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LOCALIZATION OF MONO-L-ASPARTYL CHLORIN e6 (NPe6) IN MOUSE TISSUES

KATSUO AIZAWA*, TETSUYA OKUNAKA, TAKUZO OHTANI, HIROFUMI KAWABE, YUKARI YASUNAKA, SUSUMU O'HATA, NOBUNARI OHTOMO[1], KATSUAKI NISHIMIYA[1], CHIMORI KONAKA[1], HARUBUMI KATO[1], YOSHIHIRO HAYATA[1] and TAKASHI SAITO[2]
Department of Physiology and [1]Surgery, Tokyo Medical College, Tokyo, 160 Japan, and
[2]Department of Biology, Kyorin University, Tokyo, Japan

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Abstract—It is known that HpD is retained longer by malignant tissue than normal tissue and is therefore a useful material for photodynamic therapy (PDT). Currently, vigorous research is being conducted throughout the world to discover a new material which can have greater cancer cell affinity than hematoporphyrin derivative (HpD) and will be used effectively for PDT. Investigation has been conducted to determine the spectral characteristics and cancer cell affinity of NPe6, a recently developed material.

Structurally, a double bond on the D-ring of the porphyrin ring of mono-L-aspartyl chlorin e6 (NPe6) has been reduced, thereby changing its spectral properties from that of HpD. This difference accounts for the stronger absorption bands in wavelengths longer than those of HpD. Furthermore, NPe6 in tumor showed stronger absorption at 660 nm than HpD. Absorption by hemoglobin (Hb) in the blood occurs at wavelengths in the range 500–600 nm, thereby lowering light transmittance. A compound which has a strong absorption band at wavelengths longer than 600 nm and consequently is not affected by Hb will naturally be activated by light at a greater depth in tissue than compounds which do not share this characteristic. The localization of NPe6 in sarcoma and various internal organs was examined with an endoscopic spectrophotometer using an excimer dye laser. After 72 h i.v. NPe6 injection, the results indicate that NPe6 has 10 times greater uptake in malignant tissue cells than in normal organs. Based on the above observations, it was concluded that NPe6 could be effective for PDT if toxicity is low and that this compound has a high malignant tissue affinity.

INTRODUCTION

Sarcoma tissue in rats showed greater emission of red fluorescence of porphyrin than normal tissues (Auler, 1942). This triggered research into materials in the porphyrin group which possessed an affinity for sarcoma. In 1960 hematoporphyrin derivative (HpD)† was prepared from hematoporphyrin hydrochloride treated by acetic acid and sulfuric acid (Lipson, 1961). HpD has been shown to have a higher sarcoma-oriented affinity than any other existing member of the porphyrin group. Application of laser photodynamic therapy (PDT) with HpD started in Roswell Park Memorial Institute (Dougherty *et al.*, 1978, 1979). Treatment of malignant tumors using laser irradiation injection with HpD is currently under clinical investigation in many countries. Complete remission was obtained in the first early stage lung cancer case in the world to be treated by PDT, showing PDT to have potential for localized treatment of early stage cancer (Hayata *et al.*, 1982a, 1982b; Kato *et al.*, 1984). Subsequently 5 yr disease-free survival in an early stage lung cancer case treated only by PDT has been reported (Konaka *et al.*, 1987). An endoscopic laser fluorescence spectrophotometer has been developed to measure the fluorescence spectrum of HpD under endoscopic observation (Aizawa, 1983) and the localization of HpD in cancer tissues by means of its characteristic double peak of fluorescence at 630 and 690 nm with excitation at 405 nm (Aizawa *et al.*, 1984).

Recently, Nippon Petrochemical Co. has developed mono-L-aspartyl chlorin e6 (NPe6). This material was considered to have potential for PDT due to its greater absorption bands in the wavelengths longer than those of HpD excitation (Bommer *et al.*, 1986; Svejda *et al.*, 1986; Petryka *et al.*, 1986). The results of research into this material's spectral characteristics and tumor affinity with a measurement of fluorescence spectrum is discussed in this paper.

MATERIALS AND METHODS

Structurally, mono-L-aspartyl chlorin e6 (NPe6) tetrasodium salt tetrahydrate is a compound with a mol. wt of 871.75 in which the double bond porphyrin ring has been reduced and an aspartic acid is attached to the propionic group at the 17th carbon of the tetrapyrrole ring via a peptide linkage. The NPe6 had a purity of 99% and was provided by Nippon Petrochemical Co. Because NPe6 has strong photochemical activity, it was stored in the dark. HpD was a generous gift from Queen Elizabeth Hospital, Adelaide, Australia.

Measurement of absorption spectrum. Samples with concentrations of 3.3×10^{-6}, 1.0×10^{-6}, 3.3×10^{-7}, 1.0

*To whom correspondence should be address.

†*Abbreviations*: BSA, bovine serum albumin; Hb, hemoglobin; HpD, hematoporphyrin derivative; m-KSA, mouse kidney sarcoma; NPe6, mono-L-aspartyl chlorin e6; PBS, phosphate buffer saline; PDT, photodynamic therapy; RPMI, Roswell Park Memorial Institute.

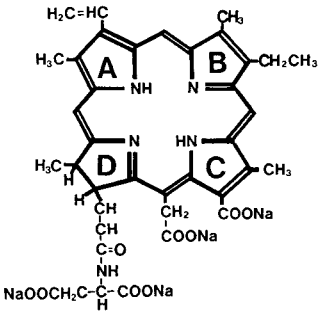

Figure 1. Structure of mono-L-aspartyl chlorin tetra sodium salt tetrahydrate (NPe6). This is a compound with mol. wt 871.75 in which the double bond of the porphyrin ring has been reduced and an aspartic acid is attached to the propionic group at the 17th carbon of the tetrapyrrole ring via a peptide linkage.

$\times 10^{-7}$, 3.3×10^{-8}, and 1.0×10^{-8} *M* were obtained by dissolving NPe6 with phosphate buffer saline (PBS) solution (Flow Laboratory, London), pH 7.2. The absorption spectrum of each sample was measured within the 300–700-nm wavelength range using a PBS solution as blank. For measurement, a 10-mm cuvette and a Hitachi U-3200 spectrophotometer were used.

Measurement of fluorescence spectrum. NPe6 samples with the same concentrations in PBS solution (pH 7.2) as those used for absorption spectrum measurement, were used to measure the fluorescence spectrum. The emission spectrum was measured within 600–750-nm wavelength range with a fixed excitation wavelength of 400 nm. The excitation spectrum of NPe6 was obtained for 300–650-nm wavelength range with a fixed emission wavelength of 660 nm. The intensity (uncorrected) of the fluorescence peak at the 660-nm wavelength set a standard for the relative intensity of fluorescence. For this measurement, a Hitachi 650–10S fluorescence spectrophotometer was used.

Measurement of spectra change by NPe6 binding. NPe6 was dissolved by PBS solution, pH 7.2, to a concentration of 2.0×10^{-6} *M*. Bovine serum albumin fraction powder (BSA; Armor Pharmaceutical Co.) (mol. wt 65 000) was dissolved in PBS solution, pH 7.2, until a concentration of 6.67×10^{-6} *M* was obtained. These two solutions were mixed until the NPe6/BSA mole ratio reached 0.3. After mixing 3 mℓ of both solutions, it was stored for 1 h in the dark at room temperature.

Four mℓ of 1.0×10^{-6} *M* NPe6 PBS solution, pH 7.2, and 4 mℓ *n*-caprylic acid $CH_3(CH_2)COOH$ (mol. wt 144.21) (Wako Pure Chemical Industries Co., Tokyo) were mixed and centrifuged for 10 min at 3000 rpm at room temperature. It was decided to employ *n*-caprylic acid as a model for lipid membrane binding. The supernatant was the NPe6–*n*-caprylic acid mixture. As a control, NPe6 was dissolved by PBS to a concentration of 1.0×10^{-6} *M*. The absorption and fluorescence spectra of the control and NPe6–BSA were measured using PBS as a blank, while *n*-caprylic acid was used as a blank to measure the spectrum of the NPe6–*n*-caprylic acid solution.

NPe6 localization in mice. Injections of 2×10^7 cells of m-KSA (mouse kidney sarcoma provided by Dr K. Kato, National Institute of Health, Japan) cultured in RPMI 1640 medium (Flow Laboratories) containing 10% newborn bovine serum (Flow Laboratories) were made into the epidermis of the back of 3-week-old Balb/c female mice to create a 10–20 mm transplanted tumor. NPe6 solution (2.0 mg $m\ell^{-1}$) was administered intravenously into the tail vein of the mice at a rate of 200 mg kg^{-1} body wt. This high dosage was employed to enable observation of fluorescence for the length of the experiment. After exsanguination, the internal organs and tumors in 3 mice were removed 3, 6, 12, 24, 48, 72 and 96 h after administration. Their fluorescence spectra were then measured by an endoscopic fluorescence spectrophotometer using an excimer dye laser to perform a time-series study of the localization of NPe6 in the organ and tumors. The fluorescence spectrum in the 600–750-nm wavelength range obtained by light excitation at 405 nm was integrated. This value was then used as the standard for evaluation of the relative intensity of fluorescence.

RESULTS AND DISCUSSION

Absorption spectrum

The absorption spectrum of NPe6 is shown in Fig. 2A. It has a characteristic Soret band at a wavelength of 400 nm and (Q bands) at four wavelengths (502, 530, 602 and 655 nm) in PBS solution, pH 7.2 (Aizawa *et al.*, 1983). The absorption spectrum of HpD is shown in Fig. 2B. HpD is characterized by a Soret band at 370–390-nm and four Q bands located at wavelengths 506, 540, 570 and 620 nm in PBS solution, pH 7.2. When absorption at the longest wavelength in the Q band for therapeutic excitation wavelength was compared, NPe6 showed an optical density of 0.295 at 654.4 nm, while HpD registered an optical density of 0.016 at 620-nm wavelength. NPe6 showed an optical density value 19 times greater than HpD. Since blood (hemoglobin) absorbs light in the 500–600-nm wavelength range, light transmittance is low in this range. Longer wavelengths, especially those greater than 600 nm, are not affected by the blood. At these longer wavelengths light transmittance through the tissues generally increases. (Therefore, NPe6, having the absorption band in the longer wavelength range, should be more useful for PDT.) The major difference in the absorption spectrum of NPe6 and HpD may be accounted for by the saturation of the

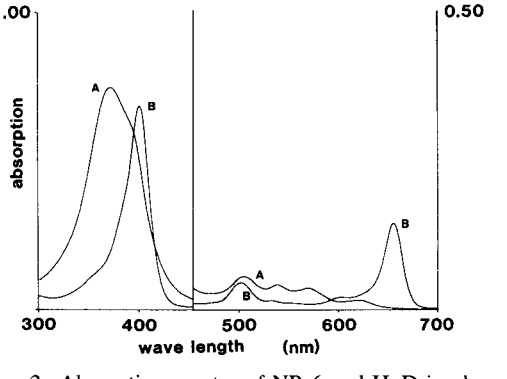

Figure 2. Absorption spectra of NPe6 and HpD in phosphate buffer saline (PBS) solution, pH 7.2. (A) HpD has a characteristic Soret band at 370–390 nm and Q bands at four absorption peaks with 506, 540, 570 and 620 nm. (B) NPe6 is characterized by a Soret band at 400 nm and four Q bands located at 502, 530, 620 and 644 nm.

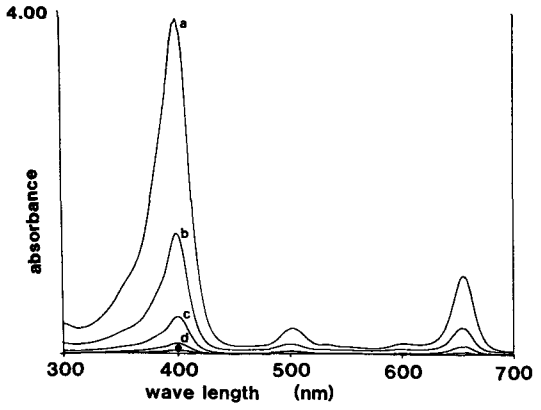

Figure 3. The variation in absorption spectra at various concentrations of NPe6 in PBS solution, pH 7.2. Both absorption peaks of NPe6 at 400 and 644 nm increased in proportion to its concentration range from 3.3×10^{-8} to 3.3×10^{-6} *M*. Concentration of NPe6 (*M*): (a) 3.3×10^{-6}, (b) 1.0×10^{-6}, (c) 3.3×10^{-7}, (d) 1.0×10^{-7}, (e) 3.3×10^{-8}.

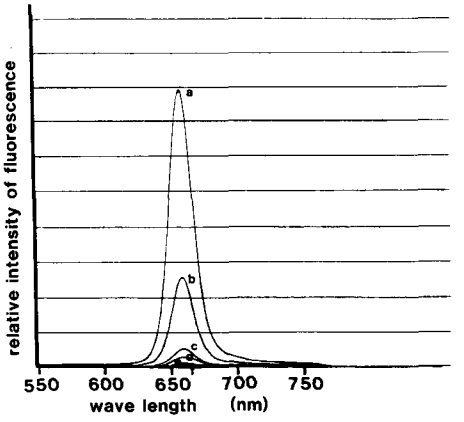

Figure 4. Fluorescence spectra of NPe6 with an excitation at 400 nm in PBS solution, pH 7.2. Emission peak of NPe6 appeared at 660 nm and its intensity increased proportionately with concentrations up to 3.3×10^{-6} *M*. Concentration of NPe6 (*M*): (a) 3.3×10^{-6}, (b) 1.0×10^{-6}, (c) 3.3×10^{-7}, (d) 1.0×10^{-7}, (e) 3.3×10^{-8}.

double bond on the D-ring of the porphyrin ring of NPe6.

The variation in light absorption at various concentrations of NPe6 is shown in Fig. 3. Absorption of NPe6 at 400 and 655 nm increased in proportion to its concentration.

Fluorescence spectrum

Fluorescence spectrum curves were obtained by excitation at 400 nm, which is the location of the Soret band of NPe6 as obtained from the results of the absorption spectrum (Fig. 4). The fluorescence peak appeared at a wavelength of 660 nm. The relative intensity of fluorescence increased proportionately with concentration, up to 3.3×10^{-6} *M*. In order to confirm this, an excitation spectrum curve was obtained with a fixed emission at 660 nm (Fig. 5). The emission appeared in each of the absorption wavelengths and the relative intensity increases proportionately with concentration of NPe6 up to a peak of 3.3×10^{-6} *M*.

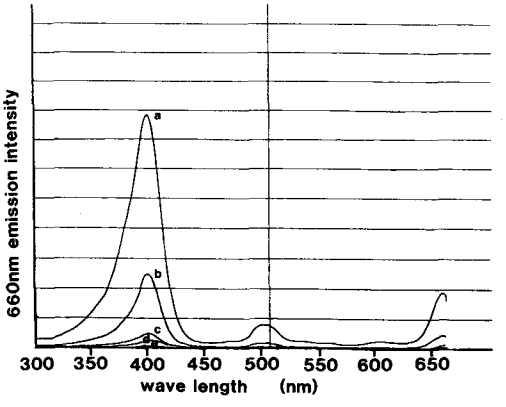

Figure 5. Excitation spectrum curve with a fixed emission at 660 nm. The emission appeared in each absorption wavelength and increased the relative intensity proportionately with concentrations of NPe6 up to a peak of 3.3×10^{-6} *M*. Concentration of NPe6 (*M*): (a) 3.3×10^{-6}, (b) 1.0×10^{-6}, (c) 3.3×10^{-7}, (d) 1.0×10^{-7}, (e) 3.3×10^{-8}.

Spectral change caused by NPe6 binding

NPe6 was dissolved at a concentration of 1.0×10^{-6} *M* in PBS solution, pH 7.2. This solution was used as a control. Its absorption spectrum showed optical density of 0.512 at a wavelength of 400 nm (Fig. 6A). NPe6 was also dissolved in PBS solution, pH 7.2, at a concentration of 2.0×10^{-6} *M*. This solution and a solution of BSA solution at a concentration of 6.67×10^{-6} *M* were mixed with equal volumes and the absorption spectrum was measured. Its optical density was 0.358 at 412 nm (Fig. 6B). Compared with the control, it was clear that absorption shifted by *ca.* 12 nm towards the longer-wavelength side and optical density decreased by a factor of *ca.* 0.15. The absorption spectrum of NPe6 dissolved in PBS solution to concentrations of 2.0×10^{-6} and 2.52×10^{-6} *M* *n*-caprylic acid showed optical density of 0.638 at a wavelength of 404.4 nm (Fig. 6C). A slight absorption peak was observed at a wavelength of 418 nm after a second-order differential absorption spectrum analysis. When this was compared with the control, there was a 20% decrease in absorption and a shift towards longer wavelength (bathochromic shift). When the absorption spectra of the NPe6/BSA mixture and the NPe6/*n*-caprylic acid mixture were compared, *n*-caprylic acid showed a shift of 6 nm towards the longer-wavelength side.

Each of the above solutions and mixtures were excited by a wavelength of 400 nm to give a fluorescence spectrum curve (Fig. 7). Although the

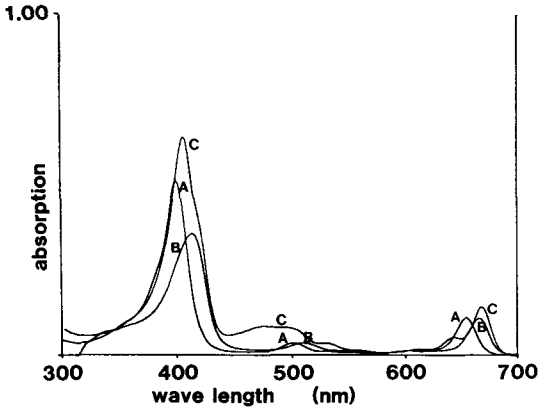

Figure 6. Difference of absorption spectrum of NPe6 with binding to other substance in PBS solution, pH 7.2. (A) NPe6 was dissolved at a concentration of 1.0×10^{-6} *M* in PBS solution, pH 7.2. Its absorption spectrum showed an optical density of 0.512 at 400 nm. (B) NPe6 with binding to bovine serum albumin showed an absorption peak at 412 nm and optical density was 0.358. (C) NPe6 with binding to *n*-caprylic acid showed an absorption peak at 404.4 nm and a shoulder at 418 nm. Optical density at 404.4 nm was 0.638.

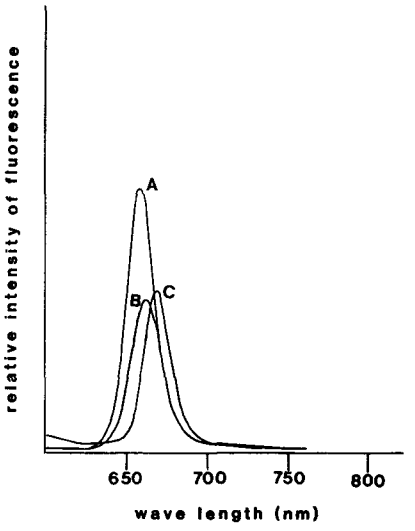

Figure 7. Fluorescence spectra change of NPe6 binding to substance. Fluorescence peak appeared at 660 nm for the control (A), BSA binding at 663 nm (B) and *n*-caprylic acid binding at 670 nm (C).

fluorescence peak appeared at 660 nm for the control, BSA solution showed a peak at 663 nm and *n*-caprylic acid solution showed a peak at 670 nm.

NPe6 localization in mice

NPe6 was injected intravenously via the tail vein of mice in which transplantation of kidney sarcoma cells had been performed, at a dosage of 200 mg kg^{-1} body wt. The localization of fluorescence was used to examine the presence of sarcoma in internal organ. NPe6 dissolved in PBS solution showed a fluorescence spectrum peak at 660 nm, while sarcoma tissue showed a fluorescence spectrum peak at about 670 nm with excitation at 405 nm. A slight 10-nm shift towards the long-wavelength side occurred in the *in vivo* fluorescence. No significant difference in this shift was observed for the surface and inside of the sarcoma tissue. The time of measurement after administration was unrelated to fluorescence spectra.

When these results were compared with the earlier observation of NPe6 binding (in which the fluorescence peak of BSA and NPe6 solution occurred at 663 nm, while the peak for *n*-caprylic acid–NPe6 solution occurred at 670 nm), NPe6 in the tumor is more likely to be bound to lipid than a protein component.

The localization of NPe6 in tumor and various internal organs is shown in Fig. 8. From this graph, it is obvious that the relative intensity of fluorescence of NPe6 in each internal organ is highest 3 h

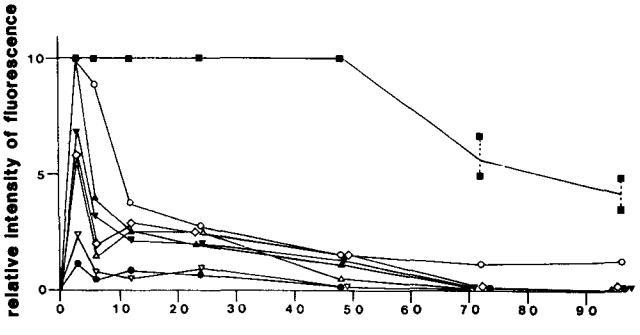

Figure 8. The localization of NPe6 in tumor and various internal organs of mice. NPe6 was injected (i.v.) via the tail vein of mice at a dosage of 20 mg kg^{-1} body wt. The fluorescence spectra were measured by an endoscopic fluorescence spectrophotometer using an excimer dye laser. Three samples were employed per data point. ■ Tumor, ○ skin, ▲ muscle, ▼ intestine, ◇ liver, △ stomach, ▽ brain, ● heart, ● kidney, ● spleen, ● lung.

after i.v. injection of NPe6. With the relative intensity of fluorescence of NPe6 in tumor at 3 h after its i.v. injection set as 100%, the relative intensity in various organs in comparison to fluorescence in the tumor was as follows: skin 100%, intestine 70%, liver 60%, stomach 50%, brain 20% and 10% for all other internal organs.

Setting the relative intensity of fluorescence of NPe6 in tumor at 3 h after i.v. injection as 100%, the decrease in the relative intensity of fluorescence of different organs 12 h after i.v. injection was as follows: skin 40%, muscle 30%, other organs *ca.* 10%. The relative intensity of fluorescence in each organ continued to drop with time and at 72 h after i.v. injection, there was little or no fluorescence remaining in any organ. In contrast, the relative intensity of fluorescence in sarcoma tissues showed no decline even after 48 h, although a 40% decrease was observed after 72 h. There was still 5 times more fluorescence in the tumor than in any organ.

The relative intensity of fluorescence within transected tumor was 1.4 times greater than that found on the tumor surface at 48 h after i.v. injection. This may be explained by a possible reduction in fluorescence due to photodegradation of NPe6 since the tumor surface was subjected to fluorescent or other light between the time of removal of tissue from the body and the subsequent measurement of fluorescence. The results of the above investigation indicate that NPe6 localized in sarcoma, and that its drainage from normal organs is rather swift.

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