Efficient photo-oxidation of phenol and photo-inactivation of bacteria by cationic tetrakis(trimethylanilinium) porphyrins
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ABSTRACT
Photo-oxidation of phenol in aqueous alkaline and neutral buffer solutions by irradiation with visible light in the presence of 5,10,15,20-tetrakis(4-N,N,N-trimethylanilinium)porphyrin (TAPP) and its zinc metal ion (ZnTAPP) photo-sensitizers is described. The inhibitory effect of these molecules on the growth of Gram-positive bacteria Bacillus subtilis and Bacillus strophaeus and the Gram-negative strain Pseudomonas aeruginosa has also been examined. Furthermore, to determine the relationship between inhibition and attachment, the degree of binding of these photo-sensitizers to these bacteria was determined. The results show that TAPP is more effective than ZnTAPP in the photo-oxidation of phenol and photo-inactivation of the bacteria. In addition, the degree of binding of TAPP to these bacteria was more than that of ZnTAPP.

INTRODUCTION
One of the most toxic substances and main pollutants in industrial wastewater is phenol. It is harmful to human beings even at low concentrations, and is therefore considered a priority pollutant (Priya & Madras 2006; García-Munoz et al. 2014). The oxidation of phenol is of special interest because phenol derivatives are known to be important chemicals in the pharmaceutical and dye industries (Takao et al. 2010). The reaction of singlet oxygen with phenol, via electron transfer (Gerdes et al. 1997; DeRosa & Crutchley 2002), mainly produces hydroperoxides that dehydrate to form benzoquinones, although some p-hydroquinone is also formed (Sergeeva & Senge 2012).

The effect of various compounds, such as activated carbon to titania catalyst (García-Munoz et al. 2014) and a composite with TiO2 (Makrigianni et al. 2015) has been studied, as a means of phenol degradation in aqueous suspensions. Mahvi et al. (2007) studied the photo-degradation of phenol with a 400 W medium-pressure mercury lamp.

Photo-oxidation of phenols by various macrocyclic systems such as porphyrins or metalloporphyrins has been reported to be a powerful process (Gerdes et al. 1997; DeRosa & Crutchley 2002; Gmurek & Miller 2012; Drozd et al. 2014).

Murtinho et al. (2000) worked on neutral porphyrins and chlorine as potential sensitizers for the photo-oxidation of phenols. Drozd et al. (2014) used a type of hybrid photosensitizer with 5,10,15,20-tetrakis(4-carboxyphenyl)porphyrin for the oxidation of phenol in aqueous solution under irradiation with visible light. Furthermore, iron porphyrin in the presence of oxygen (Sergeeva & Senge 2012) and meso-tetra-(4-benzoate,9-phenanthryl)-porphyrin and its metal complexes with Zn and Cu were used for antibacterial photo-activity and photo-oxidation of phenol by Oviedo et al. (2014).

Tetrapyrrolic macrocycle ring systems, such as porphyrins, have great potential as photo-therapeutic agents for the treatment of a variety of diseases due to absorption of
photons in the visible region (Spesia et al. 2005; Liu et al. 2008; Yu et al. 2009). Singlet oxygen is a highly electrophilic compound and capable of oxidizing phenols, sulfides, and amines (Derosa & Crutchley 2002; Mahvi et al. 2007; Drozd et al. 2014). Porphyrin derivatives are known to be efficient generators of singlet oxygen (Kim et al. 2012). The most important area of practical applications for these compounds is photodynamic therapy (PDT). The mechanism of reactive oxygen species (ROS) formation by porphyrins involves electronic excitations of their molecules to the first singlet excited state, followed by intersystem crossing with the formation of an excited triplet state which is quenched by molecular oxygen. At the end of this sequence, the porphyrin molecule returns to the ground state and singlet oxygen is formed (Drogt et al. 2012; Dossell et al. 2013; Senthilkumar et al. 2013; Mordon et al. 2015).

Photodynamic antimicrobial chemotherapy (PACT) is a developed therapeutic option that applies visible light to photosensitive molecules to induce oxidative damage to microbial pathogens via production of ROS (Peèkaitytë et al. 2005; Banfi et al. 2006; Cassidy et al. 2010; Latief et al. 2015). Various cationic porphyrins have been used as excellent antimicrobial agents in PACT (Nitzan & Ashkenazi 2001; Reddi et al. 2002; Lazzeri & Durantini 2005; Lambrecht et al. 2005; Pudžiuvytë et al. 2008; Cormick et al. 2009).

In this study, we started with the premise that tetra-cat-ionic porphyrin compounds may be used for two types of process concomitantly: photo-oxidation of phenol and photo-bactericidal activities. As a part of the water treatment process, the photo-oxidation of phenol was investigated by using 5,10,15,20-tetrakis(4-N,N,N-trimethylamminium)porphyrin (TAPP) and its zinc metal ion (ZnTAPP). The activity of these compounds against three strains of bacteria was also studied. To establish a relationship between growth inhibitions of the named bacteria and the binding of these photosensitizers, the degree of binding was determined with a UV-Vis spectrophotometer.

**MATERIALS AND METHODS**

Nutrient broth, nutrient agar and all chemicals used in this work were purchased from Merck and applied without further purification. The porphyrin TAPP and its zinc ion complex (ZnTAPP) were synthesized as reported previously (Krishnamurthy 1977; Thompson & Krishnamurthy 1979; Shamim & Hambright 1980; Rahimi 1992). Gram-positive bacteria (Bacillus subtilis and Bacillus strophaeus) and a Gram-negative bacterium (Pseudomonas aeruginosa) were obtained from the microbiology laboratory of Guilan University. For buffers with pH = 7.2 and pH = 9.2, phosphate and borate buffers were used, respectively. UV-Vis absorption spectra were measured on a T80+UV/Vis Spectrometer PG Instruments Ltd with a quartz cuvette. All the experiments were carried out in a water-jacketed reactor irradiated with a 100 W tungsten lamp as a visible light source and in a dark room to avoid light reflection. This system was set up in a shaker incubator at a rate of 80 rpm.

**Photo-oxidation of phenol by photo-sensitizers**

An aqueous solution of phenol at a concentration of $1 \times 10^{-3}$ M was mixed with equal amounts of porphyrin solution at a concentration of $1 \times 10^{-3}$ M in buffer media with the appropriate pH (7.2 or 9.2). The solution was illuminated for 120 minutes with the light source at 25 °C. At 30-minute intervals sample solutions (∼2 mL) were removed and the absorption spectrum was determined in the range of 190–340 nm. This experiment was repeated three times.

**Determination of photo-bactericidal activity, minimum inhibitory concentration and minimum bactericidal concentration**

All strains were grown aerobically at 37 °C in nutrient broth overnight. A stock solution of porphyrins was prepared in water at various concentrations. Thirty milliliters of bacterial suspension (∼$10^8$ cfu/mL) and various concentrations of porphyrins were added to nutrient broth and incubated for 20 minutes under dark conditions at 37 °C in order for the porphyrins to bind to the bacteria. The cultures were then illuminated for 30 minutes with the light source and incubated overnight. The cultures exhibiting minimum inhibitory concentration (MIC) were further analyzed to determine whether minimum bactericidal concentration (MBC) had been attained. Briefly, 100 μL of culture exhibiting MIC was spread onto the surface of a nutrient agar
plate and incubated at 37 °C overnight. Bacterial growth was examined visually and absence of growth indicated MBC. This experiment was repeated three times.

**Binding test of porphyrins to bacteria**

Thirty millilitres of bacterial suspension (∼3 × 10⁷ cfu/mL) was supplemented with porphyrins at a concentration of 1.2 μM in nutrient broth and the samples were centrifuged (10,000 rpm for 1 minute). The visible spectra of the supernatants were recorded at time zero and after 20 minutes incubation at 37 °C under dark conditions. The percent binding of porphyrins to the bacterial cells was obtained by using a UV-Vis spectrophotometer and decreasing absorption of porphyrins at the Soret band. This experiment was repeated three times.

**RESULTS AND DISCUSSION**

The absorption spectrum of ZnTAPP exhibits Soret and Q bands at 421, 556, 596 nm and that of TAPP exhibited these bands at 412, 515, 552, 580, 634 nm at neutral and alkaline pH (Figure 1).

Presently, the photo-oxidation of phenol in aqueous alkaline and neutral solutions and photo-bactericidal activity (MBC) of TAPP and its ZnTAPP against three strains of bacteria were investigated.

To study photo-oxidation of phenol, aqueous solutions were prepared at two pHs (7.2 and 9.2) and illuminated with the light source as described above. At 30-minute intervals samples (∼2 mL) were removed and the absorption spectra were measured in the range of 190–340 nm. The light is absorbed only by the porphyrin chromospheres present in the system. The irradiation of the system at pH = 7.20 resulted in the appearance of a new absorption band at λ = 238 nm for both porphyrin compounds. The rate of the process increased at pH = 9.20 and the new band was observed at λ = 245 nm. The new absorption band at pH = 9.20 for TAPP formed completely; but in the case of ZnTAPP, it was not completed (Figure 2).

According to previous studies (Gerdes et al. 1997; DeRosa & Crutchley 2002; Mahvi et al. 2007; Sergeeva & Senge 2012; Drozd et al. 2014), the new absorption bands at λ = 245 nm (pH = 9.20) and λ = 238 nm (pH = 7.20) corresponded to p-benzoquinone, although some p-hydroquinone (λmax = 288 nm) was also observed.

The lower wavelength (at pH = 7.20) can be attributed to the interaction between tetra-cationic porphyrin compounds with p-benzoquinone. It was observed that alkaline pH is an effective factor for the photo-oxidation of phenol. Alkaline pH improves the speed of the formation of p-benzoquinone, and also the degree of phenol dissociation to phenoxide can be increased at this pH (Drozd et al. 2014). For this reason, in alkaline pH, the photo-sensitizers can be more effective than at neutral pH. In addition, differences in the absorption bands of phenol in the two media corresponded to the degree of phenol dissociation and formation of phenoxide.

Figure 3 shows that at both pHs, the formation rate of p-benzoquinone for TAPP is faster than for ZnTAPP; maximum photo-oxidation of phenol takes place after 30 minutes for TAPP and 60 minutes for ZnTAPP. It seems that TAPP is more effective than ZnTAPP for photo-oxidation of phenol. Furthermore, the rate of singlet oxygen production by TAPP is also higher than that by ZnTAPP.

Photodynamic activity of both porphyrin compounds was evaluated in vitro against three strains of bacteria: Gram-positive bacteria B. subtilis and B. strophaeus and Gram-negative bacterium P. aeruginosa. Figure 4 shows the percentage of photo-inactivation in the presence of these porphyrin compounds at various concentrations. For one set of negative controls, bacterial samples were illuminated in the absence of photo-sensitizers in the same conditions. None of the strains were sensitive to illumination in the absence of the compounds. In a second set of controls, the effect of the photo-sensitizers under dark conditions, without light activation, was also determined.
although the results are not shown in this figure. According to the results, both compounds at a concentration of 15 μg/mL exhibited MBC against B. subtilis and are 100% photo-bactericidal. In these same conditions and under darkness, the percentage inactivation of B. subtilis by TAPP and ZnTAPP was 39.4% and 50.6%, respectively. TAPP at a concentration of 10 μg/mL exhibited 100% bactericidal activity against B. strophaeus. The percentage of inactivation by TAPP under dark conditions at 5 μg/mL, 10 μg/mL and 15 μg/mL concentrations was 18.1%, 71% and 72.9%, respectively. ZnTAPP had no MBC effect against this strain at any concentration. Also, both compounds have no bactericidal effect against P. aeruginosa. Percentages of photo-inactivation for TAPP and ZnTAPP against P. aeruginosa at a concentration of 30 μg/mL are 38.4% and 34.1%, respectively; under dark conditions, only 7.7% and 6.28% inactivation, respectively, were achieved. For cationic compounds, toxicity under dark conditions is probably due to the presence of the quaternary ammonium charge, known to disorganize bacterial cell walls without light irradiation (Ringot et al. 2011).

According to our experiments, TAPP was more effective than ZnTAPP in photo-inactivation of the studied bacteria. Binding tests can be used to confirm this point, as described above. This test method was designed for the first time in this work. The result from the binding tests is shown in Table 1. According to these results, the percentage binding of TAPP to B. subtilis, B. strophaeus and P. aeruginosa
was 4.43%, 7.87% and 1.8%, respectively; and that of ZnTAPP was 2.21%, 1.5% and 1.08%, respectively. It can be argued that as the percentage binding of B. strophaeus to TAPP is more than other strains, TAPP can cause photo-inactivation of this bacterium at lower concentrations. Although both strains B. subtilis and B. strophaeus are Gram-positive strains, the porphyrin compounds were not equally effective in their photo-inactivation. The results of binding tests show that not only the structure of porphyrin compounds, but also the strain of bacterium is important in the photo-inactivation process. It seems that the external structure of the cell wall of B. subtilis is more rigid than that of B. strophaeus.

In comparison with previous reports (Gerdes et al. 1997; Murtinho et al. 2000; Nitzan & Ashkenazi 2001; DeRosa & Crutchley 2002; Reddi et al. 2002; Lazzeri & Durantini 2003; Lambrechts et al. 2005; Mahvi et al. 2007; Pudžiuvytė et al. 2008; Cormick et al. 2009; Gmurek & Miller 2012; Sergeeva & Senge 2012; Drozd et al. 2014; García-Méndez et al. 2014; Oviedo et al. 2014; Makrigianni et al. 2015), TAPP had several advantages, such as the use of lower power and an inexpensive irradiation system; and a lower dose to reduce the response and irradiation time for photo-oxidation of phenol and photo-inactivation of bacteria.

**CONCLUSIONS**

In summary, the photo-oxidation of phenol by irradiation with visible light in aqueous alkaline and neutral solutions in the presence of photo-sensitizers (TAPP, ZnTAPP) and oxygen were investigated. The photo-sensitizers exhibited different activities. It seems that TAPP was a better singlet oxygen generator than ZnTAPP. This assumption was validated by UV-Vis spectroscopy via the oxidation of phenol in two reaction media. A new absorption band at
λ = 245 nm (pH = 9.20) confirmed the production of p-benzoquinone as the photochemical product. It was also found that TAPP was more effective than ZnTAPP in the photo-inactivation of bacteria. MBC was achieved for B. subtilis and B. strophaeus using the synthesized compounds. The results obtained from the binding tests clearly indicate that not only the structure of porphyrin compounds, but also the strain of bacterium is important in the photo-inactivation process.

ACKNOWLEDGEMENT

The authors gratefully acknowledge the partial support from the Research Council of the Iran University of Science and Technology.

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First received 21 February 2015; accepted in revised form 15 May 2015. Available online 1 June 2015