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Water-Soluble Quaternized Serotonin Substituted Zinc-Phthalocyanine for Photodynamic Therapy Applications

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ABSTRACT

Poor water solubility is the main drawback of phthalocyanine (Pc)-based second generation photosensitizing agents in photodynamic therapy (PDT). To resolve this, we proposed preparation of quaternized serotonin substituted zinc phthalocyanine (q-Ser-ZnPc) since the positive charge on quaternary amines could improve water-solubility and might limit self-interactions of hydrophobic aromatic surface of Pc in aqueous solutions. Briefly, serotonin substituted phthalonitrile was prepared by reaction of 4-nitrophthalonitrile with 5-hydroxytryptamine (Serotonin). Serotonin substituted zinc(II) phthalocyanine (Ser-ZnPc) was prepared by cyclotetramerization of serotonin substituted phthalonitrile. Then, q-Ser-ZnPc was prepared by the quaternization reaction of Ser-ZnPc. The synthesized compounds were characterized by ^{1}H-NMR, UV-Vis, FT-IR, fluorescence, and elemental analysis. Importantly, unlike ZnPc, which is among most widely used second generation photosensitizing agents, we report that q-Ser-ZnPc is actually water-soluble. Besides, q-Ser-ZnPc also absorbs light in the wavelengths corresponding to the therapeutic window. What's more, q-Ser-ZnPc exhibits a higher fluorescence quantum yield than that of ZnPc. Thus, the material might be useful particularly for image-guided PDT applications.

Keywords: Photodynamic therapy, Photosensitizer, Water-soluble, Phthalocyanine, Serotonin.

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Introduction

A human body consists of more than 30 trillion cells with various functions. Normally, these cells grow, perform their functions, die when they grow old or get damaged and replaced with new cells. Sometimes, damaged or abnormal cells continue to grow and divide in an uncontrollable fashion instead of dying, resulting in cancer. The cancer cells grow out of control, avoid the immune system, spread to the other parts of the body, invade normal tissues and organs, can destroy healthy cells and disrupt vital organ functions. According to the World Health Organization (WHO), cancer is the disease with the highest cause of death worldwide [1]. Even only in 2020, cancer is responsible for the loss of nearly 10 million lives, accounting for one in every six deaths [1]. These facts are leading scientists from all around the globe to work hard towards finding an efficient cancer treatment.

Currently, depending on the kind, stage, and location of cancer cells in the body, surgery, chemotherapy, and radiotherapy are the most frequently used cancer treatment methods. The problem is none of these options are actually flawless. In surgery, a reaction to medication, bleeding, internal organ damage, pain, scar tissue, and infection might occur as possible side effects [2, 3]. Regarding chemotherapy, its main drawback is that highly cytotoxic anti-neoplastic drugs are used in chemotherapy, and thus, a fraction of healthy cells are destroyed along with cancerous ones [3]. Besides, being susceptible to infections, fatigue, dizziness, hair loss, dryness of skin, change of taste, loss of memories, and diarrhea are also other common side effects of chemotherapy [3, 4]. Similar to chemotherapy, healthy cells are also damaged to some extent in radiotherapy by ionizing radiation that is targeted towards cancerous cells [2, 3].

Alternatively, photodynamic therapy (PDT) has been proposed as a promising method for cancer treatment. In PDT, a light source, photosensitizing agent and oxygen in the tissue, all of which are non-toxic alone, work together to destroy cancerous cells [2, 5]. Briefly, the photosensitizers could selectively be accumulated in only cancer cells via use of enhanced permeability and retention (EPR) effect, cancer biomarkers, functionalized nanomaterials or RNA-targeting [6-10]. Then, upon application of a long wavelength red light, photosensitizers are activated and transfer energy to molecular oxygen in the tissue, which results in the formation of reactive oxygen species like singlet oxygen [7]. Singlet oxygen is a cytotoxic agent, and can directly kill cancer cells [7, 8]. Luckily, it exhibits a very low diffusion range of 10 to 55 nm and has a short lifetime of 10 to 320 ns, thus, limiting any damage on healthy cells in the process and reducing side effects [2].

Ideally, an effective photosensitizer should be non-toxic in the dark, chemically pure, photostable, absorb

light in the wavelength of about 600-800 nm since this region is known to be the most suitable therapeutic window for PDT, exhibit high efficiency to yield reactive oxygen species, and soluble in aqueous solutions [11-13]. Photofrin is the first clinically approved photosensitizing agent for PDT, and is actually used now to cure various cancer types [14-16]. However, its main limitations are lack of purity, low absorption of light in the wavelengths of therapeutic window, which results in poor tissue penetration, and absorption of light in the wavelengths of about 400-600 nm, which cause skin phototoxicity as a side effect [2, 7, 17]. These drawbacks have led scientists to search alternative photosensitizing agents for PDT.

Eventually, in the mid-1980s, various phthalocyanine (Pc) derivatives were proposed as second generation photosensitizing agents since they meet most of the requirements of PDT [18]. First, Pcs exhibit strong light absorption in the red and deep red wavelengths of spectrum with a maximum peak at about 700 nm, and thus, they offer enhanced skin penetration. Also, since Pcs don't exhibit a significant light absorption between 400-600 nm, their use could reduce the skin phototoxicity [2]. What's more, Pcs have a large conjugated π-system, which enhances energy and charge transfer, and hence, boosting generation of singlet oxygen [19]. On the other hand, the main drawback of Pcs is low solubility in aqueous mediums, making their transport in the body difficult [20, 21]. This is due to the extended flat hydrophobic aromatic surface of Pcs that cause their aggregation in aqueous solutions through self-interactions [20, 21]. Yet, particularly Zn, Al, and Si containing Pc derivatives are among most promising second generation photosensitizers, and even some of them are commercially available [21-25]. However, an intense research effort is still ongoing to develop third generation photosensitizing agents that could not only exhibit properties of afore-mentioned second generation photosensitizers, but also have high water solubility.

In this context, we propose that serotonin substituted Pc might be a promising candidate in terms of improving solubility without deteriorating PDT properties. This is because serotonin is a non-toxic, water-soluble (25.5 mg/mL) primary amino compound with direct roles in human metabolite [26]. Further, serotonin does not exhibit light absorption peaks in the region of 400-600 nm, and one would not expect skin phototoxicity upon its attachment to Pc [27]. Although serotonin could be expected to attach Pc towards an oxygen bridge, its free NH_2 groups can be cationized for conversion to quaternary amines. The positive charge on quaternary amines could improve water-solubility and might limit self-interactions of hydrophobic aromatic surface of Pc in aqueous solutions, hence solving aggregation problem. In this sense, we prepared serotonin substituted ZnPc (Ser-ZnPc) and quaternized serotonin substituted ZnPc (q-Ser-ZnPc) compounds, since zinc (II) phthalocyanines (ZnPcs) are the most widely studied second generation photosensitizers in PDT. Then, we investigated the solubility of as-synthesized serotonin substituted ZnPc derivatives in aqueous solutions as well as their PDT performance. Strikingly, we report that not only the water solubility, but also fluorescence quantum yield is improved in case of q-Ser-ZnPc, as compared to Ser-ZnPc. These results suggest that q-Ser-ZnPc might be useful for image-guided PDT applications.

Materials and Methods

Materials

All reactions were carried out under nitrogen atmosphere and all solvents were dried by molecular sieves or proper methods [28]. Preparation of 4-nitrophthalonitrile was carried out according to previous reports in three steps [29]. In the first step, 4-nitrophthalimide was obtained as a result of the nitration reaction of phthalimide. In the second step, 4-nitrophthalamide was obtained by treating 4-nitrophthalimide and ammonia. In the last step, 4-nitrophthalonitrile was obtained by treating 4-nitrophthalamide with thionyl chloride. Solvents and all other chemical reagents were purchased from Merck.

Characterization

FT-IR spectra were recorded on a Perkin Elmer Spectrum 100 spectrometer by preparing KBr pellets. Absorption spectra were recorded by a Shimadzu 1601 UV-Vis spectrometer. Fluorescence spectra of compounds were collected by a Shimadzu RF 5301 fluorescence spectrophotometer. ^{1}H-NMR spectra were obtained by using a JEOL 400 MHz spectrometer. Melting points are recorded on Electrothermal 9100 digital melting point apparatus.

Synthesis

Synthesis procedures are described below and schematized in Figure 1.

Synthesis of serotonin substituted phthalonitrile (1)

The 4-nitrophthalonitrile (1.0 g, 5.78 mmol) and 5-hydroxytryptamine hydrochloride (1.47 g, 6.93 mmol) were dissolved in dimethyl sulfoxide (DMSO) (20 mL). Anhydrous potassium carbonate (K_2CO_3) (2.40 g, 17.34 mmol) was added to the reaction solution over period of 2 h with efficient stirring. The reaction mixture was stirred at 40°C for 2 days. The reaction was controlled by thin layer chromatography (TLC). Then the mixture was poured into a solution of salt-water (1%), and the precipitate was filtered off, washed with water and dried in vacuum oven at 40°C. Crude products were purified by column chromatography on silica gel using tetrahydrofuran (THF) and chloroform ($CHCl_3$) (50:10). Finally, a light yellow solid was obtained and found to be soluble in $CHCl_3$, THF, acetone, methanol (MeOH), N,N-dimethylformamide (DMF) and DMSO. Yield 750 mg (43%). Mp: 205°C. ^{1}H-NMR (400 MHz, $CDCl^3$, 25°C): δ= 10.7 (s, 3H, N-H); 7.3-7.0 (m, 7H, Ar-H); 2.6 (s, 4H, Aliphatic-H). FT-IR (KBr pellet) υ (cm^{-1}) 3444; 3122; 3028; 2244; 1572; 1251;760. Anal. Calc. for $C_{18}H_{14}N_4O$: C, 71.51; H, 4.67; N, 18.53%, found: C, 71.43; H, 4.60; N, 18.49%.

Synthesis of serotonin substituted zinc(II) phthalocyanine (Ser-ZnPc) (2)

Compound **1** (100.0 mg, 0.33 mmol) and dry zinc(II) acetate ($Zn(CH_3COO)_2$) (15.17 mg, 0.08 mmol) in DMF (2 mL) was heated at 180°C for 12h in the presence of 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU). After cooling, the mixture was precipitated with MeOH, filtered off and dried in vacuum. The dark green compound was dissolved in THF and filtered off. The THF solution was passed over a silica gel column. The organic phase was precipitated with diethyl ether and filtered off and dried in vacuum. Finally, a dark green solid was obtained and found to be soluble in $CHCl_3$, THF, DMF and DMSO. Yield 32.0 mg (37%). Mp: >300°C. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ= 8.2-7.00 (br m, 28H, Ar-H); 2.92 (s, 16H, $-CH_2$); 9.8 (br s, 3H, N-H, disappeared on D_2O addition). UV-Vis (DMSO) λ_{max}/nm 679, 608, 350. FT-IR (KBr pellet) υ (cm^{-1}) 3429; 3052; 2856; 1599; 1308; 764. Anal. Calc. for $C_{54}H_{56}N_{16}O_4Zn$: C, 61.27; H, 5.33; N, 21.17%, found: C, 60.95; H, 5.26; N, 21.08%.

Synthesis of quaternized serotonin substituted zinc(II) phthalocyanine (q-Ser-ZnPc) (3)

The solution of compound **2** (100.0 mg, 0.09 mmol) in $CHCl_3$ (2 mL) was added methyl iodide (CH_3I) (1.0 mL) and stirred at 40°C for 48 hours. After cooling, the organic phase was precipitated with diethyl ether and filtered. The solid was then washed with diethyl ether (2x 5mL) and dried in vacuum. Finally, a green solid was obtained and found to be soluble in water. Yield 88.0 mg (57%). Mp: >300°C. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ= 8.10-7.00 (br m, 28H, Ar-H); 2.86 (s, 16H, $-CH_2$); 9.5 (br s, 3H, N-H, disappeared on D_2O addition); 1.36 (s, 12H, $N-CH_3$). UV-Vis (DMSO) λ_{max}/nm 688, 613, 351. FT-IR (KBr pellet) υ (cm^{-1}) 3420; 3180; 2816; 1603; 1311; 767.

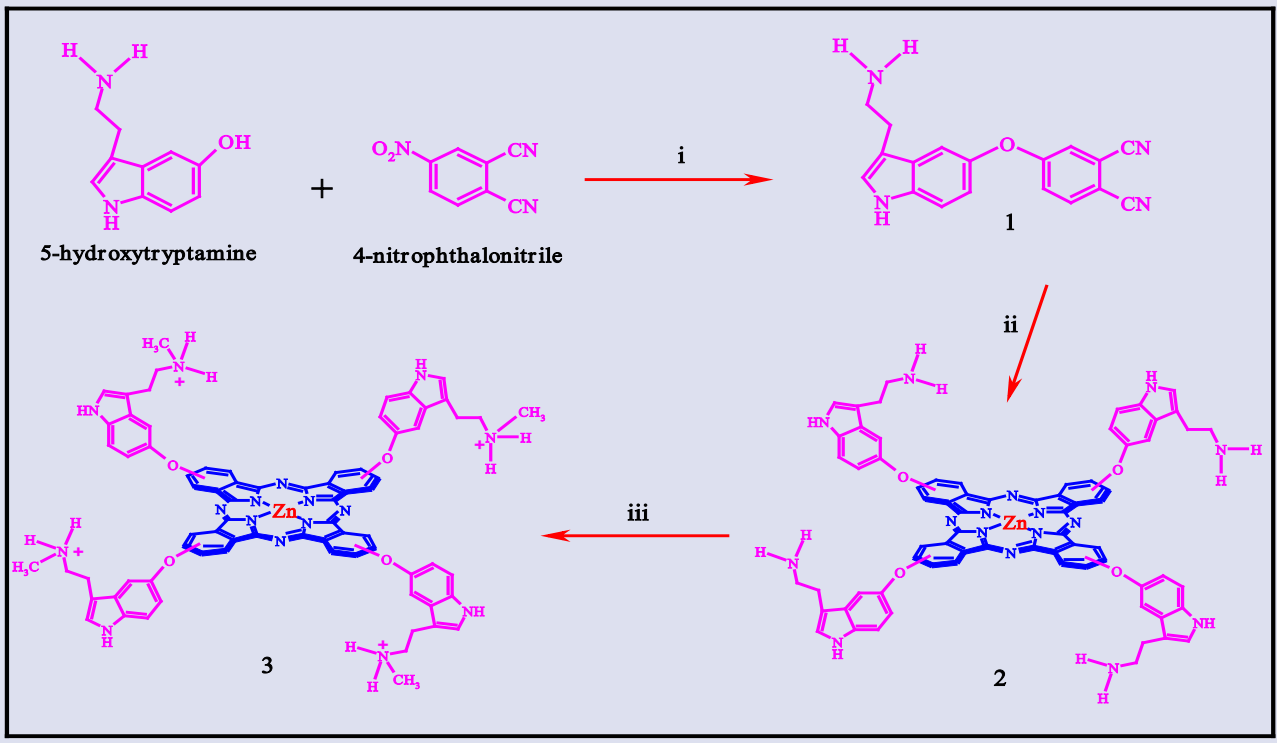

Figure 1. Synthesis of Compounds (i: dry DMSO, 2 days, K_2CO_3, 40°C, ii: dry DMF, zinc acetate, DBU, 12h, 180°C, iii: $CHCl_3$, CH_3I, 48h, 40°C).

Results and Discussion

Serotonin substituted phthalonitrile derivative (Compound 1) was synthesized by the nucleophilic substitution reaction of 4-nitrophthalonitrile and 5-hydroxytryptamine hydrochloride in DMSO in the presence of K_2CO_3. The Compound 1 was then purified by column chromatography with silica gel. The Ser-ZnPc (Compound 2) was obtained by tetramerization reaction of compound 1 with $Zn(CH_3COO)_2$. The q-Ser-ZnPc (Compound 3) was synthesized by the quaternization reaction of Ser-ZnPc. The FT-IR, UV-Vis, ^{1}H-NMR, and elemental analysis results confirmed the proposed structure of new compounds.

The characteristic vibration band of the -C≡N group appeared at 2244 cm^{-1} in the IR spectrum of compound 1 and it disappeared after conversion to Ser-ZnPc (Figure 2) [30]. The peaks corresponding to aliphatic C-H groups were realized at 3122-3028 cm^{-1}, 3052-2856 cm^{-1} and 3180-2816 cm^{-1} in the FT-IR spectra of synthesized compounds 1-3, respectively [31]. Also, the peak at 3444-3420 cm^{-1} was ascribed to the stretching vibration of N-H, the peak at 1603-1572 cm^{-1} was ascribed to the stretching

vibration of C=C, and the peak at 1311-1251 cm^{-1} was ascribed to the stretching vibration of Ar-O-C [31]. Finally, a peak for the substituted benzene ring was observed at 767-764 cm^{-1} in all compounds [31].

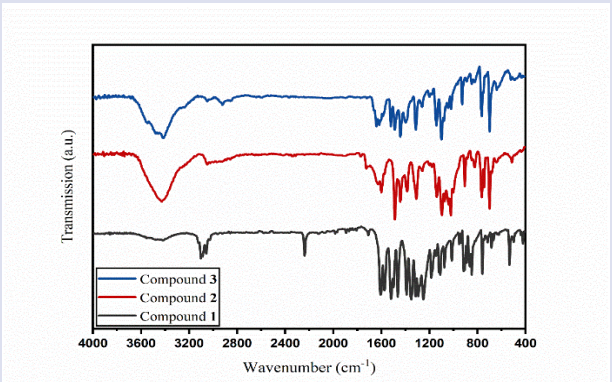

Figure 2. The FT-IR spectra of compounds 1, 2 and 3.

The characteristic absorptions were observed in the Q-band (650-700 nm) and B-band (300-400 nm) region in the UV-Vis spectra (Figure 3) of Ser-ZnPc and q-Ser-ZnPc in DMSO, as expected [31]. The characteristic Q-band is due to the π–π* transition of the Pc ring from Highest Occupied Molecular Orbital (HOMO) to Lowest Unoccupied Molecular Orbital (LUMO), while the characteristic B-band is due to deep π–π* transitions [33]. Specifically, the Ser-ZnPc and q-Ser-ZnPc show characteristic Q-bands of metallophthalocyanines at around 679 nm and 688 nm, respectively. In addition, B-bands of the compounds Ser-ZnPc and q-Ser-ZnPc appeared at around 350 nm and 351 nm, respectively. The formation of these peaks confirms the formation of Pc structure as a result of the tetramerization reaction. The UV-Vis spectra of Ser-ZnPc and q-Ser-ZnPc in DMSO show that the absorption wavelength was redshifted after the quaternization of Ser-ZnPc. Importantly, the increase in the absorption wavelength might be an indicator of a more effective PDT performance for q-Ser-ZnPc. More importantly, we report that the q-Ser-ZnPc is soluble in water. This feature is especially important in PDT applications, as it could allow easier transportation of the photosensitizer to the target in the body. Also, the redshift of the Q-band peak was found to be more pronounced in H_2O as compared to DMSO (Figure 3). This suggests that there might be a possibility of H-bond formation between q-Ser-ZnPc and water molecules.

In the ^{1}H-NMR spectrum of compound 1 which was taken in $CDCl_3$ at room temperature, the aromatic protons and aliphatic protons appeared at 7.30-7.00 ppm and at 2.60 ppm, respectively. The ^{1}H-NMR spectra of compounds Ser-ZnPc and q-Ser-ZnPc showed aromatic protons at 8.20-7.00 ppm and 8.10-7.00 ppm, aliphatic protons at 2.90 ppm and 2.86 ppm, respectively [34]. In addition, the ^{1}H-NMR spectrum of q-Ser-ZnPc showed N-CH_3 protons at 1.36 ppm [31].

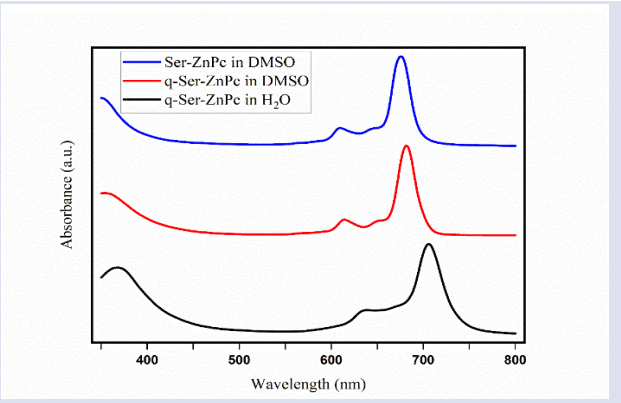

Figure 3. UV-Vis spectra of Ser-ZnPc and q-Ser-ZnPc in various solvents.

Finally, the -NH protons of compounds 1-3 appeared as singlets at 10.70 ppm, 9.80 ppm and 9.50 ppm, respectively, all of which disappeared after the D_2O exchange, as expected [34]. The integration of ^{1}H-NMR peaks are correlated with structure of compounds.

Elemental analysis was conducted experimentally and theoretically, both of which are in harmony with the proposed structures of compounds 1-3 in Figure 1.

The fluorescence quantum yield (Φ_F) is an important parameter in PDT applications. Thus, we measured the fluorescence emission spectra and calculated the fluorescence quantum yields of Ser-ZnPc and q-Ser-ZnPc by using Equation 1 [34]:

$$\Phi_F = \Phi_{F_{(std)}} \frac{F.A_{std}.n^2}{F_{std}.A.n_{std}^2} \quad (1)$$

where Φ_F symbolizes the fluorescence quantum yield of the unknown sample, and $\Phi_{F(std)}$ symbolizes the fluorescence quantum yield of the standard compound used in the measurement. The A and A_{std} in the Equation 1 symbolize the absorbance of the sample and the standard at the excitation wavelength, respectively. The n and n_{std} in the Equation 1 are the refractive indices of the solvents used for the sample and the standard, respectively. Unsubstituted ZnPc (Φ_F=0.18 in DMSO) was used as the standard in the measurements.

Table 1. Spectral data comparison for Ser-ZnPc and q-Ser-ZnPc.

Compound	Solvent	Q-band λ (nm)	Emission λ (nm)	Φ_F
Ser-ZnPc	DMSO	679	686	0.30
q-Ser-ZnPc	DMSO	688	690	0.42
q-Ser-ZnPc	H_2O	707	709	0.37

As can be seen in Figure 4 and Table 1, the absorption and emission spectra of q-Ser-ZnPc are shifted to higher wavelengths in H_2O comparing to DMSO. This shift can be explained by the tendency to H-bonding formation

between the functional groups of q-Ser-ZnPc and H_2O molecules [35]. The Φ_F of unsubstituted ZnPc, Ser-ZnPc and q-Ser-ZnPc in DMSO were found to be 0.18, 0.30, and 0.42, respectively. Also, it was observed that the Φ_F of both Ser-ZnPc and q-Ser-ZnPc are higher than that of unsubstituted ZnPc. At the same time, it was also observed that the Φ_F tended to increase after the quaternization. As it is known, a simultaneous combination of high Φ_F and singlet oxygen quantum yield (Φ_Δ) is sought for image-guided PDT applications [36-38]. However, it is difficult in most cases to reach a high Φ_Δ while concurrently maintaining the Φ_F at acceptable levels for real-time image guidance. Although we couldn't measure the Φ_Δ of q-Ser-ZnPc, a considerable increase of the Φ_F after quaternization might be beneficial for image-guided PDT, particularly if the Φ_Δ of q-Ser-ZnPc is also high. Adding this to the our observations that absorption peaks of q-Ser-ZnPc are within the therapeutic window (600-800 nm), and more importantly the q-Ser-ZnPc is water soluble, it could be beneficial to identify the Φ_Δ of q-Ser-ZnPc in the future for the progress of PDT field.

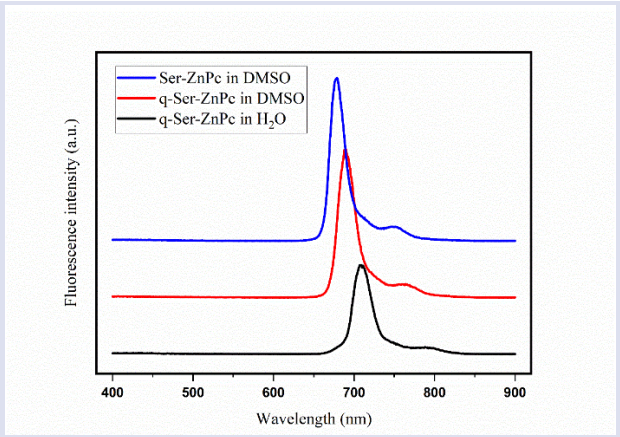

Figure 4. Emission spectra of compounds 2 and 3 in various solvents.

Regarding other factors that could affect emission spectra and fluorescence quantum yields, we also studied the effects of solvent selection and concentration. In particular, the emission spectra provide information about the fluorophore-solvent interaction, as the solvent and fluorophore molecule interactions affect the energy difference between the ground and excited states [35, 39]. Therefore, we used THF, $CHCl_3$, DMF and DMSO as solvents, in which Ser-ZnPc is readily soluble as can be seen in Figure 5. Importantly, we report that the Φ_F is increasing with a decrease of solvent polarity, since the polarities of these solvents increase in the order of THF<$CHCl_3$<DMF<DMSO, as listed Table 2 [40]. Likewise, the emission maximum shifted to shorter wavelengths with a decrease in solvent polarity [35, 39]. The variation of Φ_F with concentration was also investigated for compound 2 as illustrated in Figure 6 [39]. The results suggest no change in emission wavelength, but the Φ_F was found to be decreasing with a decrease in concentration in all of the solvents studied in this work (Figure 6).

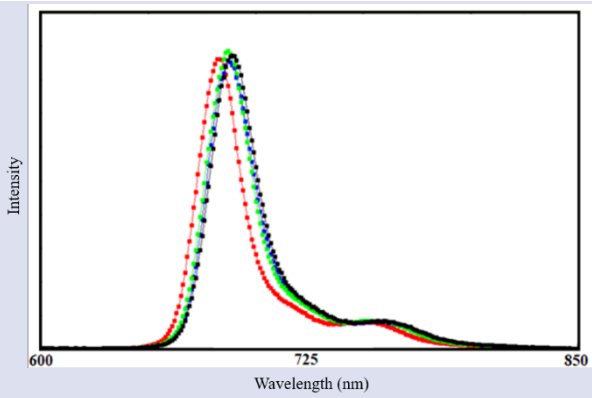

Figure 5. Emission spectra of Ser-ZnPc in various solvents (THF: red, $CHCl_3$: green , DMF: black, DMSO: blue).

Table 2. Spectral data for Ser-ZnPc in different solvents

Solvent	Dipole moment of solvents (μ)	Q-band λ (nm)	Emission λ (nm)	Φ_F
THF	1.69	673	680	0.39
$CHCl_3$	1.90	675	683	0.36
DMF	3.79	676	690	0.32
DMSO	3.96	679	686	0.30

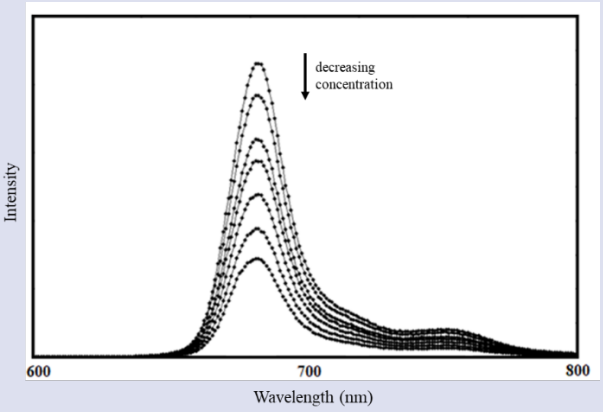

Figure 6. Emission spectra of Ser-ZnPc at various DMSO concentrations.

Conclusions

ZnPc is one of the most widely studied Pc-based photosensitizer materials in PDT applications. However, the main drawback of ZnPc that limits its application areas in PDT is the materials' poor water solubility that hinders its effective transfer to the target in the body. Accordingly, we synthesized serotonin substituted and quaternized serotonin substituted ZnPc to improve water solubility. The idea is that the positive charge on quaternary amines might limit self-interactions of the hydrophobic aromatic surface of Pc in aqueous solutions, and enhance the water-solubility. To do this, serotonin substituted phthalonitrile derivative was first synthesized by the nucleophilic substitution reaction of 4-nitrophthalonitrile and 5-hydroxytryptamine hydrochloride. Following this, serotonin substituted zinc(II) phthalocyanine (Ser-ZnPc)

was prepared by cyclotetramerization of serotonin substituted phthalonitrile. Finally, the Ser-ZnPc was quaternized to obtain q-Ser-ZnPc. The synthesized compounds were characterized by ^{1}H-NMR, UV-Vis, FT-IR, fluorescence, and elemental analysis. The results show that while unsubstituted ZnPc and Ser-ZnPc are not water-soluble, q-Ser-ZnPc is actually soluble in water. In addition, the absorption bands of q-Ser-ZnPc are within the so-called therapeutic window. Moreover, we report that the Φ_F of q-Ser-ZnPc is higher than that of both Ser-ZnPc and ZnPc. As it is known, a combination of both high Φ_F and Φ_Δ is necessary for image-guided PDT, and thus, q-Ser-ZnPc might become a promising candidate in PDT applications if it also exhibits a high Φ_Δ.

Conflict of interest

There are no conflicts to declare.

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