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Research Article

PHOTOPHYSICAL AND PHYSICOCHEMICAL PROPERTIES OF Cu(II)CHLORIN *e*$_4$ AND Cu(II)CHLORIN *e*$_6$ AS A LEAD COMPOUND OF PHOTOSENSITIZER FOR PDT

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ABSTRACT

Porphyrin derivatives are potential compounds for diagnostic agent and photosensitizer in photodynamic therapy. However, they have a weakness in molar absorptivity, especially in visible region of Q band which used to excite them. Due to incapabilities of porphyrin, other tetrapyrole derivatives, such as chlorophyllin can be alternative for a lead compound of photosensitizer. In the present research, two chlorin derivatives were isolated from commercial chlorophyllin product. Their photophysical and physicochemical properties, i.e. molar absorptivity, quantum yield of fluorescence and quantum yield of singlet oxygen were determined. Chlorophyllin carboxylic acid form, Cu(II)-chlorin *e*$_4$ and Cu(II)-chlorin *e*$_6$, were successfully isolated with recovery of 11.33% and 16.46%, respectively. The absorption spectrum of Cu(II)-chlorin *e*$_4$ showed an intense Soret band at 406 nm and two weaker Q bands at 628nm, 658nm. Fluorescence efficiency was 0.09 while efficiency for singlet oxygen at pH 6.3 and 7.4 were 0.0052±0.0017 and 0.0066±0.0012. Cu(II)-chlorin *e*$_6$ displayed soret band at 407nm and Q bands at 627nm, 663nm. Singlet oxygen at pH 6.3 was 0.0029±0.0007, while at pH 7.4 was 0.0034±0.0001. However, Cu(II)-chlorin *e*$_6$ did not show fluorescence.

Key words: Chlorophyllin, Cu(II)-chlorin *e*$_4$, Cu(II)-chlorin *e*$_6$, singlet oxygen fluorescence

INTRODUCTION

Porphyrin derivates have been widely investigated for potential use in Photodynamic diagnostics (PDD) or Photodynamic therapy (PDT) (Jichlinski and Jacqmin, 2008; Tjahjono, 2006). PDD is a technique to detect cancer by using emission photosensitizer within excitation condition (Skutnik, *et al.,* 2004) while PDT is a technique for cancer therapy that involves light, photosensitizer, and oxygen, in order to produce reactive singlet oxygen in cell. PDT actually requires two component i.e. photosensitizer and light sources. When the photosensitizer accumulated in cancer cell, light radiation in specific wavelength will be actived and changed oxygen triplet become singlet. Singlet oxygen is able to kill cancer cells through a process of random destruction of cancer cells by biochemistry reaction (Setyoko, 2010; Bonnet, 2000; Wang, 1999).

Photosensitizer can be used as PDD and PDT if this compound has high absorptivity molar in red region visible with fluorescence efficiency (Φ_f) and high efficiency singlet oxygen (Φ_Δ). Haematoporphyrin derivative, together with its commercial variants Photofrin, Photosan, Photogem, and Photocarcinorin, holds an important place in the story of tumour phototherapy. However, the compound has weakness due to their absorption in region band Q (MacDonald and Dougherty, 2001). Because of disadvantages of the compound, therefore other tetrapyrol compounds can be synthesized and developed to be the alternative guide compounds.

Another class of photosensitizers, the chlorins, are known to have intense absorption bands at wavelengths > 650nm. A chlorin is a large heterocyclic aromatic ring consisting, at the core, of three pyrroles and one pyrroline coupled through four =CH- linkages. One of the commercially available chlorin is chlorophyllin. Chlorophyllin usually used for a safety food coloring, it is easier to found and cheaper. Previous study have shown that commercially available sodium copper (II) chlorophyllins consists of a few kinds of copper (II) chlorophyll derivatives, e.g. copper (II) pheophorbide *a*, copper (II) chlorin e_6, copper (II) chlorin e_4, copper (II) rhodin g_7 and their degradation products (Inoue et al., 1994; Scotter *et al*, 2005; Ferruzzi and Schwartz, 2005). Therefore, in the present study we isolated and initiated characterization of chlorin compounds from chlorophyllin as photosensitizer candidate for anticancer therapy.

MATERIAL AND METHODS

Reagents and Instruments

Commercial grade sodium copper chlorophyllin (Sigma-Aldrich), chlorin e_6 (Frontier Scientific), chloride acid 0.1 N, organic solvents, gel silica for chromatography, and silica for thin layer (Gel silica 60 F-254).

Column for chromatography, chamber for TLC, vortex, water bath (Buchi heating bath B-490), vacuum rotary evaporator, apparatus for vacuum desicator, pH meter/pH paper, apparatus glasses, magnetic stirrer, spectrophotometer UV-Vis (Beckman Coulter DU® 720, IR (Jasco FT/IR-4200), MS (Waters LCT Premier XE ESI TOF), and Spectrofluorometer Shimadzu (Shimadzu spectrofluorophotometer RF-5301).

Procedures

Isolation and characterisation

All experiment were conducted under subdued light to minimize photooxidative degradation of chlorophyllin. A solution containing 1.0021g of commercial chlorophyllin was mixed with 350mL of HCl (0.1N) for 30min at room temperature. A further 75mL of dichloromethane added to the chlorophyllin solution. Cu(II)chlorophyllin was extracted by vigorously shaking and the dichloromethane layer was washed five times with distilled water.

Dichloromethane extract was subjected to the column then eluted with gradient polarity using n-hexane (fraction I), n-hexane: dichlorometane (fraction II), dichlorometane (fraction III), dichlorometane:isopropanol (fraction IV). Fraction I and II were not shown absorbance in Q band. Fraction III was purified further by elution with n-hexane-chloroform-methanol-acetone (5:5:1:2) (isolate III). Fraction IV was purified by elution methanol-acetone (2:1) (isolate IV). The green dark crystals were recrystallized from hexane/acetone. The structure was examined by IR, UV-Vis spectroscopy and MS.

Absorption spectra and molar absorptivity

Absorption spectra were recorded with UV-Vis spectrophotometer. The wavelength of maximum absorption (λ max) at soret and Q band was determined. A number solutions of varying concentrations were scanned for absorbance at the appropriate wavelengths.

Fluorescence spectra and fluorescence quantum yield (Φ_F)

Fluorescence spectra were acquired with Shimadzu spectrofluorophotometer RF-5301 under excitation at 431nm. Absorption spectra were recorded with UV-Vis Beckman Coulter DU® 720. Fluorescence quantum yield were calculated as the ratio of integrated fluorescence emission (420-800nm) to optical density at the wavelength of excitation (OD < 0.1). The fluorescence quantum yield for an excitation wavelength was calculated using a reference sample with a known quantum yield (chlorin e_6). Fluorescence quantum yield was determined according to Jobin Yvon's method.

Singlet oxygen quantum yields (Φ_Δ)

The absolute singlet oxygen quantum yields were determined using the same solvent and identical values for the absorbance at the excitation wavelength of the standard and the compound investigated. Φ_Δ were determined using *p*-nitrosodimethylaniline (RNO) as a selective scavenger (Kralzic and Mohsni, 1978). A certain amount sample were diluted with 1mmol imidazole, 50mmol RNO (*para*-nitrosodimethylaniline) in 100 mL buffer solution at pH 6.3. This solution was mixed and irradiated with xenon lamp with ± 8mW cm^{-2}. The temperature of reactor was ±20°C. The bleaching of RNO as followed spectrophotometrically at 440nm for 3h. It is also used in pH 7.4.

RESULT AND DISCUSSION

In this experiment we used commercial chlorophyllin from Sigma-Aldrich. This product was dominated sodium salt form complex Cu(II)chlorin *e*4 (Inoue *et al.,* 1994). Sodium Cu(II)chlorophyllin was utilized as food additive due to its high light stability and applied to medicine owing to its antioxidative effect. Within this product there were some sodium available e.g. Cu(II)chlorin *e*6, Cu(II)-rhodin *g*7, Cu(II)-pheophorbide, Cu(II) rhodoclorin and their degradation products. This chlorophyllin was coming warming process of semisyntesis chlorophyll, also aciding and cuting the phytil group to form several sodium chlorophyllin (Fahey *et al.,* 2005). This compound was stable and soluble in water (Mortensen and Geppel, 2007).

Chlorophyllin was changed into carboxyl acid form (Figure 1). A total of chlorophyllin 1.0021 g was successfully converted into a carboxyl acid form with a yield 53.19%.

The salt natrium chlorophyllin is soluble in water but the carboxyl acid form is soluble in dichloromethane. So that carboxyl form of compounds can be separated by both liquid and liquid extraction. The product contain several kinds of complex compounds Cu(II) with varying degrees of polarity so it can be separated based on polarity. Separation of purified fractions was done by different degrees of polarity column chromatography with silica stationary phase and appropriate mobile phase.

Identification and characterization of carboxylic chlorophyllin acid derivatives

Fraction III and IV were separated by column chromatography on silica gel stationary phase to obtain isolate III and IV, respectively. Monitoring isolates were performed by TLC silica gel 60 F-254.

Spectra of mass spectrometer

MS analysis for isolates III and IV were performed by EI-MS-TOF (Electrospray Ionization-Mass spectrofotometry-Time of Flight). MS fragments of carboxylic acid compounds chlorophyllin can be interpreted clearly because of the copper isotope (Cu^{63}=69% and Cu^{65}=31%) and carbon (C^{12} = 99% and C^{13}=1%). The mass spectrum of isolates have the characteristics of a carboxylic acid derivative with a typical pattern as described above. MS molecular ion isolate III showed molecular ion (negatif mode) $[m/z–H]^-$ at 612.2. Isotop distribution of isolate III showed isotop ratio 612.2/613.2/614.2=100/ 37.7/52.3 (intensity). The MS pattern indicated that isolate III could be Cu(II)chlorin *e*4. Furthermore, MS molecular ion isolate IV (negatif mode) showed molecular ion $[m/z–H]^-$ at 656.2. Isotop distribution of isolate IV showed isotop ratio 656.2/657.2/658.2=100/ 38.9/53.2. The MS pattern indicated that isolate IV could be Cu(II)chlorin *e*6.

Unfortunately, the Copper(II) chlorophyllin structure could not confirmed by ^{1}H NMR. Copper NMR is only observed for oxidation state (I) because (II) is paramagnetic. A drawback in the investigation of paramagnetic compounds is that when nuclear relaxation is too fast, signal line widths are large, resolution may become poor and, most important, coherence and magnetization transfer may be below detection (Murthy *et al.,* 1997). Therefore, absorption spectra in combination with mass spectra are powerful tools for unraveling the nature of the constituens of sodium copper chlorophyllin. Using mass spectroscopy it is possible to quickly distinguish between coppered and uncoppered compounds, because the uncoppered compounds do not show the distinctive isotopic splitting as seen with the coppered compounds (Mortensen and Geppel, 2007). Structure of Cu(II)chlorin *e*4 and Cu(II)chlorin *e*6 shown in figure 4.

Spectra of infrared

Cu(II)chlorin *e*4 infrared spectra shown in figure 3 (left). Carbonyl group absorption band (1704cm^{-1}) and the band widened the OH group (3394cm^{-1}) specifying the presence of carboxylic acid groups on the isolate III. Whereas, Cu(II)chlorin *e*6 infrared spectra shown in figure 3 (right). The absorption band of CO group appears at 1708cm^{-1} and a broad band at 3367cm^{-1} (OH group) of IR spectrum showed the presence of carboxylic group in the compound.

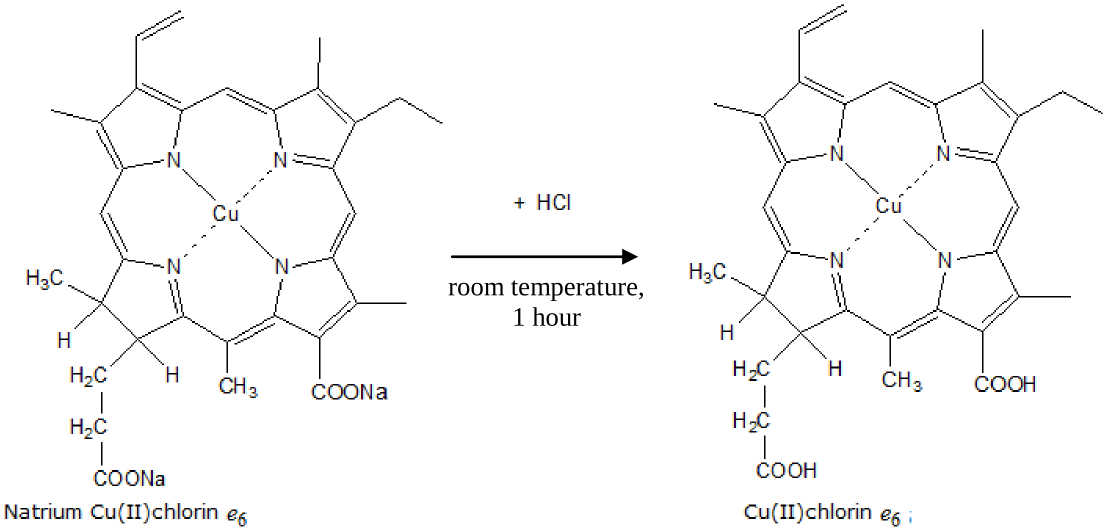

Figure 1. The scheme of chlorophyllin complexed conversion into carboxylic acid form.

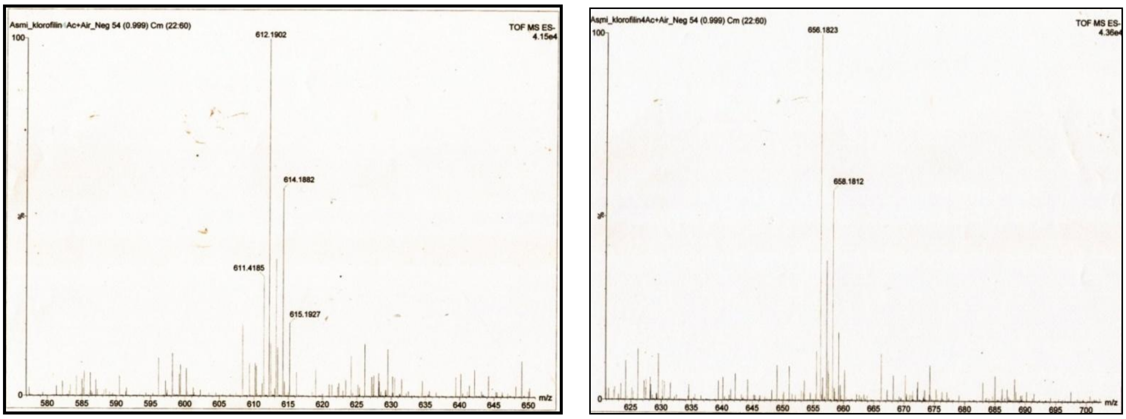

Figure 2. MS spectra of Cu(II)chlorin e_4 (left) and Cu(II)chlorin e_6 (right)

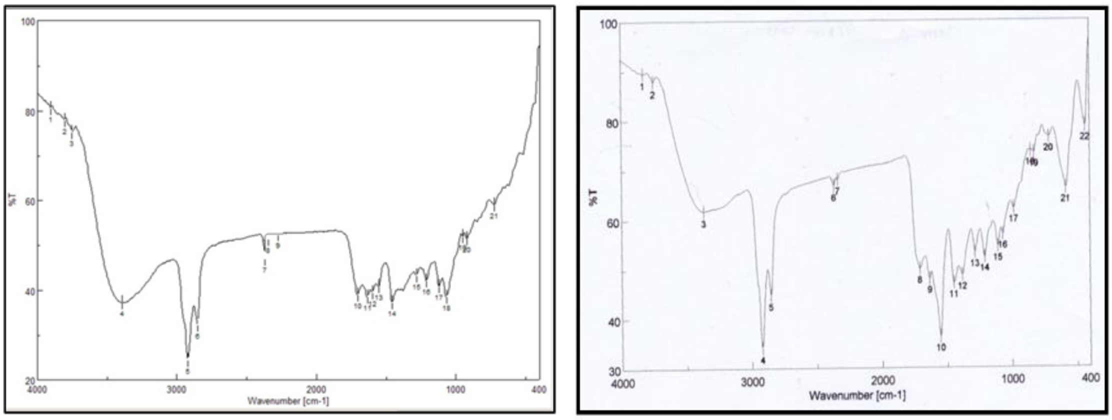

Figure 3. Infrared spectrum of Cu(II)chlorin e_4 (left) and Cu(II)chlorin e_6 (right)

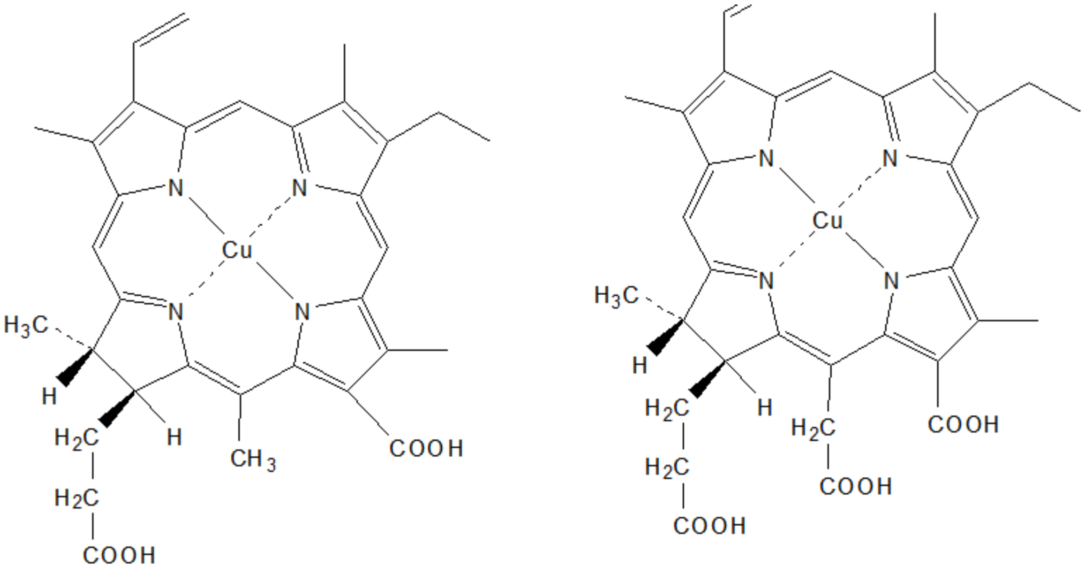

Figure 4. The structure of Cu(II)chlorin *e*$_4$ (left) and Cu(II)chlorin *e*$_6$ (right)

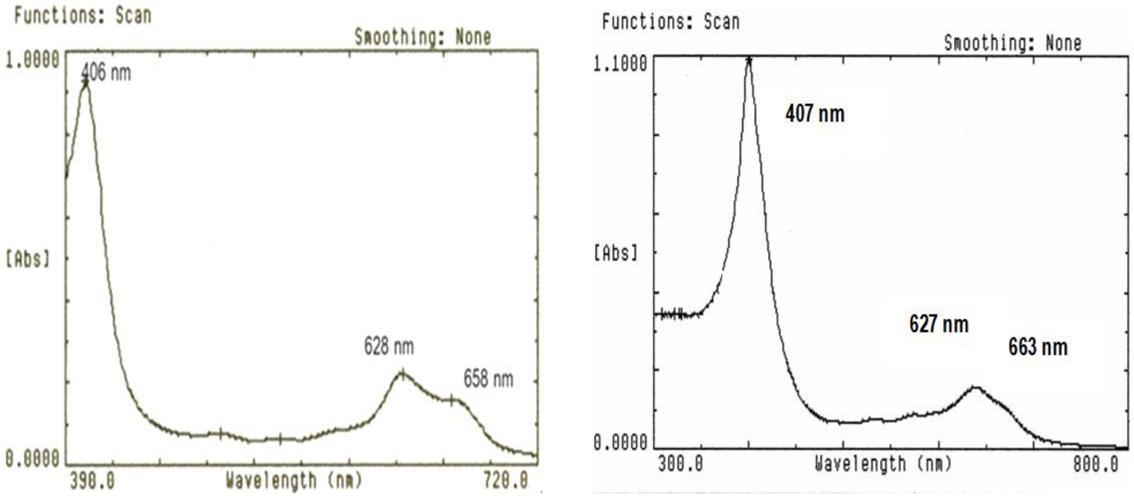

Figure 5. Absorption spectrum of Cu(II)chlorin *e*$_4$ (left) and Cu(II)chlorin *e*$_6$ (right) in dichloromethane

Table I. Molar absorptivity (ε) and λ $_{\text{maks}}$ of the compound Cu(II)chlorin *e*$_4$ and Cu(II)chlorin *e*$_6$

Compound	**λ $_{\text{maks}}$ (nm) ; ε ($cm^{-1}M^{-1}$)**	
	Soret band	**Q band**
Cu(II)-chlorin *e*$_6$	407 ; 3270	62 ; 2380
Cu(II)-chlorin *e*$_4$	406 ; 60213	628 ; 26895

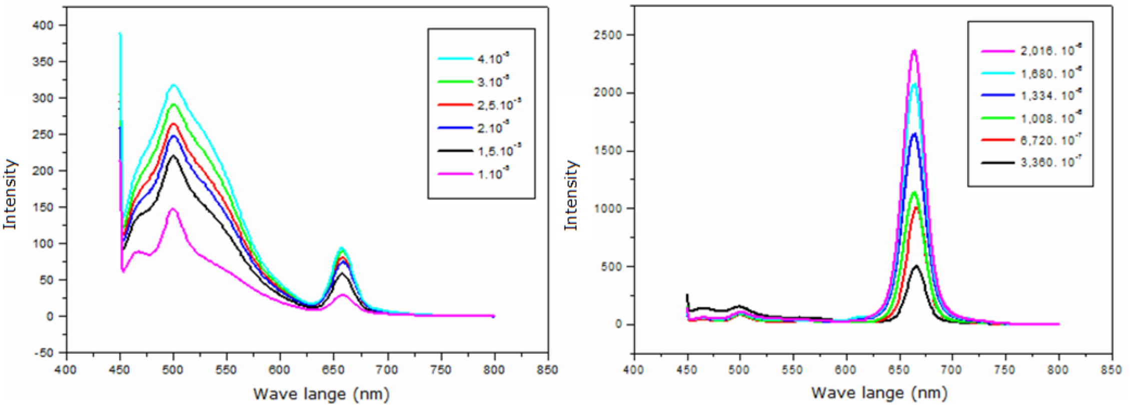

Figure 6. Fluorescence emission spectra of standard chlorin e_6 (right) and Cu(II)chlorin e_4 (left) obtained for six different concentration of sample.

Table II. Fluorescence quantum yield (Φ_F)

Compounds	Gradient	Φ_f
Chlorin e_6	16254	0.18
Cu(II)chlorin e_4	8046	0.09
Cu(II)chlorin e_6	0	0

Tabel III. Singlet oxygen quantum yields of Cu(II)chlorin e_4 and Cu(II)chlorin e_6

Compound	Φ $^1O_2$ pH=6.3	Φ $^1O_2$ pH=7.4
Chlorin e_6	0.50±0.05	0.60±0.06
Cu(II)chlorin e_4	0.0052±0.0017	0.0066±0.0012

Absorption spectra and molar absorptivity

Photosensitizer distribution has an important rules in photodynamic according to the chemical structure (Castano, 2005). With absorption in red band 675-800nm molar absortivity 5x10^4 M^{-1} cm-1 makes light penetration in skin about 2-3cm (Pandey and Zheng, 2000). Characterization of UV-Vis spectra of compounds Cu(II)chlorin e_6 and Cu(II)chlorin e_4 can be seen in figure 5. The spectrum of these compounds suggest that both compounds have characteristic spectra tetrapyrrolic macrocyclic compounds which is characterized by the presence of strong absorption is around 400nm (Soret band) and 4 bands in the 600-700nm (Q band). But the compound tetrapyrrolic macrocyclic porphyrin Q bands reduced to 2, due to the metal Cu entered and formed two covalent coordination bonds.

Soret band of the compound Cu(II)chlorin e_4 was at 406nm, while the Q bands have two unclear peaks, and the main peak appeared at 628nm wavelength. The second peak at Q band appeared at λ 658nm. Soret band of the compound Cu(II)chlorin e_6 was at 407nm, while the Q bands have two peaks, and the main peak appears at 627 nm wavelength. The second at Q band appears at λ toward the red (around 663nm). The Q band of Cu(II)chlorin e_4 and Cu(II)chlorin e_6 were comparable to those reported earlier (Inoue *et al.,* 1994). Furthermore, molar absorptivity of the compound Cu(II)chlorin e_4 was higher than Cu(II)chlorin e_6 (Table I).

Fluorescence spectra and fluorescence quantum yield (Φ_F)

Spectra emission of Cu(II)chlorin e_4 and standard chlorin e_6 shown in figure 6. Cu(II)chlorin e_4 was excitated at λ 431nm with λ emission at 664 nm. Whereas, Cu(II)chlorin *e*$_6$ was excitate at 400nm, did not show λ emission in spectra. The value of Φ_F was calculated by compare with standard chlorin *e*$_6$. The method for recording Φ_F was involved the use of well characterised standard samples with known Φ_F values. Essentially, solutions of the standard and test samples with identical absorbance at the same excitation wavelength can be assumed to be absorbing the same number of photons. Hence, a simple ratio of the integrated fluorescence intensities of the two solutions (recorded under identical conditions) will yield the ratio of the quantum yield values. Since Φ_F for the standard sample was known, it was trivial to calculate the ΦF for the test sample (Jobin, 2010).

Singlet oxygen quantum yields

Singlet oxygen was formed by irridiated the photosensitizer become triplet state and change to singlet oxygen (Mathai *et al.,* 2007). Singlet oxygen will be trapped by imidazole and then transanular imidazole-oxide was formed. Without imidazole, singlet oxygen will resistant toward RO. Transanular imidazole-oxide will break RNO as the result decreasing RNO molecule in solution. If decreasing RNO is higher the singlet oxygen will increase (Grabaw, 2005).

Singlet oxygen quantum yield was studied at two different pH (6.3 and 7.4). pH 6.3 represents a cancer cells pH, while pH 7.4 represents a physiological pH. Singlet oxygen generation was found to be higher at higher pH than those generated at lower pH. The results show in table II. The compounds have been aggregate, as the result decreasing singlet oxygen quantum yields at lower pH.

CONCLUSION

Cu(II)chlorin e$_4$ and Cu(II)chlorin e$_6$ could be isolated from sodium salt of Cu(II)chlorophyllin. Absorption spectra of Cu(II)chlorin e$_4$ and Cu(II)chlorin e$_6$ display the red-most absorption (Q band) at longer wavelengths. The spectrum were however promising candidates to be utilized in PDT. Unfortunately, the compounds have low fluorescence quantum yield and singlet oxygen quantum yield. Copper (II) in the structures cause quenching the fluorescence. Therefore, it is necessary to change the paramagnetic Cu metal into magnetic metal to increase the fluorescence and singlet oxygen quantum yields.

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