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Comparison of two strategies for conferring water solubility to a zinc phthalocyanine substituted with 1,2-diethylamino

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ABSTRACT

Polyamines are naturally occurring compounds that play important roles in multiple cell processes. In most cases, phthalocyanine-polyamine (Pc-polyamine) conjugates have good anticancer activity. However, low solubility in water limits their application in photodynamic therapy (PDT). The common method to solve this problem is quaternizing the nitrogen atoms to obtain cationic derivatives. An alternative strategy by preparing hydrochloride is easily neglected. In this paper, a Pc-polyamine conjugate substituted with 1,2-diethylamino (ZnPc1) was conferred water solubility by two different strategies, which are preparing hydrochloride (ZnPc2) and quaternizing the nitrogen atoms (ZnPc3). The synthetic methods and properties of the two derivatives were compared and discussed. Results indicated that both ZnPc2 and ZnPc3 possessed good solubility in water. However, strategy of preparing hydrochloride is much easier than quaternization process and the singlet oxygen generation ability, the photostability and anticancer activity of ZnPc2 *in vitro* were also superior to the ZnPc3.

1. Introduction

Photodynamic therapy (PDT) is a novel and promising noninvasive modality for treatment of a variety of malignant diseases [1]. It uses the combined action of photosensitizer, light and molecular oxygen to cause irreversible photodamage to tumor cells and tissues [2,3]. Among the three individually components, behaviors of the photosensitizer determine greatly the final therapeutic outcome [4]. During the past decades, a great number of potential photosensitizers for PDT have been studied. Phthalocyanines (Pcs), the two-dimensional 18 π-electron aromatic synthetic analogs of porphyrins, have attracted a great deal of interest due to their unique physical and chemical properties [5,6]. Especially, Pcs are considered as the promising photosensitizers for PDT owing to their intense absorption in the phototherapeutic window (600–900 nm), high efficiency to generate reactive oxygen species (ROS), high phototoxicity and low darktoxicity [7–10].

During the PDT administration, the drug (Pc) is injected into the patient's blood stream [4], and since the blood itself is a hydrophilic system, water solubility becomes crucial for drugs in PDT [11]. However, for most Pcs, low solubility and high aggregation are always observed in water, which has strong influences on their in vivo distribution, the photobiological and photochemical activities, and finally limit their potential applications in PDT [12]. The common means for preparing water soluble Pcs with high efficiency is to attach hydrophilic groups like polyethylene glycol, amino acid, carbohydrates [13–15], ionic groups including cationic and anionic substituent [16–18] at the peripheral and axial positions of Pc ring.

Polyamines are important compounds that play multifunctional roles in cell proliferation and differentiation processes [19]. Polyamine conjugate with photosensitizers such as porphyrins and Pcs have been reported [20,21]. In most cases, their photochemical properties, selectivity for tumor cells and anticancer activity are enhanced. However, Pc-polyamine conjugates also have low solubility in water due to the hydrophilic nature of amino groups is limited, can't suppress the intrinsic hydrophobic property of Pc macrocycle. To confer water solubility to Pcs substituted with amino groups, the common method is quaternizing the nitrogen atoms to obtain cationic derivatives. The quaternized Pcs not only have good solubility in water, but also have low aggregation and high anticancer activity [22,23]. While, the Pc-polyamine conjugates can be easily conferred water solubility by preparing hydrochloride because of the existence of amino groups in their structure. This alternative strategy takes advantages of the easily

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soluble of hydrochloride in water, has been reported rarely. Furthermore, the differences between these two strategies and the two hydrophilic derivatives have been rarely compared.

So, here, to improve water-solubility, we synthesized the quaternized form (ZnPc3) and hydrochloride form (ZnPc2) of a zinc Pc substituted with 1,2-diethylamino (ZnPc1), which was prepared in our previous studies (unpublished results). Their photochemical and photo-induced anticancer activity were also compared and discussed. Results indicate that both the two strategies, quaternizing and preparing hydrochloride, can greatly improve the water-solubility of ZnPc1. However, strategy of preparing hydrochloride is much easier than quaternization process and the singlet oxygen generation ability and *in vitro* anticancer activity of hydrochloride derivative are also superior to the quaternized form. It is expected that the comparison of the two different strategies for preparing water soluble and high efficient Pc-polyamine conjugates can promote the studies on Pc-based photosensitizers for PDT.

2. Experiments

2.1. Materials and characteristics

All necessary reagents and solvents were of analytical grade quality obtained from commercial suppliers. All reagents were purified according to reported procedures before use [24]. Disodium salt of 9,10-anthracenedipropionic acid (ADPA) and [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT) were purchased from Sigma–Aldrich. Dulbecco's modified Eagle's medium (DMEM) was from Gibco.

For all the experiments recorded in water, the water soluble Pcs (ZnPc2 and ZnPc3) were dissolved in water and diluted to the final concentrations; in the case of Pc insoluble (ZnPc1) in water, the stock solution was prepared in methanol and diluted with water to the final concentrations. IR spectra were recorded on IR-Spectrometer Nicolet Nexus 670. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Advance 400 MHz NMR spectrometer. Elemental analyses were taken with Vario MICRO, Elementar. The relative content of zinc and chlorine of the ZnPc2 were obtained using energy dispersive spectrometer (EDS, Noran Vantage, Themo Noran). UV–vis spectra were recorded on spectrophotometer Cary 5000, Varian. Fluorescence spectra were recorded on Perkin Elmer LS 50B fluorescence spectrophotometer. Zeta potential was recorded on Zetasizer Nano 90 light scattering, Malvern. The pH values were taken with FE20/EL20 pH meter, Mettler Toledo. Cell morphology changes were observed under a Zeiss Observer fluorescence microscope. A 665 nm LED lamp was used as light source.

ZnPc1 was prepared by a four-step procedure. First, N′,N′-diethylethane-1,2-diamine reacted with 4-hydroxybenzaldehyde used K_2CO_3 as the basic catalyst to obtain an imine intermediate. Then, the imine intermediate was deoxidated with $NaBH_4$ in protic solvents. The precursor of ZnPc1 was prepared by a base catalyzed nucleophilic ipso-nitro substitution reaction of 4-nitrophthalonitrile with the product of previous step. Finally, ZnPc1 was obtained by the reaction of precursor compound with zinc acetate in the presence of 1,8-diazabicyclo [5,4,0]-undec-7-ene (DBU). IR (KBr, cm^{-1}): 3440, 2970, 1660, 1580, 1460, 1223, 1080, 740. ^{1}H NMR (400 MHz, DMSO-d$_6$): δ (ppm) 9.37 (t, 4H, 23.82 Hz, Pc-H), 8.91 (t, 4H, 13.44 Hz, Pc-H), 7.81–7.89 (m, 4H, Pc-H), 7.72 (t, 8H, 11.10 Hz, Ar), 7.45–7.53 (m, 8H, Ar), 4.34 (d, 8H, 11.00 Hz, CH_2), 3.46 (s, 16H, CH_2), 3.25 (t, 16H, 6.36 Hz, CH_2), 1.25 (t, 24H, 5.66 Hz, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 11.8, 29.7, 44.2, 45.3, 47.0, 51.5, 52.5, 53.4, 120.1, 123.7 (br), 129.8 (br). Anal. Calcd. For $C_{84}H_{96}N_{16}O_4Zn$: C, 69.14; H, 6.63; N, 15.36. Found: C, 68.95; H, 6.86; N, 15.58.

ZnPc3 was obtained by methylation of ZnPc1 in CH_3OH. IR (KBr, cm^{-1}): 3438, 2990, 1595, 1468, 1230, 1080, 860, 740. ^{1}H NMR (400 MHz, DMSO-d6): δ (ppm) 9.42 (d, 4H, 2.88 Hz, Pc-H), 9.03 (d, 4H, 14.69 Hz, Pc-H), 7.94–8.03 (m, 4H, Pc-H), 7.70–7.84 (m, 8H, Ar), 7.49–7.58 (m, 8H, Ar), 4.80 (s, 8H, CH_2), 4.08(s, 16H, CH_2), 3.38–3.48 (br, 16H, CH_2), 3.12–3.18 (br, 36H, CH_3), 1.31–1.34 (m, 24H, CH_3). ^{13}C NMR (100 MHz, DMSO-d$_6$): δ (ppm) 8.1, 8.4, 9.3, 47.8, 49.9, 52.7, 55.4, 56.2, 56.5, 57.4, 67.1, 112.9, 113.4, 119.1, 119.3, 121.8, 122.7, 122.8, 124.6, 130.4, 134.2, 135.9, 140.0, 151.9, 157.4, 159.7, 159.9. Anal. Calcd. For $C_{96}H_{126}I_8N_{16}O_4Zn$: C, 43.53; H, 4.79; N, 8.46. Found: C, 42.90; H, 4.88; N, 8.40.

2.2. Hydrochloride derivative of 2(3),9(10),16(17),23(24)-tetra-(((2-(diethylamino) ethylamino) methyl) phenoxy) phthalocyaninato-zinc (II) (ZnPc2)

ZnPc1 (0.20 g, 0.14 mmol) was suspended in 5 mL redistilled water in a reaction bulb and warmed to reflux. Excess 5% HCl aqueous was added into the solution drop-wise until ZnPc1 was totally dissolved. The solution was concentrated and poured in to 20 mL acetone. The solid blue product was collected by filtration, then thoroughly washed by dichloromethane and dried in vacuum. The title product ZnPc2 was obtained as a dark blue solid. Yield: 0.23 g (93.8%). To verify the relative content of elemental chlorine of ZnPc2, the EDS was performed. Quantitative analysis shows that the mean atomic ratio of Zn/Cl of ZnPc2 is 0.120. Compared with the standard value 0.125, the results confirm the prediction made by us that one ZnPc2 molecule contains eight HCl in its structure.

IR (KCl, cm^{-1}): 3413, 2923, 2644, 1605, 1519, 1477, 1232, 1096, 1045, 834. ^{1}H NMR (400 MHz, DMSO-d^6): δ (ppm) 9.99–10.29 (br, 4H, NH·HCl), 9.02 (s, 4H, Pc-H), 8.53 (s, 4H, Pc-H), 7.78–7.90 (m, 8H, Ar), 7.73 (s, Pc-H, 4H), 7.44–7.60 (m, 8H, Ar), 4.32 (s, 8H, CH_2), 3.59 (s, 16H, CH_2), 3.24 (s, 16H, CH_2), 1.31 (d, 24H, 6.40 Hz, CH_3). ^{13}C NMR (100 MHz, DMSO-d^6): δ (ppm) 145.2, 130.1, 129.5, 124.6, 123.5, 121.0, 119.5, 54.0, 53.1, 51.9, 50.0, 47.5, 44.9, 44.5, 31.2, 29.5, 11.9. Anal. Calcd. For $C_{84}H_{104}Cl_8N_{16}O_4Zn$: C, 57.62; H, 5.99; N, 12.80. Found: C, 57.10; H, 6.08; N, 12.66.

2.3. Photodegradation studies

Photodegradation studies were carried out in water by monitoring the decrease in the Q-band absorption of the Pcs using UV–vis spectrophotometer.

2.4. Singlet oxygen generation detection

The singlet oxygen generation abilities of the Pcs were determined using the experimental described in literature [25,26]. The absorption intensity of ADPA continuously decreased as the irradiation time increasing. Pcs and ADPA were mixed and irradiated. The reaction was monitored spectrophotometrically by measuring the decrease in optical density at absorbance maximum of 378 nm of ADPA. The rate of singlet oxygen generation is calculated by the following Eq. [25]:

$$\ln([ADPA]_t/[ADPA]_0) = -kt$$

where $[ADPA]_t$ and $[ADPA]_0$ are the concentrations of ADPA after and prior irradiation, respectively. Values of k are the rate of singlet oxygen generation and t is the time of irradiation.

2.5. Zeta potential measurement (ZP)

The ZP of the HeLa cells (cervical carcinoma cell line) and ZnPcs were measured by a Malvern Zetasizer Nano 90 light scattering. For

the preparation of samples, the HeLa cells and ZnPcs were dilute with DMEM (Dulbecco's modified eagle's medium). The experiments were performed three times and an average of the results was used.

2.6. Photodynamic activity in vitro

2.6.1. Darktoxicity

The darktoxicity of the Pcs was determined by means of the colorimetric MTT assay [27,28]. HeLa cells were seeded into 96 well plates at a density of 5×10^5 cells cm^{-2} and incubated for 24 h in growth medium to allow for attachment. After 24 h, cellular survival was measured.

2.6.2. Phototoxicity

For photo-induced anticancer experiments, HeLa cells were incubated as described above. After 24 h, the old medium was replaced by fresh medium (without calf serum) with the three Pcs, separately. Cells were then incubated at 37 °C under 5% CO_2 overnight. The cells were then rinsed three times with PBS (phosphate buffered saline) and refilled with by fresh medium (without calf serum). The cells were immediately exposed to 665 nm LED light for 5 min and after 24 h incubation cellular survival was measured as described above.

2.7. Cell morphology

The HeLa cells were maintained in DMEM containing glucose supplemented with 110 mg L^{-1} sodium pyruvate, 0.1 mg mL^{-1} penicillin, 0.1 mg mL^{-1} streptomycin, L-glutamine, 10% (v/v) FBS (fetal bovine serum) and pyridoxine hydrochloride in a humidified atmosphere at 37 °C and 5.0% CO_2. The cells were treated with Pcs overnight. The cells were irradiated by 665 nm LED immediately and then washed with PBS three times. Changes of cell morphology were observed under the fluorescence microscope.

2.8. Statistical analysis

Statistical analysis was done and the mean, standard deviation, standard error, and the significant changes were expressed as the mean ± S. All biochemical experiments were performed three times and an average of the results were used. $P < 0.05$ was considered necessary as statistical significance.

3. Results and discussion

The pH values would have an important impact on ZnPc2 since protonation is reversible. So, the pH values were measured by a pH meter. The data were performed three times and the average of the results was used. At the concentration of 3×10^{-6} M (experimental concentration), the pH values of the aqueous solutions of ZnPc1, ZnPc2, ZnPc3 and redistilled water were 6.89, 6.75, 6.82 and 6.81 respectively. The results showed that there were only tiny changes after the drugs added into redistilled water, and these tiny changes would have little influence on properties and existence forms of the drugs.

3.1. Synthesis and comparison

Fig. 1 showed a brief schematic for the synthesis of quaternization and hydrochloride derivatives of ZnPc1. Cationic derivative ZnPc3 was obtained by the quaternization of aliphatic nitrogen atom of ZnPc1 in methanol. The quaternizing agent methyl iodide was commonly used. The quaternizing agent methyl iodide is potentially dangerous and must be used with care, as it is likely to methylate DNA [15]. While, the hydrochloride derivative ZnPc2 was obtained by protonating the ZnPc1 in water. The protonating agent is dilute hydrochloric acid which is commonly low toxicity. To obtain pure product, the quaternization derivative ZnPc3 was filtrated while hot, and then thoroughly washed with ethanol and methanol. ZnPc2 was poured into acetone, then, filtrated. The quaternization derivatives ZnPc3 is dark green in color, while hydrochloride derivative ZnPc2 is dark blue.

The solubility of the three compounds in some common organic and water was measured at room temperature to give some indications about their amphipathic property. The amphipathic is an important characteristic of an ideal PDT drug [29]. ZnPc1 is soluble in the common organic solvents, such as methanol, chloroform and dimethyl formamide (DMF) et al. However, it is insoluble in water due to the hydrophilic nature of amino groups is limited, can't suppress the intrinsic hydrophobic property of Pc macrocycle. By contrast, both ZnPc2 and ZnPc3 have good water solubility. Interestingly, the two derivatives also have solubility in some polarity organic solvents like DMF and dimethyl sulfoxide (DMSO). ZnPc2 also can be soluble in methanol and ethanol. To a certain degree, ZnPc2 and ZnPc3 show an amphipathic property because of their solubility both in water and organic solvents.

3.2. Ground state electronic absorption

The aim of UV–vis experiments was to provide the information about the electron density of the Pcs, their aggregation and maximum absorbance [30]. Pcs exhibit typical electronic spectra with B band at 300–400 nm and Q band at 600–700 nm, both correlate to $\pi-\pi^*$ transitions [31]. The UV–vis spectra of metallic mono-Pcs in solution display a narrow Q band, but the splitting or two bands observed in these complexes indicates the presence of both monomeric and aggregate forms of the complexes in solution [32]. For ZnPcs, they usually showed monomer behavior evidenced by a single (narrow) Q band at ~680 nm while aggregate behavior by an additional Q band at 630–640 nm [26,33]. The aggregation behavior of Pcs is dependent on the nature of solvent and substituent et al. [34].

The ground state electronic absorption spectra of ZnPc1, ZnPc2 and ZnPc3 were performed in water and shown in Fig. 2. The spectra superposed in Fig. 2 indicating that all of the three ZnPcs are typical aggregate Pcs because of the appearance of the additional Q band at 640 nm. Such spectral features are typical for Pc dimmers or aggregates [33]. Comparison studies indicated that only the quaternization derivative ZnPc3 has an obvious monomer band at 680 nm, which may be result from the effects of steric hindrance and electrical charges. It seems evident that the charged substituents induce mutual repulsion reducing the aggregation of ZnPc3. However, hydrochloride derivative ZnPc2 shows serious aggregation, with intense absorbance at 640 nm and negligible band at 680 nm. It is obvious that good water solubility and protonation did not reduce the aggregation of ZnPc2 in water.

3.3. Fluorescence spectra and properties

Since fluorescence can be used to obtain information about photosensitizer localization and distribution as well as release from tissues [35], the fluorescence properties of Pcs intended for use in PDT is especially important. In a particular solvent, the fluorescence properties of Pcs can depend on many factors, including aggregation, substituent and pH et al.

As shown in Fig. 3, the fluorescence emission spectra of the three ZnPcs were performed in water with the excitation wavelength of 600 nm. The fluorescence emission of ZnPc1 was completely quenched in water. The result can be attributed to the

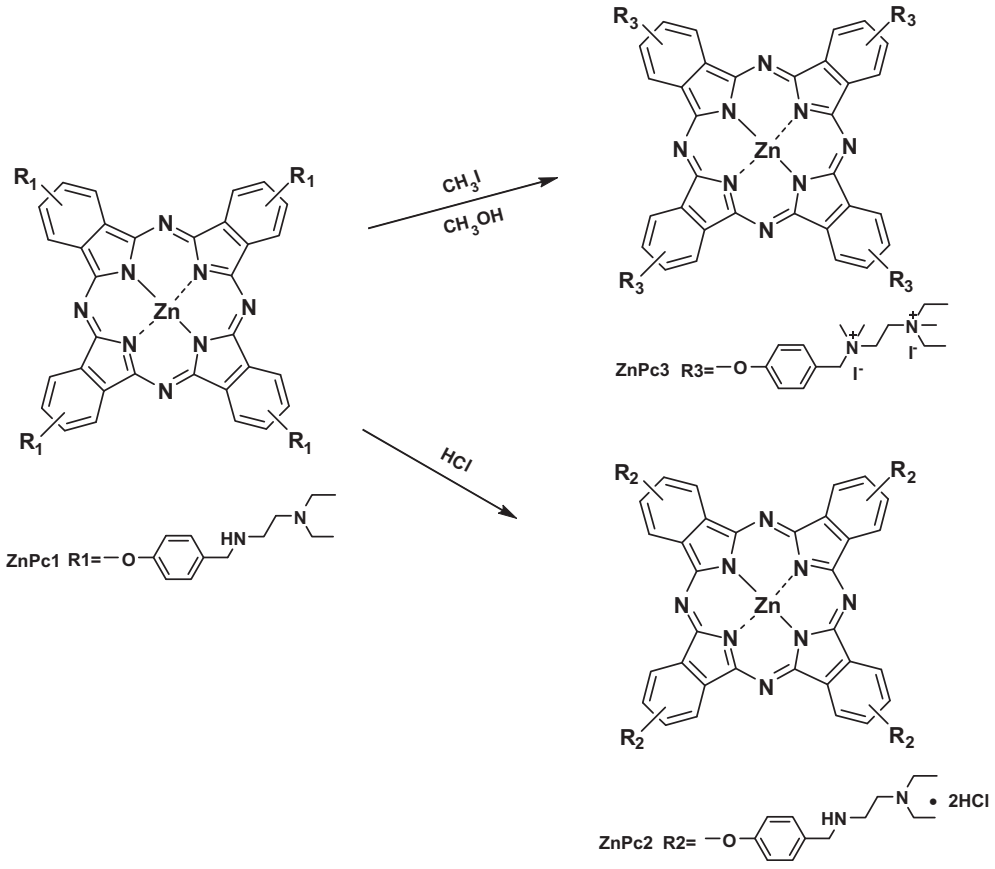

Fig. 1. Brief schematics for the synthesis of quaternization and hydrochloride phthalocyanine derivatives.

quenching of the excited singlet states by the diethylamino substituent as a result of effective intramolecular photoinduced electron transfer (PET) and aggregation [36,37]. Aggregated Pcs are not known to fluorescence since aggregation lowers the fluorescence intensity of molecules through dissipation of energy [5]. A common feature of the quaternization derivative ZnPc3 and hydrochloride derivative ZnPc2 is the strong fluorescence emission. In the case of ZnPc3, the quaternization efficiently reduces the aggregation as well as inhibits the intramolecular PET process, resulting in a stronger fluorescence emission. Contrary to most Pc aggregate [6], the hydrochloride derivative ZnPc2 show moderate fluorescence emission intensity though it is aggregate in water. The observation may be result from the competitive effect between intramolecular PET and aggregation. The formation of hydrochloride can greatly prevent the PET process by protonation, resulting in the increase of fluorescence intensity. While, it is believed that the protonation of amine groups did not reduce the aggregation evidently, resulting in the quenching of fluorescence. The final fluorescence emission spectra of ZnPc2 indicate that fluorescence quenching by aggregation is lesser compared to the fluorescence enhancement by the forbidden of PET.

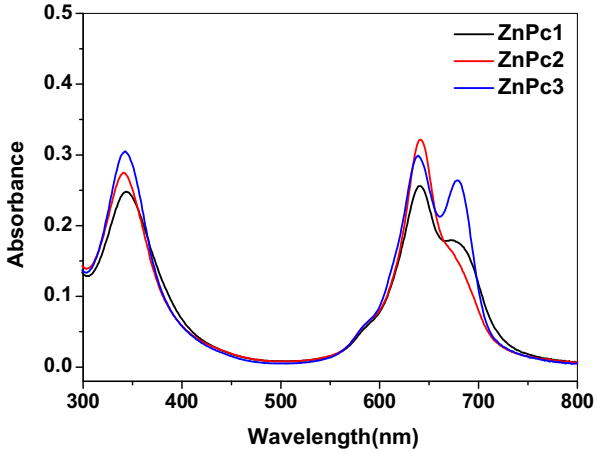

Fig. 2. Ground state electronic absorption spectra of ZnPc1, ZnPc2 and ZnPc3 in water (Concentration = 3×10^{-6} M).

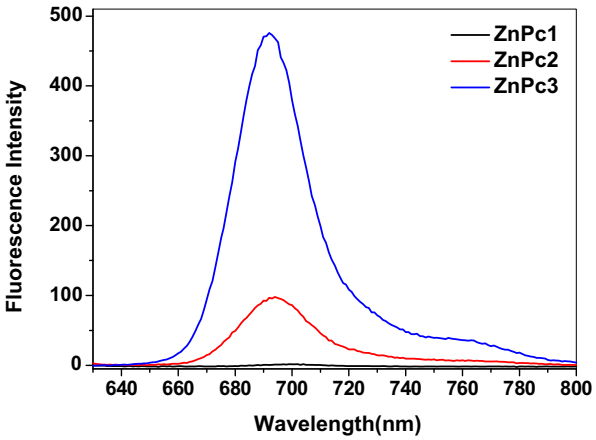

Fig. 3. Fluorescence emission spectra of ZnPc1, ZnPc2 and ZnPc3 in water (Concentration = 3×10^{-6} M; Excitation wavelengths: 600 nm).

3.4. Photodegradation studies and comparison

The photobleaching property is a measure of the photostability of a photosensitizer and this is important for those potential for use in PDT. It is indicated by a reduction in the absorbance intensity without the emergence of new peaks on photo-irradiation [38].

The decompositions of the three Pcs were analyzed following the decrease in absorption of their Q bands. Fig. 4 shows the UV–vis spectra changes of the three Pcs in water upon irradiating with 665 nm LED light. All the ZnPcs studied showed a decrease in the Q band. However, the photostability of them are different. The result can be attributed to a number of factors, including the substituent, exist form of Pcs, solvent and light intensity et al. [39]. Different substituents exhibit different effects on the photostability of ZnPcs. While some substituents stabilize the ring against photodegradation, some make it more vulnerable to oxidative attack [33,38].

As can be observed in Fig. 5, the hydrochloride derivative ZnPc2 showed better photostability than the quaternization derivative ZnPc3. Generally, the result may be attributed to the following two factors: 1) the existence form of ZnPc2 is mainly aggregate while ZnPc3 shows apparent monomer; commonly, aggregate is more stable than monomer in the same condition. 2) It is obviously that the quaternization of amine groups resulted in the decrease in the stability of the zinc Pc complexes, while the hydrochloride derivative increased the stability [5]. From the Fig. 5, we can also observe that the aggregate form of ZnPc3 is more stable than its monomer form, which agrees with the same ZnPcs reported in literature [26].

3.5. Singlet oxygen generation ability

The production of singlet oxygen is result from the energy transfer between the triplet state of photosensitizers and ground state molecular oxygen. There is a necessity of high efficiency singlet oxygen generation since the singlet oxygen generation ability is an important indicator for the potential of photosensitizers in PDT [40].

Singlet oxygen can be detected using a chemical method by the photo-oxidation of ADPA to its endoperoxide derivative. The ability of the three Pcs in generating singlet oxygen, as reflected by the rate of decay of the singlet oxygen quencher ADPA, was recorded and compared. As shown in Fig. 6, all of the Pcs can induce photooxidation of ADPA (Fig. 6A–C) and the efficiency followed the order k_{ZnPc2} (0.0036) > k_{ZnPc3} (0.0028) > k_{ZnPc1} (0.0022) (Fig. 6D). It is believed that the PET process of amino groups quench singlet oxygen excited state of ZnPc1, resulting in the reduced singlet oxygen production. In contrast, singlet oxygen generation increased when the amino groups were quaternized or protonated, as the PET pathway was switched off [41].

Comparative studies in the singlet oxygen production ability of ZnPc2 and ZnPc3 show that both of them can efficiently increase the production of singlet oxygen, while ZnPc2 is better than ZnPc3.

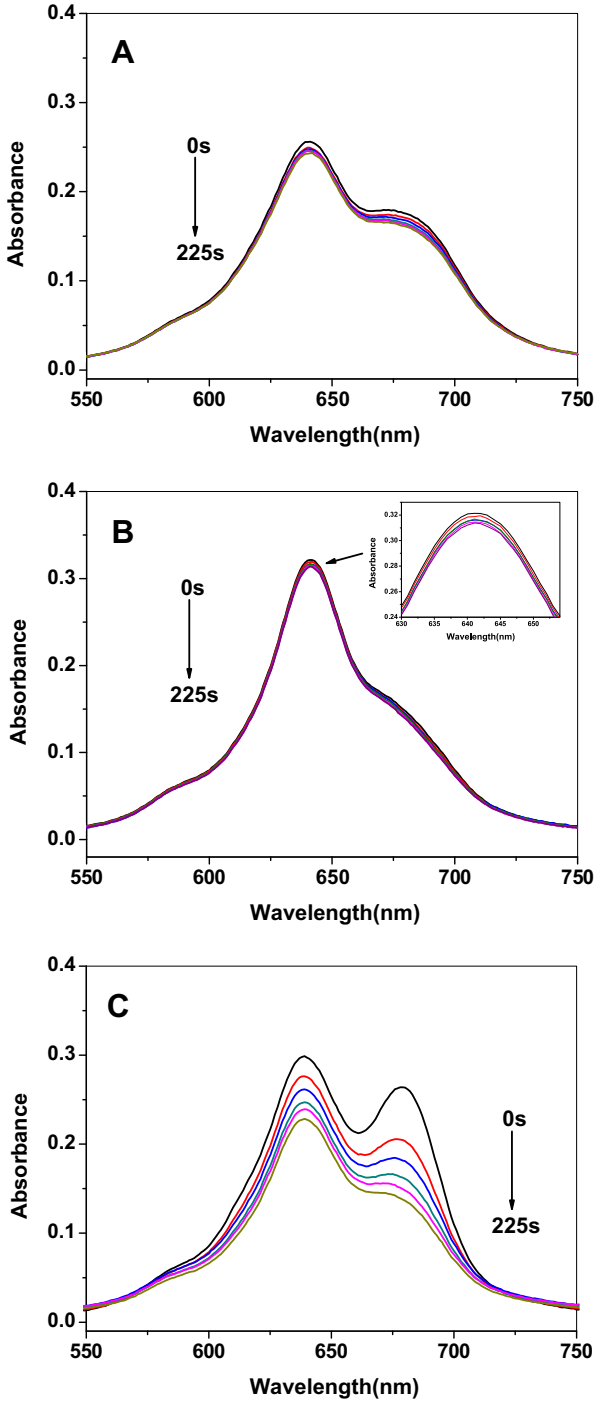

Fig. 4. Absorption spectra changes of A: ZnPc1; B: ZnPc2; C: ZnPc3 irradiated with 665 nm LED in water (Concentration = 3×10^{-6} M).

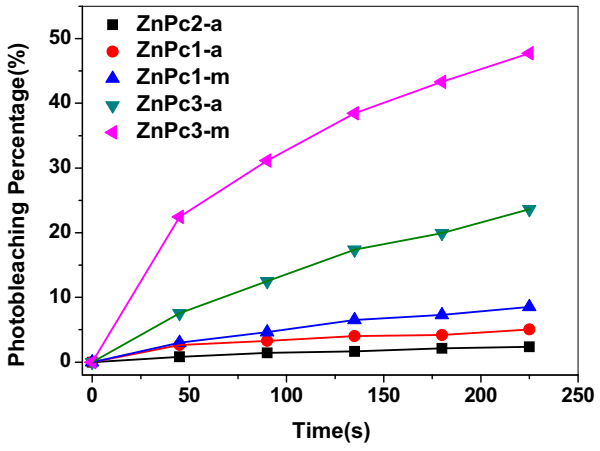

Fig. 5. Photobleaching properties of the three Pcs in water (a: aggregate form; m: monomer form; Concentration = 3×10^{-6} M).

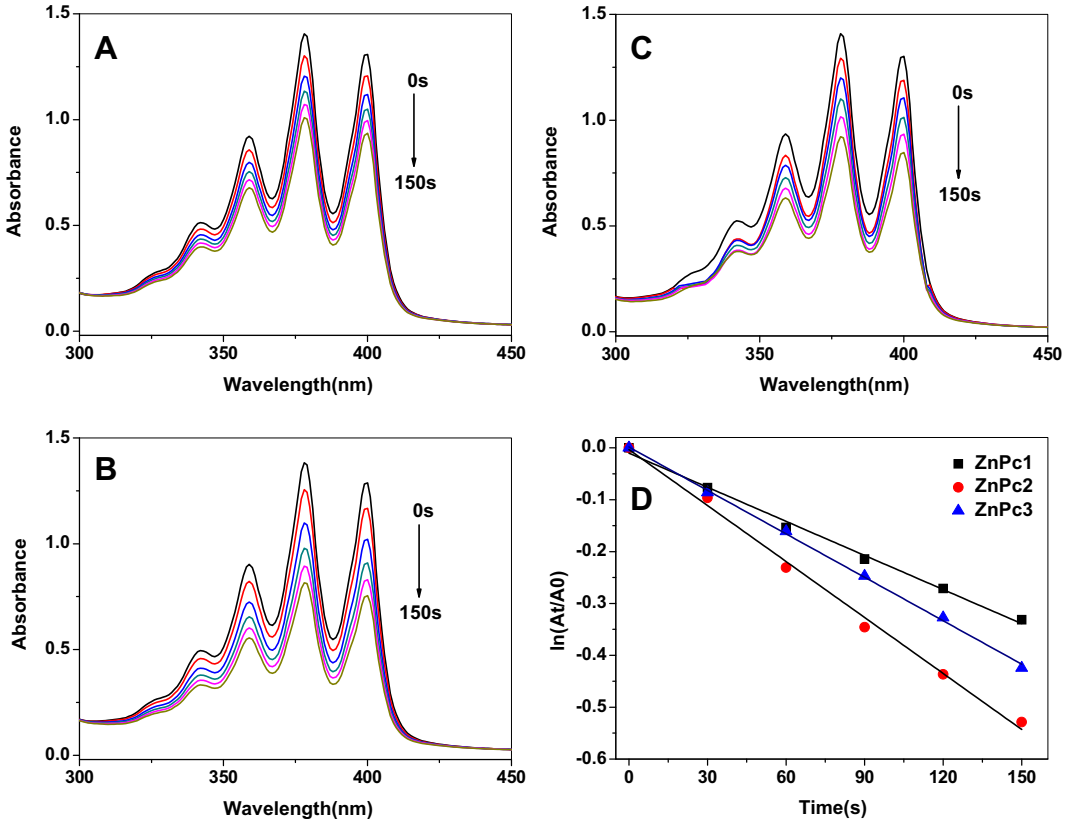

Fig. 6. UV–vis spectrum for the determination of singlet oxygen generation ability of (A) ZnPc1; (B) ZnPc2; (C) ZnPc3 use ADPA as the quencher at the concentration of 3×10^{-6} M (D) Plots of ADPA absorbance vs. time.

Many factors may be responsible for this result including: ability of substituent, triplet excited state energy and the existence form of the two Pcs. However, the predominant factor in explaining the observed trends is difficult to identify.

3.6. Zeta potential measurement

It was suggested that the zeta potential was important for photosensitizers used in PDT since the charge of drugs was closely related to cell uptake and finally affected the anticancer activity [42]. The ZP of HeLa cells and the three Pcs were measured in DMEM. The results showed that HeLa cells were electronegative (ZP = −7.34 mV), ZnPc1 was almost neutral (ZP = 0.53 mV), while ZnPc2 (ZP = 12.30 mV) and ZnPc3 (ZP = 8.76 mV) were both electropositive. This result implied the high anticancer activity of ZnPc2 and ZnPc3.

3.7. Cell morphology

The morphology of HeLa cells was observed by microscopy before and after phototreatment. Drastic changes in the morphology of HeLa cells, which was treated overnight with ZnPcs, have been observed after irradiation. The representative results are shown in Fig. 7. These results provided evidence about membrane deformation and an increase in the volume of cells, indicating a higher fragility after the photodynamic action.

3.8. Hoechst 33342 staining

PDT process will damage tumor cells as well as induce DNA destruction in nuclear. There is a necessity of DNA damage research since it is an important index in PDT. The Hoechst 33342 which is part of blue fluorescent dyes is usually used to assess changes in nuclear morphology, since it can bind to DNA. Fig. 8 showed the nuclear morphology changes of HeLa cells after phototreatment. As shown in Fig. 8A, when the cells were treated without drugs, there is no significant change in their nuclear morphology. In contrast, the cells showed obvious nuclear morphology changes, such as nuclear shrinkage, chromatin condensation and fragmentation when they were phototreated with ZnPc1 (Fig. 8B), ZnPc2 (Fig. 8C) and ZnPc3 (Fig. 8D).

3.9. In vitro anticancer activity

The toxicity of Pcs without illumination was called darktoxicity. It was an important biocompatibility indicator of Pcs. In order to study the *in vitro* anticancer activity of ZnPc1 as well as its hydrochloride derivative ZnPc2 and quaternization derivative ZnPc3, the darktoxicity and phototoxicity of them were evaluated toward HeLa cells. To study darktoxicity of the ZnPcs, HeLa cells were treated with 10 μM drugs without illumination. The phototoxicity was obtained by treating HeLa cells with 2 μM drugs and 3 min illumination by 665 nm LED lamp.

As shown in Fig. 9, all of the Pcs are essentially noncytotoxic in the absence of light, but exhibit different degrees of phototoxicity. The low darktoxicity of the three ZnPcs indicate their good biocompatibility. The phototoxicity of them follows the order ZnPc2 > ZnPc3 > ZnPc1, which is in line with the trend of singlet oxygen generation ability and ZP results mentioned above. The hydrochloride derivative ZnPc2 and quaternization derivative ZnPc3 are both particularly potent with the cell survival percentage 23.4% and 49.4%, separately.

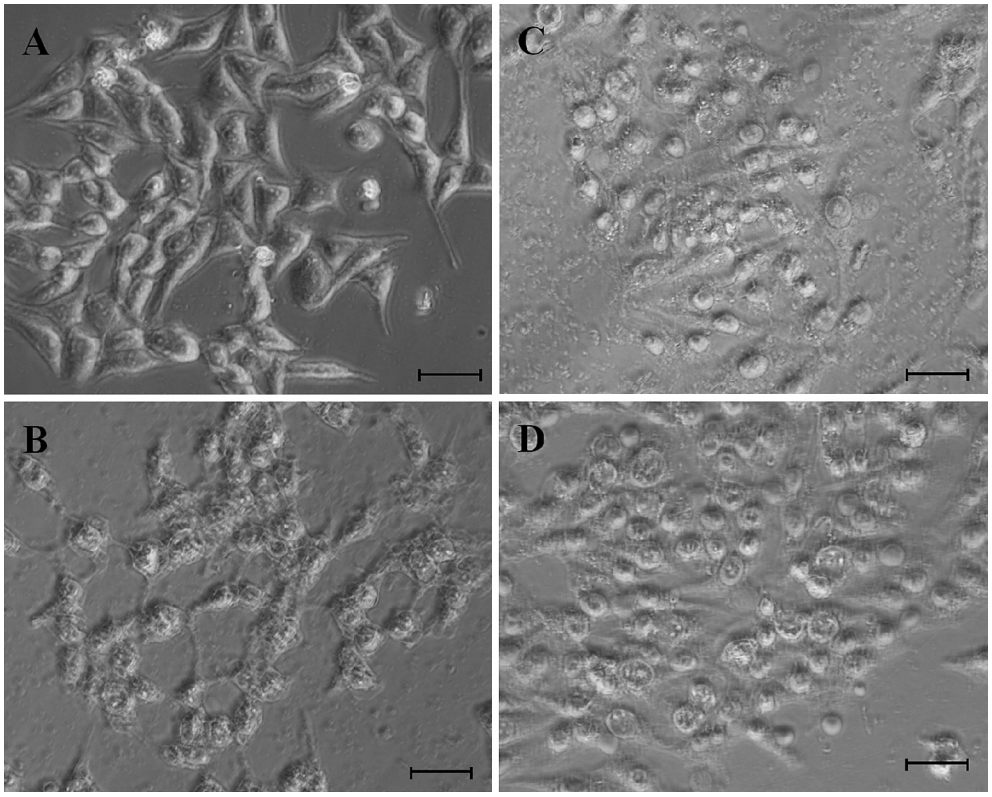

Fig. 7. Photograph showing morphology of normal HeLa cells treated with drugs and irradiated for 5 min with 665 nm LED (A) control, without drugs; (B) treated with ZnPc1; (C) treated with ZnPc2; (D) treated with ZnPc3. 40 microscope objective. Bar = 100 μm.

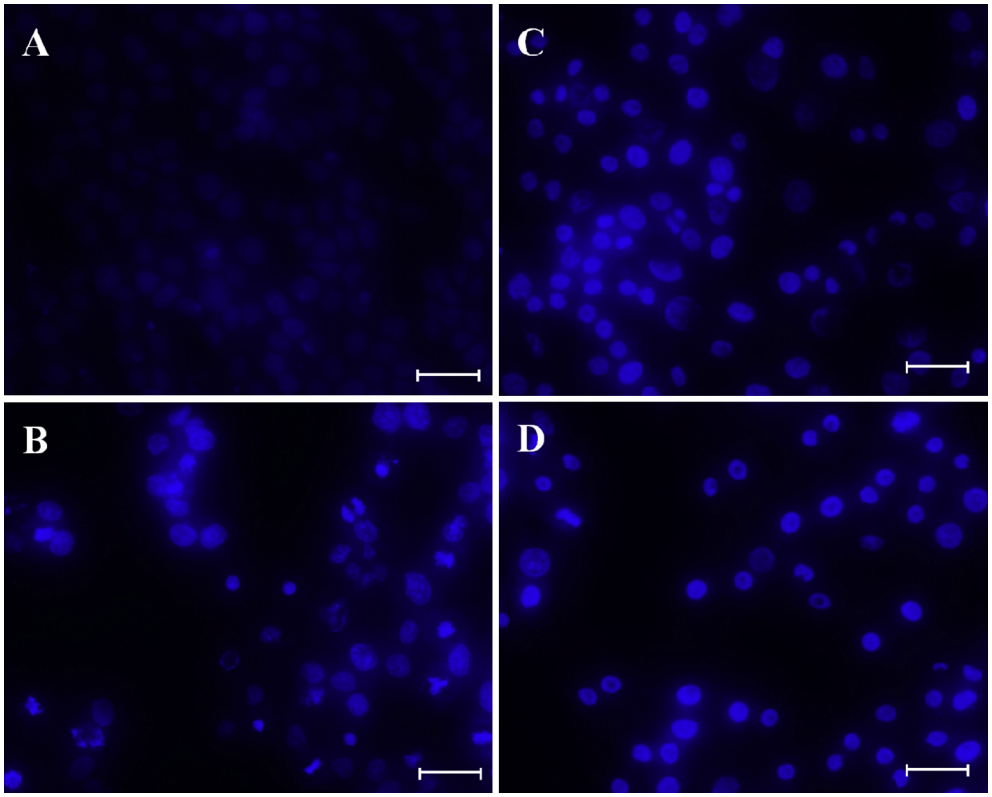

Fig. 8. Fluorescence micrographs of HeLa cells stained with Hoechst 33342 after being phototreated with ZnPcs (A) normal cells; (B) treated with ZnPc1; (C) treated with ZnPc2; (D) treated with ZnPc3. Bar = 100 μm.

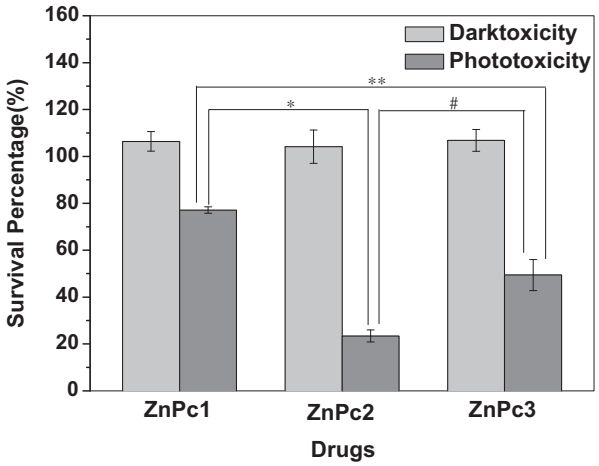

Fig. 9. Survival percentage of HeLa cells after treated with ZnPc1, ZnPc2 and ZnPc3 and subsequent irradiation/non-irradiation by 665 nm LED. ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. ZnPc1; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. ZnPc2).

4. Conclusion

In summary, we have conferred water solubility to a zinc Pc-polyamine conjugates substituted with 1,2-diethylamino by preparing hydrochloride and quaternizing the nitrogen atoms. Comparison studies showed that both of the two strategies can efficiently improve the water solubility of the Pc-polyamine conjugate. While, compare with the strategy of quaternizing the nitrogen atoms, the strategy of preparing hydrochloride is not only much easier in the preparing process but also superior in the singlet oxygen generation ability, photostability and *in vitro* anticancer activity. The results present above showed that both of the two strategies for conferring water solubility to Pc-polyamine conjugates are effectual and feasible. Prepare hydrochloride is of particular interest because of its simple prepare process and distinctive property but easily neglected.

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