European Journal of Pharmaceutics and Biopharmaceutics 76 (2010) 245–252

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

Effect of polyvinylpyrrolidone on the interaction of chlorin e6 with plasma proteins and its subcellular localization

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ARTICLE INFO

Article history:
Received 27 May 2009
Accepted in revised form 9 June 2010
Available online 15 June 2010

Keywords:
Chlorin e6
Fluorescence imaging
Lipoprotein
Photodynamic therapy
Polyvinylpyrrolidone

ABSTRACT

A photophysical study describing the effects of the polymer polyvinylpyrrolidone (PVP), on the binding interaction between chlorin e6 (Ce6) with bovine serum albumin (BSA) and human plasma proteins such as very low-density lipoprotein (VLDL), high-density lipoprotein (HDL) and low-density lipoprotein (LDL) was performed using a steady-state fluorescence technique. Combined Ce6–PVP was found to have very stable photostability at three different temperatures (4 °C, 21 °C and 37 °C) when dissolved in an aqueous solution containing 5% and 10% fetal calf serum. The partition coefficient of combined Ce6–PVP was relatively more hydrophilic than that of Ce6 alone. There was a marked increase in the emission profile of Ce6–PVP and the correlated bathochromic shift on the addition of proteins. These results also suggest that Ce6–PVP might have slightly greater association energy with VLDL in comparison to Ce6 alone. The co-localization of Ce6 and Ce6–PVP in cells was also assessed using confocal microscopy. The association of Ce6 with PVP resulted in an enhanced cellular uptake of Ce6 within the cytoplasmic compartment of cells. The present study supported the hypothesis that PVP improves the permeability of Ce6 through the biological membranes of cells.

1. Introduction

Photodynamic therapy (PDT) is a promising modality for the treatment of cancerous as well as non-cancerous diseases such as ophthalmic neovascular diseases [1]. The photoactivation of photosensitizers by laser (at an appropriate wavelength, dose and time interval) produces reactive singlet oxygen to induce irreversible destruction of tumor cells. The importance of the photosensitizer chlorin e6 (Ce6), and its derivatives for the selective and effective photodynamic destruction of human malignant tumors, has become increasingly apparent [2]. To date, many formulations of Ce6 have been developed that have significant tumor-localizing capacity. PVP is a pharmaceutical grade water-soluble polymer known to form complexes with a number of pharmacological substances. Hence, several investigations into the ability of PVP to form complexes with drugs have been described in the literature [3,4].

The original application of PVP was as a plasma substitute for trauma victims, because it is chemically and biologically inert. However, this practice has now been discontinued due to the large molecular weight fractions accumulating in the body [5]. This occurs because the excretion of PVP is inversely related to increasing molecular weight. Hence, as the molecular weight of PVP decreases, there is an increase in the rate of polymer dissolution, oral absorption and excretion [6,7]. Therefore, PVP of lower molecular weights is typically preferred for parenteral applications. PVP is now widely used as a binder for pharmaceutical drugs, while other applications utilize the polymer as a controlled release excipient and transdermal penetration enhancer [8]. Ce6–PVP was developed with the rationale to provide a novel photosensitizer formulation with high chemical and photochemical stability, good solubility in both water and biological fluids, high affinity to the tumor tissue and low phototoxicity to normal tissue [9,10].

In our previous studies, we reported the potential application of Ce6–PVP in the fluorescence diagnosis of human nasopharyngeal and human bladder carcinomas in preclinical animal models

Abbreviations: Ce6, chlorin e6; PVP, polyvinylpyrrolidone; Ce6–PVP, combined chlorin e6 with polyvinylpyrrolidone; HDL, high-density lipoprotein; HPD, hematoporphyrin derivative; LDL, low-density lipoprotein; PBS, phosphate-buffered saline; FCS, fetal calf serum; PDT, photodynamic therapy; VLDL, very low-density lipoprotein, CAM, chick chorioallantoic membrane; RT, room temperature.

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doi:10.1016/j.ejpb.2010.06.005

[11,12] and in one angiosarcoma patient [13]. We also found that Ce6–PVP exhibits a significant phototoxic effect on the bladder [14], non-small-cell lung carcinomas and small-cell lung carcinomas [15]. In addition, PVP was found to significantly enhance Ce6 concentration in tumors compared to Ce6 alone and increased the therapeutic index of PDT without any side effects in animal models [16,17]. The selective uptake of Ce6–PVP has also been confirmed in animal models, as well as in patients using in vivo fibre-optic spectrofluorometer [18]. In the chick chorioallantoic membrane model, membrane penetration of Ce6–PVP was twice as efficient compared to Ce6 alone [19]. Ce6–PVP-induced cell death was observed in a light-dependent manner that resulted in a significant production of reactive oxygen species and caused a rapid increase in lactate dehydrogenase (LDH) release, suggesting the loss of membrane integrity and subsequent cell death by necrosis [20].

The preferential accumulation of photosensitizers in neoplastic tissue has been subjected to speculations such as: (a) increased proliferation rates of neoplastic cells resulting in higher uptake, (b) reduced lymphatic drainage (c) leaky tumor vasculature, (d) specific interaction between the photosensitizer and marker molecules on neoplastic cells, and (e) specific low-density lipoprotein (LDL) receptor–photosensitizer interaction leading to increased photosensitizer concentrations in neoplastic tissue [21]. Various pharmacokinetic investigations have led to the general agreement that hydrophobic dyes are associated with lipoproteins, while their hydrophilic counterparts bind preferentially to other serum proteins, such as albumin [22]. Injected photosensitizers bind primarily to serum proteins; however, the binding of proteins differs according to the chemical characteristics of the photosensitizer and hence causes photosensitizer redistribution between plasma proteins. For this reason, the binding of proteins defines several photosensitizer parameters including its: (i) interaction with cells, (ii) intracellular localization and kinetics of accumulation in the tumor and (iii) photodynamic efficacy in vivo [23].

The objective of this study was to investigate the interactions of plasma proteins with Ce6 in the presence and absence of PVP, examining changes in both the bathochromic and hyperchromic shifts of Ce6 using spectrophotometry. The extent of binding Ce6 and Ce6–PVP to a high-density lipoprotein (HDL), a low-density lipoprotein (LDL) and a very low-density lipoprotein (VLDL) was calculated. Examination of the subcellular localization of PVP, Ce6 and Ce6–PVP was carried out using confocal microscopy. Since experimental evidence of Ce6–PVP interactions have not been previously published, this study contributes new insights into the understanding of the mechanism of the interaction of PVP with Ce6.

2. Materials and methods

2.1. Photosensitizer and serum proteins

Ce6–PVP was obtained from ORPEGEN Pharma GmbH, Heidelberg, Germany as a co-lyophilisate of Ce6 sodium salt and PVP (molecular mass ≈12,000 kDa) in a 1:1 mass ratio. Ce6 was also obtained from ORPEGEN Pharma and lyophilized without further addition of PVP. Human LDL (6.1 mg/mL; molecular weight, 3500 kDa), HDL (16.4 mg/mL; molecular weight, 175–500 kDa) and VLDL (2.37 mg/mL, molecular weight, 6000–27,000 kDa) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Determination of photosensitizer stability using fluorescence spectrometry

Ce6 (20 μM) and Ce6–PVP were separately dissolved in 1 mL of the following solutions: PBS, water and fetal calf serum (5% and 10%). Solution stability was assessed as a function of time for 27 days at three different temperatures (4 °C, room temperature (RT), i.e. 21 °C and 37 °C) by measuring the fluorescence emission spectrum using the spectrofluorophotometer RF-5301 PC (Shimadzu, Kyoto, Japan). Fluorescence emission was measured from 620 to 630 nm on excitation at 400 nm. Non-linear regression fitting using one-phase exponential decay was used to determine the half-life using Graph-Pad Prism™ version 2.0 (Intuitive Software for Science, San Diego, USA).

2.3. Preparation of photosensitizer–protein complex and measurement

The emission properties of Ce6 and Ce6–PVP, at a concentration of 0.01 mg/mL in 0.9% physiological saline, were investigated. Protein solutions were prepared at six different concentrations: 0%, 0.005%, 0.01%, 0.015%, 0.02% and 0.05%. A total of 80 μL of the photosensitizer was added into 720 μL of each concentration of the protein solutions. The solutions were mixed well and incubated for 1 h on ice in order to examine only the photosensitizer–substrate interaction in the absence of active lipolysis and to exclude the possible effects of a change in the size and/or composition of the lipoprotein particles when binding to photosensitizers. Emission intensity was measured using a HITACHI U-3010 spectrophotometer. The scan range was from 500 to 800 nm using a WI (VIS) lamp. Curves were fitted using GraphPad Prism software.

2.4. Determination of the partition coefficient

Log P (logarithm of the partition coefficient in PBS/1-octanol system) determinations were performed using equilibration techniques. Partitioning was carried out in centrifuge bottles. Partition coefficients of Ce6 were evaluated in a system of 1-octanol–PBS at four different pH values: 5.0, 6.0, 7.0 and 8.0. 50 μL of 0.01 mg/mL Ce6 and Ce6–PVP were each dissolved in 0.9% NaCl and added to 5 mL of 1-octanol (organic phase). This was followed by the addition of an equal volume of PBS (aqueous phase) at each specified pH. After the two phases were presaturated with each other, the tubes were vortexed for 2 min at room temperature (21 °C). Tubes were then centrifuged for 2 min at 3000 rpm to separate the octanol and PBS phases. After centrifugation, the solute in both phases was analyzed. A volume of 1.8 mL of the buffer phase was pretreated with 0.2 mL of 10% Triton X-100 to ensure de-aggregation of the drug. Absorption was measured with a UV–Vis absorption spectrometer. The partition coefficients (P) were calculated as:

$$P = C_{oct}/C_{PBS} = abs_{oct}/abs_{PBS}$$

where C_{oct} and C_{PBS} represent the photosensitizer concentrations in the organic and the aqueous phase, respectively, abs_{oct} represents the absorbance of the compound measured in the octanol and abs_{PBS} represents the absorbance in the PBS solution.

2.5. Labelling of PVP with fluorescein isothiocyanate (FITC)

PVP (1.25 g) and KOH (1.25 g) were dissolved in 50 mL water. The solution was transferred to a 100-mL pressurized bottle. The pressurized bottle was closed with a Teflon screw cap, then placed

Table 1
The influence of different concentrations of three lipoproteins on the protein binding capacity (B_{max}) of Ce6 and Ce6–PVP. The value of B_{max} is expressed in terms of percentage.

B_{max} (%)	Ce6			Ce6–PVP		
	HDL	LDL	VLDL	HDL	LDL	VLDL
Low concentration (0.01 mg/mL)	1.66	1.84	3.10	1.68	2.23	3.13
High concentration (0.06 mg/mL)	2.74	2.23	1.19	5.71	4.58	9.53

on a heating mantle and heated to a surface temperature of 135 °C for 15 h. The assembly was then cooled, the bottle opened and the solution was filtered. The pH of the remaining solution was adjusted to pH 7 using HCl. The solution was then transferred to a dialysis bag (MWCO = 3000 Da, Spectrapore) and dialyzed against milliQ water (4L × 6) for three days. The contents of the dialysis bag were then transferred to a 250-mL round bottomed flask and lyophilized to obtain a pure opened ring PVP (PVPRO) (approximately 1 g). Potentiometric titration showed that the percentage of ring opening was 3.5 ± 0.3%. PVPRO (20 mg) was dissolved in 20 mL 0.1 M sodium carbonate buffer and 2 mL of FITC (1 mg/mL) was added at room temperature and stirred gently with a magnetic stirrer bar. After 2 h, the solution was transfer red to a dialysis bag (MWCO = 3000 Da, Spectrapore) and dialyzed against deionized water for 3 days. The content of the dialysis bag was then transferred to a 100-mL round bottomed flask and lyophilized to produce PVP–FITC (16.8 mg). This was dissolved in 16.8 mL PBS. A concentration of 10 μM of PVP–FITC was then prepared in Roswell Park Memorial Institute (RPMI) medium and used for cell culture studies.

2.6. Intracellular localization by confocal laser-scanning microscopy

MGH (human bladder carcinoma cells) were maintained in RPMI medium containing 10% fetal calf serum (FCS), sodium pyruvate (110 mg/mL), penicillin (100 U/mL) and streptomycin (100 mg/mL) under 5% CO_2. The cells were seeded at 1×10^5 per well onto the 8-well Labtek chamber slide (Nunc, Naperville, CT) in 0.5 ml of RPMI medium. After a 24-h attachment and growth period at 37 °C, the cells were incubated with Ce6, Ce6–PVP and PVP-FITC (10 μM) for 30 min. Cells were then washed and fixation was performed in 3.7% formaldehyde and 0.05% Triton X-100 in PBS for 5 min. The cells were then stained with DAPI (λ_{ex} = 378 nm, emission filter = BP 400–440 nm) rhodamine-phalloidin (λ_{ex} = 543 nm, emission filter = LP 590 nm) using the procedure adapted from the experimental protocol (Subcellular Structure Localization Kit, Chemicon, USA). The coverslips were then mounted onto a slide, using antifade mounting medium (Vectashield, Vector) before imaging, using a confocal laser-scanning microscope (CLSM). Confocal parameters are listed as follows: 40×/0.55 and 63×/1.3 objective lens; 488 nm exciting wavelengths for Ce6/Ce6–PVP and FITC; 488/568 nm beam-splitter for FITC; 660–670 nm bandpass barrier filter for Ce6/Ce6–PVP fluorescence detection.

3. Results and discussion

3.1. Partition coefficient

One of the challenges in formulating photosensitizers is to optimize their amphiphilic properties. This ensures that they are sufficiently hydrophilic enough to be administered as aqueous solutions and are rapidly eliminated from the body following photodynamic therapy. In addition, photosensitizers must also be sufficiently lipophilic enough to be preferentially retained within the tumor tissue. The lipophilicity of photosensitizers is considered important in controlling their penetration and uptake through the cellular membrane. In this context, this study was conducted to assess the influence of pH on the lipophilicity of Ce6 and Ce6 combined with PVP (i.e. Ce6–PVP).

One traditional way of evaluating the amphiphilicity of medicinal compounds is to determine the partition coefficients between 1-octanol and PBS. This is a simplistic way of calculating the manner in which an organic compound is distributed in the body. It is termed as "two-compartment" or "two-phase" system, comprising of aqueous (water) and organic (lipid) layers. The molecular structures of Ce6 and PVP are presented in Fig. 1. In the current study, it was found that both Ce6 and combined Ce6–PVP had strong absorption at 400 and 665 nm. Binding of PVP to Ce6 did not affect the Ce6 absorption spectra. Fig. 2 shows the partition coefficients of the Ce6 and Ce6–PVP at a range of different pH values. Comparison of the partition coefficients of Ce6 versus Ce6–PVP showed that binding of PVP caused an expected decrease in the hydrophobicity of Ce6 at pH 6 and pH 8. The partition co-efficient increased as the pH of the aqueous buffer was lowered below 7 indicating that a larger fraction of Ce6–PVP was incorporated into the 1-octanol phase under more acidic conditions. It has been documented that a blood pH of 7.4 may cause protonation of the photosensitizer [24]. Existing research showed an increase in the Ce6 fraction when it was bound to low-density lipoproteins (LDL), whereas when bound to human serum albumin, the Ce6 fraction decreased with decrease in pH from 7.4 to 6.5 [25]. Hence, while Ce6 existed in the amphiphilic form at pH 7.4, a proportion of Ce6 may have been lost because the hydrophilic part of the Ce6 may have interacted with other serum proteins. Thus, the results of the current study implied that there might be enhanced passive diffusion of Ce6 or combined Ce6–PVP into tumor cells due to the extracellular tumor tissue being slightly acidic.

3.2. Fluorescence properties of Ce6–PVP in various biological media

Qualitative and quantitative changes in emission spectra, resulting from a change of reaction medium, can provide information about the interaction between excited states and the environment. Such information is potentially important towards explaining the photoreactivity of a photosensitizer, in a certain formulation. Fluorescence emission of Ce6–PVP in water, PBS and fetal calf serum (5% and 10%), were recorded at three different temperatures (4 °C, 21 °C and 37 °C) over a period of 27 days. The stability of Ce6–PVP deteriorated drastically when dissolved in water (Fig. 3A). By day 27, the

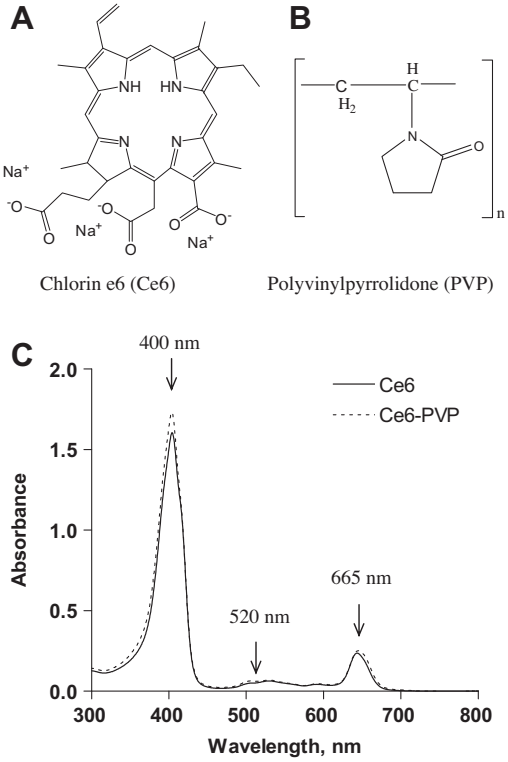

Fig. 1. The molecular structure of (A) Ce6 and (B) PVP (C_6H_9NO), with (C) the absorption spectra of Ce6 (solid line) and Ce6–PVP (dashed line) in 0.9% NaCl measured from 400 to 800 nm.

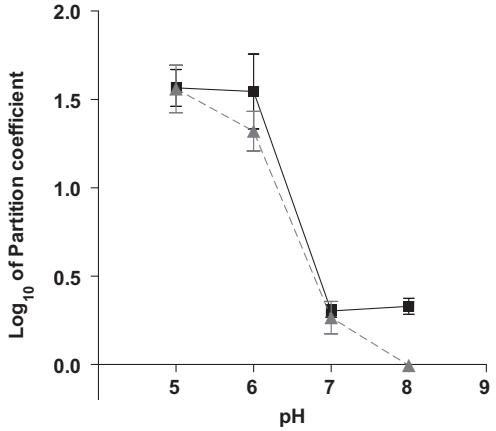

Fig. 2. The partition coefficients of Ce6 (■) and Ce6–PVP (▲) between 1-octanol and PBS at various pH values.

emission intensity of Ce6–PVP had reduced by 98%, 72% and 100% when dissolved in water at a temperature of 37 °C, room temperature (RT, i.e. 21 °C) and 4 °C, respectively. On the other hand, when Ce6–PVP was dissolved in PBS, the emission intensity of Ce6–PVP had reduced by 27% at a temperature of 37 °C by day 27 (Fig. 3B). During the same time period, the emission intensity of Ce6–PVP had reduced by 45% and 44% when dissolved in PBS at RT and 4 °C, respectively. Ce6–PVP dissolved in 5% and 10% FCS was found to be very stable throughout the 27-day period at all the three temperatures (Fig. 3C and D). When the fluorescence emission data of Ce6–PVP dissolved in water was fitted to non-linear regression equation using one phase exponential decay, the half-life of the fluorescence intensity was found to be 12 day (95% CI 10.8–14.3) at RT, 7.7 days (95% CI 6.8–8.9) at 37 °C and 3.0 days (95% CI 2.5–3.5) at 4 °C. The half-life of Ce6–PVP dissolved in PBS was found to be 24.2 days (95% CI 20.3–30.0) at RT, 114 days (95% 71.4–285.0) at 37 °C and 30.7 days (95% CI 25.2–39.3) at 4 °C. Ce6–PVP appeared to remain stable in the presence of 5% and 10% FCS throughout the 27-day survey period. It was interesting to observe that at lower temperature of 4 °C, in the absence of 5% and 10% FCS, the fluorescence emission of Ce6–PVP was lower than in RT and 37 °C. This could be due to the increase in molecular aggregation at low temperature and thus could have led to a reduction in the fluorescence yield. This effect had been observed for other photosensitizers where aggregation could drastically reduce the fluorescence yield of tetrapyrrolic drugs [26]. Further study is warranted to elucidate the mechanism of degradation of Ce6–PVP.

3.3. Effect of plasma lipoproteins on the spectral properties of Ce6 and Ce6–PVP

Lipoproteins are complex particles that consist of spherical hydrophobic cores of triglycerides or cholesteryl esters surrounded by amphipathic (polar and non-polar) mono-layers of phospholipids, cholesterol and apolipoproteins [30]. Lipoproteins are usually classified by their size and density as 'very low-density lipoprotein' (VLDL), 'low-density lipoprotein' (LDL) and 'high-density lipoprotein' (HDL). Their main function is to transport endogenous and dietary fats in the blood and lymphatic vessels. Lipoproteins are also the key components that regulate the transport of photosensitizers in the body's circulatory system. The association of photosensitizers to lipoproteins is dependant on the non-polar moieties of the photosensitizers and lipids, aggregation properties, polarity, pH effects and the chemical nature of side-groups in the photosensitizer. In this study, it was observed that the peak of the spectral wavelength of both Ce6 and Ce6–PVP increased with the increase in concentration of LDL (Fig. 4). The emission spectra of Ce6 with different concentrations of LDL exhibited a major shift in wavelength peaks. For example, Ce6 with 0% LDL showed a peak at 664.8 nm. Ce6 with 0.005% LDL showed a peak at 674.4 nm, producing a shift of 9.4 nm in the spectral wavelength. Ce6 with 0.05% LDL showed a peak at 680.8 nm, producing a total shift of 16 nm from Ce6 with 0% LDL.

Ce6–PVP with LDL showed equivalent wavelength shifts in comparison to Ce6 with LDL. Ce6–PVP with 0% LDL exhibited a peak at 664.2 nm wavelength. Ce6–PVP with 0.005% LDL showed a peak at 675.4 nm, producing a shift of 11.2 nm. Ce6–PVP with 0.05% LDL exhibited a peak at 680.2 nm, producing a total shift of 16 nm from Ce6–PVP with 0% LDL. A similar observation was noted in which both Ce6 and Ce6–PVP emissions increased with increasing concentration of LDL. Ce6–PVP combined with LDL produced double the emission intensity to that of Ce6 with LDL at the same wavelength.

The emission spectra of Ce6 with different concentrations of HDL exhibited major shifts in wavelength peaks. Ce6 with 0% HDL exhibited a peak at 664.8 nm. Ce6 with 0.005% HDL had a peak at 678.2 nm, producing a shift of 13.4 nm. Ce6 with 0.05% HDL had a peak at 679.8 nm, giving a total shift of 15 nm. Overall, Ce6–PVP exhibited a slightly higher wavelength shift in comparison to that of Ce6 in general. Ce6–PVP with 0% HDL showed a peak at 664.2 nm. Ce6–PVP with 0.005% HDL had a peak at 678.4 nm, giving a shift of 14.2 nm. However, Ce6–PVP with 0.005% HDL exhibited a slight decrease in fluorescence emission. Ce6–PVP with 0.05% HDL had a peak at 679.4 nm, giving a total wavelength shift of 15.2 nm. Here, the emission intensity for Ce6–PVP was twice as high as that of Ce6.

The emission spectra of Ce6–PVP with different concentrations of VLDL exhibited a slight shift in wavelength peaks. Ce6–PVP 0% VLDL showed a peak at 664.2 nm. Ce6–PVP with 0.005% VLDL had a peak at 666 nm, giving a shift of 1.8 nm. Ce6–PVP with 0.05% VLDL had a peak at 671.4 nm, giving a total shift of 7.2 nm. The Ce6–PVP with 0%, 0.005% and 0.05% VLDL produced an almost threefold increase in emission intensity.

Previous research has shown that the bathochromic shift of the porphyrin Soret band indicates a π–π interaction between the porphyrin and binding protein [31]. In fact, the induction of a bathochromic shift as a result of the formation of a porphyrin–protein complex is well documented [32]. There are two possible factors that may lead to bathochromic shifts. The first possibility is related to an increase in p-electron orbitals at the periphery of the porphyrin when the tetrapyrrolic macro-cycle associates with adjacent aromatic ligands in serum proteins via ionic binding and van der Waals forces [33]. Both Ce6 and Ce6–PVP may form complexes via p–p interaction between the aromatic ligands of the lipoprotein and the tetrapyrrolic macro-cycle of Ce6. Another possible factor that may lead to bathochromic shift is the monomerization of porphyrins from dimers or aggregated states. Porphyrinic compounds that have an amphiphilic structure such as Ce6 tend to self-associate in aqueous solution, the degree of which depends on factors such as the nature of the polar side chains, the pH of the surrounding medium and the stereochemistry of the porphyrin molecule [34]. It is possible that the addition of lipoprotein might induce monomerization and thus lead to bathochromic shift. Hence, the lipoprotein may actually function as an endogenous drug carrier for Ce6 via lipoprotein receptor mechanisms in tumors [35].

3.4. Association binding measurement

The systemic administration of photosensitizers primarily depends on their binding to various serum proteins, such as HDL, LDL, VLDL and albumins, for transport and distribution. Hence,

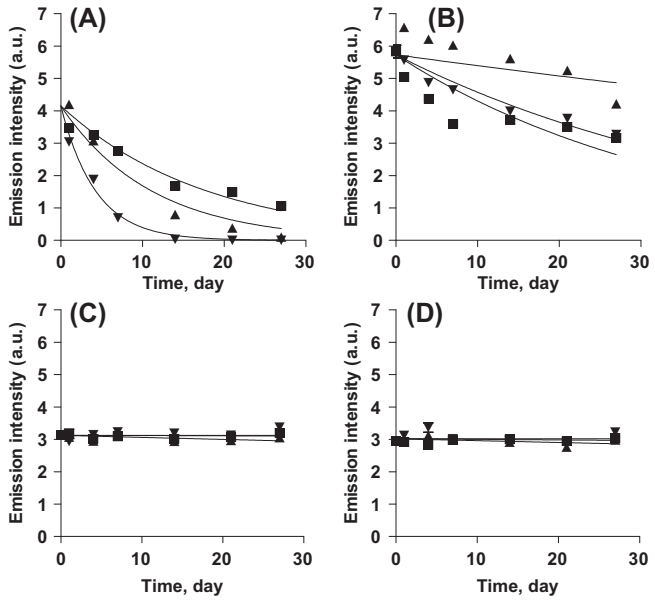

Fig. 3. The effect of (A) water, (B) phosphate-buffered saline (PBS), (C) 5% fetal calf serum (FCS) and (D) 10% fetal calf serum (FCS) on the emission intensity of Ce6–PVP (0.5 mg/mL) at room temperature (RT 21 °C) (■), 37 °C (▲) and 4 °C (▼) for a period of up to 27 days. Each data point represents the average of three measurements. Experiments were repeated twice and showed similar trends.

the presence of these serum proteins in the blood may improve PDT to target tumors by enhancing the intracellular accumulation of the photosensitizer via receptor-mediated endocytosis [36]. A spectrophotometry method was used to study the binding of lipoprotein with Ce6 and Ce6–PVP, in order to understand the influence of PVP on the distribution of Ce6 with various lipoproteins. Non-specific binding was measured by incubating Ce6 and Ce6–PVP with various concentrations of human lipoproteins. The association of Ce6 and Ce6–PVP was analyzed by plotting the emission peaks against increasing concentrations of HDL, LDL and VLDL (Fig. 5). Data analysis was performed using a one-site binding equation:

$$Y = (B_{max} \cdot X)/(K_d + X)$$

This equation describes the binding of a photosensitizer to a lipoprotein receptor following the law of mass action. 'Y' in the above equation is the fluorescence intensity of the photosensitizer, which is assumed to be linearly proportional to the concentration of the photosensitizer, 'B_{max}' is the maximal binding (binding capacity). 'X' is the concentration of the lipoprotein and 'K_d' is the concentration of photosensitizer required to reach half-maximal binding (binding affinity). The absorbance level of Ce6 was measured to confirm that all the photosensitizer concentrations used for this experiment were consistent with one another. A preliminary binding affinity of different lipoproteins to both Ce6 and Ce6–PVP were calculated and presented in Table 1. In general, when the concentration of the lipoproteins increased, the fluorescence intensity of both Ce6 and Ce6–PVP increased until a plateau was reached. The plateau occurred because the free binding sites of the lipoproteins were no longer free to interact with Ce6. It was observed that the B_{max} of all three lipoproteins binding with Ce6–PVP was slightly higher compared to that of Ce6, at the photosensitizer concentration of 0.01 mg/mL. When the binding capacity for various lipoproteins was measured in the presence of high Ce6 concentration (i.e. 0.06 mg/mL), the B_{max} of HDL increased by 1.7-fold, the B_{max} of LDL increased by 1.2-fold and the B_{max} of VLDL decreased by 2.6-fold in comparison to the values obtained for the low Ce6 concentration. For the high concentration of Ce6–PVP, the B_{max} of HDL, LDL and VLDL increased by 3.3-, 2.1- and 3.0-fold, respectively, compared to the values obtained for the low concentration of Ce6–PVP. If these preliminary results could be further confirmed, it may have important indication that the binding capacity of Ce6 to the lipoproteins is influenced by PVP as well as by the concentration levels of the photosensitizer.

It is well established that porphyrins have a high affinity for human serum proteins, albumin, HDL, LDL and VLDL. However, this selective mechanism is yet to be elucidated [28]. It is possible that an over expression of LDL receptors on the tumor cell surface or on neovascular endothelial cells may result in the enhanced uptake of LDL-bound photosensitizer by receptor-mediated endocytosis [37]. Since LDL is the main component of lipoprotein in the blood, it is the most likely lipoprotein to interact with photosensitizer. It has been shown that lypophilic photosensitizers have a high affinity for LDL and VLDL whereas amphiphilic photosensitizers have a higher affinity for HDL. In contrast, a very hydrophilic photosensitizer has been reported to have a high affinity for albumin [31]. However, in this experiment, it was observed that low concentrations (i.e. 0.01 mg/mL) of Ce6 and Ce6–PVP had high binding affinities for LDL and VLDL, while only a low binding affinity was observed with HDL. This suggests that the LDL and VLDL are the principal carriers for Ce6, which supports the existing literatures demonstrating the role of LDL receptors as carrier molecules to Ce6 and thereby improving the photodynamic therapy [38]. Thus, hydrophilic Ce6–PVP bound to LDL may preferentially accumulate in proliferating endothelial cells through the LDL receptor-mediated endocytosis pathway.

A significant increase in fluorescence yield was observed when low concentration of Ce6–PVP was bound with VLDL. The fluorescence yield increased even higher at high concentration of Ce6–PVP. However, no association of VLDL was observed for high concentration of Ce6 alone. This suggests that Ce6 could be aggregated at high concentrations thereby blocking it from binding to the lipoproteins. The VLDL receptors (VLDLR) have been found distributed in several tissues like heart, skeletal muscle, adipose tissue, kidney, placenta and brain. VLDLR was also found in gastric adenocarcinoma cells [39] and breast carcinomas [40]. This inevitably suggests a possible uptake of Ce6–PVP in cancer cells via VLDLR.

3.5. Subcellular localization

In vitro experiments were conducted to compare the cellular uptake of Ce6 and Ce6–PVP. MGH human bladder cancer cells were incubated with 10 μM Ce6 and 10 μM Ce6–PVP separately for 30 min. The fluorescence images were displayed in pseudo colors for organelle markers and photosensitizer, respectively (Fig. 6). The co-localization study indicated that the intracellular deposition of both Ce6 and Ce6–PVP was localized in the cytoplasm and nucleus. The precise step involving the transfer of Ce6 from PVP carriers to cellular membranes is still unknown. Therefore, PVP was labeled with FITC to study the uptake dynamics of PVP into cells. The fluorescence of PVP-FITC was observed in the cytoplasm of the cells with no nuclear co-localization. This result is in good agreement with the intracellular transport of ^{125}I-labeled PVP in isolated rat hepatocytes [41]. Another study demonstrated that drug co-precipitated with PVP was transported across membranes in high-energy amorphous phase [42]. Hence, in the current study, the cellular uptake of Ce6–PVP in MGH cells was observed to be higher compared to that of Ce6. This implies that PVP may increase the rate of membrane transport of Ce6 in the cells. We also hypothesize that because of the presence of numerous lipoprotein receptors on the tumor cells, Ce6–PVP may accumulate more efficiently within the tumor cells than in non-tumor cells.

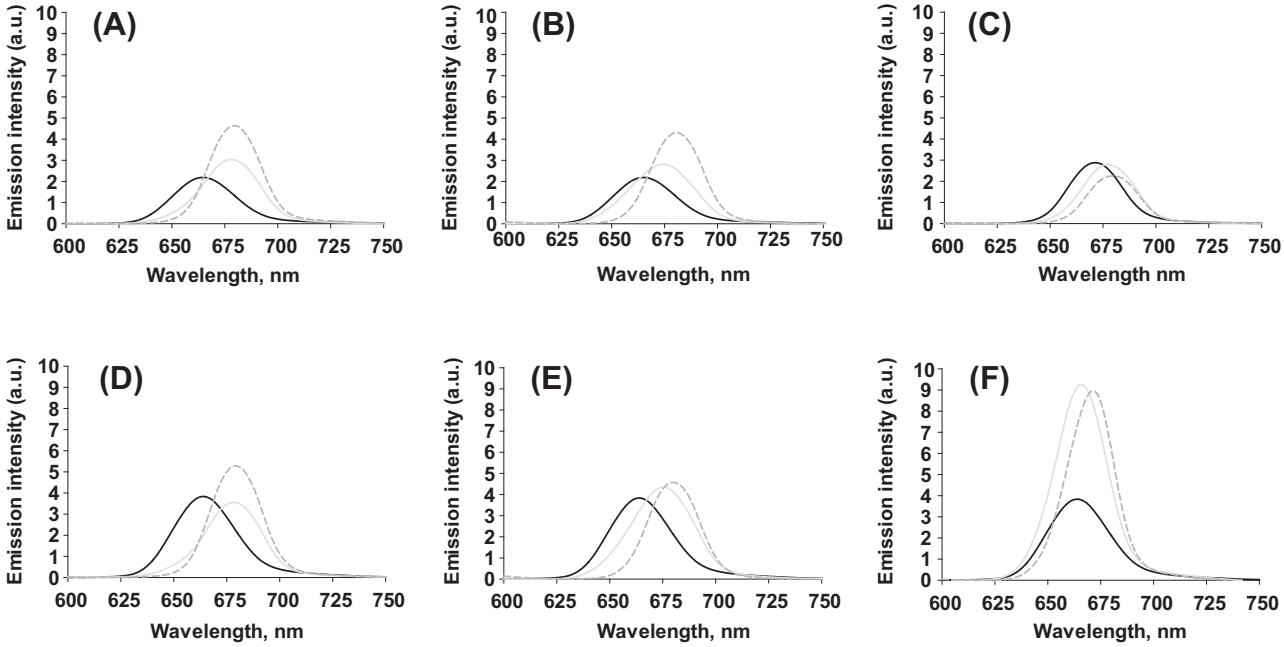

Fig. 4. The fluorescence emission spectra of Ce6 dissolved in saline with (A) HDL, (B) LDL and (C) VLDL at concentrations of 0% (black), 0.005% (grey) and 0.05% (dashed grey). The fluorescence emission spectra of Ce6-PVP dissolved in saline with (D) HDL, (E) LDL and (F) VLDL at concentrations of 0% (black), 0.005% (grey) and 0.05% (dashed grey). λ_{ex} = 400 nm.

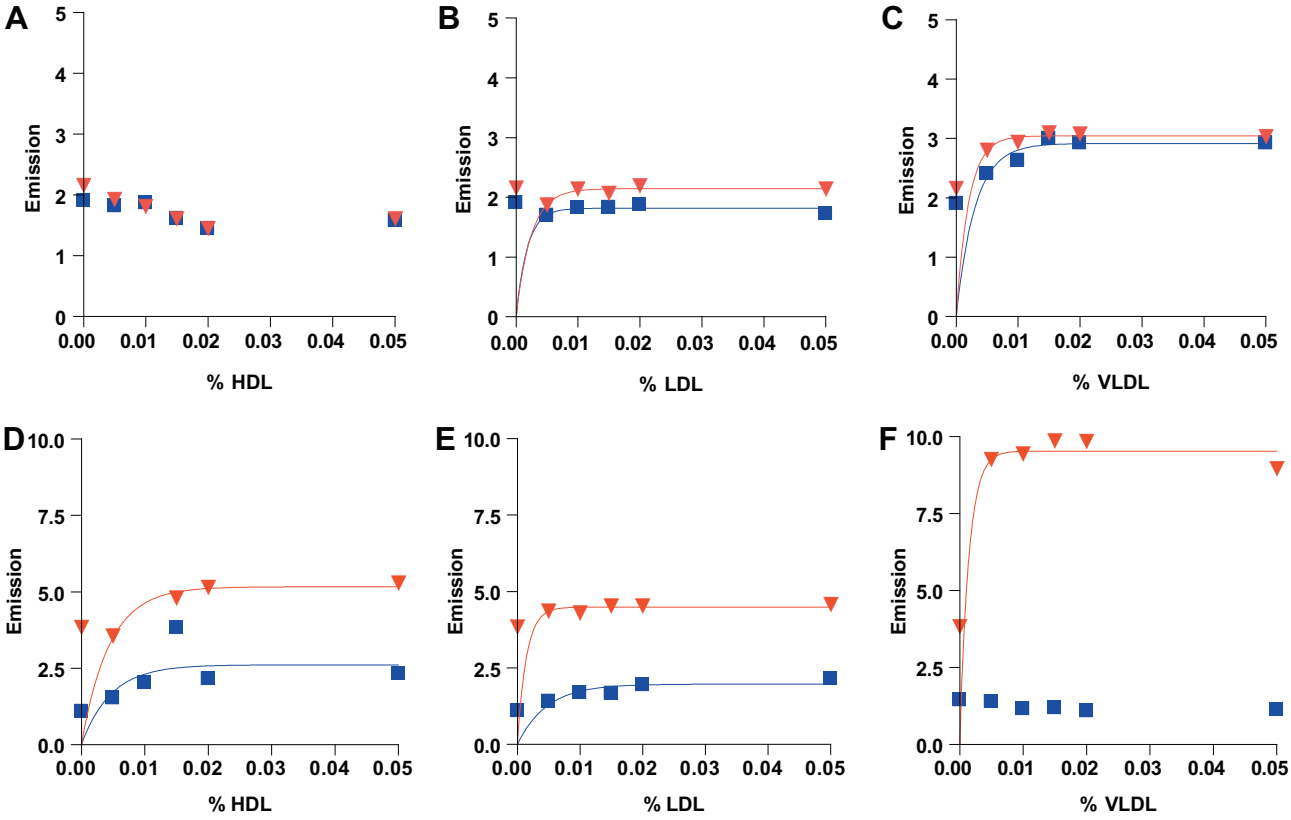

Fig. 5. The equilibrium titration of Ce6 (■) and Ce6–PVP (▼) in various lipoprotein buffer solutions at pH 7.0. Low concentration (0.01 mg/mL) of the photosensitizers combined with (A) HDL, (B), LDL and (C) VLDL and high concentration (0.06 mg/mL) of the photosensitizers combined with (D) HDL, (E), LDL and (F) VLDL. Curves were fitted and constants derived from one-site binding equation and are presented in the main text. *Y*-axis units are relative fluorescent units per mL of the solution. On graphs (A) and (F) trendlines were not presented for some of the data sets because the data did not fit in the binding equation. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

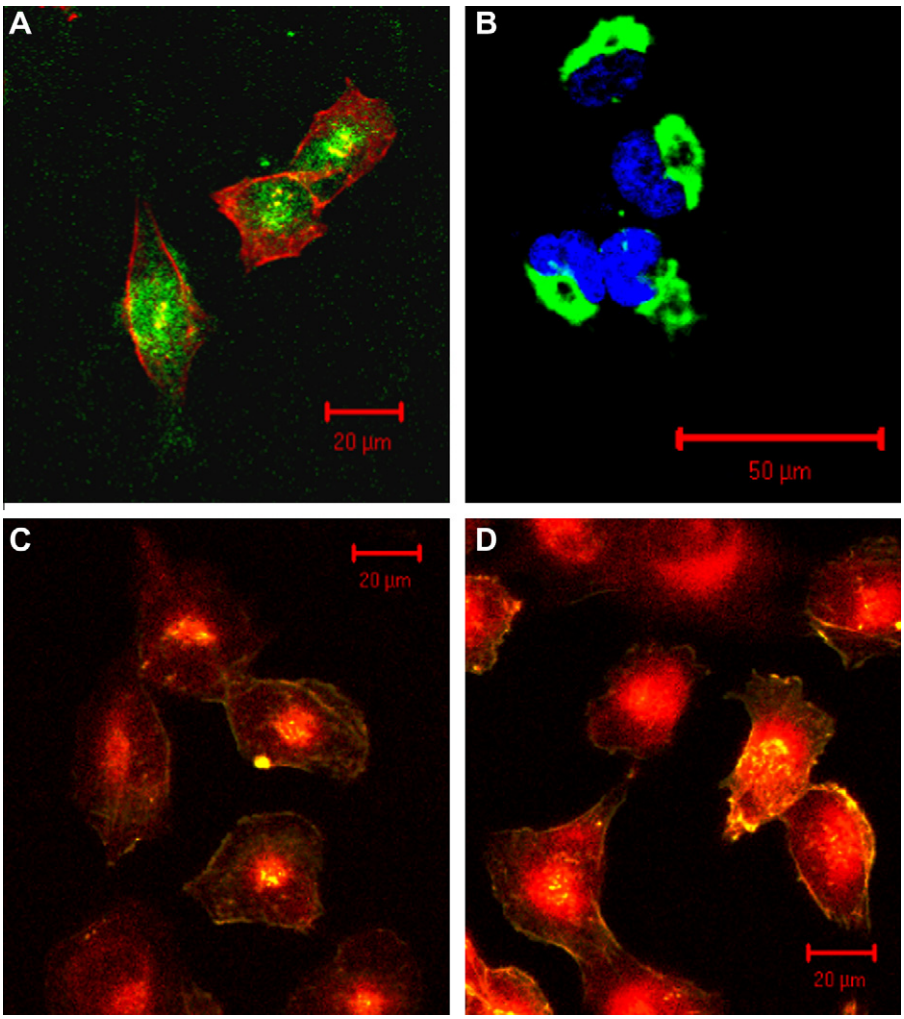

Fig. 6. MGH cells were incubated with PVP-FITC (green) for 30 min and co-stained with (A) rhodamine-phalloidin (red) and (B) DAPI (blue). Cells were also incubated with (C) Ce6 (red) and (D) Ce6–PVP (red) for 30 min and co-stained with rhodamine-phalloidin (yellow).

4. Conclusion

Ce6–PVP was found to be stable in a protein environment. A decrease in pH from 7 to 6 caused the partition coefficient of both Ce6 and Ce6–PVP to become more lipophilic. However, Ce6–PVP was slightly less lipophilic than Ce6. The cellular internalization of Ce6–PVP was higher than Ce6, and fluorescence microscopy revealed the penetration of PVP into the intracellular compartment of the cells. This supported the hypothesis that PVP improves the permeation and solubility of Ce6 through biological membranes in cells. The fluorescence intensity of Ce6–PVP, when bound with HDL, LDL and VLDL, was significantly higher compared to Ce6 alone. This indicated that PVP affects the level of association of Ce6 with lipoproteins, which would also be expected to influence the intracellular distribution of Ce6. This further supports the hypothesis that the externally added macromolecule PVP improves the uptake of Ce6 in cells. In particular, Ce6–PVP was found to interact more with VLDL, suggesting a role for the VLDL-receptor pathway with regard to Ce6–PVP biodistribution.

Acknowledgement

W.W. Chin is a recipient of the Singapore Millennium Foundation scholarship.

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