CHAPTER 4

N-Confused Porphyrin Derivatives as PDT Sensitizers

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4.1 Abstract

In recent years, photodynamic therapy (PDT) has emerged as a promising and noninvasive treatment for various types of cancer. The technique involves controlled generation of short-lived cytotoxic agents within a cell on irradiation of a prodrug or photosensitizer, which in turn destroys the affected cells. This chapter describes the PDT application of two NCP derivatives, meso-hydroxy (NCPH) and p-sulfonato-phenyl substituted (NCPS) derivatives, which have better molar extinction coefficient in the red region of visible light compared with normal porphyrin derivatives. Photophysical studies of the molecules have been conducted in polar solvents such as deionized water and MeOH, which gives promising values for parameters required for PDT such as triplet quantum yield and singlet oxygen quantum yield. In vitro anticancer studies were conducted with both the derivative, where a series of cancer cell lines showed promising IC₅₀. Upon illumination, the NCPS exhibited more photocytotoxicity to adenocarcinomas than the other epithelial cell lines, and maximum activity has been attributed toward breast adenocarcinoma MDA-MB-231 cells, with an IC₅₀ value as low as 6 µM, where corresponding value for NCPH was 12 µM. Generation of reactive oxygen species and apoptosis induced pathway of cell death was established through various experiments such as flow cytometry, fluorescent imaging, mitochondrial membrane potential assay and PARP cleavage.
4.2 Introduction

Photodynamic therapy (PDT) is a non invasive technique for treatment of cancer, in which cell death and tissue eradication are achieved on irradiation of a photosensitizer and the subsequent production of cytotoxic species, in particular singlet molecular oxygen [168]. The technique works as combination of three components; photosensitizer (drug), light and oxygen. Controlled generation and deactivation of short-lived cytotoxic agents within a cell on irradiation of a prodrug or photosensitizer is the key step in PDT. Light excitation of a dye causes an intermolecular triplet-triplet energy transfer that generates the highly reactive cytotoxic agent, singlet oxygen molecule ($^1O_2$), within a target region, which in turn destroys the affected cells. The whole photochemical process during PDT is described in Figure 4.1.

![Figure 4.1. Modified Jablonski diagram for the formation of singlet oxygen [168].](image)
4.2.1. History of Photodynamic therapy

The history of usage of light for the treatment of various diseases is starting from antiquity, where the light energy has been employed as such or coupled with a drug. Even thousand years before people in Egypt, China and India utilized light for different skin diseases such as psoriasis, vitiligo, rickets and even psychosis and known as phototherapy [169-171]. The ancient Greeks employed whole-body sun exposure or heliotherapy in the treatment of disease and the Greek physician Herodotus is known as the father of this treatment. Light was used for treatment of various diseases, including tuberculosis, rickets, scurvy, rheumatism, paralysis, edema and muscle weakness in France [172].

However, the technique of using light as a treatment tool attained phenomenal development and popularity in the medicinal field only in the last few decades. Phototherapy was further developed by the Danish physician, Niels Finsen, who described the successful treatment of smallpox using red light and was rewarded with Nobel Prize in 1903 for his contributions [173].

In 1907, two German scientists, Herman Von Tappeiner and his student Oscar Raab, introduced the concept of cell death by the interaction of light and chemicals. During their investigation on the effects of acridine on malaria causing protozoa, Raab found that the combination of acridine red and light had a lethal effect on infusoria, a species of paramecium [174]. However, the first medical application of the interaction between a compound or a drug and light was demonstrated by von Tappeiner along with Jesionek, in which they used a combination of topical eosin and white light to treat skin tumors and also explained the requirement of oxygen for the treatment. And it was Von Tappeiner, who called the technique for the first time as ‘Photodynamic therapy’ [175-177].
4.2.2 Sensitizers in PDT

A suitable sensitizer is the primary requirement in PDT, and it should have the following criteria [178-179].

a) Maximum absorption in the 600-800 nm region and minimum in the 400-600 nm: The incident intensity of light irradiance is reduced by absorption by chromophores in tissue (400-600 nm) or by scattering. This efficiency loss can be minimized by using higher wavelength light sources. On the other side, absorption by water molecules increases at wavelengths above 800 nm. Consequently, the window for optimum penetration lies between 600 and 800 nm, i.e. in the region of red light, and in PDT it is known as the therapeutic window.

b) High singlet oxygen quantum yields ($^1\text{O}_2$): Singlet oxygen is the reactive oxygen species that destroys the cancerous tissues in PDT. Hence high efficiency of a sensitizer to generate singlet oxygen is related to the possibility to use that sensitizer as a PDT drug. The efficiency of singlet oxygen generation by the sensitizer in vivo depends on various factors such as interaction of the sensitizer with surrounding biopolymers, aggregation of the sensitizer, oxygen depletion and other side reactions. However, the photophysical calculation of quantum yield should not be overestimated.

c) Specific retention in the malignant tissue: The specific retention of a sensitizer in the malignant tissue and effective removal from the malignant and healthy tissue are other important criteria.

d) Photostability, non-toxicity and phototoxicity: A suitable sensitizer should be stable against photodegradation or photobleaching and oxidation by $^1\text{O}_2$ or other reactive oxygen species generated in situ during the therapy. Photobleaching of sensitizers in biological systems is a complex process, decreases the skin sensitization and the specificity of phototreatment. Also, low “dark” toxicity (non-toxic in the absence of
light) is desirable so as to avoid unnecessary strain on the organism prior to irradiation, but the destructive photodynamic effect only in the presence of light, phototoxicity. In addition, high solubility in water, purity and fluorescence properties are other criteria desirable for the therapy. Sensitizers used in PDT can be classified as porphyrinoid sensitizers and non-porphyrin sensitizers. The technique became popular and is used widely in medicinal applications recently, only because of some important porphyrin based photosensitizers. However, there are many non-porphyrin based photosensitizers tested and used for the therapy even from ancient times.

### 4.2.2.1 Non-porphyrin photosensitizers

The non-porphyrin based photosensitizers such as psoralens, anthracyclines, hypericin and hypocrellin, methylene blue, acridine and nile blue analogue were used for treatment of various skin diseases and tumors [180]. For example, the oral administration of the psoralen drug leads to its uptake by malignant T-cells in the blood stream. The blood aliquot taken was illuminated with white light and found that the light illumination leads to DNA damage via photoadduct formation and thus to direct cytotoxicity. A member in the anthracyclines family, Doxorubicin has also shown phototoxicity *in vitro* with high selectivity. Sensitizers in Hypericin and Hypocrellin family were found to be inhibitors of protein kinase C (PKC), a key enzyme in the proliferation of tumour cells. Methylene blue (MB) on the other hand was not used much in direct application in PDT but found applications in the clinical diagnosis of a variety of diseases and as a tumour marker in surgery. Also, Nile blue is taken up extremely well by tumour cells, thus making it an excellent tumour marker. However, these compounds suffer from a major drawback due to their inherent dark cytotoxicity [180-181].

Recently, many borondipyrrromethane (BODIPY) based sensitizers were reported with promising PDT activity. Nagano and co-workers demonstrated the synthesis of a
diiodo substituted BODIPY molecule with enhanced singlet oxygen quantum yield and PDT application, which is explained by heavy atom effect and subsequently enhanced the intersystem crossing efficiency from singlet to triplet state that controls the singlet oxygen production [182]. A series of azadipyromethenes were synthesised by O’Shea and co-workers with high molar extinction coefficient in the far-red region and demonstrated their efficacy in light-induced toxicity in a panel of tumor cell line [183, 184].

4.2.2.2 Porphyrin photosensitizers

Majority of sensitizers investigated and approved for medical use were with porphyrinoid structure. Even though porphyrins were identified in the mid-19th century, until the early 20th century there was no report for their medicinal application, when hematoporphyrin, a mixture of porphyrin derivative was first isolated by Scherer in 1841 during investigating the nature of blood [185]. Dried blood was heated with con.H₂SO₄ the precipitate was washed free of iron and then treated with alcohol to isolate the porphyrin derivative. But, it was only in 1911, Hausmann performed the first studies of the biological effects of hematoporphyrin, where he investigated the effect of hematoporphyrin and light on paramecium and red blood cells and described skin reactions in mice exposed to light after hematoporphyrin administration [186]. However, the first report of human photosensitization by porphyrins was in 1913 by the German, Friedrich Meyer-Betz. He injected himself with 200 mg of hematoporphyrin and subsequently noticed prolonged pain and swelling in light-exposed areas [187].

In 1955, Schwartz et al. revealed the multi component nature of hematoporphyrin, where after partial purification, the pure hematoporphyrin produced localized only very poorly in tumors, whereas the residue left behind had great affinity for tumor tissues. Schwartz continued to purify the non-hematoporphyrin fraction, finally end up with a
single fraction, hematoporphyrin derivative (HpD). Interestingly, this substance was found to be approximately twice as phototoxic as crude hematoporphyrin [188]. Later, Lipson along with Baldes demonstrated effective in tumor localization property of HpD [189, 190].

The milestone in the history of PDT development was achieved in 1975, when Dougherty and co-workers reported the first successful complete tumor cure with HpD activated with red light in the treatment of experimental animal tumors [191]. In the same year, J. F. Kelly demonstrated that human bladder tumor cells transplanted into mice could be destroyed using PDT [192]. After a year Kelly tested the PDT activity of HpD in human with bladder cancer [193], followed by other report on human for various carcinoma such as skin and esophageal using HpD as the photosensitizer [194-196].

In 1983 Dougherty proposed that the active component of HpD was composed of two porphyrin units linked by an ether bond and the compound was given the abbreviated name as dihematoporphyrin ether (DHE). Further analysis suggested that the active component of HpD comprised of a mixture of porphyrin rings, linked by a number of ether and ester bonds, which is now commercially known as ‘Photofrin®’(Figure 4.2) and is the first photosensitizer approved for clinical use by different health organizations [197-199]. Followed by Photofrin,® there were few more drugs approved for clinical use, such as Foscan®, Protoporphyrin IX, Visudyne®, Motexafin lutetium, Podoporfin and Padeliporfin etc (Figure 4.2). Among these, Temoporfin (Foscan®) was the first sensitizer used in a formal clinical study of PDT for prostate cancer [200, 201]. Endogenous Protoporphyrin IX photosensitization induced by exogenous administration of ALA led to the FDA approval in 2000, which marked another historic event for PDT. Another clinically used porphyrin sensitizer, a benzoporphyrin derivative, Verteporfin, commercially known as Visudyne®, has been used primarily for ocular PDT and
Figure 4.2. Clinically used porphyrin photosensitizers.
approved for age-related macular degeneration (AMD) worldwide [203]. Motexafin lutetium, a texaphyrin derivative, has been used in the post radiotherapy, breast cancer and ophthalmic disease [204-206]. Padoporfín and Padeliporfín are two palladium based photosensitizers, where Padoporfín was lipophilic and Padeliporfín was water soluble. Both the sensitizers were investigated for prostate clinical studies [207, 208]. The historical developments of PDT sensitizers were summarized in Figure 4.3.

**Figure 4.3.** Timeline of selected milestones in the historical development of PDT [209].
Curiosity and enthusiasm of researchers all over the world facilitates the introduction of many more photosensitizers including normal, expanded, core-modified, metalloporphyrins, phthalocyanines, chlorin derivatives and porphycene, which shows enhanced singlet oxygen quantum yield and photocytotoxicity [210-214]. The photosensitizers reported were classified as first, second and third generation photosensitizers. The first generation sensitizers were the early stage sensitizers where mixture of porphyrin derivative used as such for therapy, which includes the clinically approved Photofrin®. The major drawbacks of these sensitizers were their complex nature, long retention in tissues and low molar extinction coefficient at the therapeutic window, which in fact, encourage the introduction of second generation photosensitizers [215]. The second generation photosensitizers, on the other hand were chemically pure and exhibit high absorbance in the red region of visible light with high singlet oxygen quantum yield, which include majority of porphyrins and phthalocyanines based sensitizers reported recently [210-214]. Still, selectivity towards the malignant tissues remained unsolved, which is tried to rectify in the third generation sensitizers. The third generation sensitizers were combination of a carrier and sensitizer, where the drug was released only at the respective sites [216, 217].

4.2.3 Light sources in PDT

As discussed earlier, light is an important component in PDT and have a critical role in determining the efficiency of the therapy, which in turn related to penetration depth. The penetration depth is the distance that light can travel through body and dependant on power and wavelength of the light used, and optical properties of the tissue including reflection, scattering, transmission and absorption. Light with wavelength shorter than 580 nm is not suitable because of strong absorption by haemoglobin and pigment-rich tissues, such as those of melanoma and typically, the depth of penetration is from 3 to 8.
mm for light in the range from 630 to 800 nm. Light dosimetry, which includes two parameters, fluence and fluence rate is an important vector to determine the efficiency of PDT treatment. Fluence is the total energy of exposed light across a sectional area of irradiated spot (energy per unit area of exposed light, J/m²), whereas fluence rate is the radiant energy incident per second across a sectional area of irradiated spot (power per unit area of light, W/m²). It has been reported by Wilson et al. that an increased fluence rate can reduce the photodynamic effect due to depletion of oxygen [218].

Accurate light dosimetry in internal organs can be achieved only by appropriate light sources. For some time, lasers have been considered as the perfect light sources for PDT, as they could provide monochromatic light corresponding to different absorption maxima of photosensitizers. But, these ‘old generation’ laser sources were rather complicated to use, to install and were very expensive compared to the new available sources. For the last fifteen years diode lasers coupled with optical fibres emerged as new light source for PDT, which are simple to use, easy to transport, due to their relatively small sizes. Recently, an innovative diode laser with dedicated software has been developed for interstitial PDT, which enables real time, patient specific and optimized interstitial PDT [219]. The weakness of diode lasers’ systems is that they can offer only one wavelength and are not tunable.

In recent years, improvements in semiconductor technology have substantially increased the light output of light emitting diode (LED) chips. An aluminium gallium arsenide (AlGaAs) based semiconductor was developed to emit light with peak wavelengths of 680 to 730 nm which are optimal wavelengths for the absorption spectrum of second generation photosensitizers used for cancer PDT. When large surfaces are to be treated, LED light sources are a safer and lower cost source of light. In modern technology, optical delivery system carries the light to the respective organs for
the therapeutic action. Here, optical fibres fitted with specific devices at distal end so as to distribute a defined, controlled and homogeneous subthermal light dose to organs to be treated. The core size of such fibres varies from 200 to 600 microns with overall sizes around 1 to 1.5 mm and is used through the working channel of endoscopes [219]. Also, better distribution of light was assured by using diffusers of same geometry of the organ to be treated (skin, lung, uterus; Figure 4.4).

Figure 4.4. Different shape diffusers for PDT.

4.2.4 Role of oxygen in PDT

The crucial step in PDT is generation of molecular singlet oxygen from ground state oxygen, which is in triplet state. The singlet molecular oxygen was discovered in 1924, but attained the interest of many researchers only after 1963 mainly by the research of Khan and Kasha [220]. The growing interest in the formation and deactivation of singlet oxygen revealed the mechanism and amount of energy involved in the whole process of generation and deactivation as shown in Figure 4.5.

Figure 4.5. Electronic transitions involved in the generation of singlet oxygen in a solution [179].
Molecular oxygen has two low-lying singlet excited states, $^1\Delta_g$ (95 kJ mol$^{-1}$ or 22.5 kcal mol$^{-1}$) and $^1\Sigma_g^+$ (158 kJ mol$^{-1}$ or 31.5 kcal mol$^{-1}$) above the triplet state (Figure 4.5). For ground-state oxygen, the two highest energy electrons reside separately in the outermost antibonding orbitals with same spin: $(\sigma_{2p})^2(\pi_{2px})^2(\pi_{2py})^2(\pi_{2px}^*)^1(\pi_{2py}^*)^1$, while in the singlet oxygen, the two highest energy electrons reside together in the same antibonding orbital with opposite spin: $(\sigma_{2p})^2(\pi_{2px})^2(\pi_{2py})^2(\pi_{2px}^*)^2$. The first excited state, O$_2(^1\Delta_g)$ is more stable compared to the second excited state O$_2(^1\Sigma_g^+)$ due to spin allowed transition to the first excited state, where the transition from the first singlet to ground excited state is spin forbidden [221].

In fact, generation and quenching of the highly reactive singlet oxygen is the key step in PDT. In photosensitized generation of $^1$O$_2$, the sensitizer excitation is generally achieved via a one photon excitation, followed by transition from ground state ($S_0$) to singlet excited state ($S_n$). Energy transfer through intersystem crossing makes available a long lived triplet state ($T_1$) of the sensitizer, which can avail various paths to deactivate including radiative and non-radiative. In non radiative relaxation it can follow two mechanisms, Type I and type II mechanisms. Type I mechanism involves the energy transfer to a substrate molecule by hydrogen-atom abstraction or electron-transfer between the excited sensitizer and a substrate, yielding free radicals. These radicals can react with oxygen to form an active oxygen species such as the superoxide radical anion. Where in Type II mechanism, a triplet-triplet energy transfer from the excited triplet state of the sensitizer to the ground state triplet oxygen to generate singlet [221].

Once, after the generation of $^1$O$_2$ it can follow mainly two channels for deactivation, physical and chemical. In physical quenching there will be no conception of oxygen or product formation, but in chemical quenching the $^1$O$_2$ react with the quencher to form a new product. The stability of singlet excited state is expressed in terms of lifetime values,
which are 45 min and 7-12 sec in the gas phase and $10^{-6}$-$10^{-3}$ and $10^{-11}$-$10^{-9}$ sec in the solution for the first and second excited states respectively [221-223]. Hence, normally the term singlet oxygen refers to the first excited state of molecular oxygen ($^1\Delta_g$) and can be represented as $^1\text{O}_2$. However, the lifetime of $^1\text{O}_2$ is considerably shorter in cellular systems, ranging from 100 ns in the lipid regions of membranes to 250 ns in the cytoplasm and hence, the diffusion range of $^1\text{O}_2$ is predicted to be limited to approximately 45 nm in cellular media [224]. On the other hand the diameter of human cells ranges from approximately 10 to 100 mm, which limit the action of this reactive oxygen species within the cell limit [224].

4.2.5 Apoptosis; mechanism of cell death

Apoptosis is defined as programmed cell death, and is a normal physiological process essential for the control of tissue development and homeostasis. This tightly regulated cell suicide process is controlled by both intracellular and extracellular signals. Apoptosis contributes towards a characteristic sequence of morphological and biochemical changes for the systematic dismantling of the cell and preparation of the residual cell component. Most importantly, no tissue inflammation occurs due to the process as the intracellular leakage limits to the immediate environment [225-227].

In fact, during PDT the cell death is an outcome of induced apoptosis, and mitochondrion, one of the major targets inside the cell for PDT induced cell death. Mitochondrial damage occurs during PDT through inhibition of electron transport components, including succinate dehydrogenase and cytochrome c oxidase, and also disrupted the mitochondrial electrochemical gradient [228]. Apoptotic cells exhibit distinct morphological change, such as shrunken cells with condensed nuclear chromatin, mitochondrial potential difference, cleavage of poly(ADP-ribose) polymerase (PARP), which is a DNA repair protein etc. During physiological cell turnover, apoptosis is
initiated by depletion of a growth factor or by the interaction of cytokines or other ligands with cell surface receptors [227].

**4.3 Objective of the work**

The increased popularity of PDT in recent years, intensify the urge for developing new photosensitizers. In light of the above discussion, there are a few necessary requirements for such sensitizers for practical application, which include solubility in polar solvents and high molar extinction coefficient in the red region. On the other hand, the porphyrin isomer, NCP is known for their highly red-shifted absorption compared to the normal porphyrin derivative. Here, this chapter introduces two novel NCP derivatives as PDT sensitizer, hydroxyl and sulfonated derivatives of NCP. The compounds were highly soluble in polar solvents, especially sulfonated derivative, which is even water soluble. Various photophysical analyses such as UV-Vis absorption spectroscopy, triplet absorption spectra, energy transfer calculations, lifetime calculation and singlet oxygen generation quantum yield calculation reveals the potential candidacy of these derivatives as PDT drug. *In vitro* analyses of the sulfonated derivative was promising with competitive IC₅₀ values.

**4.4 Result and discussion**

**4.4.1 NCPH as a PDT sensitizer**

Synthesis and characterization of *meso*-tetrakis(3,5-dihydroxyphenyl)N-Confused Porphyrin (NCPH) is described in the previous chapter. Red-shifted absorption spectrum of NCPH, compared to the normal porphyrin derivative was encouraging enough to investigate the PDT application of the molecule. Molar extinction coefficient of NCPH at 443 nm and 730 nm was $9.07 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ and $5636 \text{ M}^{-1}\text{cm}^{-1}$ respectively. As we discussed, a stable triplet state of the sensitizer is one of the preliminary criterion that a
sensitizer should have for being PDT active. Triplet state lifetime and triplet energy transfer efficiency are two parameters that really contribute towards the energy transfer to the ground state oxygen to generate singlet oxygen. Transient state properties of NCPH were calculated by using a nanosecond laser flash photolysis with 355 nm laser pulse. The efficiency of energy transfer was calculated with respect to the energy transfer efficiency of tris(bipyridyl)ruthenium(II)complex [Ru(bpy)$_3^{2+}$] to β-carotene, whose efficiency is considered as hundred percent [229, 230]. For transient absorption studies a 6 μM solution of NCPH in MeOH has taken and degassed with argon. A 355 nm laser pulse was used for excitation, where the excited state was probed by a xenon lamp. The triplet decay profile and absorption spectrum is given in Figure 4.6. Lifetime of NCPH was calculated as 1.44 μs with rate of decay is 0.69 μs$^{-1}$. Triplet absorption of NCPH gives maxima at 500 nm with bleach at 450 nm, where ground state absorption of the molecule predominates. Existence of triplet state was confirmed by the quenching of triplet absorption in the presence of dissolved oxygen.

![Figure 4.6.](image)

Figure 4.6. Triplet absorption of NCPH in methanol. Inset shows the transient decay at 500 nm.
Optically matched solutions of NCPH and Ru(bpy)$_3^{2+}$ were taken at the irradiation wavelength (355 nm) with equal volume of known concentration of β-carotene, where the end concentration of β-carotene was $7.48 \times 10^{-5}$ M. Here, the assumption was that the transient absorbance ($\Delta A$) of the β-carotene triplet was monitored at 520 nm for both reference and the compound, which was formed by the energy transfer from Ru(bpy)$_3^{2+}$ and NCPH triplet respectively. The quenching of the sensitizer’s triplet absorption indicates the complete energy transfer to the β-carotene system. Comparison of plateau absorbance following the completion of sensitized triplet formation, properly corrected for the decay of the donor triplets in competition with energy transfer to β-carotene, enabled to estimate the triplet quantum yield ($\Phi_T$) of NCPH based on the following equation.

\[
\Phi_T^{\text{comp}} = \Phi_T^{\text{Ref}} \cdot \frac{\Delta A^{\text{comp}}}{\Delta A^{\text{Ref}}} \cdot \frac{K^{\text{comp}}_{\text{obs}}}{K^{\text{Ref}}_{\text{obs}}} \cdot \frac{K^{\text{comp}}_{\text{obs}} - K^{\text{Ref}}_{\text{obs}}}{K^{\text{comp}}_{0}}
\]

(i)

Here, superscripts ‘comp’ and ‘Ref’ can be substituted by the corresponding value of NCPH and Ru(bpy)$_3^{2+}$ respectively. $K_{\text{obs}}$ is the pseudo-first-order rate constant for the growth of the β-carotene triplet at 520 nm and $K_0$ is the rate constant for the decay of the donor triplets at their respective triplet maximum for both the compound and reference, in the absence of β-carotene. $\Delta A$ represents the plateau absorbance and the quantum yield of the reference was taken as unity in methanol. The triplet quantum yield obtained for NCPH in methanol was $0.69 \pm 0.05$.

Presence of stable triplet state and triplet energy transfer efficiency encouraged to calculate the singlet oxygen generation ability of the molecule, which is quantified in terms of singlet oxygen quantum yield, $\Phi(^1O_2)$. An indirect method was adopted to calculate the $\Phi(^1O_2)$, where a singlet oxygen scavenger, 1,3-diphenylisobenzofuran (DPBF) was used along with the compound, whose initial absorption was noted [231].
The generation of singlet oxygen will cause a decrease in the absorption spectrum of quencher which was followed by an absorption spectrometer. $\Phi(1O_2)$ of NCPH in MeOH was determined by monitoring the photooxidation of DPBF during the formation of singlet oxygen using the absorption spectrometer. Concentration of the photosensitizer was adjusted with an optical density of 0.02-0.03 at the irradiation wavelength (600 nm) to minimise the possibility of singlet oxygen quenching at higher concentration. The solution containing the sensitizer and the scavenger was saturated with oxygen before irradiation. The photooxidation of DPBF was monitored at 411 nm, where the quencher exhibit maximum absorption, with an interval of 10 sec up to one and half minutes. No thermal recovery of DPBF (from a possible decomposition of endoperoxide product) was observed under the conditions of these experiments. In order to calculate the quantum yield, a reference compound with known quantum yield was also investigated with the same procedure. Here, in this investigation meso-tetrakis(p-sulfonatophenyl)porphyrin tetrasodium salt (TPPS) was used as the reference. The following equation was used to calculate the singlet oxygen quantum yield of the sensitizer with respect to the reference.

$$
\Phi(1O_2)^{\text{comp}} = \Phi(1O_2)^{\text{Ref}} \frac{m^{\text{comp}}}{m^{\text{Ref}}} \frac{F^{\text{Ref}}}{F^{\text{comp}}} \quad (\text{ii})
$$

Where, $\Phi(1O_2)$ is the quantum yield of singlet oxygen, superscripts ‘comp’ and ‘Ref’ represents NCPH and TPPS respectively, $m$ is the slope of a plot of difference in change in absorbance of DPBF (at 411 nm) with the irradiation time and F is the absorption correction factor, which is given by $F = 1 - 10^{-OD}$ (OD at the irradiation wavelength). Change observed for the absorption spectra of DPBF in a MeOH solution of NCPH when irradiated with xenon lamp source with a long pass filter ($\lambda_{irr} > 600$ nm) in different time interval was shown in Figure 4.7. A reference solution of TPPS in MeOH with similar optical density at the irradiating wavelength (600 nm) also irradiated in similar
conditions. The singlet oxygen quantum yield obtained for NCPH in MeOH solution was 0.63 ± 0.05 and the yield obtained was comparable with other sensitizers reported in the literature.

Figure 4.7. (a) Change in the absorption spectra of DPBF upon irradiation with (i) NCPH (ii) TPPS in MeOH and (b) Plot of change in absorbance of DPBF at 411 nm vs irradiation time (λ_{irr} > 600 nm) in the presence of NCPH against TPPS as the standard in MeOH.

Aggregations of photosensitizer normally decrease the efficacy of singlet oxygen generation [232, 233]. Singlet oxygen generation quantum yield of NCPH was
investigated in ACN/MeOH (9:1) mixture to analyze the aggregation effects (Figure 4.8). Interestingly, there was no decrease in the singlet oxygen quantum yield, more over observed slight enhancement in the quantum yield (0.67±0.03) possibly due to the tautomeric change.

Figure 4.8. (a) Change in the absorption spectra of DPBF upon irradiation with (i) NCPH (ii) TPPS in MeOH/ACN and (b) Plot of change in absorbance of DPBF at 411 nm vs irradiation time (λirr> 600 nm) in the presence of NCPH against TPPS as the standard in MeOH/ACN.
4.4.2 NCPS as a PDT sensitizer

4.4.2.1 Photophysical investigations of NCPS for PDT application

Eventhough NCPH was showing promising singlet oxygen quantum yield, the compound was lacking water solubility. Search for new sensitizer with high water solubility and better absorption in the red region ended up in designing a tetrasulfonated N-confused porphyrin derivative, *meso*-tetrakis(*p*-sulfonatophenyl)N-confused porphyrin tetrasodium salt (NCPS). The synthesis of NCPS was achieved by following synthetic route as given in Scheme 4.1.

![Scheme 4.1. Synthetic route for NCPS.](image)

The synthesis of *meso*-tetrakis(*p*-sulfonatophenyl)N-confused porphyrin tetrasodium salt (NCPS) involves a two step process, starting from tetraphenyl N-confused porphyrin (NCTPP), which was prepared by using Lindsey’s method [64]. To synthesize NCPS, we adopted the known method of sulfonation for normal porphyrin [234], where the tetraphenyl derivative was dissolved in con.H₂SO₄ and heated for 5 h at 90 °C, then stirred overnight at room temperature. After neutralizing the excess acid with sodium hydroxide solution, the reaction mixture was washed many times with MeOH to extract the compound and filtered to remove the sodium sulphate salt formed during neutralization. The MeOH solution was evaporated and the solid obtained was extracted
through a soxhlet apparatus using MeOH to derive NCPS in 56% yield from the corresponding tetraphenyl derivative [235].

**Figure 4.9.** (a) $^1$H NMR spectrum of NCPS in DMSO-$d_6$, (b) Expanded $^1$H NMR spectrum of NCPS in the aromatic region.
The compound was highly soluble in water and characterized by using $^1$H NMR, $^{13}$C NMR, MALDI-TOF and IR analysis. Presence of two sharp peaks at the negative region of $^1$H NMR (Figure 4.9) assigned to the inner CH and NH protons of NCPS, which has been confirmed with deuterium exchange studies with D$_2$O. Peak resonating at $\delta$ = -1.07 was assigned to the inner NH proton and $\delta$ = -2.76 was from the inner CH proton, where the outer NH resonates at the extreme downfield shifted region of $^1$H NMR spectra.

![Figure 4.10](image)

**Figure 4.10.** (a) Absorption spectra of NCPS in water and MeOH, (b) Emission spectra of NCPS with respect to TPP.
Absorption spectra of NCPS (Figure 4.10.a) was recorded both in deionized water and MeOH, and showed the Soret band at 444 nm and 440 nm respectively, with Q-bands ranging from 550-800 nm. The better absorption of NCPS compared to the counter part of normal porphyrin after 700 nm (1400 M$^{-1}$cm$^{-1}$ at 747 nm in water for NCPS, where the absorbance of normal sulfonated derivative ends at 630 nm) enhances the utility of the sensitizer as a photodynamic therapeutic drug. On the other hand, the fluorescence quantum yield calculated (Figure 4.10.b) for NCPS with respect to tetraphenylporphyrin was too low and the yield obtained in MeOH is 0.0032, which is comparable with the similar N-confused systems [235, 236]).

![Figure 4.11.](image)

Figure 4.11. Triplet absorption of NCPS in deionized water recorded at 7.2 μs. Inset shows the transient decay at 490 nm.

As discussed in the case of NCPH, triplet-triplet energy transfer is the key step to generate singlet oxygen, which is the cytotoxic agent in photodynamic therapy. Figure 4.11 shows the triplet absorption of NCPS in deionized water with the decay profile at
490 nm inset, also the formation of triplet state was confirmed by the absorption quenching in the presence of dissolved oxygen. Transient absorption of the compound shows maxima at 490 nm in deionized water with bleach at 440 nm range where the compound has significant ground state absorption. Triplet absorption profile of the compound in MeOH was similar to that of in water. Triplet lifetime of the compound was measured both in deionized water and MeOH and found to be 16 and 1.2 µsec respectively. Since, low water solubility and triplet lifetime remain as the main barriers for many sensitizers on their real application, comparatively better triplet lifetime of the sensitizer in water was promising [235].

The efficiency of triplet-triplet energy transfer was quantified by calculating triplet quantum yield, using energy transfer method to β-carotene with tris(bipyridyl)ruthenium (II) complex, Ru(bpy)$_3^{+2}$. However, the insolubility of β-carotene in water prevents to determine the triplet quantum yield of the sensitizer in water, but the respective value in methanol was obtained as 0.70 ± 0.05 [235].

The singlet oxygen quantum yield of NCPS both in MeOH and water was quantified by indirect method using DPBF. Irradiation of the sensitizer was done with a xenon lamp with a 600 nm long pass filter at different time intervals from 10-90 s. The decrease in the absorption of DPBF was monitored at 411 nm as shown in Figure 4.12, which is due to the dye sensitized generation of singlet oxygen followed by photooxidation of DPBF. Absorbance at the irradiating wavelength was adjusted to 0.02 for both the sensitizer and the reference. From the slope of the graph obtained by plotting change in optical density against the time interval (Figure 4.12) the singlet oxygen quantum yield was calculated as 0.70 ± 0.03 in MeOH and 0.55 ± 0.05 in water [235].
Figure 4.12. Change in the absorption spectra of DPBF upon irradiation with (a) NCPS, (b) TPPS in water (c) with NCPS (d) TPPS in MeOH. Plot of change in absorbance of DPBF at 411 nm vs irradiation time ($\lambda_{irr} > 600$ nm) in the presence of NCPS against TPPS as the standard in (e) water and (f) MeOH.
4.4.3 *In Vitro* Studies

4.4.3.1 Cytotoxicity Studies of NCPS in Different Cell Lines.

Photocytotoxicity of sulfonated derivatives of different porphyrin and phthalocyanines derivatives have been studied extensively during the past years. These sulfonated derivatives were known for their excellent membrane permeability and lysosomal accumulation in cells with high selectivity towards carcinomas [237]. The *in vitro* photodynamic activity of NCPS was evaluated against eight different cell lines, namely, human colon cancer cells (HCT-116), human breast cancer cells (MCF7-ER, PR positive and MDA-MB-231-ER, PR negative), human pancreatic cancer cells (MIA-PaCa-2), human cervical cancer cells (HeLa & SiHa) and human oral cancer cells (SCC-172 and SCC-131). The cytotoxicity of NCPS in these cell lines were investigated both in the presence and absence of light using MTT assay as shown in Figure 4.13. For the analysis a stock solution of NCPS was prepared in DMSO and diluted to appropriate concentrations with the culture medium. The cells, after being rinsed with phosphate buffered saline (PBS), were incubated with different concentration of NCPS in Dulbecco's Modified Eagle Medium (DMEM) solutions for 1 h at 37 °C before being illuminated at an ambient temperature. Growth inhibition was determined by means of the colourimetric assay called MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Approximately $5 \times 10^3$ cells were seeded in two 96 well cluster plate and allowed to reach the exponential phase of growth and then NCPS was added at analysed for cytotoxicity. Cytotoxic studies revealed that NCPS is essentially noncytotoxic in the absence of light but interestingly on the other hand exhibits high photocytotoxicity. The comparative study of IC$_{50}$ values for NCPS on the above cell lines showed that the IC$_{50}$ value of MDA-MB-231 cells (6 µM,) are about five
fold lower than those for the HeLa cells (25 µM). Oral and cervical cancer showed an increase in IC$_{50}$ values than the breast,

**Figure 4.13.** MTT assay was done on a panel of cancer cells and shows cytotoxicity of NCPS in the presence and absence of light. NCPS shows significant cytotoxicity in the presence of light in all cells but shows negligible cytotoxicity in the absence of light.
pancreatic and colon cancer cells (Table 4.1). Our observations suggest that NCPS exhibits more photocytotoxicity towards adenocarcinomas over the other epithelial cancer cell lines studied [235].

**Table 4.1.** Comparison of IC\(_{50}\) Values of NCPS in a panel of cancer cells.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>IC(_{50}) in (\mu)M</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>25</td>
</tr>
<tr>
<td>SiHa</td>
<td>20</td>
</tr>
<tr>
<td>SCC-131</td>
<td>13</td>
</tr>
<tr>
<td>SCC-172</td>
<td>11</td>
</tr>
<tr>
<td>MIA-PaCa-2</td>
<td>8</td>
</tr>
<tr>
<td>HCT-116</td>
<td>8</td>
</tr>
<tr>
<td>MCF-7</td>
<td>12</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>6</td>
</tr>
</tbody>
</table>

**4.4.3.2 NCPS Sensitized ROS Generation.**

Generation of singlet oxygen (Reactive oxygen species-ROS), during light irradiation has a central role in photodynamic cytotoxicity. The formation of cellular ROS after PDT with NCPS was determined using CM-H\(_2\)DCFDA, which is a fluorescein derivative and ROS detector. The probe emits a green fluorescence after the oxidation reaction with reactive oxygen species and the diacetate groups are removed by cellular esterase [238]. NCPS induced accumulation of ROS in the cells, resulting in a substantial increase in the number of fluorescent cells as detected by flow cytometry and fluorescent imaging. MDA-MB-231 cells showed the least IC\(_{50}\) value (6 \(\mu\)M), hence selected as model system for further investigations. Flow cytometry analysis (Figure 4.14) shows the efficacy of NCPS on generating ROS during PDT and the concentration dependent increase in cellular ROS content after PDT with NCPS. High fluorescence intensity could observe in higher concentration of NCPS (12\(\mu\)M) by flow cytometry and fluorescence microscopy.
Figure 4.14. (a) Flow Cytometry analysis, (b) Fluorescence images of MDA-MB-231 cells shows enhanced fluorescence after PDT with 6 µM and 12 µM of NCPS.
analysis as an indicative of ROS generation. In flow cytometry, the population (P2) shows background fluorescence which represents cells with low ROS and the population at right hand side (P3) shows cells with enhanced fluorescence indicating cells with high ROS [235].

Cellular damage during photodynamic therapy is mediated through apoptosis. Apoptosis is a normal physiological process essential for the control of tissue development, involution and for tissue homeostasis. The earliest hallmark of apoptosis is the loss of plasma membrane asymmetry. In apoptotic cells, the membrane phospholipid phosphatidylserine is translocated from the inner to outer leaflet of the plasma membrane, thus exposing phosphatidylserine to the external cellular environment. AnnexinV (a cellular protein) tagged with FITC (Fluorescein isothiocyanate) has high affinity for phosphatidylserine and therefore serves as a sensitive probe for identifying apoptotic cells by fluorescence microscopy and flow cytometry [239].

Generally, Annexin V-FITC is used together with propidium iodide (PI), which is another fluorescent probe to distinguish viable cells from dead cells, as the former can penetrate through the intact and viable cells but the latter only with dead cells. The cell death mechanism induced by PDT was studied by using NCPS by examining the dual fluorescence of Annexin V-FITC/PI using flow cytometry as shown in Figure 4.15. The cell populations at different phases of cell death, namely, viable (AnnexinV-FITC-/PI-), early apoptotic (Annexin V-FITC+/PI-), and necrotic or late-stage apoptotic (Annexin V-FITC+ / PI+) were examined at different drug doses.

It was observed that most of the cells were negative for Annexin V-FITC and PI after treatment with NCPS (12 µM) in the absence of light. This indicates that NCPS is non-cytotoxic toward MDA-MB-231 cells in darkness. However, upon illumination, the percentage of cells at the early apoptotic stage (i.e., externalization of phospholipid
phosphatidylserine but not membrane leakage, Annexin V-FITC (+/PI-) increased from 3.5 ± 0.41% to 83.9 ± 7.8% when the concentration of NCPS increased from 0 to 12 µM. From these results, it can be concluded that NCPS on PDT induces apoptosis extensively [235].

Figure 4.15. Flow cytometric analysis of the cell death mechanism induced by NCPS upon PDT treatment.

Chromatin condensation is another sensitive marker of apoptosis along with a decrease in membrane potential across the mitochondrial inner membrane. In the present study, Hoechst stain was employed to observe chromatin condensation and cells were viewed under a fluorescent microscope, and found that PDT with NCPS in MDA-MB-231 cells at 6 µM resulted in 72 ± 4.46% chromatin condensation, whereas at 12 µM showed 91 ± 2.6%. Whereas in light and dark controls only 10.4 ± 1.5% and 7.6 ± 1.2%
chromatin condensation was observed (Figure 4.16). This demonstrates that NCPS induces apoptosis in a concentration dependent manner [235].

![Figure 4.16. Characterization of cytotoxic actions of NCPS in PDT using Hoechst stain after PDT with NCPS (6 µM and 12µM). In Hoechst staining, chromatin condensation was visualized using fluorescence microscope.](image)

Also, the changes in mitochondrial membrane potential were monitored using the JC-1 cationic fluorescent dye, as shown in Figure 4.17. The accumulation of JC-1 in mitochondria yields both a green and red fluorescence. However, a decrease in the mitochondrial inner membrane potential due to apoptosis causes a decrease in red fluorescence that can be easily monitored by fluorescent microscopy. PDT with NCPS in MDA-MB-231 cells at 6 µM resulted in about 69.9 ± 5.4% decrease in membrane...
potential where as at 12 µM, 88.5 ± 4.9% decrease in mitochondrial membrane potential was observed. Whereas in light and dark controls only 12.1 ± 4.9% and 10.3 ± 1.3% chromatin condensation was observed, which illustrate mitochondrial mediated cell death pathway through apoptosis during PDT with NCPS [235].

**Figure 4.17.** Characterization of cytotoxicity of NCPS during PDT using JC1 Fluorescence cytochemistry. In JC1 staining, cells with red colour indicate healthy cells with high mitochondrial membrane potential and green coloured apoptotic cells with low mitochondrial membrane potential.

Another confirmation for the NCPS induced apoptosis was from the cleavage of PARP, a 116 KDa nuclear poly (ADP-ribose) polymerase which is involved in DNA repair reaction to cellular stress. This protein can be cleaved by many ICE-like caspases *in vitro* and is one of the main cleavage targets of caspase-3 *in vivo*. In human PARP, the cleavage occurs between Asp214 and Gly215, which separates the PARP
amino-terminal DNA binding domain (24 kDa) from the carboxy-terminal catalytic domain (89 kDa) [240, 241]. PARP helps cells to maintain their viability; cleavage of PARP facilitates cellular disassembly and serves as a marker of cells undergoing apoptosis. During our experiment, no PARP cleavage was observed after treatment with NCPS (12 µM) in the absence of light, which again confirms the non-cytotoxic nature of NCPS in the absence of light (Figure 4.18). However, upon illumination, NCPS induces PARP cleavage at both the concentrations of 6 and 12 µM, thus confirms that NCPS induces cell death via apoptosis [235].

![Figure 4.18. PARP cleavage observed after PDT with NCPS. Here there is no PARP cleavage observed in both light and dark controls but there is significant cleavage at a concentration of 6 and 12 µM of NCPS.](image)

### 4.4.3.3 Cytotoxicity Studies of NCPH

In order to compare the cytotoxicity of NCPS and NCPH, cytotoxicity of NCPH was evaluated with human breast cancer cell (MDA-MB-231-ER, PR negative). The cytotoxicity of NCPH in MDA-MB-231 was investigated both in the presence and absence of light using MTT assay as shown in Figure 4.19. For the analysis a stock solution of NCPH was prepared in DMSO and diluted to appropriate concentrations with
the culture medium. The cells, after being rinsed with phosphate buffered saline (PBS), were incubated with different concentration of NCPH in Dulbecco's Modified Eagle Medium (DMEM) solutions for 1 h at 37 °C before being illuminated at an ambient temperature. Growth inhibition was determined by means of the colourimetric assay MTT. Approximately $5 \times 10^3$ cells were seeded in two 96 well cluster plate and allowed to reach the exponential phase of growth and then NCPH was added at analysed for cytotoxicity. Cytotoxic studies revealed that the cytotoxicity of NCPH in the absence of light was negligible, but interestingly on the other hand exhibits high photocytotoxicity. The IC$_{50}$ value obtained for NCPH in MDA-MB-231 was 12 μM, where that of NCPS was only 6 μM, which indicate better photocytotoxicity of NCPS against NCPH.

![Graph](image.jpg)

Figure 4.19. MTT assay of NCPH in the presence and absence of light. NCPH shows significant cytotoxicity in the presence of light but shows negligible cytotoxicity in the absence of light.

As discussed earlier, the membrane phospholipid phosphatidylserine translocation from the inner to outer leaflet of the plasma membrane is a significant indication of the apoptotic path way of cell death. AnnexinV (a cellular protein) tagged with FITC (Fluorescein isothiocyanate) used as a sensitive probe for identifying apoptotic cells by fluorescence microscopy and flow cytometry.
Annexin V-FITC was used together with propidium iodide (PI), which is another fluorescent probe to distinguish viable cells from dead cells, as the former can penetrate through the intact and viable cells but the latter only with dead cells. The cell death mechanism induced by PDT was studied by using NCPH by examining the dual fluorescence of Annexin V-FITC/PI using flow cytometry as shown in Figure 4.20. The cell populations at different phases of cell death, namely, viable (Annexin V-FITC-/PI-), early apoptotic (Annexin V-FITC+/PI-), and necrotic or late-stage apoptotic (Annexin V-FITC+ / PI+) were examined at different drug doses.

**Figure 4.20.** Flow cytometric analysis of the cell death mechanism induced by NCPH upon PDT treatment.
It was observed that most of the cells were negative for Annexin V-FITC and PI after treatment with NCPH (20 μM) in the absence of light (dark control). This indicates that NCPH is non-cytotoxic toward MDA-MB-231 cells in darkness. However, upon illumination, the percentage of cells at the early apoptotic stage (i.e., externalization of phospholipid phosphatidylinerine but not membrane leakage, Annexin V-FITC + /PI-) increased from 3.3% to 21.5% when the concentration of NCPH increased from 0 to 10 μM, however further increase in the concentration of NCPH (20 μM) increases the value to 27.9%, which is only a slight increase from the lower concentration. A comparative analysis shows less rate of apoptosis with NCPH with respect to NCPS, which shows 83.9% increase in the apoptotic cell population with 12 μM concentration of NCPS.

**Figure 4.21.** Characterization of cytotoxic actions of NCPH in PDT using Hoechst stain after PDT with NCPH (10 μM and 20 μM). In Hoechst staining, chromatin condensation was visualized using fluorescence microscope.
In order to confirm the apoptotic pathway of cell death during PDT with NCPH, chromatin condensation analysis has been conducted where Hoechst stain was employed to track the apoptotic cells (Figure 21.). Hoechst stain was employed to observe chromatin condensation and cells were viewed under a fluorescent microscope, and found that PDT with NCPH in MDA-MB-231 cells at 10 µM resulted in 26% chromatin condensation, whereas at 20 µM showed 56% condensation. However, light and dark controls showed only 6% and 8% chromatin condensation (Figure 4.20) respectively. This demonstrates that NCPH also induces apoptosis in a concentration dependent manner as in the case of NCPS. However, the overall PDT activity of NCPH was found to be less when compared with NCPS.

4.5. Conclusions

In conclusion, a tetra sulfonated derivative of N-confused porphyrin derivative, NCPS with better molar extinction coefficient in the red region of visible light compared to normal porphyrin derivatives have been synthesized and characterized. The PDT applications of NCPS and NCPH were investigated. NCPH was highly soluble in methanol but not in water, where NCPS derivative was highly soluble in MeOH as well as water. Photophysical studies of the molecules have been conducted which shows promising singlet oxygen quantum yields. *In vitro* analysis of NCPS in a series of cancer cell lines showed promising IC$_{50}$ values. Upon illumination NCPS exhibited more photocytotoxicity to adenocarcinomas over the other epithelial cell lines and maximum activity has been attributed toward breast adenocarcinomas MDA-MB-231 cells, with an IC$_{50}$ value as low as 6µM. As shown by flow cytometry and fluorescent imaging using ROS probe CM-H2DCFDA, NCPS induces accumulation of ROS in the cells in a concentration dependant manner. Apoptosis induced cell death during PDT with NCPS was found to be mediated in a mitochondrial dependent manner as evidenced by JC1
mitochondrial membrane potential assay. Apoptotic potential of NCPS was also confirmed by DNA condensation, Annexin V apoptotic assay and PARP cleavage. In vitro analysis of NCPH showed that the photocytotoxicity and overall PDT activity of the sensitizer is low in comparison with NCPS.

4.6 Experimental Section

4.6.1 Materials and methods

Tetrasulfonated derivative of N-confused porphyrin (NCPS) was prepared from corresponding tetraphenyl N-confused porphyrin which in turn was prepared by using Lindsey’s method [64]. The reagents for the synthesis as well as photophysical studies were obtained from Sigma-Aldrich and Merck, India and used as such. All solvents were distilled and dried before use. Deionized water was from Millipore.

$^1$H and $^{13}$C NMR spectra were recorded on a Bruker Biospin 400 MHz spectrometer. NMR experiments were done in DMSO-$d_6$. $^1$H NMR spectra was obtained in 512 scans, where $^{13}$C in 17000 scans. Spectra were referenced internally by using the residual solvent ($^1$H, $\delta = 2.5$ and $^{13}$C, $\delta = 39.4$ for DMSO-$d_6$) resonances relative to SiMe$_4$. Matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectra was recorded in Shimadzu Biotech Axima mass spectrometer. Elemental analysis have conducted using Perkin Elmer 2400 seriesII instrument and the purity of the compound was determined as $> 95\%$. Infrared spectrum of the compound was recorded on a Perkin Elmer FT-IR spectrometer, spectrum RXI.

Electronic absorption spectra and steady state fluorescence spectra were recorded on Agilent diode array UV-Visible spectrophotometer (model 8453) and Perkin Elmer LS55 Fluorescence spectrometer respectively. The transient absorption studies were carried out using a nanosecond laser flash photolysis experiments by employing an Applied Photophysics model LKS-20 laser kinetic spectrometer using OCR-12 Series Quanta Ray.
Nd:YAG laser. The analyzing and laser beams were fixed at right angles to each other. The laser energy was 62-66 mJ at 355 nm during the experiments. Energy transfer method to $\beta$-carotene was employed to calculate the triplet yields ($\Phi_T$) of NCPS using Ru(bpy)$_3^{2+}$ as the reference, assuming the 100% energy transfer from the reference to $\beta$-carotene. Optically matched solutions of NCPS and Ru(bpy)$_3^{2+}$ were taken at the irradiation wavelength with equal volume of known concentration of $\beta$-carotene, where the end concentration of $\beta$-carotene was $7.48 \times 10^{-5}$ M. Here, the assumption was that the transient absorbance ($\Delta A$) of the $\beta$-carotene triplet was monitored at 520 nm for both reference and the compound, which was formed by the energy transfer from Ru(bpy)$_3^{2+}$ and NCPS triplet respectively. The quenching of the sensitizer’s triplet absorption indicates the complete energy transfer to the $\beta$-carotene system. Comparison of plateau absorbance following the completion of sensitized triplet formation, properly corrected for the decay of the donor triplets in competition with energy transfer to $\beta$-carotene, enabled us to estimate the quatum yield.

In order to find the singlet oxygen quantum yield, a steady state method was adopted using 1,3-diphenylisobenzofuran (DPBF) as scavenger of singlet oxygen and meso-tetrakis($p$-sulfonatophenyl)porphyrin tetrasodium salt (TPPS) as the reference. The experiments were carried out with a light source 200 W xenon lamp (model 3767) on an Oriel optical bench (model 11200) with a grating monochromator (model 77250). The intensity of light was maintained constant throughout the irradiations by measuring the output using an Oriel photodiode detection system (model 7072). Quantum yield for singlet oxygen generation of NCPS in deionized water and methanol were determined by monitoring the photooxidation of DPBF during the formation of singlet oxygen using the absorption spectrometer. Concentration of the photosensitizer was adjusted with an optical density of 0.02-0.03 at the irradiation wavelength (600 nm) to minimise the
possibility of singlet oxygen quenching at higher concentration. The solution containing
the sensitizer and the scavenger was purged with oxygen before irradiation. The
photooxidation of DPBF was monitored with an interval of 10 sec up to one and half
minutes. No thermal recovery of DPBF (from a possible decomposition of endoperoxide
product) was observed under the conditions of these experiments

4.6.2 Synthesis of NCPS

To synthesize NCPS, tetraphenyl N-confused porphyrin (800 mg, 1.3 mmol) was made
into paste with 5 ml con. sulfuric acid, then transferred to a 100 ml round bottom flask
using another 15 ml acid. The mixture was then heated at 90 °C for 5 h and then at room
temperature for 12 h. Dil. sodium hydroxide solution was added slowly to the reaction
mixture at 0 °C to neutralize the excess acid present. The formed sodium sulfate salt was
filtered, and washed many times with methanol to extract the compound. The solution
was evaporated to dryness and soxhlet extracted using methanol to obtain pure NCPS in
56% yield as greenish black solid, m.p > 300 °C: H NMR (400 MHz, DMSO-d6) δ 8.7
(s, 1H, Pyrrolic α-CH), 8.36 (s, 2H, Pyrrolic β-CH), 8.28-8.29 (d, J=4 Hz, 1H, Pyrrolic
β-CH), 8.25-8.26 (d, J=4 Hz, 1H, Pyrrolic β-CH), 7.86-7.88 (d, J=8 Hz, 2H, Pyrrolic β-
CH), 7.92-8.01(m, 16H, Phenyl), -1.07(s, 1H, Exch. D2O, Pyrrolic NH), -2.76(s, 1H, Pyrrolic β-CH). C {H}NMR (100 MHz, DMSO-d6): δ 167.29, 158.43, 146.18, 134.84,
134.57, 133.62, 128.82, 124.86, 124.47, 124. IR (KBr): 3448 (br), 2925, 2372, 2345,
1648, 1459, 1178, 1123, 1040 cm⁻¹. MALDI-TOF MS: m/z 1016.58 (C44H25N4Na3O12S4
+ H2O), 1000.98 (C44H27Na3N4O12S4), 936.84 (C44H30N4O12S4 + 2H), 930.04
(C44H26N4O12S4)², 792.75 (C44H25N4NaO6S2)². Anal. Calcd. for C44H26N4NaO12S4: C,
51.66; H, 2.56; N, 5.48; Found: C, 51.01, H, 2.12, N, 5.11.
4.6.3 Cell Lines and Culture Conditions

Human cervical cancer (HeLa&SiHa), breast cancer cells (MDA-MB-231 & MCF7), colorectal cancer cells (HCT-116), pancreatic cancer cells (MIA-PaCa-2), were purchased from ATCC (USA) and Human oral cancer cells (SCC-131 and SCC-171) were obtained as a gift from Dr. Susanne M Gollin, University of Pittsburgh-USA and were maintained in DMEM (Sigma, USA) containing 10% foetal bovine serum (Sigma, USA) and 1% antibiotic antimycotic cocktail (Invitrogen, USA). All experimental steps, after seeding the cells, including photosensitizer incubation, illumination and post-illumination incubation were performed in the same medium. For measurement of dark and light cytotoxicity, cells were seeded (5 x 10^3 per well in 100 µL medium) in 96-well microplates (BD-Falcon, USA).

4.6.4 Photocytotoxicity Assay

NCPS was first dissolved in DMSO to give 106.9 mM solution and diluted to appropriate concentrations with the culture medium. The cells, after being rinsed with phosphate buffered saline (PBS), were incubated with different concentrations of NCPS in DMEM solutions for 1 h at 37 °C before being illuminated at an ambient temperature. A 70 W sodium vapour lamp was used as the light source with fluence rate (λ>590 nm) of 55 mWcm^{-2}. Illumination for 30 min led to a total fluence of 100 Jcm^{-2}.

Growth inhibition was determined by means of the colourimetric MTT assay. Approximately 5 x 10^3 cells were seeded in two 96 well cluster plate and allowed to reach the exponential phase of growth. Then NCPS was added in serial dilution 3.34 µM to 53.45 µM. Out of two plates, one plate was kept in the dark for studying dark cytotoxicity. The second plate was photoirradiated using sodium vapour lamp and kept in an incubator. After illumination, the cells were incubated at 37 °C under 5% CO₂ for 24 h. MTT (Sigma Aldrich) solution in PBS (10 mg mL^{-1}, 10 µL) was added to each well.
followed by incubation for 4 h under the same environment. Later the media was replaced by 100 µL of isopropanol. The plate was agitated on a Bio-Rad microplate reader at ambient temperature for 10 s before the absorbance at 570 nm for each well was taken. The cell viability was then determined by the following equation, percentage growth inhibition = (OD value of control-OD value of test/OD value of control) x 100.

### 4.6.5 Detection of cellular ROS using CM-H2DCFDA assay

For ROS Stress studies approximately $10^6$ MDA-MB-231 cells were plated in 60 mm and 96 well (BD falcon) plates with serum containing media. After 24 h the cells were treated with 6 and 12 µM NCPS for one hand photoirradiation was done using sodium vapour lamp for 30 min. To one plate 12 µM NCPS added and kept in dark as taken as dark control. After 24 h of PDT with NCPS cellular ROS content was determined using the CM-H2DCFDA probe according to the manufacturer’s instructions (Invitrogen) and a flow cytometric analysis was then carried out using FACS Aria II (BD, USA). Images were taken using pathway imager (BD, USA).

### 4.6.6 Chromatin condensation analysis by Hoechst staining.

To study chromatin condensation, approximately $10^5$ MDA-MB-231 cells were seeded in 35 mm culture dishes and incubated for 18 h. Cells were incubated with 6 µM and 12 µM NCPS for 1 h followed by photoirradiation using sodium vapour lamp. Light and Dark control were taken as previously described. After 24 h of treatment MDA-MB-231 cells were rinsed twice with PBS, cells were stained with 5 µg/ml Hoechst dye 33342 (Invitrogen) for 15 min at room temperature. Cells were then washed twice with PBS and visualized under an inverted fluorescence microscope.
4.6.7 Mitochondrial membrane potential assay using JC1 Dye.

For Mitochondrial membrane potential assay, approximately $10^5$ MDA-MB-231 cells were seeded in 35 mm culture dishes and incubated for 18 h. Cells were incubated with 6 µM and 12 µM NCPS for 1 h followed by photoirradiation using sodium vapour lamp. Light and Dark control were taken as previously described. After 24 h of treatment MDA-MB-231 cells were rinsed twice with PBS and cells were stained with JC1 dye mitochondrial membrane potential Detection Kit (Sigma Aldrich) according to the manufacturer’s instruction, and the cell were examined under an inverted fluorescence microscope.

4.6.8 Flow cytometric Annexin V apoptotic studies and Immunoblot analysis

Approximately $10^6$ MDA-MB-231 cells were seeded on 60 mm dishes and incubated for 24 h at 37°C under 5% CO₂. Cells were incubated with 6 µM and 12 µM NCPS for 1 h followed by photoirradiation using sodium vapour lamp. In this experiment light and dark control were taken as previously described. Cells were stained with FITC-labelled Annexin using Annexin V-FITC Apoptosis Detection Kit (Sigma Aldrich) according to the manufacturer’s instruction, and a flow cytometric analysis was then carried out using FACS Aria (BD, USA). For immunoblot analysis, after light irradiation, cells were lysed and the total protein content was measured using Bradford’s reagent. 50 mg of total protein was loaded for SDS-PAGE and immunoblotting was carried out using PARP antibody (cell signalling) Horseradish peroxidase-conjugated secondary antibodies (Santa Cruz) were used followed by detection using enhanced chemiluminescence (ECL) method.