two general directions: to discover new specific CL emitters and to broaden the analytical scope of the existing CL techniques. As chemiluminescence emitters, luminol, lucigenin, 1,10-phenanthroline, the Ru(II) complex are widely used to determine protein.[11-13] Reactive oxygen species (ROS, singlet oxygen, hydroxyl radical and superoxide anion) are involved in these chemiluminescence mechanisms.

It is well known that singlet oxygen is extremely oxidative and this is often employed in biochemistry and biomedical analysis.[14,15] Fluoresceinyl cypridina luciferin analog (FCLA) is a CL probe that can specifically interact with singlet oxygen and superoxide; it thus has a less complicated mechanism compared to other CL probes with poorer selectivity. Nearly all existing CL protein detection techniques are based on the principle that, once the protein reaches certain concentration levels, it can quench CL. Yet, we have previously reported that, by carefully controlling the ratio of HSA and FCLA concentration, the presence of HSA can actually enhance the production of FCLA CL.[16] The main contents of previous work were to discover the phenomenon that CL from FCLA-$^1O_2$ can be greatly enhanced by addition of HSA. Developing a new approach to detect singlet oxygen during the process of photodynamic therapy (PDT) was the purpose of former research. In this work, the FCLA CL enhancement by the presence of HSA has been investigated as a novel and highly sensitive means for low-level protein detection. Compared to that conventional quenching method, the technique based on CL enhancement provides a new means for quantitative measuring extreme low concentration of HSA. To our knowledge, this is the first reported attempt to use the CL enhancement as a means to determine HSA.

In our experiment, singlet oxygen, produced by the chemical reaction between hydrogen peroxide and sodium hypochlorite, can react with FCLA and emit 532 nm light. Meanwhile, it was confirmed that both bovine serum albumin (BSA) and lysozyme can also enhance CL of FCLA-$^1O_2$. For comparison, the sensitivity of FCLA is much higher than that of the widely used CL emitter luminol. Therefore, FCLA would be a good CL

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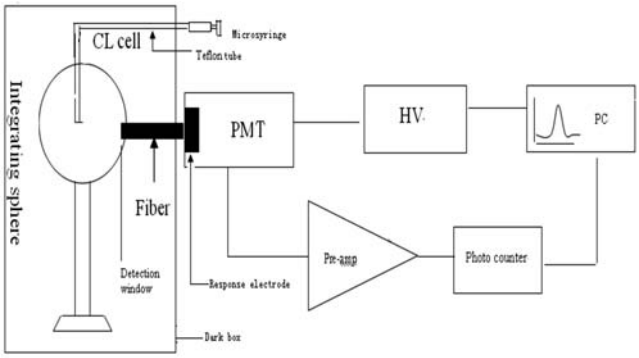

Fig. 1 Schematic diagram of chemiluminescence analysis. PMT, photomultiplier; HV, high voltage power; PC, computer.

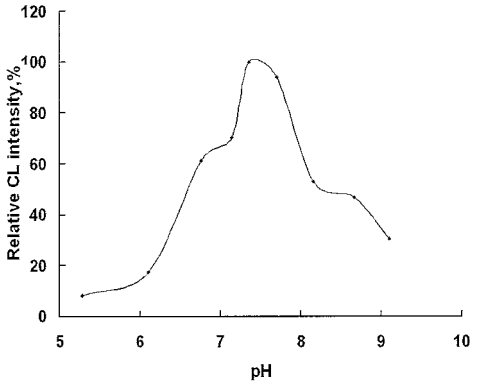

Fig. 2 Effect of pH on CL reaction. Conditions: FCLA, 1×10^{-6} mol L^{-1}; H_2O_2, 4×10^{-3} mol L^{-1}; NaClO, 1×10^{-2} mol L^{-1}; HSA, 8×10^{-8} mol L^{-1}; temperature, 25°C. Every experiment was repeated five times.

emitter, which has an application prospect to determine proteins and drugs.

We have designed a chemiluminescence analysis apparatus for determination of HSA based on its enhancement of FCLA-CL phenomenon as described above. Factors of experiments were investigated including pH, temperature, concentration of FCLA, interference of coexisting substances as well as metal ions, amino acids and proteins which affected the determination. Based on results, a simple, effective, and highly sensitive method to quantify HSA with novel CL emitter was developed. The method was low in cost and had a high sensitivity. Results were consistent with those from conventional methods.

Experimental

Reagents

HSA, purchased from Sigma (St. Louis, MO, USA), was used without further purification. Stock HSA solution was prepared by directly diluting 0.069 g of HSA in 100 mL of water to a concentration of 1×10^{-5} mol L^{-1} and this solution was stored at 4°C. Each working solution was freshly prepared by appropriate dilution with water.

Hydrogen peroxide and sodium hypochlorite were prepared by dissolving 228 μL of hydrogen peroxide (30%) and 6.77 mL of sodium hypochlorite (Guangzhou Chemical Reagent Co., China) in 100 mL doubly distilled water. The stock solution of 1×10^{-4} mol L^{-1} FCLA (Tokyo Kasei Kogyo Co., Tokyo, Japan) was prepared by dissolving 1 mg of FCLA in 15 mL of water deoxygenated by N_2 bubbling and the solution was stored at –20°C. Phosphate buffered saline (PBS) was prepared by dissolving 8.5 g of sodium chloride, 2.2 g of sodium hydrogen phosphate, and 0.2 g of sodium dihydrogen phosphate in 1 L of water. The pH value of this solution was titrated to 7.4 ± 0.1. All reagents were of analytical reagent grade or the best grade commercially available and were used without further purification. Water used throughout was doubly deionized. Urine samples were collected from volunteers.

CL apparatus

All chemical reactions to generate singlet oxygen and subsequent FCLA CL occurred within an optical integrating sphere (IOI Technology Co., Ltd., Shanghai, China) with windows for chemical-sample feeding and CL signal collection. Each CL signal was collected from the sphere using a photon multiplier tube (PMT, Model MP-952, Perkin Elmer, USA) running on single photon counting mode. The signal collection was controlled and analyzed with a custom Labview program. The pH value was measured by acid measurer PB-10 (Sartorius, Germany). Fluorescence spectra and CL spectra were obtained using spectrofluorometer LS-55 (Perkin Elmer, USA).

Procedure

As shown in Fig. 1, for each sample analysis, a volume of 480 μL of a solution, comprised of sodium hypochlorite (60 μL), FCLA (6 μL), and PBS (414 μL) was injected into an air-tight thin-layer reaction cell inside the integrating sphere. The CL reaction was initiated by further injection of hydrogen peroxide (120 μL). CL signal, without the addition of HSA, was recorded as a control. The procedure was repeated, now with addition of HSA at various concentrations to construct the figure curve, or with addition of actual urine samples for the protein measurement. The CL signal was integrated for 5 s for each experiment. After subtracting the control CL signal, we plotted the CL values as a function of HSA concentration to generate a titration curve. The CL signal from the actual urine samples was then calibrated against this curve for its HSA concentration.

Results and Discussion

Effect of pH on CL reaction

The CL intensity was greatly affected by pH, as shown in Fig. 2. With the presence of HSA, CL was examined over a pH range between 5.3 – 9.1. Given otherwise identical conditions, the CL signal was maximum at pH 7.4. Around this peak value, the signal was relatively stable within the pH range of 7.3 – 7.7. Thus, pH 7.4 was selected for the subsequent experiments.

Effect of concentration of reaction reagents

With consideration of both acquired CL intensity and *S*/*N*, for the given apparatus, the concentrations of FCLA, hydrogen peroxide, sodium hypochlorite selected for the analysis were 1×10^{-6}, 4×10^{-3}, and 1×10^{-2} mol L^{-1}, respectively.

Interferences of coexisting foreign substances

In order to assess the feasibility and reliability of the proposed method for quantitative measurement of HSA concentration in urine, we examined the influences of substances commonly found in clinical urine samples. CL was measured with HSA (8×10^{-8} mol L^{-1}) only and then with various mixed concentrations of these substances. The results are listed in Table 1.

A variation greater than 8% from the average CL value would be considered as a significant interference. We found that, within clinically possible concentrations, the majority of these substances gave less than 10% of interference to the CL measurement results. In addition, tryptophan, bovine serum albumin and lysozyme were investigated and their interference to the measurement could be substantial (> 10%). Yet, for all practical purposes, these three compounds are not normally found in human urine samples.

Titration curves

A standard titration curve was constructed by plotting the CL measurement value against HSA concentrations, as shown in Fig. 3. Within the HSA value investigated, the curve obeys a first-order equation: $\Delta I_{CL} = (0.05 \pm 0.03) + (0.19 \pm 0.01)C_{HSA}$ (C_{HSA}: 10^{-8} mol L^{-1}, $R^2 > 0.99$, $n = 5$), where ΔI_{CL} is the net change in CL intensity (10^5 cps as unit), C_{HSA} is the concentration of HSA (10^{-8} mol L^{-1}). The titration curve is effective with a range between 5×10^{-9} - 8×10^{-8} mol L^{-1} with a detection limit of 2.5×10^{-9} mol L^{-1} (3σ). The limit of detection (LOD) was given by equation LOD = KS_0/S,[17] where K is a numerical factor chosen according to the confidence level desired, S_0 is the standard deviation (SD) of the blank measurements and S is the slope of titration curve. The relative standard deviation (RSD) at 8×10^{-8} mol L^{-1} of HSA is 5.8% (n = 11).

Table 1 Effect of coexisting substances

Substance	Concentration/ 10^{-6} mol L^{-1}	Clinical rang/ $\times 10^{-6}$ mol L^{-1}	Change of ΔCL, %
K^+NO^{3-}	35	0.65 - 1.31	–6.2
$Mg^{2+}SO_4^{2-}$	9	0.08 - 0.34	–3.8
Na^+Cl^-	100	2.24 - 4.5	6.6
$NH_4^+SO_4^{2-}$	10	1.11 - 3.89	4.8
$Zn^{3+}SO_4^{2-}$	10	0.04 - 0.28	–7.7
$Pb^{2+}CH_3COO^-$	20	—	–9.8
Glucose	20	0.28 - 1.5	2.5
Xanthine	83	—	4.5
d-Sorbitol	12	—	5.9
l-Hydroxyproline[a]	30	0.18 - 1.8	–8.5
l-Tyr[a]	30	—	1.2
l-Trp[a]	10	—	12
Bovine serum albumin	0.1	—	17.8
Lysozyme	0.1	—	13.3

Conditions: FCLA, 1×10^{-6} mol L^{-1}; H_2O_2 , 4×10^{-3} mol L^{-1}; HSA, 8×10^{-8} mol L^{-1}; NaClO, 1×10^{-2} mol L^{-1}; pH 7.4; temperature, 25°C. Results of clinical range are diluted a 1000 fold.
a. mg/L.

Comparison of several analysis methods

The current method has a lower opertational cost and a higher sensitivity than that of flurescence spectra analysis, total internal reflected resonance light scattering, synchrony fluorescence scanning technique, Rayleigh light scattering technique, as shown in Table 2.

Application: determination of HSA in urine

The proposed method was tested to quantitate HSA in human urine samples. As the assay was intended for extremely low levels of HSA in urine, fresh samples pre-evaluated with conventional technique were diluted appropriately to be within the linear range of the current method. The standard titration curve, as described above, was used to calculate the concentration of HSA in a sample, based on the results from the corresponding CL measurements. The HSA concentration from the measured sample was corrected for its dilution factor and compared to its corresponding value from conventional clinical assay using the paired student *t*-test. The result indicates that there is no statistically significant difference between the values (confidence level of 95%), thus confirming the reliability of the current method. The results are summarized in Table 3.

Possible Mechanism of Increased CL

CL spectra of FCLA-$^1O_2$

In our chemical reaction system, hydrogen peroxide reacts with sodium hypochlorite and generates singlet oxygen ($^1O_2$). The interaction between H_2O_2 and FCLA as well as NaClO and FCLA was investigated and no CL was observed. Adding hydrogen peroxide to the mixture of FCLA and sodium

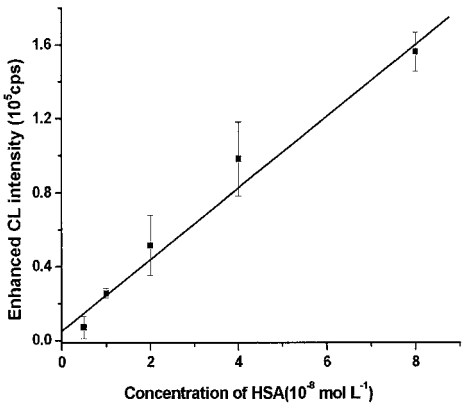

Fig. 3 The titration curve. Conditions: FCLA, 1×10^{-6} mol L^{-1}; H_2O_2, 4×10^{-3} mol L^{-1}; NaClO, 1×10^{-2} mol L^{-1}; temperature, 25°C.

Table 2 Comparison of this proposed technique and several conventional methods

Method	Linear range/10^{-6} mol L^{-1}	LOD/μg L^{-1}	Regression coefficient	RSD, [a] %	Ref.
Fluorescence spectrum	0 - 0.14	64	0.9948	3.7 (0.07)	18
	0.14 - 0.51	115	0.9987		
Total internal reflected-resonance light scattering	0.00221 - 0.015	14.4	0.9981	2.9 (0.14)	10
Synchronous fluorescence-scanning technique	0.0015 - 0.15	22	0.9977	3.2 (0.11)	19
Rayleigh light scattering	0.103 - 0.530	882	0.9988	0.76 (0.29)	9
This proposed method	0.005 - 0.08	17.32	0.9919	5.8 (0.08)	

Table 3 Determination results for urine

Sample	This methodfound[a]/mg L^{-1}	Clinical data[b]/mg L^{-1}	*t*-test[c]
1	3.87 ± 0.32	3.73	0.94
2	10.97 ± 1.05	9.17	2.07
3	5.48 ± 0.58	5.34	0.54
4	8.08 ± 0.65	7.07	2.17

a. Mean of five determinations ± standard deviation.
b. The date provided by Nanfang hospital which detected a 24 h microalbumin in urine.
c. One-sample *t*-test, $t_{0.95,4} = 2.776$.

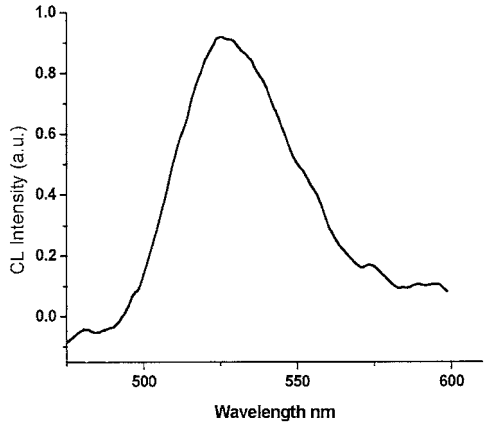

Fig. 4 A representative CL spectrum of FCLA-$^1O_2$. Conditions: FCLA, 1×10^{-6} mol L^{-1}; H_2O_2, 4×10^{-3} mol L^{-1}; NaClO, 1×10^{-2} mol L^{-1}. Emission slit width is 5 nm.

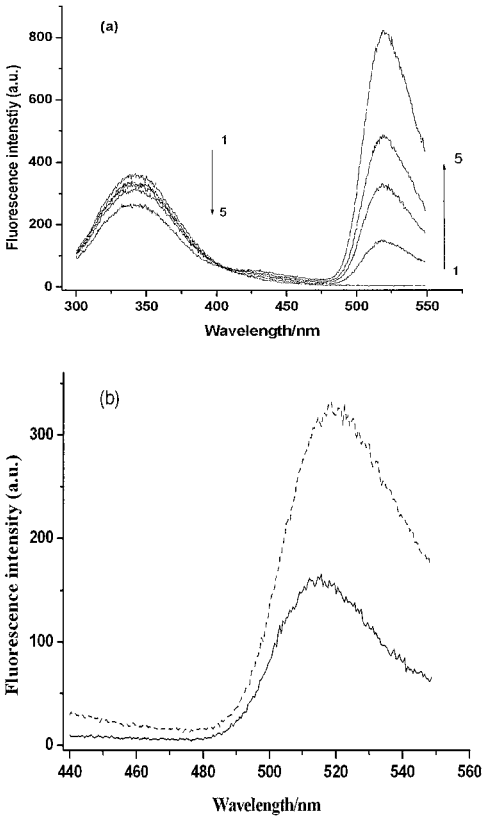

Fig. 5 (a) Fluorescence spectra of HSA (2×10^{-6} mol L^{-1}) as a function of FCLA concentrations (λ_{em} = 280 nm). Curves 1 → 5 correspond to 0, 2, 4, 12, and 16×10^{-6} mol L^{-1} FCLA concentrations. Slit widths are both 5 nm. (b) Comparison of FCLA (4×10^{-6} mol L^{-1}) fluorescence intensity in the presence of HSA and in the absence of HSA. The excitation wavelength for emission spectra is 288 nm. Dashed line represents the presence of 2×10^{-6} mol L^{-1} HSA; solid line means the absence of HSA. Slit widths are both 5 nm.

hypochlorite resulted in the observed CL. The CL emission spectrum was obtained immediately after the excitation light source was turned off, as shown in Fig. 4. The spectrum is consistent with that of FCLA-$^1O_2$ reported previously.[16] Therefore, we confirmed that the CL emission was due to FCLA-$^1O_2$.

Intermolecular energy transfer in chemiluminescence system

According to Förster's theory, the efficiency of energy transfer depends mainly on the following factors: (I) the extent of overlap between the donor emission and the acceptor absorption, (II) the orientation of the transition dipole of donor and the acceptor, and (III) the distance between the donor and the acceptor.[20] Figure 5a reveals that fluorescence of HSA at 348 nm decreases with an increasing of FCLA concentration. The energy from excited-state HSA is likely transferred to FCLA or is lost by non-irradiation. Figure 5b shows that fluorescence intensity of FCLA with the presence of HSA increases greatly compared with that without the presence of HSA in the conditions of Fig. 5a. This provides strong evidence for the occurrence of Förster's type energy transfer from the tryptophan moiety (donor) in HSA to the FCLA molecule (acceptor). Following Zhou, we know that the FCLA probe is located in the inter domain region of HSA (near Trp-214) and that the distance from bonded-FCLA to Trp214 is less than 5 nm.[16] We consider that efficient energy transfer can exist between HSA (donor) and FCLA (acceptor).

Interaction force and reaction microenviroment changes

Owing to some hydrophobic group such as a hetero-nitrogen ring, benzene ring, the water solubility of FCLA is poor. The reported pK_a values of the benzene carboxy group and phenolic hydroxyl group of FCLA in water are in the range of 4.2 – 4.4 and 10.0, respectively.[21-23] FCLA is negatively charged in pH 7.4 buffer solution. HSA is an amphipathic molecule in water solution. Its surface is hydrophilic, while the intra-molecular structure is hydrophobic. Therefore, we propose that HSA bonds to FCLA by electrostatic interaction. HSA plays a role as a nonionic surfactant and attracts FCLA to the hydrophobicity domain of HSA. The contribution of HSA is to enhance the solubility of FCLA, and thus change the reaction microenvironment. This enhances the CL quantum yield and is better for intermolecular energy transfer.[24,25]

Conclusion

A highly ROS-selective CL probe FCLA is, for the first time, employed to determine proteins. With proper conditions, FCLA-$^1O_2$ CL can be significantly enhanced by the addition of HSA. The extent of the enhancement can be proportional to the concentration of HSA. The probe combined with our custom made CL analyzer has been tested to quantify low level HSA in human urine samples, and the result is in excellent agreement with that by conventional method. The proposed method is technically simple and highly sensitive. The mechanism of CL

enhancement in the presence of HSA was also investigated. Intermolecular energy transfer between the chemiluminescence probe and HSA and changes in microenvironment due to the combination of the two, are likely to be the main factors contributing to the CL enhancement.

Acknowledgements

This research is supported by the National Natural Science Foundation of China (30470494; 30627003; 60678050) and the Natural Science Foundation of Guangdong Province (015012) and the US NIH grant PO1-43892.

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