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Photosensitized Formation of Singlet Oxygen by Phycobiliproteins in Neutral Aqueous Solutions

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Phycobiliproteins (PBPs) are a type of promising sensitizers for photodynamic therapy (PDT). Upon irradiation (λ>500nm) of an oxygen-saturated aqueous solution of phycobiliproteins, particularly, C-phycocyanin (C-PC), allophycocyanin (APC) or R-phycoerythrin (R-PE), the formation of singlet oxygen ($^1O_2$) was detected by using imidazole in the presence of *p*-nitrosodimethylaniline (RNO). The bleaching of RNO caused by the presence of imidazole in our system showed typical concentration dependence with a maximum at about 8mM imidazole, which is in agreement with the formation of $^1O_2$. In addition, the generation of $^1O_2$ was verified further in the presence of D_2O and specific singlet oxygen quencher – 1,4-diazabicyclo [2,2,2] octane (DABCO) and sodium azide (NaN_3). Our experimental results indicated that APC possesses high ability to generate reactive oxygen species and the relative quantum yields of photogeneration of $^1O_2$ by PBPs are as follows: APC > C-PC > R-PE.

Keywords: Phycobiliprotein; Singlet Oxygen; Photobleaching

Abbreviations: PBPs, Phycobiliproteins; PDT, Photodynamic Therapy; C-PC, C-phycocyanin; APC, allophycocyanin; R-PE, R-phycoerythrin; RNO, *p*-nitrosodimethylaniline; DABCO, 1,4-diazabicyclo [2, 2, 2] octane; PEC, phycoerythrocyanin; HpD, hematoporphyrin derivatives; NaN_3, sodium azide

1. INTRODUCTION

Since the Federal Food and Drug Administration of the USA gave its approval to photodynamic therapy (PDT) as an alternative form of therapy against cancer in 1995, PDT has increasingly been used as a routine clinical application. It possesses combined advantages of the radiotherapy and the chemotherapy as well as low systemic toxicity. Recently, this approach has also been used in the purification of blood-made products[1–3] and photo-induced inactivation of virus[4]. The primary photo-induced processes leading to cell damage were suggested as two principal pathways: Upon light absorption, a photosensitizer molecule is excited and then changed into the triplet state, which transfers energy to a molecular oxygen ($^3O_2$) to form a singlet oxygen ($^1O_2$) (Type II mechanism)[5]. Alternative mechanism involves generation of radical species and are generally grouped as the type I mechanism[6].

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Phycobiliproteins (PBPs), including phycoerythrin (PE), phycoerythrocyanin (PEC), phycocyanin (PC) and allophycocyanin (APC), are a type of pigment-protein complexes playing the role of light-harvesting antenna in photosynthesis of cyanobacteria, red alga and cryptomonads. Recently, Morcos et al. and Zheng et al. demonstrated that C-phycocyanin (C-PC) exerted much stronger photodynamic action on tumor cells compared with hematoporphyrin derivatives (HpD) and might be used as a new type of photodynamic therapeutic agents[7–10].

Now, more and more results support the hypothesis of $^1O_2$ – mediated impairment of the cellular biochemistry. However, detection of $^1O_2$ in aqueous solutions is not easy to achieve since $^1O_2$ has a rather short lifetime in aqueous and some other solutions. For this reason, its formation is often inferred from quenching experiments, such as bleaching of Trp[11], reaction with a sterically hindered amine[12], destruction of RNO in the presence of imidazole derivative[13] and disappearance of ADPA[14].

In previous work[15], we used ESR with spin-trapping techniques to detect hydroxyl radical (·OH) generated by irradiation of C-PC from *Spirulina platensis* and suggested that $^1O_2$ was involved in formation of ·OH.

In the present paper, a simple but sensitive spectrophotometric method was applied to neutral aqueous solution of PBPs for detecting the singlet oxygen in aqueous solution by using imidazole in the presence of *p*-nitrosodimethylaniline (RNO)[13]. In this work, the formation of rather short-lived $^1O_2$ was detected by the characteristic dependence of the RNO bleaching (reaction 4) on the concentration of imidazole.

$$S + h\nu \rightarrow {}^1S \rightarrow {}^3S \quad (1)$$

$${}^3S + {}^3O_2 \rightarrow S + {}^1O_2 \quad (2)$$

$${}^1O_2 + A \rightarrow [AO_2] \rightarrow AO_2 \quad (3)$$

$$[AO_2] + RNO \rightarrow -RNO + \text{Products} \quad (4)$$

^1S and ^3S are the first excited singlet and lowest triplet state of sensitizer (S) respectively; $[AO_2]$ is an intermediate singlet-oxygen adduct which decomposes or rearranges into a final oxygenation product AO_2, but which can also be intercepted by a suitable scavenger. – RNO represents bleached RNO as followed at 440nm. The degree of bleaching of the yellow RNO dye is directly proportional to the overall production of singlet oxygen generated in the photodynamic reaction.

2. EXPERIMENTAL

2.1 Chemicals

C-phycocyanin (C-PC) and allophycocyanin (APC) was isolated from laboratory cultures of the blue alga *Spirulina platensis*, according to Wang et al. and Xia et al[16,17]. R-phycoerythrin (R-PE) was isolated from the red alga *Polysiphonia urceolata*[18], which was collected at the seacoast of Qing Dao, China. The purity of the proteins was examined by absorption spectra ($A_{max}/A_{280} > 4.5$) (Fig. 1). The powders of lyophilized proteins were stored at –20°C. *p*-nitrosodimethylaniline (RNO) and 1,4-diazabicyclo [2,2,2] octane (DABCO) were Aldrich products. Sodium azide (NaN_3), heavy water (D_2O), NaH_2PO_4 and Na_2HPO_4 (analytic grade reagent) were commercially available. Imidazole (analytic grade reagent) was used after recrystallization from triply distilled water, until it did not show any impurity absorption above 300nm.

2.2 Experimental Conditions

All samples were freshly prepared with triply distilled water and buffered with 50mM phosphate buffer saline (pH7.0). The solutions were aerated with high-purity oxygen by bubbling for 20min prior to irradiation with a medium-pressure sodium lamp (450W) on a

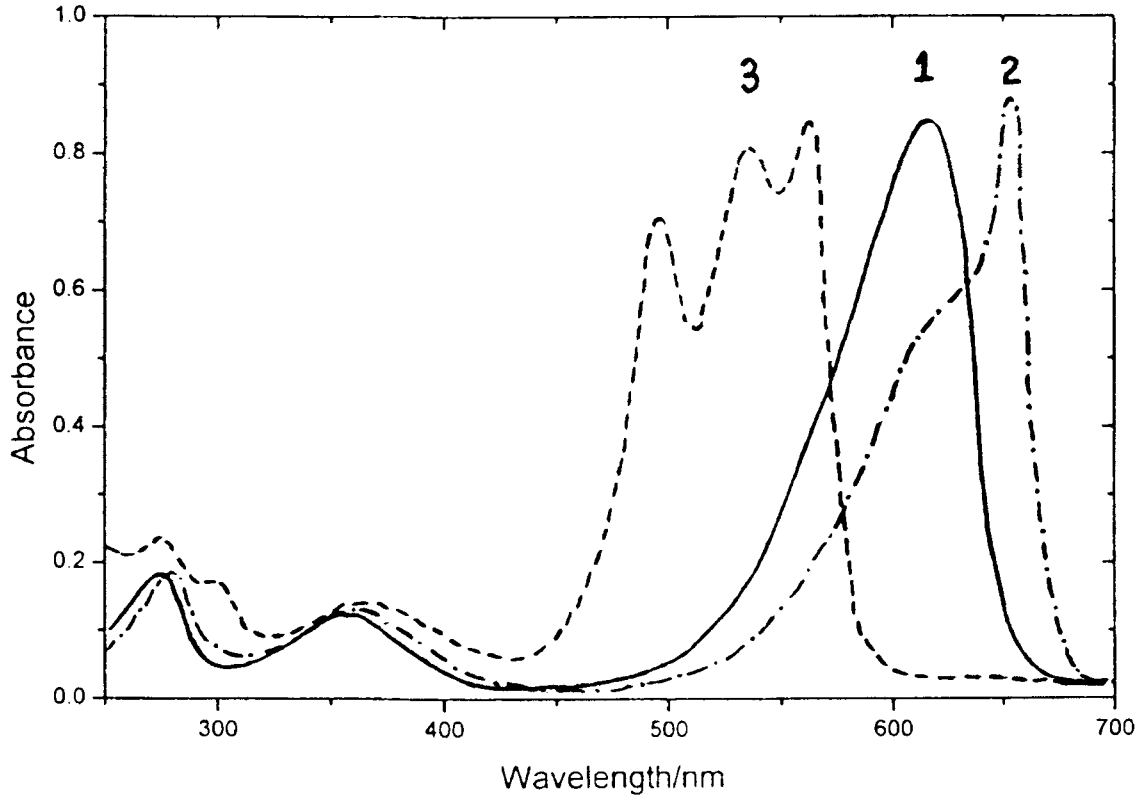

FIGURE 1 Absorption spectra of C-PC, APC and R-PE dissolved in phosphate buffer saline (pH=7.0). The maximal absorption peaks of C-PC (1), APC (2) and R-PE (3) are located at 618, 650 and 566nm, respectively

"merry-go-round" apparatus. The wavelength less than 500nm was cut off by a long-pass filter, and the apparatus was immersed in running water in a thermostat at 20°C.

The detection of singlet oxygen was performed by the method developed by Kraljic and El Mohsni[13]. The maximum absorption of protein solution was adjusted to 1.0 before the experiments. The phycobiliprotein solution in the presence of imidazole (10mM) and RNO (45μM) in a 50mM phosphate buffer saline (pH=7.0) was exposed to light. Bleaching of RNO was monitored spectrophotometrically at 440nm, the absorption maximum of RNO. The formation of singlet oxygen was also verified by reaction with $^1O_2$ quencher such as DABCO and NaN_3 and the effect of D_2O. UV-visible absorption spectra were recorded by means of Shimadzu UV-160A spectrophotometer.

3. RESULTS AND DISCUSSION

3.1 The influence of imidazole concentration on the bleaching of RNO

The bleaching of RNO caused by the presence of imidazole in our aqueous system (Fig. 2) showed typical concentration dependence with a maximum at around 8mM imidazole, the shape of the curves was identical to that published by Kraljic and El Mohsni[13], which implied the formation

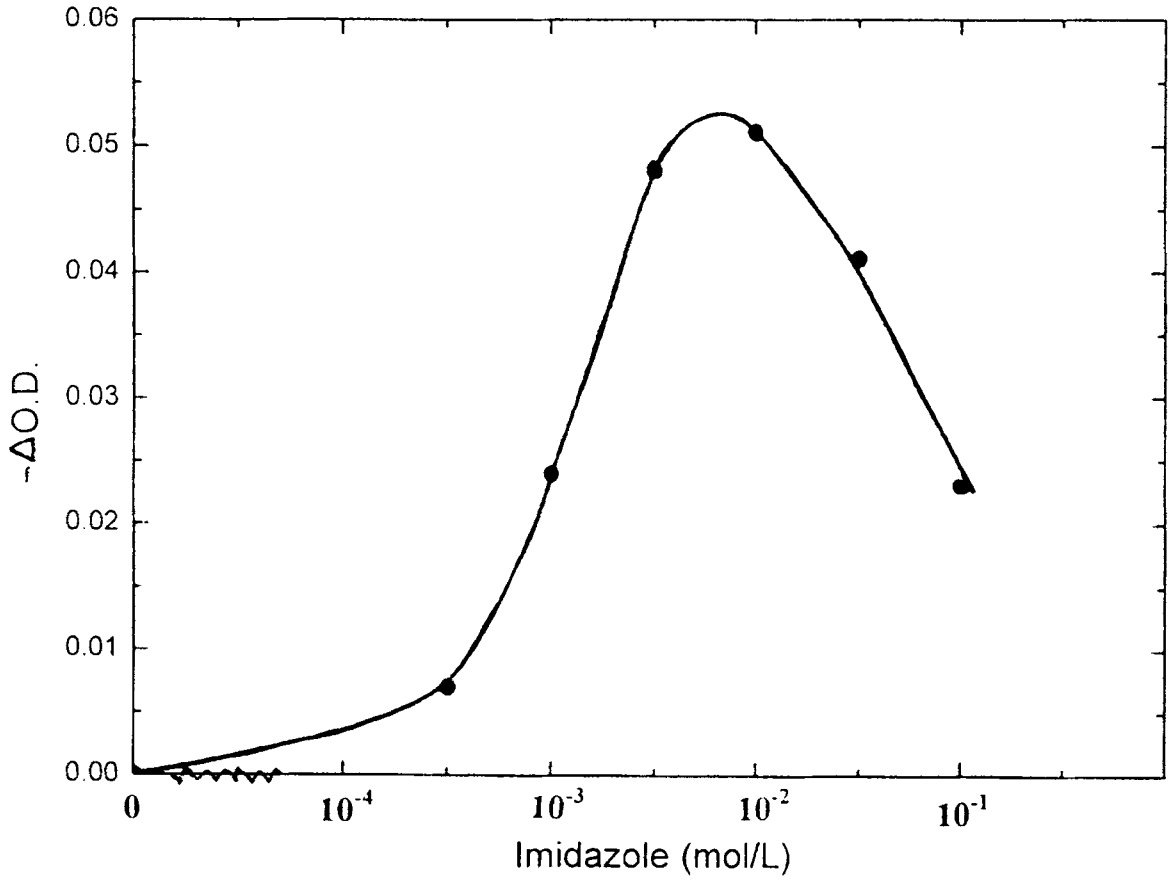

FIGURE 2 The influence of imidazole concentration on the bleaching of RNO as a test of singlet oxygen in the system: C-PC – RNO – imidazole. ΔO.D. represents the difference in optical density at 440nm (an absorption maximum of RNO) between irradiated and non-irradiated samples. λ of irradiation > 500nm; irradiation time = 10min; pH=7.0; Concentration of RNO = 45 μ M

of $^1O_2$. The decreased bleaching of RNO at higher imidazole concentrations may be explained by the competition between RNO and imidazole itself for the reactive intermediate [AO_2]. On the other hand, the reaction probability of the sensitizers in their triplet state with imidazole in singlet state must be very low because of the symmetrical rule[19,20], especially in oxygen saturated conditions, since the reaction $^3S + O_2$ (producing $^1O_2$) occurs with a very high rate, of the order of 10^9 $M^{-1}s^{-1}$[21]. Following experiments can further confirm our assumption that the interception of $^1O_2$ takes place in our aqueous system.

3.2 The effect of $^1O_2$ quenchers and D_2O on the yield of $^1O_2$

To confirm the production of $^1O_2$, experiments were carried out in the presence of specific $^1O_2$ quenchers (DABCO and NaN_3). By taking C-PC as the example, the rate of bleaching of RNO, studied in the presence of equimolar amount of imidazole and DABCO (10mM), was given in Fig. 3. The ratio of the slopes of RNO bleaching in the presence and absence of DABCO from Fig. 3 was derived to be nearly half, because imidazole and DABCO quench $^1O_2$ with similar rate constants [k (imidazole / $^1O_2$) =2.0×10^7 $M^{-1}s^{-1}$, k

(DABCO / $^1O_2$) =1.5×10^7 $M^{-1}s^{-1}$][22]. The replace of DABCO by NaN_3 (0.1 mM) produced similar amount of the inhibition (Fig. 3), because k (NaN_3 / $^1O_2$) was estimated as 2.2×10^9 $M^{-1}S^{-1}$[22], while some different values of the rate constants for azide quenching were also reported in literatures, such as, 2.0×10^8 $M^{-1}S^{-1}$[23a]. However, inhibitation of RNO oxidation by NaN_3 did indicate that $^1O_2$ was generated. On the other hand, it was ever reported that the rate constant for spontaneous decay of $^1O_2$ in H_2O was $2.4\times10^5s^{-1}$[24]. Therefore, at the moment, some conclusions can be drawn from the results. At first, $^1O_2$ was generated during photosensitization of phycobiliproteins, secondly, the rates for the three processes, i.e., imidazole and DABCO quench $^1O_2$ as well as spontaneous decay of $^1O_2$ were comparable.

Another important evidence for the C-PC photosensitized formation of $^1O_2$ comes from the D_2O effect on the yield of $^1O_2$. The lifetime of $^1O_2$ was about ten times longer in the deuterated solvent than that in the protonated solvent[25]. As shown in Fig. 3, when measurements were repeated with D_2O instead of H_2O, the rate of photobleaching of RNO increased sharply.

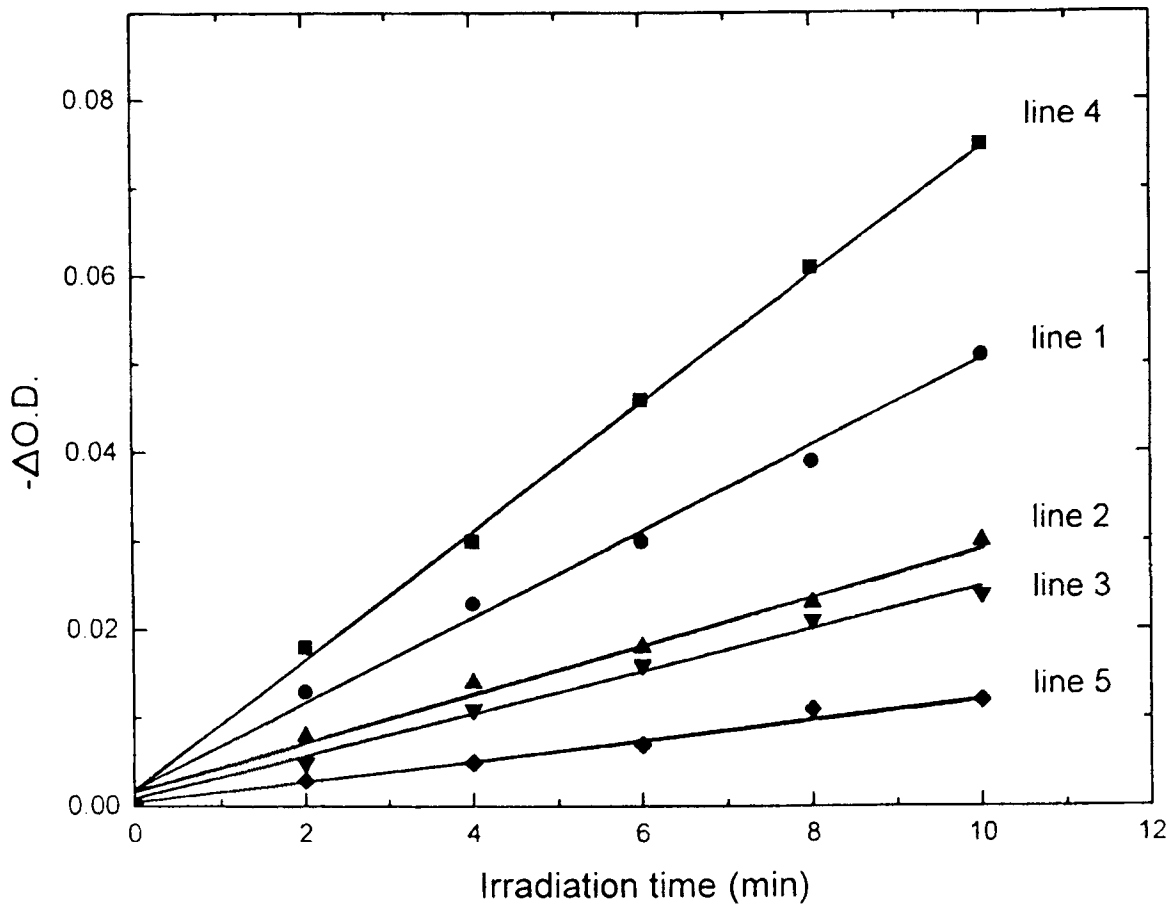

FIGURE 3 Photosensitized RNO bleaching measured at 440nm in the presence of imidazole (10mM) in 50mM phosphate buffer (pH7.0) with C-PC (initial A_{618}=1.0) as a function of irradiation time. Line 1, C-PC in oxygen-saturated aqueous solution; line 2, same as line 1, but in the presence of 10mM DABCO; line 3, same as line 1, but in the presence of 0.1mM NaN_3; line 4, same as line 1, but the solvent using D_2O instead of H_2O; line 5, same as line 1, but in the absence of imidazole

All these experimental results confirmed the generation of $^1O_2$ during the photosensitization process.

Since $^1O_2$ cannot bleach RNO directly[13,19], the blank in the absence of imidazole can serve for the detection of some other strongly oxidizing intermediates capable of destroying the RNO chromophore, such as OH radical and some organic free radicals[26]. In such a case, the bleaching of RNO would indicate that the system produces some reactive intermediate of high oxidation potential. In the experiments with aqueous solution of C-PC, there was measurable bleaching of RNO in the absence of imidazole (Fig. 3). This positive "blank" indicates that strongly reactive intermediates in measurable amount generated under our experimental condition. This species was most likely free hydroxyl radicals [RNO reacts very rapidly with the OH radical (reaction 5)][26].

$$^{\bullet}OH + RNO \rightarrow -RNO \qquad (5)$$

This was also agreement with our previous work[15] that ·OH was indeed generated in the aqueous system. But, the bleaching of RNO is strongly increased by the presence of imidazole with a characteristic dependence on their concentration (Fig. 2 and Fig. 3). The dependence of RNO bleaching on the imidazole concentration contributes to the selectivity of this test for $^1O_2$.

3.3 The relative yield of $^1O_2$ generated by C-PC, APC and R-PE

When APC or R-PE was used instead of C-PC as a sensitizer, the similar photosensitized formation of $^1O_2$ and its involvement in the photobleaching of RNO was also observed (Fig. 4). Under our experimental conditions, imidazole being in large excess (10mM) and variation of RNO being kept under 10% of its initial concentration, The slope B of the plots of -InA against the irradiation time, their standard deviation SD and correlation coefficients R were found by linear regression. The calculated slopes B are proportional to the relative quantum yield of $^1O_2$ [27,28]. From Fig. 4, it can be inferred that the relative quantum yield of $^1O_2$ generated by phycobiliproteins are as follows: APC > C-PC > R-PE. The marked differences in the sensitizing properties among C-PC, APC and R-PE may be explained on the basis of intersystem crossing efficiencies (Φ_{isc}) of these phycobiliproteins. Since the $^1O_2$ is most likely produced *via* the triplet quenching, a decreased Φ_{isc} can also lead to a lower yield of $^1O_2$. No published value of Φ_{isc} for APC, C-PC and R-PE is available yet, however, according to our previous work[29,30], the triplet states of C-PC and APC were detected while that of R-PE not, which maybe due to its lower Φ_{isc}. On the other hand, Stadnichuk[31] indicated that the phosphorescence quantum yield increased by 100 times after R-PE was denatured, which might imply that quantum yields of the triplet states would be dependent on aggregate states of phycobiliproteins. In addition, the relative phorsphorescence yields of APC, CPC and R-PE are determined as follows: APC > C-PC > R-PE[32], which may be another evidence to support our experimental results.

4. CONCLUSION

In summary, a simple but sensitive spectrophotometric method for the detection of singlet oxygen in aqueous solutions using imidazole in the presence of *p*-nitrosodimethylaniline (RNO) has been successfully applied to neutral aqueous solution of PBPs. This method has found extensive use especially in monitoring the production of $^1O_2$ in biological systems. From our experimental results, the relative quantum yield of $^1O_2$ generated by phycobiliproteins are as follows: APC > C-PC > R-PE. Of course, further experiments have to be undertaken to get quantitative results.

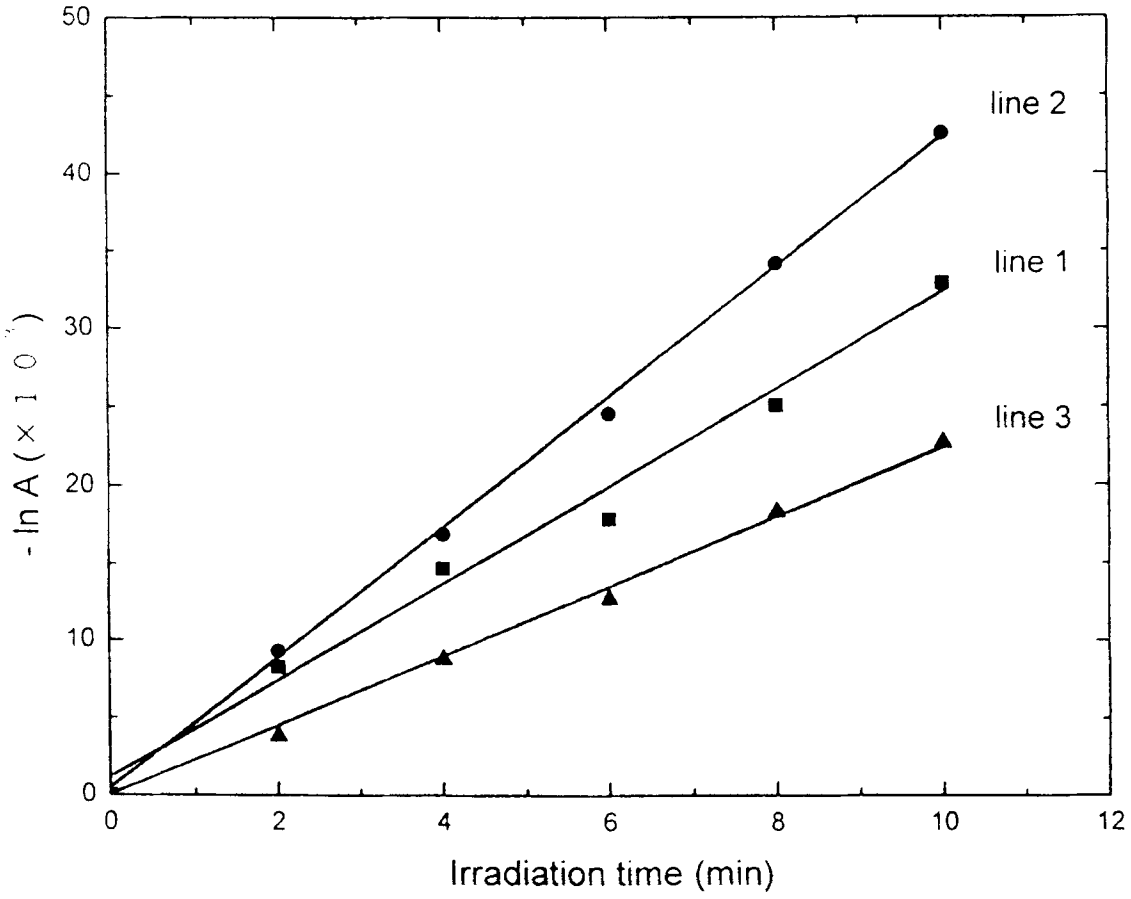

FIGURE 4 Photosensitized RNO bleaching measured at 440nm in the presence of imidazole (10mM) in 50mM phosphate buffer (pH7.0) with C-PC (line 1), APC (line 2) and R-PE (line 3) as a function of irradiation time. The initial absorbance at maximum absorption of all samples was all adjusted to 1.0 with triply distilled water

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References

[1] Matthews, J.L., J.T. Newman, F. Sogandares-Bernal, M.M. Judy, H. Skiles, J.E. Leveson, A.J. Marengo-Rowe and T.C. Chanh (1988) Photodynamic therapy of viral contaminants with potential for blood banking applications. Transfusion, 28, 81–83.

[2] Moor, A.C., A. Van der Veen, A.E. Wagenaars van Gomple, T.M. Dubbelman, J. VanSteveninck and A. Brand (1997) Shelf-life of photodynamically sterilized red cell concentrates with various number of white cell. Transfusion, 37, 592–600.

[3] Margolis-Nunno, H., E. Ben-Hur, P. Gottlieb, R. Robinson, J. Oetjen and B. Horowitz (1996) Inactivation by phthalocyanine photosensitization of multiple forms of human immunodeficiency virus in red cell concentrates. Transfusion, 36, 743–750.

[4] Sieber, F., J.M. O'Brien and D.K. Gaffney (1992) Merocyanine-sensitized photoinactivation of enveloped viruses. Blood Cells, 18, 117–127.

[5] Ochsner, M. (1997) Photophysical and photobiological processes in the photodynamic therapy of tumours. Journal of Photochemistry and Photobiology, B: Biology, 39, 1–18.

[6] Foote, C.S. (1991) Definition of type I and type II photosensitized oxidation. Photochemistry and Photobiology, 54, 659.

[7] N.C. Morcos, W.L. Henry (1987) Medical uses for phycocyanins. US Patent, 4,886,831.
[8] S. Zheng, X.H. Cai, L.M. He (1994) A new type of photosensitizer using as phototherapy of cancer. China Patent, CN 1,091,976.
[9] X.H. Cai, L.M. He, J.L. Jiang, L.L. Yu. Z.M. Xu, S. Zheng (1995) Experimental study on the sensitization of phycobiliproteins from *Spirulina platensis* to phototherapy of cancer. Chinese Journal of Oceanic Drugs, 1, 15–18.
[10] L.M. He, X.H. Cai, S. Zheng, L.L. Yu (1995) The photodynamic effect of two photosensitizers on Colon Cancer Line HR-8348 as measured with MTT assay. Chinese Journal of Lasers, 22, 847–849.
[11] Nilsson, R., P.B. Merkel and D.R. Kearns (1972) Unambiguous evidence for the participation of singlet oxygen ($^1\Delta$) in photodynamic oxidation of amino acids. Photochemistry and Photobiology, 16, 117–124.
[12] Lion, Y., M. Delmelle and A. Van de Vorst (1976) New method of detecting singlet oxygen production. Nature, 263, 442–443.
[13] Kraljic, I. and S. El Mohsni (1978) Determination of singlet oxygen rate constants in aqueous solutions. Photochemistry and Photobiology, 28, 577–581.
[14] Lindig, B.A., M.A.J. Rodgers and A.P. Schaap (1980) Determination of the lifetime of singlet oxygen in D_2O using 9,10-anthracenedipropionic acid, a water-soluble probe. Journal of the American Chemical Society, 102, 5590–5593.
[15] S.P. Zhang, J. Xie, J.P. Zhang, J.Q. Zhao, L.J. Jiang (1999) ESR studies on photosensitized formation of hydroxyl radical by C-phycocyanin from *Spirulina platensis*. Biochimica et Biophysca Acta, 1426, 205–211.
[16] G.C. Wang, B.C. Zhou, C.K. Zeng (1996) Construction of energy transfer model of C-phycocyanin and allo-phycocyanin from *Spirulina platensis*. Chinese Science Bulletin, 41, 741–743.
[17] A.D. Xia, J.C. Zhu, H.J. Wu, L.J. Jiang, X.Y. Zhang (1993) Fluorescence lifetimes, quantum yields and spectral properties of phycobiliproteins from blue-green alga *Spirulina platensis* Photographic Science Photochemistry, 11, 35–40.
[18] J.S. Ma, H.Z. He, Y.Y. Liu, L.J. Jiang (1981) Seperation and characterization of R-phycoerythrin from *Polysiphonia urceolat.*, Chinese Science Bulletin, 26, 933–938.
[19] El Mohsni, S., Kraljic, I., M. Arvis and F. Barat (1976) On the primary reactions in sensitized photooxidations. In Excited States of Biological Molecules (Edited by J.B. Birks), John Wiley, New York. pp. 34–38.
[20] Kraljic, I. and L. Lindqvist (1974) Laser photolysis study of triplet eosin and thionine reactions in photosensitized oxidations. Photochemistry and Photobiology, 20, 351–355.
[21] Mathis, P. and J. Kleo (1973) Triplet state of β-carotene and of analog polyenes of different length. Photochemistry and Photobiology, 18, 343–346.
[22] E. Wilkinson, J.G. Brummer (1981) Rate constants for the decay and reactions of the lowest electronically excited singlet state of molecular oxygen in solution. Journal of Physical and Chemical Reference Data, 10, 809.
[23] a) N. Hasty, P. B. Merkel, P. Radlick and D. R. Kearns (1972) Role of azide in singlet oxygen reactions: reaction of azide with singlet oxygen. Tetrahedron Letters, 1, 49–51.
b) The reviewer gave another more recent determination result [$k\ (^1O_2 / N_3^-) \sim 5.8\times10^8 M^{-1}s^{-1}$.
[24] M. A. J. Rodgers, (1983) Solvent-induced deactivation of singlet oxygen: additivity relationships in nonaromatic solvents. Journal of the American Chemistry Society, 105, 6201–6205.
[25] P.B. Merkel and D.R. Kearns (1972) Radiationless decay of singlet molecular oxygen in solution. An experimental and theoretical study of electronic-to-vibrational energy transfer. Journal of the American Chemical Society, 94, 7244–7253.
[26] Kraljic, I. and C.N. Trumbore (1965) *p*-Nitrosodimethylaniline as an OH radical scavenger in radiation chemistry. Journal of the American Chemistry Society, 87, 2547–2550.
[27] E. Gandin, Y. Lion and A. Van de Vorst (1983) Quantum yield of singlet oxygen production by xanthene derivatives. Photochemistry and Photobiology, 37, 271–278.
[28] J. Mosinger, Z. Micka (1997) Quantum yields of singlet oxygen of metal complexes of meso-tetrakis (sulphonatophenyl) porphine. Journal of Photochemistry and Photobiology, A: Chemistry, 107, 77–82.
[29] S. P. Zhang, S. P. Qian, J. Q. Zhao, S. D. Yao. L. J. Jiang (1999) Characterization of the transient species generated by the photoexcitation of C-PC from *Spirulina platensis*: a laser photolysis and pulse radiolysis study. Biochimica et Biophysica Acta, 1472, 270–278.
[30] S. P. Zhang, Z. H. Han, J. Q. Zhao, S. D. Yao, L. J. Jiang, unpublished data.
[31] I. N. Stadnichuk, Y. V. Kovalev and A. A. Krasnovski Jr. (1993) Phosphorescence spectra of R-phycoerythrin. Journal of Photochemistry and Photobiology, B: Biology, 19, 15–18.
[32] Y. Li, J. P. Zhang, J. Xie, J. Q. Zhao, L. J. Jiang. Detect of the photochemical intermediate processes in phycobiliproteins. Submitted.