In vivo monitoring of singlet oxygen using delayed chemiluminescence during photodynamic therapy

Yanchun Wei
Jing Zhou
Da Xing
South China Normal University
MOE Key Laboratory of Laser Life Science and
Institute of Laser Life Science
Guangzhou 510631 China
E-mail: xingda@scnu.edu.cn

Qun Chen
University of Colorado at Denver and
Health Sciences Center
Department of Radiation Oncology
Aurora, Colorado 80262
and
South China Normal University
MOE Key Laboratory of Laser Life Science and
Institute of Laser Life Science
Guangzhou 510631 China

Abstract. It is known that singlet oxygen ($^1O_2$) is the main factor mediating cytotoxicity in photodynamic therapy (PDT). The effectiveness of a PDT treatment is directly linked to the $^1O_2$ produced in the target. Although the luminescence from $^1O_2$ is suggested as an indicator for evaluating photodynamic therapy, the inherent disadvantages limit its potential for *in vivo* applications. We have previously reported that chemiluminescence can be used to detect $^1O_2$ production in PDT and have linked the signal to the cytotoxicity. We further our investigation for monitoring $^1O_2$ production during PDT. The lifetime of 3,7-dihydro-6-{4-[2-(*N*′-(5-fluoresceinyl)thioureido)ethoxy]phenyl}-2-methylimidazo {1,2-a} pyrazin-3-one-chemiluminescence (FCLA-CL) is evaluated, and the results show that it is much longer than that of direct luminescence of $^1O_2$. A gated measurement algorithm is developed to fully utilize the longer lifetime for a clean measurement of the CL without the interference from the irradiation light. The results show that it is practically feasible to use the technique to monitor the $^1O_2$. Compared to the direct $^1O_2$ luminescence measurement, our new technique is sensitive and can be realized with a conventional optical detector with excellent signal-to-noise ratio. It thus provides a means for real-time *in vivo* monitoring of $^1O_2$ production during PDT.
[DOI: 10.1117/1.2437151]

Keywords: photodynamic therapy; chemiluminescence (CL); singlet oxygen; skin.

Paper 06236R received Sep. 6, 2006; revised manuscript received Oct. 27, 2006; accepted for publication Nov. 9, 2006; published online Feb. 2, 2007.

1 Introduction

Photodynamic therapy (PDT) is a cancer treatment modality that utilizes light energy to activate a preadministered photosensitizer drug to achieve localized tumor control.[1–3] It is well established that PDT cytotoxicity is mainly mediated via singlet oxygen produced during the photochemical reactions.[4–7] Similar to other types of radiation therapies, proper dosimetry is critical to the success of a PDT treatment. Given a target with its intrinsic sensitivity to PDT, the effectiveness of the treatment depends on the interplay of three basic factors: The concentration of the photosensitizer in the target, the light absorption by the photosensitizer, and the presence of molecular oxygen.[1,3] There are other factors, such as bleaching of the photosensitizer and oxygen concentration decrease, that can affect the treatment outcome.[8,9] Current clinical PDT dosimetry is largely based on two parameters, delivered optical and drug "doses." The optical dose is often described as the energy fluence and fluence rate per unit area (for superficial irradiation) or per unit length (for interstitial irradiation). The parameters do not consider the large variations in optical inhomogeneities in each specific target. The photosensitizer concentration in the target tissue is determined by the pharmacokinetics of the drug and, similar to the optical properties, varies greatly both intrapatient and interpatient. In the current PDT dosimetry, these variations are neglected, largely due to the complexity of the issue.

It is known that monitoring the production of singlet oxygen by measuring its luminescence at 1270 nm would provide the ultimate marker for PDT cytotoxicity and the ideal dosimetry technique,[10,11] yet it is hampered by several issues. The luminescence is extremely weak and is interfered with by the infrared light of the body, it has a half-life on the order of ns.[12,13] and its wavelength is beyond most conventional optical detectors. Although technically it is possible to overcome these disadvantages, these characteristics of singlet oxygen luminescence do make it difficult and too expensive as a method for practical applications.

Reactive oxygen species (ROS)–specific chemiluminescence (CL) probes have been used to evaluate ROS production. A probe molecule can interact with ROS and, after a series of reactions that result in the release of additional chemical energy, release photons.[14,15] Depending on the probe used, the photons are typically in the visible wavelength range and are easily measurable with conventional optical detectors such as a photon multiplier tube (PMT). We have previously

Address all correspondence to Da Xing, South China Normal University, MOE Key Laboratory of Laser Life Science and Institute of Laser Life Science, Guangzhou 510631, PR China. Fax: 86-20-85216052; Tel: 86-20-85210089; E-mail: xingda@scnu.edu.cn.

1083-3668/2007/12(1)/014002/7/$25.00 © 2007 SPIE

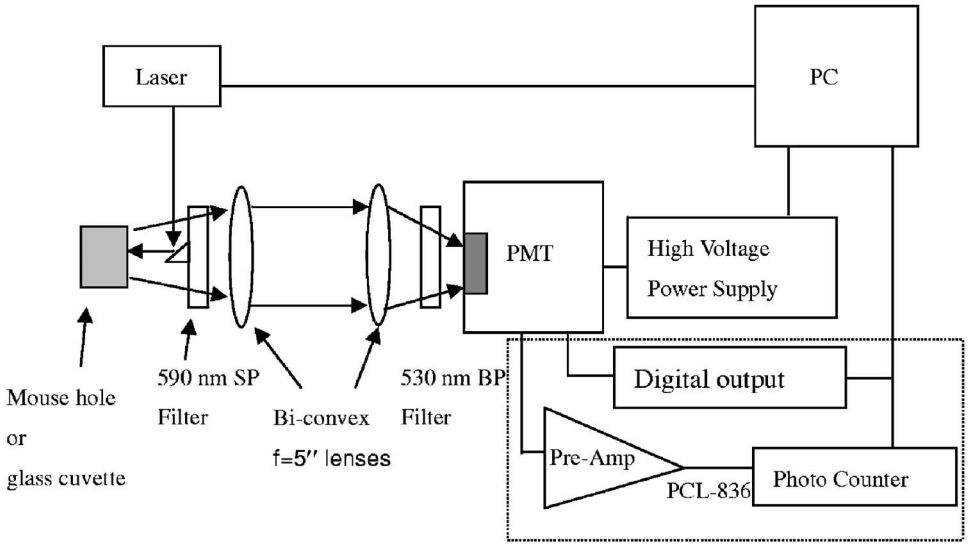

Fig. 1 Schematic of the experimental system for chemiluminescence measurements.

reported that, in an *in vitro* system, (FCLA-CL) is directly related to PDT treatment parameters.[16,17] More importantly, without differentiating the treatment protocols, the total CL observed during a PDT treatment is a direct marker for the cell viability. This establishes the base of the current study, that ROS-induced CL may be used as a marker for real-time PDT dosimetry monitoring.

There are many issues that need to be addressed before this technique can have any practical application. Most important is the issue of whether it be used *in vivo*, as that is the ultimate goal of PDT dosimetry. Compared to an *in vitro* study in which the experimental conditions and optical properties of a sample can be well controlled, a real tissue sample poses several problems. PDT irradiation light, scattered by the target sample, is an overwhelming source to diminish the signal-to-noise ratio in the measurement. In the *in vitro* study, the samples were nearly optically transparent, and the excitation light was introduced to the sample in a perpendicular way to the optical axis of the detector system, yet there was still a significant amount of light reaching the PMT that required the use of band-limiting filters for both background suppression and PMT protection. This is a far more severe problem for *in vivo* measurement, as most tissues are highly scattering at wavelengths used for PDT.

The solution to this problem lies in the mechanism of CL photon production. Because CL photons are produced as a result of chemical reactions, the intensity decay upon termination of the singlet oxygen source (namely, turning off the PDT excitation) is governed by the chemical reaction speed. We thus hypothesize that CL produced in PDT has a long lifetime. It can be gated for *in vivo* measurement without the interference of the excitation light source.

2 Materials and Methods

2.1 *Chemicals*

For the ROS-specific chemiluminescence probe, FCLA (free acid FCLA, Tokyo Kasei Kogyo Co., Tokyo, Japan) was dissolved in double-distilled water at a concentration of 100 μM and stored at $-80\,^{\circ}$C until needed. Upon reaction with either singlet oxygen or superoxide, the probe produces a luminescence at 532 nm.[18,19] For the photosensitization reaction, photosensitizer Protoporphyrin IX disodium salt (PpIX) (Aldrich Chemical Co., Milwaukee, Wisconsin) was prepared according to the manufacturer's directions to a concentration of 200 μM. Human serum albumin (HSA) (purity $>96\%$, Sigma, St. Louis, Missouri) was prepared in double-distilled water to a concentration of 50 μm. The stocks were stored in the dark at $4\,^{\circ}$C until needed. When the *in vitro* experiment was done, the room temperature was $25\,^{\circ}$C.

2.2 *Apparatus for Chemiluminescence Detection*

The schematic for chemiluminescence detection is shown in Fig. 1. The irradiation light source for the photosensitization reaction is a custom-built gated diode laser system (maximum power 100 mW, 635 nm laser diode controller, LDC 2000, ThorLab, USA). The chemiluminescence was measured using a photon multiplier tube (PMT, Model MP 952, PerkinElmer Optoelectronics, Wiesbaden, Germany) with a counter (PCL-836, Advantech Co., Ltd., Taiwan). The irradiation and CL measurement system is synchronized and controlled by LabVIEW (LabVIEW version 6.1, National Instruments, USA). The time sequence and length of the irradiation and data acquisition are both controlled by the program. To avoid damage and saturation of the measurement system, the PMT is turned off during light irradiation. A pair of band filters [590-nm short-pass (SP) and a 530-nm bandpass (BP) filter] is used to further protect the PMT from scattered irradiation light.

2.3 *Chemiluminescence in Solution*

The basic PDT-CL measurement system is based on measurement of chemiluminescence from FCLA when activated by PDT-generated ROS. Stock FCLA and PpIX solutions were diluted in phosphate-buffered saline (PBS, pH 7.4: 0.8 g N_aCl, 0.02 g KCl, 0.02 g KH_2PO_4, 0.349 g $N_aHPO_4 \cdot H_2O$

were dissolved in 100 ml double-distilled water) to concentrations of 5 and 10 μM, respectively. Considering sufficient serum being *in vivo*, we also did the same experiment with HSA to test the character of CL. The samples were prepared either with (5 μM) or without the addition of HSA. The prepared samples were transferred to a 1-ml glass cuvette and irradiated with laser (635 nm, 20 mW/cm^2). The CL signals were collected before and immediately after the laser irradiation.

2.4 *Quenching of Singlet Oxygen*

Since FCLA can interact with either or both singlet oxygen and superoxide, it is important to identify the nature of the CL generated in the study. A well-established singlet oxygen quencher, NaN_3, was used to quench the singlet oxygen produced by PDT.[20] The CL was measured five times with 1-s irradiation followed by a 2-s CL signal collection prior to the addition of the quencher. NaN_3 (0.05 mol/1.20 μl) was then added to the sample, and the next five irradiation and CL signal collection cycles were resumed immediately. The same experiment was repeated three times, and all the data were averaged.

2.5 *CL and Light Irradiation Parameters*

Samples (10 μM PpIX, 5 μM HSA, 5 μm FCLA in PBS) in the 1-ml glass cuvette were irradiated with light fluence rate ranging from 5 to 40 mW/cm^2, in 5 mW/cm^2 increments. The excitation time was 1 s followed by a 1-s CL signal recording. The excitation and data collection from each sample were repeated five times. Each data point represents an average from six independent samples.

2.6 *Effect of the Excitation Time on CL Production*

The data collection for this study was controlled via a custom-programmed LabVIEW system. For each experiment, a sample (as described earlier) was irradiated with a sequence of various excitation times (0.05 s to 25.6 s). Immediately after each irradiation period, CL was collected for 2 s. The excitation and data collection were conducted in a continuous way without any interruption. The procedure was repeated four times for each sample, and the CL signals after each irradiation time period were compared and analyzed. If there was no significant difference between signals after each complete time sequence, the data for each irradiation time was then averaged.

2.7 *Feasibility of CL Detection from In Vivo Mouse Skin During PDT*

To test whether CL can be adequately detected *in vivo*, CL was measured from normal skin of BALB/CA mice (Center of Experimental Animal Sun Yat-Sen University, Guangzhou, China) during PDT. The mice were housed in an environmentally controlled animal facility with a regular 12/12 cycle. Before each experiment, a hind leg of a mouse was molted by depilatory (Na_2S 8% aqua-solution). We have previously demonstrated that there was no observable *in vitro* FCLA toxicity up to 100 μM.[21] To further test the *in vivo* toxicity of FCLA, 100 μl of FCLA (with 0.9% N_aCl) at concentrations from 0 (saline only) up to 100 μM was injected subcutaneously at the PDT-CL measurement site. The mice were observed daily for potential changes in their life pattern for one week. For PDT irradiation and CL measurement, a mouse was restrained inside a custom holder without anesthesia. A hind leg of the animal was positioned outside the holder while care was taken not to compress its blood flow. Mixed PpIX (0.02 μmol) and FCLA (0.01 μmol) in 200-μl physiologic saline (0.9%) was injected subcutaneously into a leg, one hour prior to the light irradiation to allow adequate absorption.[21,22] The previously described PDT and CL apparatus was modified to accommodate the animal holder. For the PDT treatment, the leg was irradiated with a 635-nm laser at 20 mW/cm^2 for a fluence of 10 J/cm^2. The light was delivered in a 3 s/1 s light/dark cycle so that CL signals could be collected during the dark periods.

2.8 *Data Analysis and Statistics*

Each experiment was repeated at least three times, and the results are analyzed accordingly. Numerical data are presented as average and standard deviation. The statistical evaluation was done by the t-test.

3 Results

3.1 *Delayed Chemiluminescence and CL Quenching Experiment*

The temporal profiles of CL were analyzed for its delayed luminescence property. Figure 2 shows the CL decays, and the curves are fitted with the single exponential component. The lifetimes are 0.18 s and 0.07 s for samples with and without HSA, respectively. The results also indicate that in solution of PBS/HSA, the intensity of CL is higher[23] and the lifetime is longer than in PBS. The t-test about lifetime between PBS/HSA and PBS was made, and $p \leqslant 0.01$.

Overall, the CL decay is considerably slower compared to that of $^1O_2$ fluorescence at 1270 nm. It has been reported that PpIX PDT in PBS is a Type II reaction.[24,25] To confirm that $^1O_2$ is the main ROS product in our experiment, a well-established ROS quencher NaN_3 was used to selectively quench the $^1O_2$ in both PBS and PBS/HSA solution. With NaN_3 added to the samples, CL production was significantly quenched by over 70%, with or without the presence of the HSA (Fig. 3). This proved that the dominant CL in the study was due to $^1O_2$.

3.2 *Relationship Between CL Rate and Light Fluence Rate*

By evaluating the maximum CL production rate immediately after each irradiation period, the relationship between the irradiation light fluence rate and CL production rate was compared. The result is shown in Fig. 4. There is a strong linear relationship between the light fluence rate and the CL rate.

3.3 *Experiment of Changing Irradiating Time*

As in any photochemical reaction, CL production requires a certain time periods to reach its dynamic equilibrium upon the onset of light irradiation. To establish a reasonable irradiation time (pulse width), CL was measured immediately after various light irradiation periods. The results show that, at 20 mW/cm^2, it takes approximately 1 s of irradiation for the CL production to reach its dynamic equilibrium (Fig. 5). The

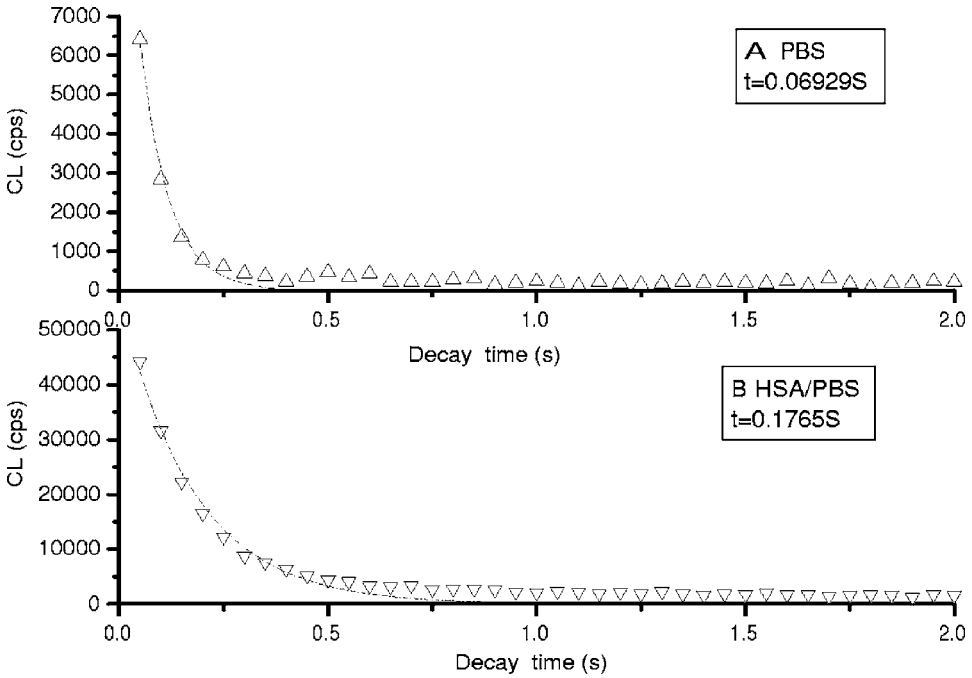

Fig. 2 The decay CL and the single-exponential fit. In PBS and PBS/HSA solution (PpIX 10 μM, FCLA 5 μM, HSA 5 μM), the photosensitizer was excited by laser (20 mW/cm^2, 635 nm). The irradiation and detection times are 1 s and 2 s, respectively. The correlation coefficient is A: R^2 0.95101; B: R^2 0.95965.

CL lifetime was also analyzed for the effect of irradiation time. The results show that the irradiation pulse width had little effect on the CL lifetime (Fig. 6).

3.4 *Experiment In Vivo*

The preliminary toxicity study results show that mouse can easily tolerate the subcutaneous FCLA injection. After injection of saline either alone or with FCLA, the skins responded identically: each injection caused a skin lump that was absorbed completely within an hour. Throughout the experiment, there was no erythema due to the injection. The injection alone, with or without FCLA, caused absolutely no observable behavioral change in the animals. CL signals can be readily detected from *in vivo* animal skin during PDT with excellent signal-to-noise ratio. As shown in Fig. 7, the CL signal obtained from the *in vivo* animal has a very similar temporal profile to that from PBS/HSA solution. Figure 8 shows the integrated CL from the *in vivo* skin as a function of PDT irradiation fluence (time). The curve shows that the total amount of CL does not linearly increase with the irradiation time, but rather shows a trend of gradually slowing down.

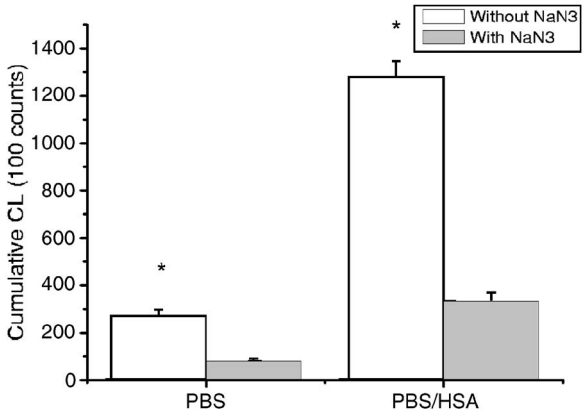

Fig. 3 Effect of 20-μl (0.05 mol/l) sodium azide (NaN_3) on fluoresceinyl cypridina luciferin analog chemiluminescence (FCLA-CL) in PBS solution and PBS/HSA solution, respectively. The PDT parameters are PpIX, 10 μM; FCLA, 5 μM; laser, 20 mW/cm^2; irradiation and detection time 1 s and 2 s). * indicates $p < 0.01$.

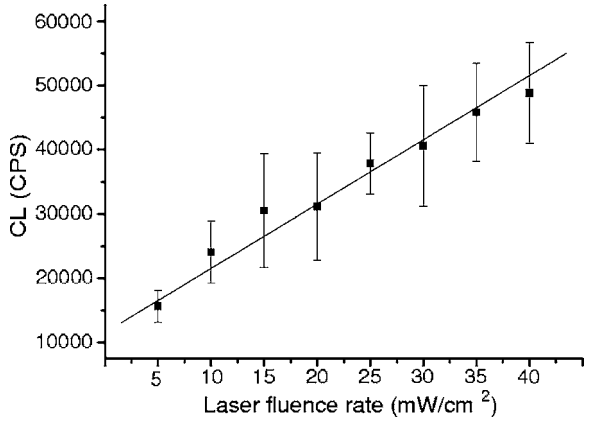

Fig. 4 The relationship between CL rate and laser fluence rate in PBS/HSA solution The fluence rate is 5, 10, 15, 20, 25, 30, 35, and 40 mW/cm^2. The linear fit was done, and the correlation coefficient is 0.98998.

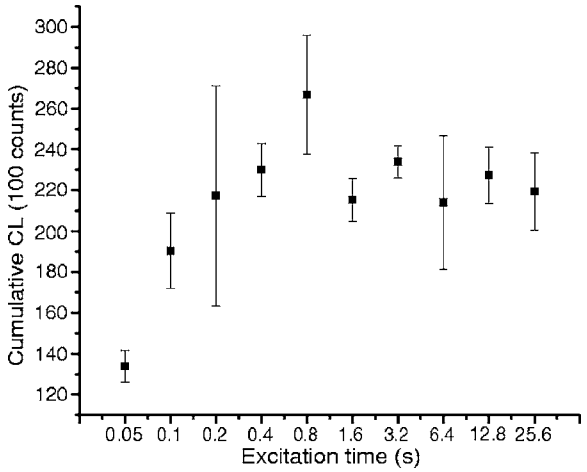

Fig. 5 Cumulative CL in PBS/HSA solution after different laser excitation times. The irradiation time is 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, and 25.6 s. The other PDT parameter is the same as shown in Fig. 3.

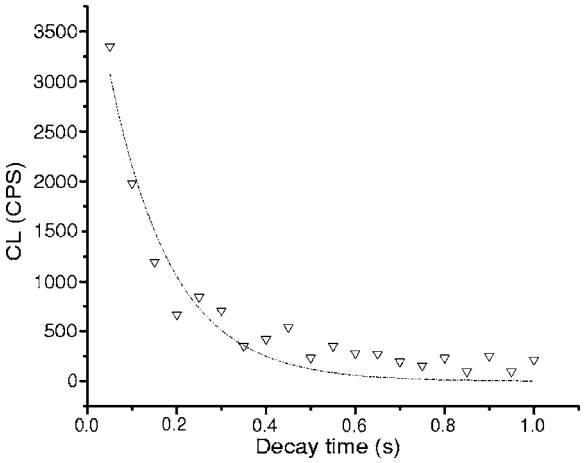

Fig. 7 The decay CL of the sensitized mouse. PpIX (0.02 μmol) and FCLA (0.01 μmol) mixture 200 μl (NaCl, 0.9%) was transfused into mouse skin. About 1 h later, the mouse was fixed and irradiated by laser (20 mW/cm^2, 635 nm; irradiation and detection times, 3 s and 1 s).The lifetime is about 0.14 s.

4 Discussion

Controlling the dose is key during PDT. Many methods have been reported to estimate the PDT dose, for example, to predict the PDT outcome by monitoring the oxygen concentration in tissue or its change induced by PDT.[26,27] The fluorescence of the photosensitizer was also detected to estimate the dosimetry of PDT.[28] But to estimate the subsequent effect of PDT by monitoring the oxygen concentration or the flouresence of the photosensitizer is indirect. It is known that singlet oxygen is the main reactant in PDT, and the detection of singlet oxygen is key to predicting therapeutic effect. Many methods of detection of $^1O_2$ have been explored. Chemiluminescence has been reported as a sensitive means to detect singlet oxygen. We have previously reported using FCLA-CL as an alternative dosimetry technique to evaluate $^1O_2$ production in PDT *in vitro*. The results indicate that FCLA-CL is a sensitive marker for PDT-induced biological outcome, regardless of the treatment protocols. With *in vivo* PDT applications, Sakurai et al. have proposed a detection and imaging method *in vivo* by a sensitive and specific chemiluminescence probe.[29] However, the interference from the scattered excitation light source inevitably would become a major deterrent for *in situ* treatment monitoring, causing a major decrease in the signal-to-noise ratio. The current study has developed a new approach of using CL techniques as a means to measure PDT $^1O_2$ production *in vivo*.

It has been observed that FCLA-CL has a long lifetime. By gating the excitation light source, CL measurement can be made in a "dark" environment with minimum background interference. The results show that, with the new gated technique, the delayed CL intensity is proportional to the irradiation light fluence rate. The finding is similar to that observed *in vitro*. Yet a simple relationship between CL intensity and PDT treatment protocol is not a full guarantee of the validation of the technique for *in vivo* applications. To evaluate the feasibility of the technique, we have investigated various fac-

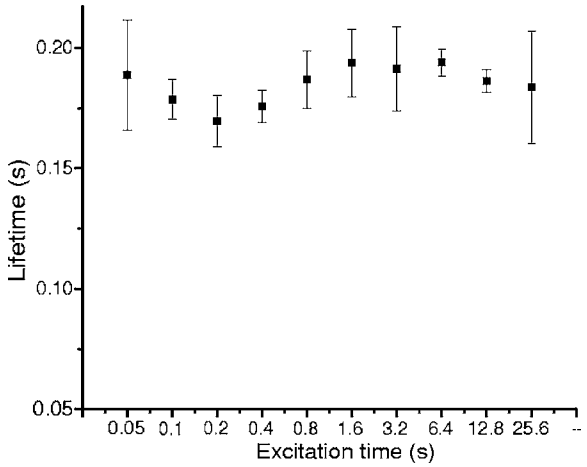

Fig. 6 In PBS/HSA solutions, the delayed CL lifetime with different excitation times. The samples were the same batch treated with PDT shown in Fig. 5.

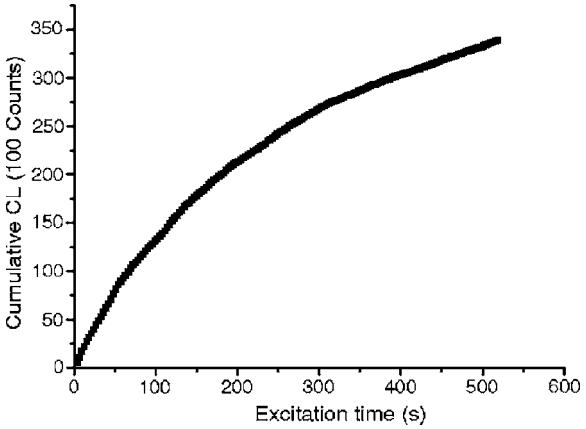

Fig. 8 The course of cumulative CL on mouse skin. All the CL data for the mouse from Fig. 7 were accumulated. The total laser fluence is about 10 J/cm^2.

tors that may impact the CL measurement results.

The single-exponential fit was made and the half lifetime was given to indicate the decay rate in Fig. 2. Since the back of the fit curve was not well fitted, a double-exponential fit was done. The analysis indicates that the average lifetime of the slow components of the fit is about 2 s, and the initial intensity is only about 10% of the fast components. The selectivity of the delayed CL technique was evaluated by using a highly selective $^1O_2$ quencher, NaN_3, in the PDT-CL process. The results clearly demonstrate that the majority of the CL signal was due to $^1O_2$, the well-known dominant agent that mediates PDT biological effect. But the double-exponential fit and the proportion of the CL quenching also reveal that superoxide may contribute, to a degree, to the CL production, and the fast component must come from singlet oxygen. Nevertheless, as shown in our previous *in vitro* study, integrated CL provides a marker directly linked to the PDT-induced biological effect.

In a practical PDT treatment, it is likely that the changes in singlet oxygen production occur in a gradual way that does not require continuous monitoring that in turn requires frequent interruption to the treatment process. With minimal interruption of the treatment, the CL measurement can be made periodically every few seconds or minutes, depending on the treatment protocol and progress. The impact of the irradiation length to the subsequent delayed CL signal was studied. The CL intensity increased gradually with the irradiation light period and eventually reached a plateau (approximately after 1 s irradiation with our experimental protocol). Variation in the irradiation time has minimum effect on the delayed CL lifetime. These findings are true both *in vitro* and *in vivo*. It is likely due to the fact that CL is a continuous excitation and de-excitation process that takes a certain time to reach its dynamic balance. The results suggest that the delayed CL measurement is a reliable monitoring of the PDT process, given that each measurement follows an adequate irradiation light period.

Our results show that the delayed CL monitoring technique can be adopted easily for *in vivo* study. Direct $^1O_2$ fluorescence measurement has been reported recently *in vivo* using a specialized PMT and pulse laser system.[25] In comparison, the CL technique can be realized with a conventional optical system with minimum modification to the existing PDT system. It provides a new approach for *in vivo* $^1O_2$ monitoring during PDT with significantly improved signal-to-noise ratio at a lower cost. As shown in the results, CL did not increase linearly with increased irradiation light fluence. This may not indicate a true decrease in $^1O_2$ production rate but is likely due to combined local chemical consumption/redistribution of FCLA and PpIX and photo-bleaching of PpIX. Among these factors, only changes in local PpIX concentration and photobleaching would lead to true changes in $^1O_2$ production. More detailed study is necessary to identify the contributions of each factor. The major disadvantage of the new technique is that the $^1O_2$ measurement is made through the FCLA probe that itself has to be introduced into the target. It is obvious that quantitative measurement of FCLA-CL is more complex compared to that of direct $^1O_2$ fluorescence, with the added pharmacokinetics and irreversible consumption of the FCLA. Our results clearly show that the technique is feasible in $^1O_2$ detection *in vivo*. It is thus critical to understand the characteristics of FCLA. One possible approach to resolve this issue is to use the fluorescence signal of FCLA that is readily detectable both *in vivo* and *in vitro* as an internal marker for the probe. This is currently under investigation.

In summary, this work has shown the feasibility of using delayed CL to detect singlet oxygen *in vivo* and also to obtain some useful data about detection *in vivo*. Based on these data, we will study the relation between CL and the curative tumor response.

Acknowledgments

This research is supported by the National Natural Science Foundation of China (Grant nos. 60378043 and 30470494), the Natural Science Foundation of Guangdong Province (Grant nos. 015012, 04010394, and 2004B10401011), and the US NIH (Grant no. PO1-43892).

References

1. K. Jiang, Y. Cheng, and S. M. Wu, "The photodynamic therapy of tumor," *Laser J.* **25**(1), 77–99 (2004).
2. J. M. Wu, J. W. Xiong, and H. Xiao, "The photodynamic therapy used in the treatment of tumor," *Laser J.* **24**(1), 70–73 (2003).
3. S. B. Brown, E. A. Brown, and I. Walker, "The present and future role of photodynamic therapy in cancer treatment," *Lancet Oncol.* **5**, 497–508 (2004).
4. C. S. Foote, "Definition of type I and type II photosensitized oxidation," *Photochem. Photobiol.* **54**, 659–661 (1991).
5. J. Moan, Q. Peng, R. Sorensen, V. Iani, and J. M. Nesland, "The biophysical foundations of photodynamic therapy," *Endoscopy* **30**, 387–391 (1998).
6. W. M. Sharman, C. M. Allen, and J. E. van Lier, "Photodynamic therapeutics: basic principles and clinical application," *Drug Discovery Today* **4**(11), 507–517 (1999).
7. B. W. McIlroy, T. S. Mann, J. S. Dysart, and B. C. Wilson, "The effects of oxygenation and photosensitizer substrate binding on the use of fluorescence photobleaching as a dose metric for photodynamic therapy," *Vib. Spectrosc.* **28**, 25–35 (2002).
8. J. C. Finlay, S. Mitra, M. S. Patterson, and T. H. Foster, "Photobleaching kinetics of Photofrin *in vivo* and in multicell tumour spheroids indicate two simultaneous bleaching mechanisms," *Phys. Med. Biol.* **49**, 4837–4860 (2004).
9. L. W. Ma, S. Bagdonas, and J. Moan, "The photosensitizing effect of the photoproduct of protoporphyrin IX," *J. Photochem. Photobiol., B* **60**, 108–113 (2001).
10. M. J. Niedre, M. S. Patterson, and B. C. Wilson, "Direct near-infrared luminescence detection of singlet oxygen generated by photodynamic therapy in cells *in vitro* and tissues *in vivo*," *Photochem. Photobiol.* **75**, 382–391 (2002).
11. M. J. Niedre, A. J. Secord, M. S. Patterson, and B. C. Wilson, "In vitro tests of the validity of singlet oxygen luminescence measurements as a dose metric in photodynamic therapy," *Cancer Res.* **63**, 7986–7994 (2003).
12. C. Schweitzer and R. Schmidt, "Physical mechanisms of generation and deactivation of singlet oxygen," *Chem. Rev. (Washington, D.C.)* **103**, 1685–1757 (2003).
13. J. W. Snyder, I. Zebger, Z. Gao, L. Poulsen, P. K. Frederiksen, E. Skovsen, S. P. McIlroy, M. Klinger, L. K. Andersen, and P. R. Ogilby, "Singlet oxygen microscope: from phase-separated polymers to single biological cells," *Acc. Chem. Res.* **37**, 894–901 (2004).
14. K. Teranishi and T. Nishiguchi, "Cyclodextrin-bound 6-(4-methoxyphenyl)imidazo[1,2-a]pyrazin-3(7H)-ones with fluorescein as green chemiluminescent probes for superoxide anions," *Anal. Biochem.* **325**, 185–195 (2004).
15. X. H. Li, G. X. Zhang, H. M. Ma, D. Q. Zhang, J. Li, and D. B. Zhu, "4,5-dimethylthio-4¢-[2-(9-anthryloxy)ethylthio]tetrathiafulvalene, a highly selective and sensitive chemiluminescence probe for singlet oxygen," *J. Am. Chem. Soc.* **126**, 11543–11548 (2004).

16. M. Hao, D. Xing, Q. Chen, and J. Wang, "A high sensitivity detection method of singlet oxygen and superoxide anion," *Chin. Chem. Lett.* **15**(6), 679–682 (2004).
17. Y. F. Qin, D. Xing, J. Zhou, S. M. Luo, and Q. Chen, "Feasibility of using fluoresceinyl cypridina luciferin analog in a novel chemiluminescence method for real-time photodynamic therapy dosimetry," *Photochem. Photobiol.* **81**, 1534–1538 (2005).
18. M. Nakano, K. Sugioka, Y. Ushuima, and T. Goto, "Chemiluminescence probe with cypridina luciferin analog estimating the ability of human granulocytes to generate O2-," *Anal. Biochem.* **159**, 363–369 (1986).
19. Y. H. He, D. Xing, S. Tan, Y. H. Tang, and K. Ueda, "*In vivo* sonoluminescence imaging with the assistance of FCLA," *Phys. Med. Biol.* **47**, 1535–1541 (2002).
20. L. K. Verna, D. Chen, G. Schluter, and G. M. Williams, "Inhibition by singlet oxygen quenchers of oxidative damage to DNA produced in cultured cells by exposure to a quinolone antibiotic and ultraviolet A irradiation," *Cell Biol. Toxicol.* **14**, 237–242 (1998).
21. Y. X. Wu and D. Xing, "Permeating efficiency and localization of fcla and hpd through membrane of lung, cancer cell," *Acta Laser Biology Sinica* **14**(4), 287–291 (2005).
22. K. K. L. Yee, K. C. Soo, B. H. Bay, and M. Olivo, "A comparison of protoprophyrin IX and protoporphyrin IX dimethyl ester as photosensitizer in poorly differentiated human nasopharyngeal carcinoma cells," *Photochem. Photobiol.* **76**(6), 678–682 (2002).
23. J. Zhou, D. Xing, and Q. Chen, "Enhancement of fluoresceinyl cypridina luciferin analog chemiluminescence by human serum albumin for singlet oxygen detection," *Photochem. Photobiol.* **82**(4), 1058–1064 (2006).
24. N. S. Soukos, S. Som, A. D. Abernethy, K. Ruggiero, J. Dunham, C. Lee, A. G. Doukas, and J. M. Goodson, "Phototargeting oral black-pigmented bacteria," *Antimicrob. Agents Chemother.* **49**, 1391–1396 (2005).
25. M. J. Niedre, C. S. Yu, M. S. Patterson, and B. C. Wilson, "Singlet oxygen luminescence as an *in vivo* photodynamic therapy dose metric: validation in normal mouse skin with topical amino-levulinic acid," *Br. J. Cancer* **92**, 298–304 (2005).
26. H. W. Wang, M. E. Putt, M. J. Emanuele, D. B. Shin, E. Glatstein, A. G. Yodh, and T. M. Busch, "Treatment-induced changes in tumor oxygenation predict photodynamic therapy outcome," *Cancer Res.* **64**, 7553–7561 (2004).
27. H. J. C. M. Sterenborg, J. W. de Wolf, M. Koning, B. Kruijt, A. van den Heuvel, and D. J. Robinson, "Phosphorescence-fluorescence ratio imaging for monitoring the oxygen status during photodynamic therapy," *Opt. Express* **12**(9), 1873–1878 (2004).
28. L. A. Boere, D. J. Robinson, H. S. de Bruijn, J. van den Boogert, H. W. Tilanus, H. J. C. M. Sterenborg, and R. W. F. de Bruin, "Monitoring in situ dosimetry and photoporphyrin IX fluorescence photobleaching in the normal rat esophagus during 5-aminolevulinic acid photodynamic therapy," *Photochem. Photobiol.* **78**(3), 271–277 (2003).
29. H. Sakurai, H. Yasui, Y. Yamada, H. Nishimura, and M. Shigemoto, "Detection of reactive oxygen species in the skin of live mice and rats exposed to UVA light: a research review on chemiluminescence and trials for UVA protection," *Photochem. Photobiol. Sci.* **4**(9), 715–720 (2005).