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Microwave-assisted synthesis of 5,10,15,20-tetrakis(4-nitrophenyl)porphyrin and zinc derivative and study of their bacterial photoinactivation

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Abstract

In this study, 5,10,15,20-tetrakis(4-nitrophenyl)porphyrin (TNPP) and its zinc porphyrin complex (ZnTNPP) were synthesized in situ using the microwave method and identified by UV-Vis, FT-IR and ^{1}H NMR. The photostability and photodynamic antimicrobial activity (PACT) of these compounds were investigated on *Pseudomonas aeruginosa* and *Bacillus subtilis* under visible light irradiation. MIC, MBC and inhibition zones produced by these compounds were determined and the number of bacteria counted. The results indicated that both compounds have significant stability when illuminated for various illumination periods in nutrient broth media. Both compounds exhibited more effective activity against *P. aeruginosa* than *B. subtilis* in nutrient agar.

Keywords: Antimicrobial activity; *Bacillus subtilis*; Photoinactivation; *Pseudomonas aeruginosa*; TNPP.

Introduction

In recent years the preparation of molecules with antibacterial properties for use in a wide range of products, such as sanitary materials, household goods, medical equipment and food packaging, as well as other commodities

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has been constantly growing. Commonly, antibacterial agents with low molecular weight are used; for example, agents based on phenols, halogens (e.g., iodine), biguanides, heavy metals (e.g., silver, tin, and mercury), phosphonium salts and quaternary ammonium salts [1-3].

One group of these chemicals used as antibacterial agents, are porphyrin compounds. Porphyrins are important instances of macrocyclic complexes and have attracted much interest in the study of various processes. These compounds have low toxicity, as they are easily cleared from blood fluids and tissues [4,5]. Porphyrin derivatives are known to be efficient generators of singlet oxygen [6]. Hence, one of the applications of porphyrins is photodynamic therapy (PDT). Photodynamic antimicrobial chemotherapy (PACT) or antimicrobial photodynamic therapy (antimicrobial PDT), is a developed therapeutic option that applies visible light to photosensitive molecules to induce oxidative damage to microbial pathogens. PDT is based on the principle that porphyrins can selectively concentrate in tumor cells, and upon subsequent illumination in the presence of oxygen, they induce the production of reactive oxygen species. [4,5]. Upon reaction between these highly toxic oxygen radicals and cellular components, various biomolecules are rendered inactive and cell death results[3,7-9]. In many articles, cationic porphyrins have been used as antimicrobial agents in PACT [10-18].

On the other hand, the nitro group has a high oxidative reactivity. Some nitro containing compounds such as nitrofurans and nitroimidazoles are operative antibacterial agents; and are used extensively to battle anaerobic and protozoal infections [19]. Also, nitro-substituted aromatic compounds have been found to be effective electron affinity radiosensitizers. Nitro and amino groups can be easily functionalized and conjugated with bioactive molecules, such as monoclonal antibodies, oligomeric carboranyl phosphate diesters, polymer backbones and cyclodextrins [20,21]. Porphyrins substituted with electron withdrawing groups, such as nitro group(s), have been studied for tuning redox and photophysical properties and for further functionalization of the macrocycle [22,23]. Various approaches have also been used for photoactivation of porphyrin compounds [24-28]. Our aim in the present work was to investigate the effect of 5,10,15,20–tetrakis(4-nitrophenyl)porphyrin (TNPP) and its zinc porphyrin compound as neutral compounds having a nitro group, on photoinactivation of *P.aeruginosa* and *B. subtilis* by means of an irradiation source of

visible light. In addition, the photoinactivation efficiency of these compounds on the two strains was compared.

Experimental

General

Electronic spectra were measured on a UV-1700 pharma Spec UV-Vis spectrophotometer (Shimadzu) with a quartz cuvette. The FT-IR measurements were carried out with the help of Shimadzu FT-IR spectrometer in the form of KBr pellets. ^{1}HNMR spectra were obtained at 300 MHz with a Bruker instrument. Nutrient broth, nutrient agar and all chemicals used were purchased from Merck, without further purification. Pyrrole was distilled under vacuum.

Synthesis of porphyrins

TNPP was synthesized by microwave irradiation similar to article [29] with little changes. A Samsung domestic microwave was used for microwave irradiation. Lactic acid and nitrobenzene with 4-nitrobenzaldehyde (1.511 g, 0.01mol) and pyrrole (708 µL, 0.01mol) were mixed in a 100 mL Erlenmeyer flask. The flask was then transferred to the microwave and cooked for 15 minutes at 300 watts. Every 2 minutes the flask was taken out and shaken. After completion, the reactants were cooled to room temperature. Purple crystals were separated by suction filtration and a small amount of CH_3OH (20 mL) was added to the mixture and shaken vigorously. This mixture was allowed to stand overnight and then washed using a mixture of CH_2Cl_2 and C_2H_5OH (1:1). The purity of the product was confirmed by TLC. The crystals were vacuum dried (product yield was 30%).^{1}HNMR ($CDCl_3$) δ: -2.73 (s, 2H, NH), 7.76, 8.24 (d,d, 16H, C_6H_4), 8.87 (S, 8H, β-Pyrrole).

The isolated product (0.200 g, 0.28 mmol) along with zinc acetate, $Zn(OAc)_2.2H_2O$ (0.100 g, 0.45 mmol) were dissolved in DMF and heated to boiling for 30 min. After cooling, the resulting mixture was diluted with water (20 mL), yielding a dark precipitate. The product was filtered, air-dried and extracted with chloroform to yield the zinc porphyrin compound. The crystals were vacuum dried (product yield was 90 %).

Irradiation system

A 100 watt tungsten lamp was used as the illumination source. To absorb heat, a plate filled with water was used. The wavelength range for the lamp was between 350-800 nm. This system was setup in a shaker incubator at a rate of 80 rpm. All the experiments were carried out in a dark room to avoid light reflection.

Photostability study of prepared porphyrins

The photostability of prepared porphyrins was determined in DMF and nutrient broth upon illumination with a tungsten lamp. During irradiation, the solution was magnetically stirred at room temperature. The concentration of the porphyrin samples was monitored spectrophotometrically at times 0, 10, 20 and 30 min and the photostability was expressed as the percentage residual absorbance compared to absorbance measured before irradiation.

Photoinactivation of *Bacillus subtilis* and *Pseudomonas aeruginosa* on agar surface

Bacillus subtilis and *Pseudomonas aeruginosa* were obtained from University of Guilan. Both strains were grown aerobically at 37 °C in nutrient broth or nutrient agar overnight. 30 µL of these broths (~10^9 cfu/mL) were aseptically transferred onto nutrient agar plates and spread on the surface with a sterile spreader. Wells (diameter 0.5 cm) were made in nutrient agar seeded with the target strain, using sterile Pasteur pipette ends. A stock solution of TNPP and ZnTNPP was prepared in pure DMF at various concentrations and kept in the dark until use. Aliquots of 30 µL of different concentrations of porphyrins were added to these wells. The plates were incubated at 37 °C for 20 minutes in the dark and illuminated with the tungsten lamp (100 Watts) for 30 minutes. They were then incubated at 37 °C overnight in the dark. One well was filled with DMF in the same conditions, as negative control. A second negative control was also employed using porphyrins that were not photocativated. The experiments were carried out in triplicate. Bacterial growth was examined visually by measuring inhibition zones around the wells and also by viable colony counts of serially diluted samples of overnight incubated cultures inoculated onto nutrient agar plates. A diameter larger than 10 mm was considered as a positive response formally.

MIC determination for TNPP and ZnTNPP

Minimum inhibitory concentration (MIC) was determined by preparing nutrient broth inoculated with the bacterial strain and adding varying concentrations of photo-activated porphyrin compounds. The culture was incubated at 37 °C overnight and examined for bacterial growth under the microscope. Cultures exhibiting MIC were further analyzed to determine whether minimum bactericidal concentration (MBC) had been attained. To compare the effect of minimum inhibitory concentration of TNPP and ZnTNPP, ampicillin was used as a positive control for determining MIC.

MBC determination for TNPP and ZnTNPP

MBC was estimated for porphyrin concentrations giving a negative culture reaction in the MIC assay. 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 the presence of MBC.

Results and discussion

The absorption spectra of two porphyrin compounds were recorded in DMF (Figure 1). The absorption spectrum of TNPP and ZnTNPP exhibit a Soret band at 423 nm and 429 nm, respectively.

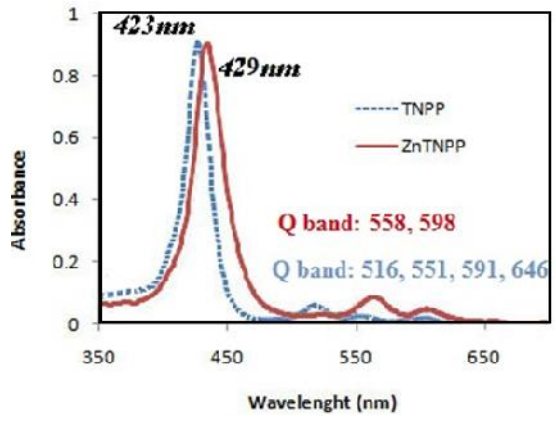

Figure 1. The UV-Vis spectra of TNPP and ZnTNPP in DMF

The FT-IR spectra of two porphyrin compounds are shown in Figure 2. The peaks present are due to: at 704 and 711 cm^{-1}, the presence of γCH meso phenyl for TNPP and ZnTNPP respectively; the peak around 1487 cm^{-1} is due to the presence of the aromatic skeleton for both compounds; the peaks at 1595 and 1647 cm^{-1} are due to the presence of ring C=N for TNPP and ZnTNPP respectively; the strong peaks around 1342-1518 cm^{-1} indicate the presence of nitro groups in the porphyrin structures [30, 31]. The short peaks at 3317 cm^{-1} and 964 cm^{-1} are due to NH groups and these specific peaks were missing in FT-IR spectra of ZnTNPP. The peak around 1000 cm^{-1} is due to the presence of Zn-N bonding.

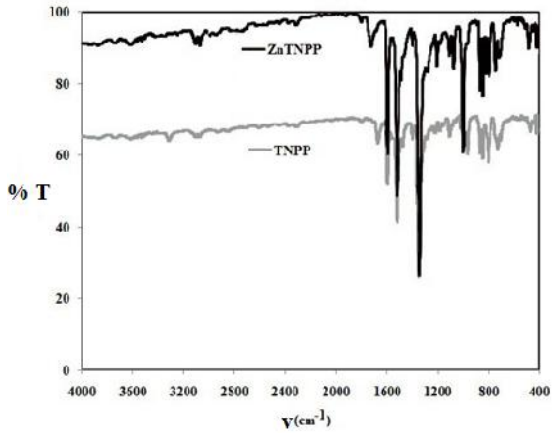

Figure 2. FT-IR spectrum of TNPP and ZnTNPP

The photostability study of these porphyrins was determined in DMF and nutrient broth upon illumination after 10, 20 and 30 min with the irradiation system; the results are shown in Figure 3. Photostability is expressed as the percentage residual absorbance relative to the absorbance

measured before irradiation. In DMF after 30 min irradiation, the residual absorbance of TNPP and ZnTNPP was determined to be 87.67% and 91.89%, whereas in nutrient broth under the same conditions, the residual activity for these compounds were 94.5% and 98.02%, respectively. Therefore the results show that both porphyrin compounds are generally more stable in nutrient broth. This can be attributed to the muddy color of nutrient broth.

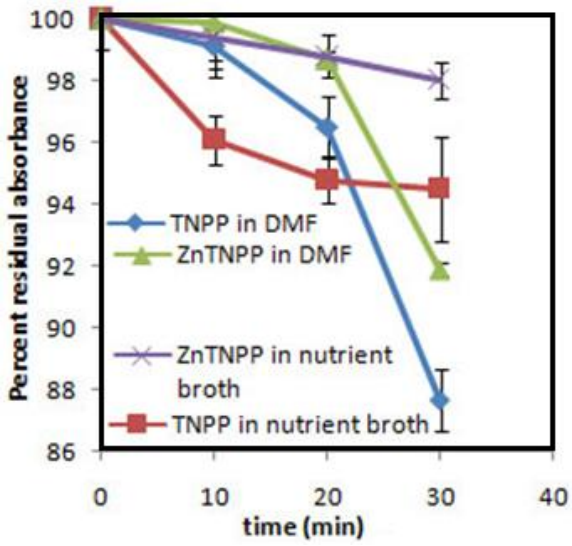

Figure 3. The photostability of TNPP and ZnTNPP in DMF and nutrient broth

The effect of various concentrations of TNPP and ZnTNPP against two strains of bacteria on agar surface, are shown in Table 1. Inhibition zones are shown in Figure 4; zones larger than 10 mm were considered as a positive response formally. The plates containing inactivated porphyrins did not show photoinactivation of the bacteria, with complete growth over the agar surface. As shown in Table 1, TNPP and ZnTNPP were more effective against *P. aeruginosa* than *B. subtilis*; in addition, TNPP made bigger inhibition zone than ZnTNPP.

Table 1. The effect of various concentrations of TNPP and ZnTNPP on selected strains

Diameter of inhibition zone (mm)

Concentration (µg/well)	***P. aeruginosa***		***B. subtilis***	
	TNPP	**ZnTNPP**	**TNPP**	**ZnTNPP**
60	19	19	20‡	15‡
45	18	18	20‡	15‡
30	15	16	19‡	15‡
15	11	14	19‡	15‡
3	8	10	18‡	10‡

‡A number of colonies started growing around the wells

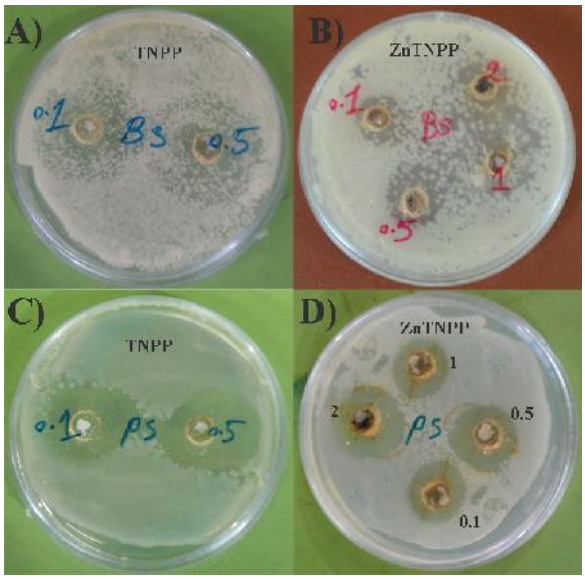

Figure 4. Inhibition zones of various concentrations of TNPP and ZnTNPP Pagainst *P. aeruginosa* and *B. subtilis*. a) TNPP against *B. subtilis*. b) ZnTNPP against *B. subtilis* c) TNPP against *P. aeruginosa* and d) ZnTNPP against *P. aeruginosa*

As shown in Figure 4, inhibition was complete inside the zone for *P. aeruginosa* whilst, sporadic colonies of *B. subtilis* started growing inside the inhibition zones, indicating the rapid emergence of resistant *B. subtilis* strains.

MIC determinations for both compounds were carried out at concentrations of 10, 30 and 60 µg/mL against the two bacterial strains. The results are shown in Table 2 and Figure 5. The number of viable colony forming units (cfu/mL) was determined after overnight incubation. Increasing the

concentration of activated porphyrins resulted in a decrease in the numberof bacteria.

At 60 µg/mL of ZnTNPP and TNPP, the number of *P. aeruginosa* decreased by ~1 log and ~2 log respectively, whereas the number of *B. subtilis* with both compounds decreased by ~0.7 log. However, inoculating the treated cultures onto agar plates resulted in bacterial growth, and therefore MBC was not reached at the above mentioned concentrations. Ampicillin showed MIC at 60 µg/mL for both isolates. Thus the potency of both porphyrins is similar to ampicillin.

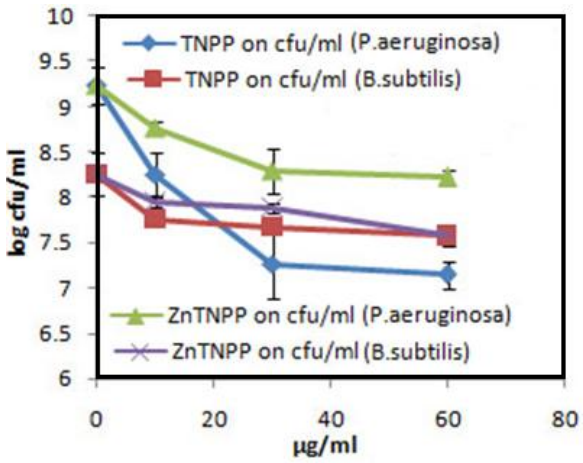

Figure 5. The effect of various concentrations of TNPP and ZnTNPP on bacteria after overnight incubation

Table 2. The effect of various concentrations of TNPP and ZnTNPP on log (cfu/mL) of two strains of bacteria after overnight incubation

	P. aeruginosa		***B. subtilis***	
Concentration (µg/mL)	**TNPP log(cfu/mL)**	**ZnTNPP log(cfu/mL)**	**TNPP log(cfu/mL)**	**ZnTNPP log(cfu/mL)**
10 µg/mL	8.25(±0.237) ‡	8.77(±0.075) ‡	7.75(±0.075) ‡	7.95(±0.050) ‡
30 µg/mL	7.26(±0.370) ‡	8.29(±0.241) ‡	7.67(±0.085) ‡	7.89(±0.055) ‡
60µg/mL	7.14(±0.150) ‡	8.22(±0.071) ‡	7.57(±0.110) ‡	7.58(±0.11) ‡
Untreated	9.23(±0.204) ‡	9.23(±0.204) ‡	8.25(±0.237) ‡	8.25(±0.237) ‡

‡Standard deviation measured for 3 experimental

The mechanism of radical oxygen singlet formation by porphyrins involves electronic excitations of their molecules to the first singlet excited state, followed by intersystem crossing with the formation of 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 [8, 32-34].The production of singlet oxygen plays an important role in the mechanism of action of porphyrins under light conditions.This molecule is able to react with almost every cellular ingredient, bringing about irreversible

damage that ultimately leads to cell death [35].

Conclusion

In this work, TNPP and ZnTNPP were synthesized and their application was considered in PACT. The results show that photoactivated TNPP and ZnTNPP have effective inhibitory activity against *B. subtilis* and *P. aeruginosa*, as compared with ampicillin. The interesting thing about TNPP and ZnTNPP antibacterial activity is that it seems to be more active against the Gram negative *P. aeruginosa*, than the Gram positive *B. subtilis*. Most novel antibacterial compounds tend to be more active against Gram positive isolates. In conclusion, preliminary results indicate that both porphyrins studied are potential antimicrobial agents which may be used at surfaces against bacteria, especially *P. aeruginosa*.

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