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Picosecond Fluorescence Lifetimes of Hematoporphyrin Derivatives in Solutions and in vitro

Hyun Soo Kim, Ph.D., Sung Sil Chu, Ph.D., and Gwi Eon Kim, MD.
Won Young Lee, Ph.D*, Ung Kim, Ph.D**

Department of Radiation Oncology, Yonsei University, College of Medicine, Seoul, Korea
Department of Microbiology,*
Department of Physics,** Yonsei University,

Abstract

The picosecond time resolved fluorescence spectra of Hematoporphyrin Derivative (HPD)in both solutions and cancer cell are measured by a time correlated single photon counting system with a synchronously mode locked dye laser. Two exoponential decay components in the fluorescence spectra were observed. The slow decay(6.3 ns)and the fast one(350 ps)are attributed to be originated from monomers and dimers, respectively. The absorption and fluorescence measurements in steady state also showed the presence of a monomeric and dimeric forms of HPD molecules. The monomer lifetime in the cancer cell was measured to be longer than that in solution, which was expected from the blue shift and narrowing of the absorption spectra for HPD-treated in vitro. The relative amplitude of the fast component was found to be enhanced in cancer cell, strongly indicating the higher affinity of the dimer for the cancer cell.

Key Words : picosecond spectra, hematoporphyrin derivative, dimer, photodynamic therapy

1. INTRODUCTION

Recently, there has been an increasing interest in the photosensitizers for early cancer detection and photodynamic therapy. The former is based on the property of selective accumulation in tumor cells[1, 2]. The latter is related to the broad absorption band of photosensitizers in the laser excitation region[3].

Since it has been shown that Hematoporphyrin Derivative(HPD) is effective photosensitizer in photodynamic therapy(PDT) for treatment of various types of cancers, its photophysical, photochemical and biolgical behaviors have been widely investigated.[4] Barbara[5] showed that the photosensitizing efficiency of HPD was controlled by various parameters such as the polarity of the surrounding medium and the aggregation state of HPD. From clinical and biological studies of HPD[3, 6, 7], it was found that the treatm

ent. The monomer is only cytotoxic but do not have tumor localization ability.

However, mechanisms of the energy relaxations and the photochemical reactions of the HPD treatment are not yet well understood. In order to understand the role of HPD aggregaters, its fluorescence decay kinetics in phosphate buffer saline solution was investigated in the picosecond and nanosecond time region[8,9]. From those studies, it has been revealed that the fluorescence decay time of aggregates in the HPD solution is around few hundred ps and the fluorescence band is shifted to the low energy side.

In this paper, we report a systematic investigation of the picosecond dynamic behavior of HPD in the solution and caner cell *in vitro* . For various water-methanol solut ion, the amplitude of fast decay and energy transfer efficiency were increased with increasing the amount of water due to the aggreagtion of the HPD molecules. The monomer lifetime in the cancer cell was measured to be longer than in solution, which was expected from the blue shift and narrowing of the absorption spectra for HPD-treated *in vitro*. The relative amplitude of the fast component was found to be enhanced in cancer cell, strongly indicating the higher affinity of the dimer for the cancer cell.

2. MATERIALS AND METHODS

The fluorescence decays were measured using a time-correlated single photon counting(TC-SPC)technique. Further information regarding our TC-SPC system can be found in other literature[10]. The decay curves recorded in a multichannel analyzer after obtaining pulses from a TAC(time-to-ampltude converter) were transfered to a computer for theoretical analysis. In particular, we employed TC-SPC system equipped with MCP-PMT as a photodetecter of about 10 picosecond time resolution. Since the fluorescence intensity in the ps time scale is very weak, the detector with high sensitivity, high te-

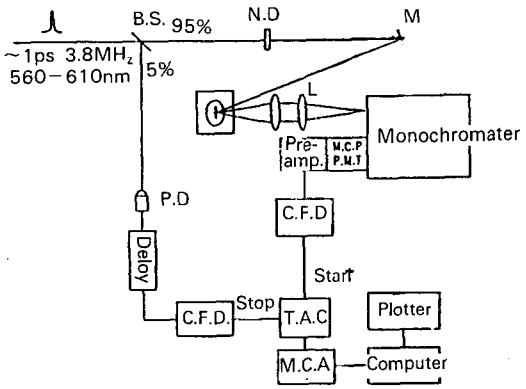

Fig. 1. Schematic diagtam of TC-SPC system
PD:pin diode, MCP-PMT:micro channel plate photomultiplier tube, CFD:constant fraction discriminator, TAC:time to amplitude converter, MCA:multi channel analyzer BS:beam splitter, ND:neutral density filter, M:mirror, L:lens

mporal resolution and fast response time are required. The samples were excited with 10 ps(FWHM) at 588nm from synchronously mode-locked cvity dumper dye laser. A block diagram of our TC-SPC system is shown in Fig.1.

Hematoporphyrin Derivative was obtained from Sigma Co., USA. The solutions of HPD were carefully prepared based on Lipson's method[11]. It is generally known[8] that the slow decays(several ns) and the fast one(few hundred ps) of HPD fluorescence behavior in aqueous solutions are mainly originated from monomers and dimers, respectivesly. The higher affinity of dimer for the cancer cell was also reported[2].

We have prepared various water-methanol solvent mixture solution at fixed HPD co ncentration to control the amount of dimer. In addition, maligant tumor cultured cells, MOLT-4 cells, were grown from a human T-cell lymphoma line. These cells wer maintained in minimum essential medium(MEM, CLS, Australia)containing 10% fetal calf serum(FCS, CSL, Australia), penicillin and streptomycin(100IU and 100μg/ml).Uptake of HPD was done by incubating MOLT-4cells in the medium containing a 100μg/ml HPD solution for one hour[12]. The cells suspended in solution(6×10^5 cells/ml) were kept in a quartz cell for the experiment.

The reabsorption effect of the fluorescence at high concentration was carefully avoided by measuring the front fluorescence near the surface of the sample cell. Steady-state absorption and emission spectra were recorded by using a UV/VIS spectrophotometer and common photoluminenscence equippment. The deconvolution techique was adapted to analyze the measured decay curves.

3. ANALYSIS OF TIME-RESOLVED FLUORESCENCE DECAY SPECTRA

According to Förster kinetics of energy transfer[15], the fluorescence decay mechanisms of the the donor are described by

$$I(t)=Io\ \exp(-t/\tau)\ \exp[-2\gamma(t/\tau)^{1/2}] \qquad (1)$$

wher is τ the lifetime of the donor in the absence of energy transfer and $\gamma=C_A/C_{oA}$ where C_A is the acceptor concentration and $C_{oA}$is the critical acceptor concentration.

On the basis of the above consideration, we can try to describe quantitatively the total fluorescence deay I(t), assuming the linear summation of the slow decay from the monomer and the fast decay from the dimer. The monomer can be dynamically quenched through the Förster resonance energy transfer[15] from the monomer as donor to the dimer as acceptor since the fluorescence spectra from the monomer partially overlap with the dimer absorption spectrum[16]. Therefore, the total decay I(t)can be expressed by

$$I(t)=A_1\ \exp(-t/\tau_1)\ \exp[-2\gamma(t/\tau_1)^{1/2}]+A_2\ \exp(-t/\tau_2) \qquad (2)$$

where A_1 and A_1 are relative initial amplitudes. $\tau_1$and τ_2 are the fluorescence lifetime of the monomer and dimer in the adsence of energy transfer, respectively. γ is the reduced concentration given by $\gamma=C_d/C_{od}$ that is relatived with the energy transfer efficiency from monomer to dimer.

We measured the time-resolved fluorescence decay curve of HPD in the mixture solution and cancer cell *in virto* by using a time correlated single photon counting system equipped with a synchronously mode locked dye laser.

The decay curves recorded in a multichannel analyzer after obtaining pulses from a TAC(time-to-ampltude converter) were transfered to a computer for theoretical anlysis The deconvolution techique was adapted to analyze the measured decay curves[10].

4. RESULTS

Firstly, we investigated absorption and fluoresence spectra of HPD in steady state. Fig.2 shows the absorption spectra of 0.1mM HPD in a phosphate buffer saline aqueous solution(PBS solution)and in a methanol solution. Fig.3 shows the typical fluorescence spectra of HPD in both aqueous solution and methanol soultion by using common photoluminenscence equippment.

The measured picosecond decays, I(t), of the total fluorescence spectra for 0.1 mM HPD in various water-methanol solvent mixtures(100% metanol, 50%water-methanol. 75% water solution)are shown in Fig.4. In order to clarify the mechanism of the fluorescence

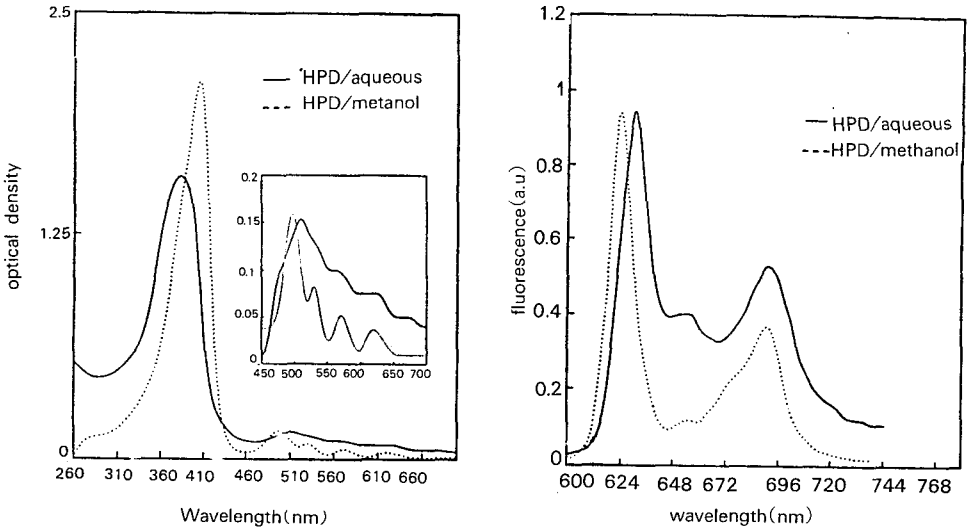

Fig. 2. Absorption spectra of HPD in steady state

relaxation in solution, the time-resolved fluorescence curves at 625 nm and 650 nm were carefully measured agin at the same medium. The ps decays of I(t) are shown in Fig.5.

Moreover we have also investigated the picosecond time-resolved fluorescence curves from the HPD in cancerous cell *in vitro* at both band as shown in Fig.6 Fig.7 shows absorption spectra of HPD in solution and in vitro.

5. DISCUSSIONS

A)STEADY-STATE ABSORPTION AND FLUORESCENCE SPECTRA

Fig.2 shows the absorption spectra of 0.1mM HPD in a phosphate buffer saline aqueous solution (PBS solution)and in a methanol solution. The absorption increase at the high energy and the broadened absorption maximum at the low energy for the PBS solution indicate the prescence of large amounts of aggregated species(possibly dimer)in the aqueous solution. This behaviour can be understood since the dielectric constant of water is higher than that of methanol[13, 14].

Fig.3 shows the typical fluorescence spectra of HPD in both aqueous solution and methanol solution. Two peaks located at 625 and 650 nm and red-shift fluorescence in the PBS solution indicate the presence of a monomeric and dimeric form of the HPD molecule. The increase of the fluorescence intensity around 650 nm in the PBS solution suggests that the dimer band is dominant at 650 nm rather than at 625 nm where the monomer band dominated. Note also that incerase of the fluorescence intensity in the PBS solution is more remakable at 650 nm than that of at 690 nm.

B)TIME-RESOLVED FLUORESCENCE SPECTRA

The measured picosecond decays, I(t), of the total fluorescence spectra for 0.1 mM HPD in various water-methanol solvent mixtures(100% metanol, 50% water-methanol, 75% water solution)are shown in Fig.4. Solids lines represent the best fitted function given by eq.(2). The mean value of the $x^2$is 1.2 which is small enough to indicate a good fit. The decay curves have fast and slow components and the amplitude of the fast component increses with the increase of the water. It has been believed thought that the fast(350 ps)and slow(6.3 ns) decay components are due to dimers and monmers, respectively[8].

In order to clarify the mechanism of fluorescence relaxation, the time-resolved fluorescence curves at 625 nm and 650 nm were carefully measured again at the same medium. The ps decays of I(t) are shown in Fig.5. The fast component at 650 nm is dominant compared with the one at 625 nm as expected from the steady-state fluorescence specta. This tendency becomes more remarkable as the amount of the water in water-methanol solution is increased. The observed total fluorescence behaviour of HPD in the water solution at 625nm(Fig.5(a))is very similar to that of the methanol solution at 650 nm. Therefore, we interpret that the fast component arises from the dimers which can be accumulated more in the water solution.

From the numerical fitting of the measured total fluoerscence decays at different solvent mixtures, A_1, A_2 and γ are determined as given in Tab.1. The fluorescence lifetimes of the dimer and monomer are obtained to be 350 ps and 6.3 ns, respectively. For various water-methanol solvent mixtures, the amplitude of fast decay(A_2) at the dimer band are increased with increasing the amount of water while it is almost invaiant at monomer band. The energy transfer efficiency(γ)are also increased with increasing the amount of

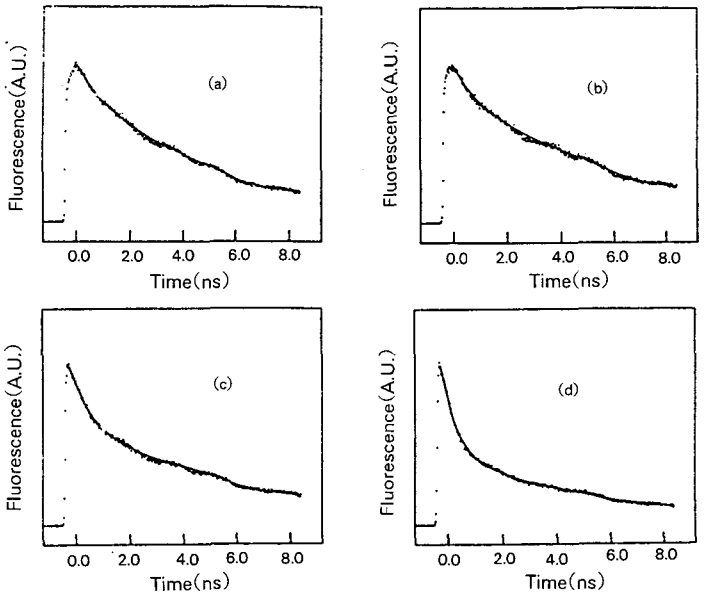

Fig. 4. Total fluorescence decay I(t)of HPD for the different percentages of the aqueous solution at 650nm(HPD conc. 05,mg/ml)for varing (a) 0%(100% methanol), (b) 25%, (c)50%, (d) 75%, The solid lines are fittied by li near summation of slow and fast decay with $\tau_1=6.3$ ns, $\tau_2=350$ ps($x^2=1.2$).

water at both band, resulting from the dipole-dipole interations between monomers and dimers.

We have also investigated the picosecond time-resolved fluorescence curves from the HPD in cancerous cell *in vitro* at both band as shown in Fig.6. The effective fluorescence lifetime for HPD in the cancer cell is different from the results in solution. The effective fluorescence lifetime of the slow component in the cell is longer than that in solution at 625 nm. According to Förster kinetics, the fluorescenc decay of the monomer is quenched by nonradiative energy transfer from monomer to dimer. Since the monomer life time in the cell becomes longer, therefore, mechanisms of the energy transfer from any upperstate of the cell to monomer state should be considered. Note that this is consistent with the blue-shift of the absorption spectra for HPD *in vitro*(Fig.7). The relative amplitude of the fast component is also observed to be enhanced in the cell only at 650nm, strongly implying the higher affinity of dimer for the cancer cell. From the above consideration, we can confirm that aggreagtion states have important role for the selective accmulation properties of HPD molecules which may be localized around tumor as aggregate states(i.e.,dimer state).

Here, we note that the fluorscence decay signals of HPD-treated *in vitro* were true ones. Any fluorescence signals were not practically found in the medium alone without HPD. In our experiment we have no unfortunately definite localization about which components of the cell are connected with accumulative in demonstrating the preferential accumulation

of a photodynamically active incorporation of HPD.

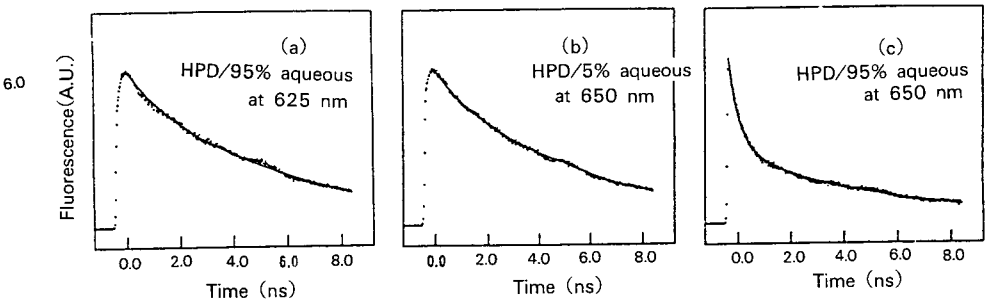

Fig. 5. Total fluorescence decay I(t)of HPD in the mixture solution(conc.0.1 mg/ml)for varing (a) 95% aqueous solution at 625 nm, (b) 5% aqueous solution at 650nm, (c) 95% aqueous solution at 650nm. The solid lines are fitted by linear summation of slow and fast decay with $\tau_1 = 6.3$ns, $\tau_2 = 350$ ps ($x^2 = 1.2$)

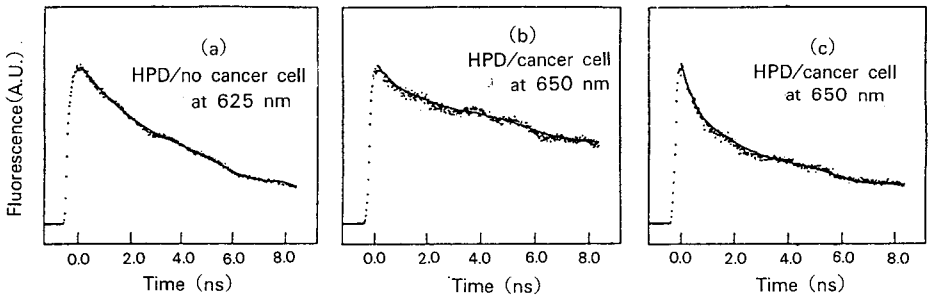

Fig. 6. Total fluorescence decay I(t) of the cancer cell from HPD-treated in vitro (HPD conc. 0.2mg/ml, cell density:5×10^5). (a)at 625nm without cancer cell, (b)at 625 nm with cancer cell, (c)at 650nm with cancer cell

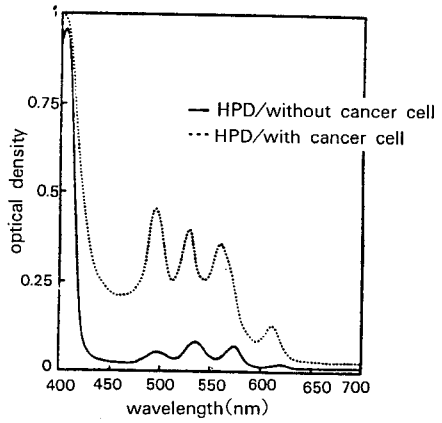

Fig. 7. Absorption spectra of HPD in solution and in vitro

In conculusion, two exoponential components in the fluorescence decays and steady-stayte absorption and fluorescene spectra show the presence of a monomeric and dimeric forms of HPD molecules. The slow decay(6.3 ns) and the fast one(350 ps) are attributed to be originated from monomers and dimers, respectively. The monomer lifetime of the slow component in the cancer cell was measured to be longer than that in solution, which was expected from the blue shift and narrowing of the absorption spectra for HPD-treated *in vitro*.

The relative amplitude of the fast component was found to be enhanced in cancer cell, strongly indicating the higher affininty of the dimer for the cancer cell. From the above consideration, we can reconfirm that aggeragtion states have important role for the selective accmulation properties of HPD molecules which may be localized around tumor as aggregate states(i.e.,dimer state).

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용액 및 시험관 실험에서의 헤마토포르피린 유도체 분자의 피코초 형광수명시간 분석

김현수, 추성실, 김귀언, 이원영*, 김 웅,**
연세대학교 의과대학 방사선 종양학과, 미생물학과*
연세대학교 물리학과**

초 록

헤마토포르피린 유도체는 광역학 치료를 위한 활성감응제로 이용되고 있다. 본 연구는 헤마토포르피린 유도체 분자의 암세포와의 에너지 전달과정을 조사하기 위하여 동시 모드록킹된 색소 레이저와 ~70피코초(10^{-12} sec)시산 분해능을 갖는 단일광자 계수장치 장치를 이용하여 용액에서와 암세포에서 각각 시간분해 형광 스펙트럼을 측정 분석하였다. 측정된 스펙트럼은 deconvolution 방법으로 곡선맞춤되었는데 단량체의 느린 소멸(τ_1=6.3 ns)과 이량체의 빠른 소멸(τ_2=250 ps)로 분석되었으며 정상상태에서의 흡수 및 형광 스펙트럼의 결과와 일치하였다. 농도를 일정하게 하고 극성 분자를 5%에서 95%까지 증가시키면서 형광 소멸 곡선을 측정하여 곡선 맞춤과 결과 빠른 소멸성분(A_2) 값이 증가하였으며 그 증가 폭이 비극성 분자인 메탄올에서 보다 현저하게 나타났다. 헤마토포르피린 유도체 용액에 암세포를 자연 부유시켜 형광 소멸 곡선을 비교 분석한 결과 암세포에 흡착되었을 때 유효 형광 수명 시간이 감소하였다. 이것은 단량체에 비하여 이량체가 암세포의 흡착에 관여하고 있다는 증거로 분석되었다.