

NANOSCOPIC ZINC PHTHALOCYANINE: PHOTODYNAMIC ACTIVITY

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Introduction

Although photofrin is the most commonly used photosensitizer in PDT it has significant side effects [1]. Therefore, major effort has been invested in the development of new sensitizers. In particular, there is a need for new compounds that absorb light at longer wavelengths to assist tissue penetration, greater PDT efficiency, selective tissue localization and self-limiting minor skin photosensitivity.

Bown and Coworkers [2] showed that aluminium chlorophthalocyanine sulfonate (ClAlSPc) produced more prolonged photosensitization than HpD, but less skin sensitivity in ambient light. Chan et al [3] showed that AlSPc was eliminated from the deeper muscle layers more quickly than the superficial layers of the bladder wall leading to an increased concentration in the mucosa and lamina propria at 24h.

Wrenn et al and Frigerio [4,5] reported great affinity of neoplastic tissues for Pc. However, only the comprehensive research of the last few years has boosted these dyes to the position of viable candidates for PDT.

Pcs are synthetic porphyrins with a well-defined identity, whose pyrrole moieties are extended by condensation with extra benzene rings, and bridged by aza nitrogens instead of methine groups. The conjugated chromophore enables a substantial increase in the extinction coefficient of the Q absorption bands in the visible range of 675-700nm ($\epsilon > 10^5 \text{ M}^{-1} \text{ cm}^{-1}$) [6]. As with other large, planar molecules, most Pc substituted with hydrophilic groups form stacked

aggregates when dissolved in water as a result of the propensity of the hydrophobic skeleton to avoid contact with the water molecules. The disaggregation to monomeric dye can be achieved by addition of organic solvents, detergents or serum proteins to aqueous solution [7].

Pc macrocycle can chelate with virtually any element in the periodic table and can also be substituted at the periphery with diverse substituents. However, from the abundant data accumulated on in vitro and in vivo systems, ClAlPcS and ZnPc have emerged as the most common photosensitizers of this class. Since a long-life triplet state is a prerequisite for efficient photosensitization, a Pc dye containing a diamagnetic metal is better suited for this function than an analog compound containing a paramagnetic metal [8]. Diamagnetic metals such as In, Al, Zn etc which extend the triplet lifetime enhance phototoxicity. Metal free -Pc is inactive in sensitizing the photohemolysis of red blood cells [9] and shows only a very weak cell killing effect in experiments with human glioblastoma in vitro [10] and several other tissue culture cells (11) as well as with EMT – 6 [12] and C3H mammary carcinoma [13] in mice, suggesting that a central metal is a prerequisite for a good sensitizer.

Phthalocyanines are second generation photosensitizers for PDT of cancer [14,15]. Recently, Pc has been studied for use in PDT of blood for viral inactivation prior to transfusion to enhance the safety of blood supply [16,17]. The singlet oxygen quantum yield of ZnPcs is larger (0.45) than that of AlPcs (0.32)[18].

The sulfonate substitution of the Pc ring determines the dye solubility, the hydrophilic or lipophilic character, the amount of peripheral charges, the molecular size and consequently the cellular uptake and retention [20-26]. Inverse relationships between in vitro phototoxic effect and the degree of sulfonation were reported for ZnPcS [27-32]. Moreover large variations in photo reactivity were observed among the four isomeric disulfonated derivatives with the most hydrophobic isomer exhibiting highest reactivity.

Phthalocyanines tend to accumulate in membrane fractions and following photodynamic treatment, morphological changes in membrane organelles have been observed. This leads to the suggestion that plasma membrane, microsomes and mitochondria are the major sites of photodynamic action [33-36]. Therefore PDT induced cellular damage could be enhanced by membrane active drugs. The hemolysis of red blood cells photosensitized by ClAlPcs is a convenient model system for studying membrane aspects of photodynamic damage [9]. The phototoxicity of Pc is dependent on the presence of molecular oxygen [18] and these dyes are known to photosensitize the formation of $^1\text{O}_2$ [37-41]. ZnPc was a more potent dye for inactivating HSV per absorbed photon than HpD, polyhemato porphyrin esters or proflavine sulfate [42-45]. It was shown by Brasseuer et al [20] that mice receiving upto 11.3 mg/kg ZnPcS showed no apparent toxic reaction.

The very intense light absorption of Pc dyes at the red end of the light spectrum which is the region of effective tissue penetration, combined with a substantial chemical simplicity and stability, insignificant dark toxicity, preferential retention in malignant tumors minimal skin photosensitization and, last but not least, outstanding photodynamic activity solicits further consideration for this class of dyes in PDT. In the present study it was aimed to prepare nanoscopic ZnPc, characterize them and evaluate the photodynamic activity of them on human erythrocytes.

Experimental section

Materials and methods

Zinc phthalocyanine was purchased from Sigma Aldrich, and all the other reagents used in this experiment were Analar grade.

Preparation of nanoscopic zinc phthalocyanine colloids

Nanoscopic zinc phthalocyanine was prepared by the method reported by Weijun Liu et al [25]. In brief, a known quantity (2.5g, 6.7mmol) of surfactant, hexadecyltrimethyl ammonium

bromide was added to 400ml double de-ionised water and heated to 50°C under stirring to get a transparent solution. Zinc phthalocyanine 0.5mmol was prepared in 15 ml of concentrated sulphuric acid and added in drops to the surfactant solution under vigorous stirring between 0 and 5°C to give a transparent blue colloidal solution. The colloidal solution was then washed to neutral pH with water by using an ultra filter. A trace amount of H₂SO₄ was removed by anion - exchange resins. The neutral colloidal solution was then concentrated by using an ultrafilter and stored in dark at 4°C as 10µg/ml prior to use.

Sample irradiation

Light from xenon source filtered at 630nm with 20nm band pass filter was used as the light source for irradiating the sample. The microtitre plates having wells (2.5cm dia) containing 1ml of the sample were irradiated at different light doses with different concentrations of ZnPc nanoparticles. The irradiated cell suspension was centrifuged at 1500 rpm for 10min and the supernatant was pipetted out and its O.D at 413nm was measured using spectra photometer (Perkin Elmer Lamda 35) to quantify the percent hemolysis. The same procedure was repeated to study the role of scavengers such as sodium azide and glutathione reduced (GSH) by adding 1ml of each scavenger separately to 1ml of nanoscopic ZnPc in PBS during hemolysis. During irradiation the temperature of the samples did not increase by more than 1°C .

Results and Discussion

Characterization of nanoscopic zinc phthalocyanine

UV-Visble absorption spectrum of nanoscopic zinc phthalocyanine

The absorption spectrum of nano ZnPc between 500-750nm is shown in Fig. 1. There is a broad absorption maximum covering the entire visible range. In the broad absorption there are three overlapping absorbance maxima lying at about 610 675 and 775nm. Hence there might be three groups of different nanosize ZnPc particles. The intermolecular force in each group and the force by which each associates with solvent might be the cause for broadening of absorption.

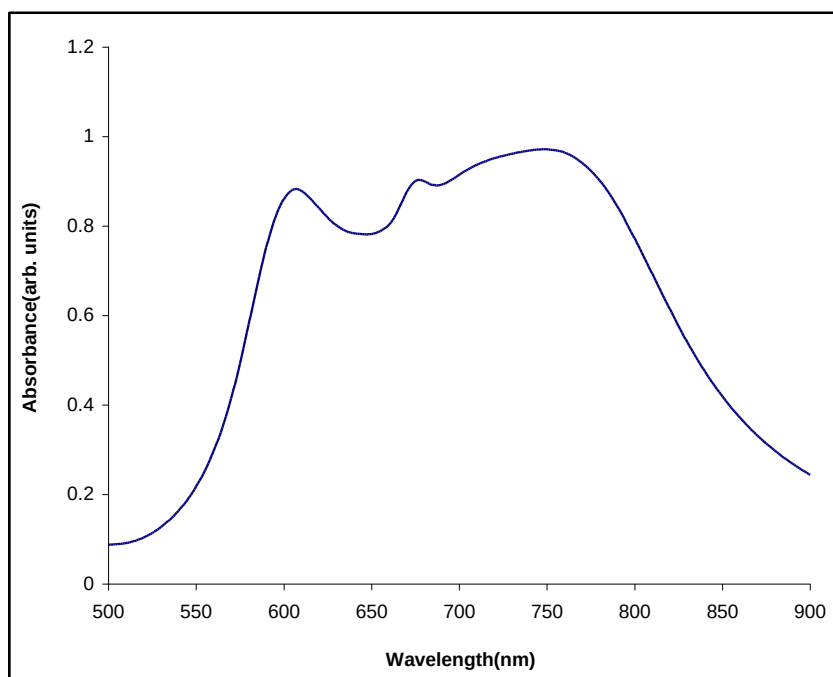


Fig.1 UV-Visible Absorption Spectrum of Nanoscopic Zinc Phthalocyanine

UV-Visible Absorption Spectrum of Zinc Phthalocyanine

Fig. 2 illustrates the absorption spectrum of ZnPc. In this spectrum two absorption maximum are clearly distinct, with the one occurring close to 675nm and the other at about 610nm. Change of this sharp absorption maxima to a broadened envelope in Fig. 3.1 is a clear indication for the formation of nanoscopic ZnPc [26].

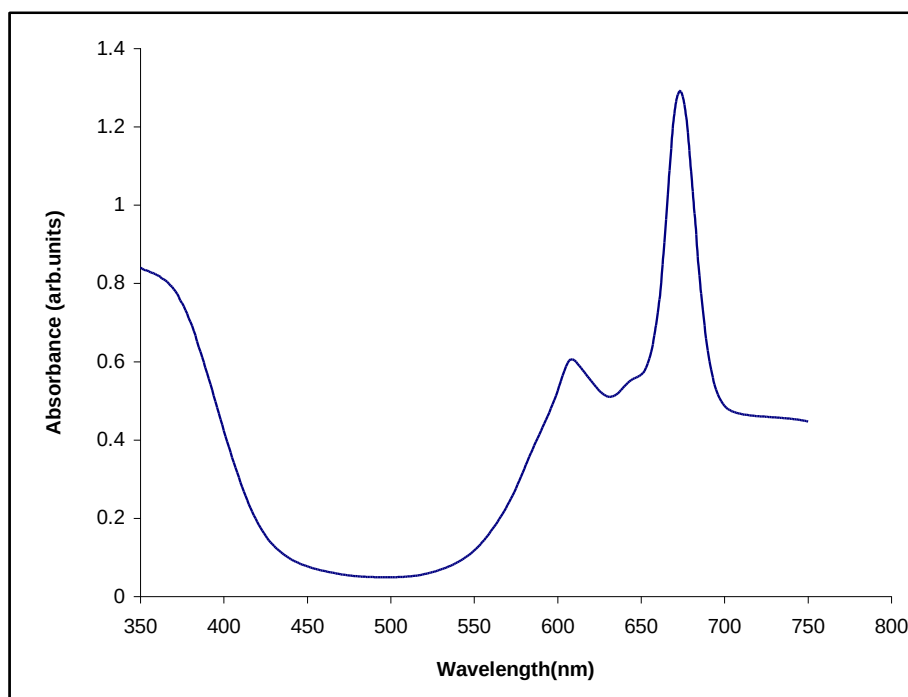


Fig.2. UV-Visble absorption spectrum of zinc phthalocyanine

Fluorescence characteristics of nanoscopic zinc phthalocyanine

The fluorescence spectrum of nanoscopic Pc excited at 610nm is shown in Fig.3. It gives an intense emission with the maximum at 680nm. Though the absorption spectrum is very much broad the emission spectrum gives a sharp absorption maximum, hence irrespective of the size distribution in nanoscopic Pc, emission appears to be independent of the size of the clusters. Therefore the emission is suggested to occur from the lowermost level of the excited state. There might be fast relaxation to the lowermost vibrational level of the excited state in each clusters. There might be rapid relaxation for a cluster with larger size but slow relaxation for those with smaller size.

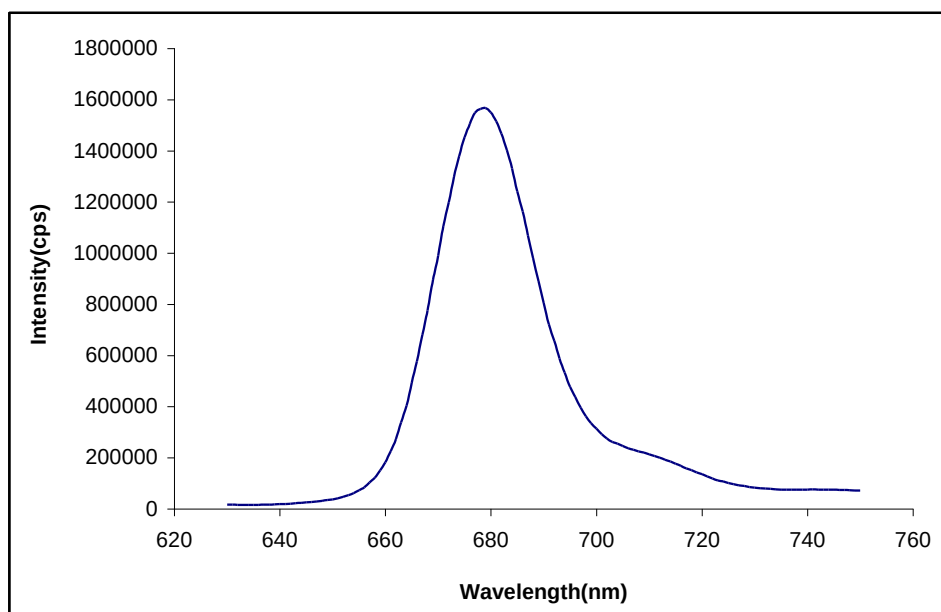


Fig. 3 The Fluorescence Spectrum of Nano-ZnPc excited at 610nm.

Fluorescence characteristics of ZnPc excited at 610nm

The fluorescence spectrum of ZnPc is shown in Fig.4. It yields two maxima at about 720 and 750nm. So there is significant shift to lower wavelength than the absorption spectrum. Hence the excited ZnPc might be enough relaxed by vibrations before emitting fluorescence. The absorption spectrum is nearly very sharp, whereas the emission spectrum appears to be broad due to overlap of two emission bands. Both absorption and emission spectra are comparable with that reported in literature [26]. Since the intensity appears to be same for both the states they could be equally populated.

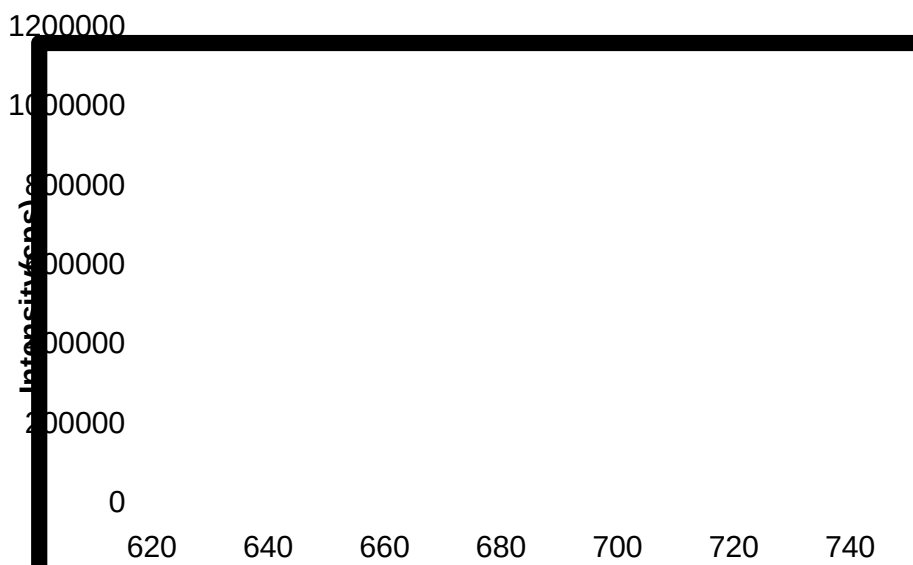


Fig. 4 The Fluorescence Spectrum of ZnPc excited at 610nm

The fluorescence spectrum shows sufficient intensity in the region extending from 700-750 nm. It is important for PDT, if precautions are taken for its insolubility in water.

FTIR spectrum of zincphthalocyanine.

The FTIR spectrum of ZnPc is shown in Fig 5. It shows aromatic C-H and N-H vibrations above 3000cm^{-1} . The C=N and aromatic ring skeletal vibrations show a peak at 1576cm^{-1} . The band between 1400 and 1408cm^{-1} are also due to aromatic ring skeletal vibrations. The intense peak at 1332cm^{-1} is due to C-N vibrations. The aromatic ring C-H in-plane bending modes give peaks between 1059 and 1117cm^{-1} . The characteristic out of plane bending vibration due to adjacent hydrogens of the aromatic ring gives a peak at 726.9cm^{-1} . The above details are also in accordance with those reported in the literature [26].

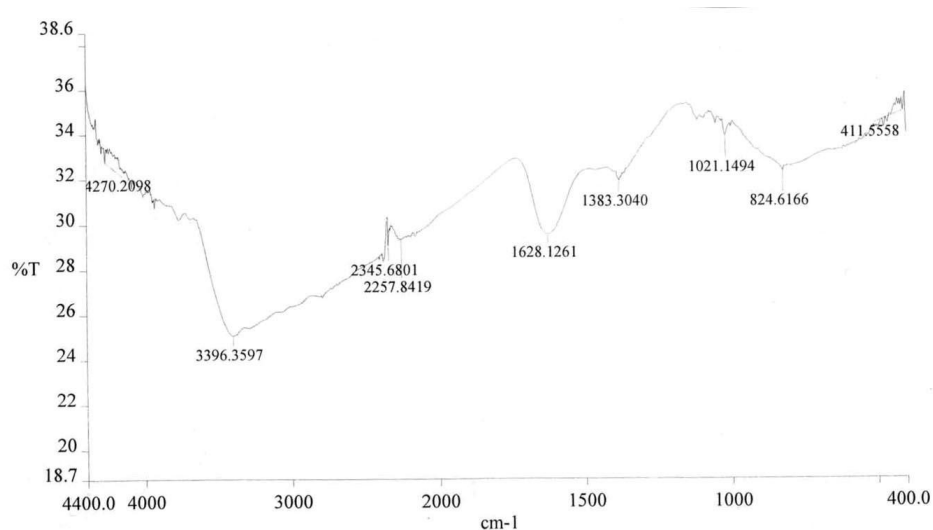


Fig.5 FTIR spectrum of Zincphthalocyanine

Atomic force microscopy

The AFM image of nanoscopic ZnPc shown in Fig.6. The picture clearly indicates clusters of Pc with different sizes. The clusters appears to be isolated with irregular morphologies without sharp edges all of them appear to be nearly spherical particles.

Photohemolysis using nano-ZnPc as photosensitizer

The photohemolysis studies were carried out under two different experimental conditions: first one at different light doses (38.5, 77.03, 115.5Jcm⁻²) at fixed concentrations of nanoscopic ZnPc, and the second one at different concentrations of nanoscopic ZnPc (2,4,6,8,10μg/ml) at fixed light dose.

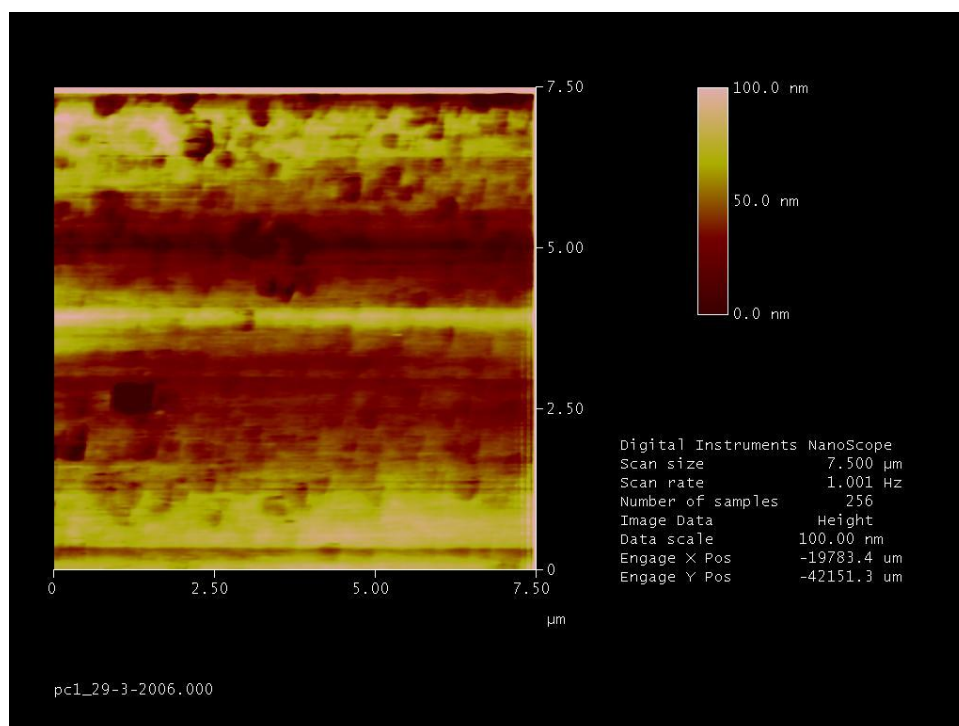


Fig.6 AFM picture of nano ZnPc

Photohemolysis as the function of fluences

The erythrocytes were irradiated as the function of fluence (light dose) at fixed concentrations of drugdose (nanoscopic ZnPc). From Fig.7. it is observed that the percent hemolysis increases with increase in fluence. The rate of photohemolysis also increases with increase in the concentration of nano ZnPc. The percent hemolysis for lower concentrations (2,4μg/ml) is found to be below 50% for all light doses. 50% hemolysis is achieved only for higher concentrations. The LD₅₀ (Lethal for 50% hemolysis) is found to be 85,70,65 Jcm⁻² for 6,8,10μg/ml concentrations respectively. From this it is clearly observed that the light dose is reduced when the sensitizer concentration is increased.

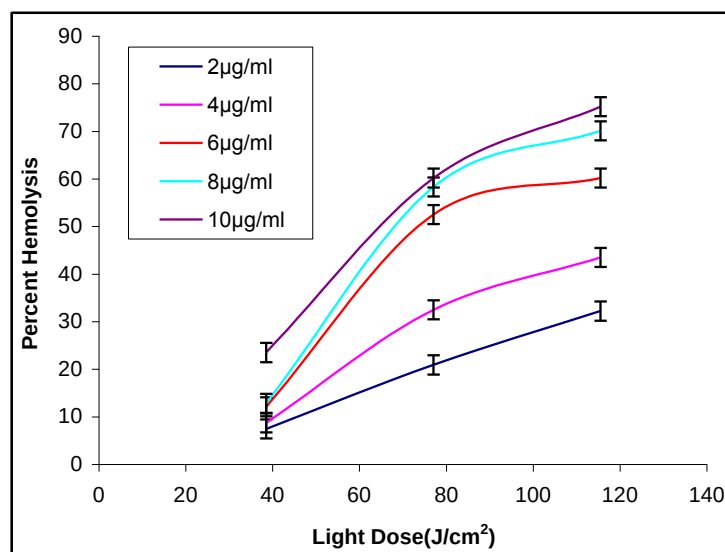


Fig.7 Effect of light dose on photohemolysis of nanoscopic ZnPc.

Photohemolysis as the function of concentration

The red blood cells were also irradiated as the function of concentration of nanosensitizer and lysis was determined at three different fluences viz 36.5, 77.03, 115.5 Jcm⁻². From Fig.8. it is observed that the lysis increases with sensitizer concentration as well as fluence. The LC₅₀ (Lethal concentration of nanoZnPc for 50 % hemolysis) is achieved only at 77.03 and 115.5 Jcm⁻² fluences and not at the light dose of 36.5 Jcm⁻². The LC₅₀ values for 77.03 Jcm⁻² fluence is 6µg/ml and that for 115.5 Jcm⁻² is found to be 5µg/ml. From Fig.8 it is clearly observed that the nanoscopic ZnPc induces photolysis effectively even at lesser light doses than that required for conventional photosensitizers ZnPc [26].

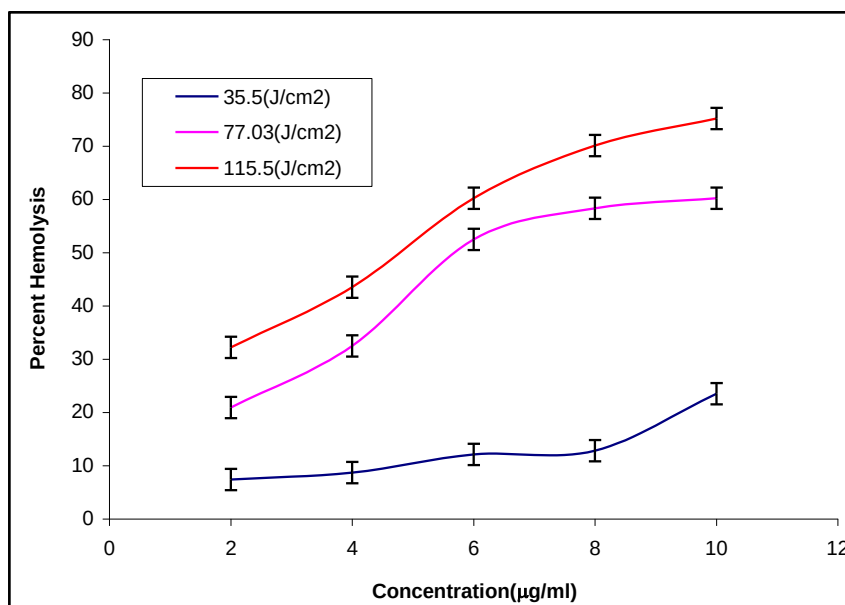


Fig.8 Effect of concentration on photohemolysis of nano-ZnPc

As the lysis depends both on concentration of nanosensitizer and light dose, the nature of photohemolysis is purely a chemical mediated one, which follows Type I /Type II mechanism.

Effect of scavengers

As it is clearly shown that photohemolysis depends on both drug and light dose, and the nature of mechanism is purely chemical mediated one, it is also aimed to study whether the hemolysis is due to Type I or Type II mechanism. In order to establish whether nanoscopic zinc phthalocyanine favours Type I and/or Type II mechanism photohemolysis was carried out in the presence of scavengers such as NaN_3 and GSH which are the best known quenchers for singlet oxygen and superoxide anion respectively. The inhibitions of photohemolysis were calculated by taking the corresponding percent hemolysis without scavenger as 100%. Fig. 9. shows the role of scavenger, NaN_3 , at various concentrations of nano-ZnPc. For all the concentrations (2-10 µg/ml) the percent hemolysis was considerably reduced indicating the formation of singlet oxygen during the photohemolysis process.

Moreover, inhibition of hemolysis increased as the concentration of NaN_3 was increased from 45 to 90mM.

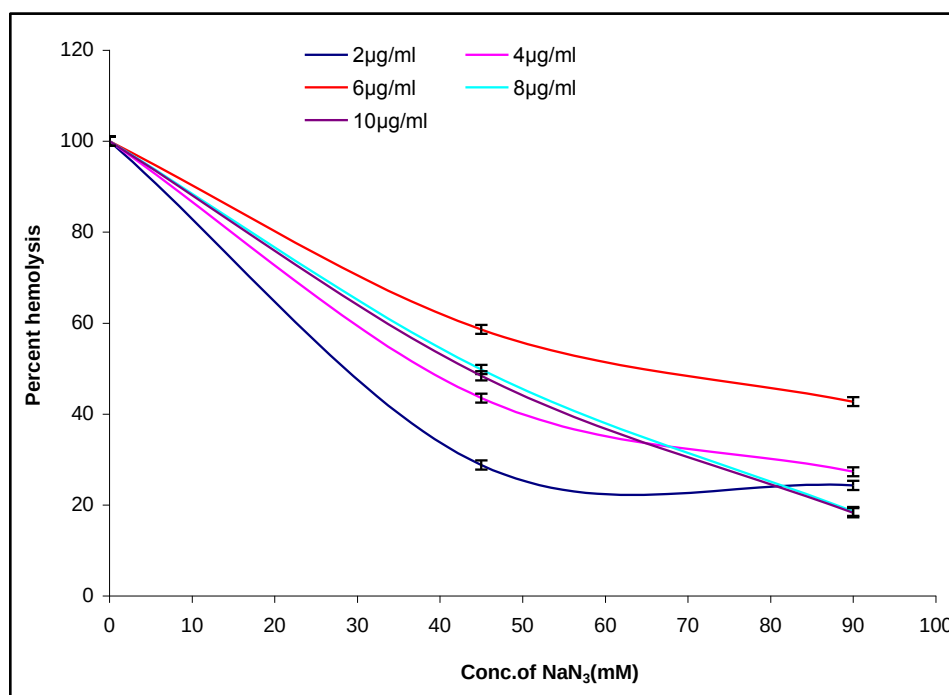


Fig.9 The effect of scavenger, NaN_3 on photohemolysis of nano-ZnPc

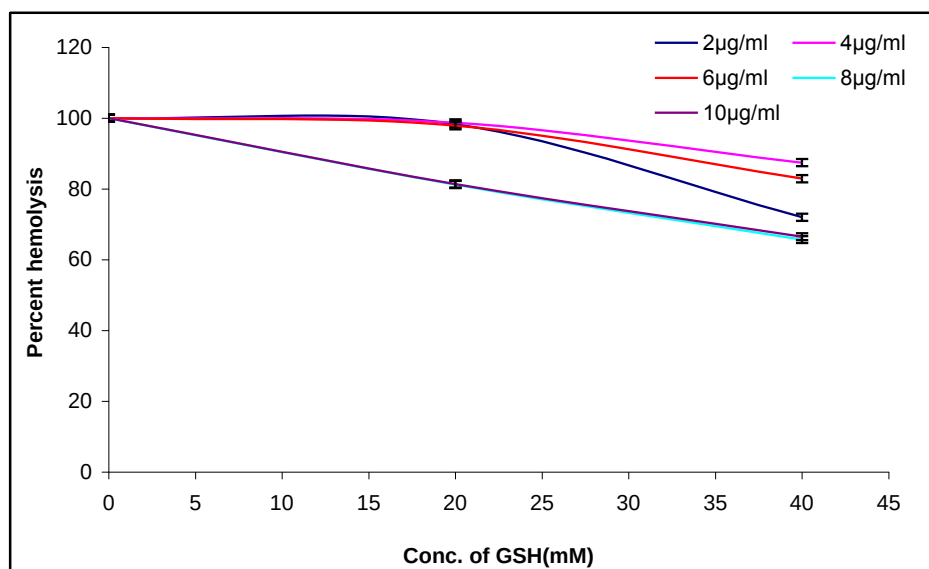


Fig. 10 The effect of scavenger, GSH on photohemolysis of nano-ZnPc

Fig.10. shows the role of scavenger GSH on the photohemolysis of various concentrations of nanoscopic ZnPc. The percent hemolysis largely remained invariant due to the presence of GSH. From Fig.9&10 it is observed that the formation of singlet oxygen is dominant over superoxide anion which is quenched by NaN_3 . A control experiment was carried out with different concentrations of nanoscopic ZnPc without irradiation and it is confirmed that the non-irradiated nanosensitizer was not toxic to red blood cells. Similarly non-irradiated nano ZnPc with scavengers GSH or NaN_3 showed no cell killing effect. The standard errors in triplicate measurements of the same suspension were 2-3%.

Conclusion

In the present study human erythrocytes were effectively killed by photoexcited nanoscopic ZnPc photosensitizer in vitro. The percent hemolysis was considerably increased by both increase in concentration of the sensitizer and fluence. When the concentration is increased from 2 to 10 µg/ml 80% hemolysis was achieved for higher fluences. The role of scavenger

NaN_3 shows that there is considerable formation of singlet oxygen during photoexcitation of nanoscopic ZnPc. The effect of GSH on photohemolysis is less when compared to that of NaN_3 . It indicates the formation of singlet oxygen is dominant over that of superoxide anion. Therefore it is concluded that even though the PDA of nanoscopic ZnPc favours both Type I and Type II mechanism the latter one predominates than the former. The unexposed nano-photosensitizer is found to be non toxic towards red blood cells. Therefore it is concluded that the use of Zinc phthalocyanine in the nanoscopic state might be a convenient route to study its photodynamic activity and light irradiated nanoscopic ZnPc can be a convenient substitute for the conventional photosensitizers.

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