CHARACTERIZATION AND EFFECT OF Sn(IV) CHLORIN e6 DICHLORIDE TRISODIUM SALT IN PHOTODYNAMIC THERAPY

By

KHALED ABDU BDAIWI AL-KHAZA'LEH

Thesis submitted in fulfillment of the requirements
for the degree of
Doctor of Philosophy

April 2009

I would like to express my heartfelt gratitude to my

father ...and father in law…

may Allah rest their souls in peace

To their souls and to my lovely mother and lovely wife

I dedicate this thesis

ACKNOWLEDGMENTS

"All praises and thanks to ALLAH"

I would like to express my sincere appreciation and heartfelt thanks to my supervisors, Dr. Khalid M. Omar and Associate Professor Dr. Mohamad Suhaimi Jaafar for their creative guidance, intellectual support, stimulating discussions and inspiring words. I am grateful for their excellent hospitality and wonderful attitude.

Great thanks for Univirsiti Sains Malaysia for providing me financial support for this research. Also, I would like to express my gratitude to the School of Physics, Universiti Sains Malaysia and the Medical Physics lab assistant Yahya Ibrahim.

My grateful thanks to the School of Biological Science and the School of Chemistry in Universiti Sains Malaysia for use of their equipments.

Great thanks from my heart to my mother for her prayers, unlimited support and true love had maimed the geographical distances between us. She stands by me, raised me, supported me, and loves me.

I would like to express my warmest thanks to my wonderful wife Hind and our children Qutaiba, Hudaifah, Abdullah and Yaqin for their understanding, patience and support throughout the period of my research.

I would also like to thank my lovely brothers Mohammad, Ahmad, Ali and Amer and beloved sisters Amal, Eiman, Njoud, Aidah and Taghreed for their support and love during my study abroad. And I want to thank my mother in law, my brothers and sisters in law chiefly Nawwaf Khwaileh and Khaleel Abu Shagra, my uncles and aunts as well.

Finally, I would like to thank my colleagues, Dr. Ahmad Al-Zoubi, Dr. Eissa Al-Khutaba, Dr. Abdullah Al- Fawwaz, Dr. Ahmad Musleh and Dr. Guat-Siew Chew

for their support and help. I also want to thank my dearest friends Dr. Qasem Awagleh, Maamoon Shbool, Ali Khazalih, Muwafaq Azaizeh, Esam Draiseh, Khaldoon Radaideh, Khaldoon Al-Sraiheen, Mahdi Al-Alzobi, Dr. Khalid Al Hussen, Dr. Eid Abdel_Munem, and Qais Alhourani, for their care and support.

Khaled Abdu Bdaiwi Al-Khaza'leh

Penang, Malaysia. April 2009

ABBREVIATIONS

FBS	Foetal bovine serum
PBS	Phosphate buffer saline
DMSO	Dimethyl sulfoxide
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (methylthiazolyl diphenyl-tetrazolium bromide)
PDT	Photodynamic therapy
FCS	Foetal calf serum
BSA	Bovine serum albumin
HAS	Human serum albumin
HpD	Hematoporphyrin
mTHPC	Meta-tetra (hydroxyphenyl) chlorin
mTHPP	Meso-tetra(3-hydroxyphenyl) porphine
UV	Ultra violet
ALA	5-aminolaevulinic acid
$3P^*$	Excited triplet state
ROS	Reactive oxygen species
PpIX	Protoporphyrin IX
HpIX	Hematoporphyrin IX
mTHPBC	Meta-tetra (hydroxyphenyl) bactriochlorin
ZnTPPS4	tetrakis(4-sulfophenyl)porphinatozinc
TAPP-4Val	Meso-tetra(4-aminophenyl) porphyrin **(**tapp**)** with valine
$TPPS_{2a}$	Disulfonated meso-tetraphenylporphine
MC540	Merocyanine 540
PDD	Photodynamic diagnosis

EMEM	Eagle's minimal essential medium
RPMI 1640	Roswell park memorial institute (series 1640)
BCA	Bacteriochlorin *a*
RME	Receptor-mediated endocytosis
ISC	Inter system crossing
CCD	Central composite design
RSM	Response surface methodology

LIST OF SYMBOLS

ξ	Natural variable
β	Regression coefficient
ε	Molar extinction coefficient
P	Power
D	Light dose
A	Area of irradiation
t	Time of irradiation
c	concentration
α	Absorption coefficient
I	Transmitted intensity
I_0	Incident intensity
l	Optical length
λ_{em}	Emission wavelength
λ_{ex}	Excitation wavelength

TABLE OF CONTENTS

CHAPTER 2 – LITERATURE REVIEW

CHAPTER 3 – MATERIALS AND INSTRUMENTATIONS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

ABSTRAK

PENCIRIAN DAN KESAN GARAM TRINATRIUM Sn(IV) KLORIN e6 DIKLORID DALAM TERAPI FOTODINAMIK

Kajian ini dilakukan bagi menyelidik kesan pH dan serum ke atas foto pemeka garam Sn(IV) klorin e6 diklorid trinatrium dan mengkaji kesannya dalam terapi fotodinamik.

Penyelidikan ini merangkumi empat tahap. Dalam tahap pertama, kekutuban foto pemeka dikaji dan didapati menjadi lebih larut dalam pelarut kutub dengan pekali pemadaman molar tinggi. Pengasidan medium yang mengandungi foto pemeka menghasilkan perubahan kimia terhad dalam cecincin pirol. Tidak terdapat kesan bererti serum ke atas struktur kimia foto pemeka.

Dalam tahap kedua penyelidikan ini, foto pemutihan bagi foto pemeka telah dipengaruhi oleh kehadiran serum di dalam medium itu. Penambahan kepekatan serum meningkatkan kadar foto pemutihan foto pemeka Sn(IV) klorin e6. Faktor utama yang menyebabkan foto pemutihan adalah oksigen singlet.

Tahap ketiga penyelidikan ini adalah mengkaji pengambilan bersel Sn(IV) klorin e6 oleh sel kanser. Adalah didapati pengambilan dadah bersel bertambah dengan pengurangan pH medium. Sebaliknya, kehadiran serum di dalam medium menghasilkan pengurangan pengambilan dadah bersel. Menggunakan sel kanser HepG2 dan sel normal CCD 18 CO, didapati pengambilan dadah bersel oleh sel kanser adalah lebih banyak berbanding sel normal.

Tahap terakhir penyelidikan ini adalah mengkaji kesan Sn(IV) klorin e6 dalam terapi foto dinamik *in vitro* menggunakan MCF7, T47D dan garis sel kanser HepG2. Keputusan yang diperolehi menunjukkan foto pemeka mempunyai kurangnya ketoksikan gelap. Masa inkubasi, kepekatan dan dos cahaya berlainan telah

digunakan bagi mengkaji kesan foto pemeka dialam terapi foto dinamik. Masa inkubasi optimum adalah lebih kurang 6 jam. Manakala, kepekatan optimum adalah sekitar 30 µg/ml dan bertambahnya dos cahaya akan meningkatkan foto ketoksikan foto pemeka.

CHARACTERIZATION AND EFFECT OF Sn(IV) CHLORIN e6 DICHLORIDE TRISODIUM SALT IN PHOTODYNAMIC THERAPY

ABSTRACT

This study was performed to investigate the effect of pH and serum on Sn(IV) chlorin e6 dichloride trisodium salt photosensitizer and studying its effect in photodynamic therapy.

This research consists of four stages. In the first stage the polarity of the photosensitizer was examined and found to be more soluble in polar solvents with high molar extinction coefficient. Acidification of the medium containing the photosensitizer resulted in limited chemical changes in its pyrrole ring. There was no significant effect of serum on the chemical structure of the photosensitizer.

In the second stage of this research the photobleaching of the photosensitizer was affected by the presence of serum in the medium. The increasing of the concentration of serum in the medium increases the photobleaching rate of Sn(IV) chlorin e6 photosensitizer. The main factor responsible for the photobleaching was found to be the singlet oxygen.

The thired stage of the research was to investigate the cellular uptake of Sn(IV) chlorin e6 by cancer cell. It was found that the cellular drug uptake increases with the decreasing of pH of the medium. On the other hand, the presence of serum in the medium resulted in a reduction of the cellular drug uptake. Using HepG2 cancer cells and CCD 18 CO normal cells it was found that the cellular drug uptake by cancer cells is more than that in normal cell.

The final stage of this research was to study the effect of Sn(IV) chlorin e6 in photodynamic therapy *in vitro* using MCF7, T47D and HepG2 cancer cell lines. It

was found that the photosensitizer has a lack of dark toxicity. Different incubation times, different concentrations and different light doses were used to investigate the effect of the photosensitizer in photodynamic therapy. The optimum incubation time was about 6 hours while the optimum concentration was around 30 μg/ml and the increasing of light dose increases the phototoxicity of the photosensitizer.

CHAPTER 1

INTRODUCTION

1.1 History of photodynamic therapy

Solar light was used thousands of years ago by some ancient civilizations in treating some kinds of diseases such as vitiligo, psoriasis, and skin cancer. Egyptians, Indians, Chinese, and Greeks have applied such techniques three thousands year ago. The Greeks Herodots called such treatment heliotherapy (Ackroyd *et al.* 2001). Indians used the seeds of plant (Psoralea Corylifolia) combined with sunlight while Egyptians used extracts of plant (Ammi Majus) in treating vitiligo. Psoralens are considered as the photoactive components of the plants (Briffa and Warin 1979).

In the early twentieth century, the term photodynamic action was used to describe photosensitization of biological effects. A photodynamic action is a light-activated process which requires the presence of molecular oxygen. Photodynamic action was replaced by photochemotherapy then known as photodynamic therapy (PDT).

PDT is the type of photochemical therapy that depends on the presence of oxygen. Light - chemical interactions induced cell damage was initially obtained by the German medical student Oscar Raab (Dolmans *et al.* 2003). During one of his experiments to investigate the toxic effect of acridine on paramecium, Raab concluded that an interaction between light and acridine caused toxic effects that destroyed paramecia (Grossweiner 2005). The researcher explained that the toxicity depends on the amount of light in the laboratory proving that a small amount of acridine which is not toxic in the dark can rapidly damage paramecia in the presence of light (Juarranz *et al.* 2008).

In 1901, Niels Finsen discovered that red light could discharge smallpox pustules and prevent their formation (Schneider *et al.* 2008) and UV sunlight could be used to treat cutaneous tuberculosis. For these findings, Finsen was awarded the 1903 Nobel Prize in medicine (Juarranz *et al.* 2008).

PDT had been used for the first time in 1903 by Tappeiner and Jesionok, when they applied eosin to basal cell carcinomas (BCCs) and irradiated the area by white light (Dolman *et al.* 2003). Hausmann was the first scientist who studied the biological properties of hematoporphyrin in 1908 (Urbach *et al.* 1976)**.** Hematoporphyrin was produced (in an impure form) by Scherer, who removed iron from dried blood in 1841 (Grossweiner 2005)**.** During the period 1908 - 1913 there were a lot of studies to demonstrate the mechanism of sensitiveness of hematoporphyrin by light. In 1913 Meyer Betz applied a study on himself by injecting 200 mg of hematoporphyrin. He remained sensitized to light for two months**.** Meyer and Fischer studied the efficiency of porphyrin structure in photodynamic therapy (Dolmans *et al.* 2003). It was difficult to purify the hematoporphyrins, so research for pure substances started. Auler and Banzer reported that hematoporphyrin injected in rats accumulated in primary and metastatic tumours as well as lymph nodes (Ackroyd *et al.* 2001). Rasmussen, Ward and Figge reported that the hematoporphyrin had a tumour-localizing ability in a variety of human malignancies (Rasmussen *et al.* 1955). Between 1960 and 1966, Samuel Schwartz and his group found that low or medium concentrations of hematoporphyrin have good sensitizing effect (Cohen and Schwartz 1966).

Schwartz found that pure hematoporphyrin has a poor tumour – localization and some components had better localization. These were known as

hematoporphyrin derivatives HpD, and are used for diagnostic and therapeutic purposes (Huang 2005).

In 1974, treatment of psoriasis was performed by using a combination of psoralens and UV (320-400 nm) radiation (Briffa and Warin 1979). In 1975, PDT was used by Dougherty, who reported that HpD can be used as a photosensitizer with red light and can completely destroy mouse mammary tumour. Then, HpD photosensitizer was used for treating bladder cancer and skin cancer (Schouwink 2001). The research attempted to use new photosensitizers such as ALA Luvulan, ALA Metvix, and mTHPC Foscan, and PDT became an established treatment method for localized cancers.

1.2 Photodynamic Therapy

PDT is a promising treatment for early stages of various neoplastic and non-neoplastic diseases. It is an effective single treatment for small superficial tumours and an adjuvant treatment in debulking surgery for more advanced diseases.

PDT is based on using a combination of a photosensitizer and light with appropriate wavelength with the presence of oxygen to cause selective damage to the unhealthy tissue. The photosensitizers make cells more responsive to light. PDT treatment could be applied through two steps as follows:

Firstly, the patient is injected with the light sensitive drug which accumulates in the tumour more than intact tissue, and stays longer in the cancer cells. Secondly, after injection the tumour area will be irradiated by light of proper wavelength which causes cancer cell damage.

The destruction of cells by necrosis and apoptosis can be observed within a few hours after PDT. After about 24 hours, the cell will breakdown (Korbelik and

Dougherty 1999). This cell death causes a modulation of the immune system where number of cytokines and inflammatory mediators are released to attack the cancer cells (Tuner and Hode 2004). The activity of the immune system is very important to complete the tumour destruction (Korbelikl *et al.* 1996) . There are different types of these drugs and each one is activated by light of different wavelength of light.

Researchers have used different photosensitizing agents and different wavelengths to treat different areas of the body by PDT. The therapy can affect cancer cells by three ways: Direct killing of cancer cells, shrinking or destroying cancer cells by damaging the blood supply to the tumour, which prevents the cancer from growing, and it may trigger the immune system to attack the cancer cells.

1.3 Principles of Photodynamic Therapy

Photodynamic therapy protocol depends on three important parameters: The administration of a photosensitizer in tumour tissue, light with a suitable wavelength, and the presence of oxygen in the tumour tissue.

When the photosensitizer is exposed to light with a particular wavelength the molecule of photosensitizer absorbs the photon of light and is excited from its ground state to the excited singlet state. The life time of the molecule in the excited state is very short. The decay of molecule to its ground state occurs by more than one way. It may decays directly by emitting a photon (fluorescence) after an internal conversion with loss of excess energy, or by taking the inter-system crossing and transferring into the lowest triplet state, which has a lower energy level than the first excited state. A therapeutic photodynamic effect can be obtained when the photosensitizer undergoes electron spin conversion to triplet state (3P*) which has a lower energy but has longer lifetime than singlet state (1 – 10 μs) (Ochsner 1997). The increase of

the molecules lifetime increases the probability of transferring the energy to neighboring molecules, and the probability of the molecule to interact with other molecules such as molecular oxygen would also increase. There are two mechanisms for this energy to transfer termed by Type I and Type II reactions.

1.3.1 Type I Reaction

In type I process, the excited photosensitizer directly interacts either with the surrounding molecules, substrates and biological solvents to form radicals by hydrogen atom extraction, or with radical ions by electron transfer. Most of these radical species can react with molecular oxygen to form reactive oxygen species (ROS) such as superoxide anions, hydroxyl radicals, and hydrogen peroxide. These reactions cause a cytotoxic effects and subsequently cell death (Neto *et al.* 2006).

1.3.2 Type II Reaction

In this type of reaction, the energy of the photosensitizer molecule in its excited triplet state may directly transfer to molecular oxygen to generate excited state singlet oxygen. Singlet oxygen can react with a large number of biological substrates to cause the most damage species during photodynamic treatment as shown in Fig. 1.1 (Hermann *et al.* 2006).

1.4 The Mechanism of Light Excitation

Quantum theory explains the transitions between levels. When incident light on molecule has energy corresponding to the different energy between two levels, it can be absorbed and an electron moves to a higher level. The molecule will be in an

excited state, and remains for a short period of time at the excited state before returning to its ground state. The electron can return to its ground state by:

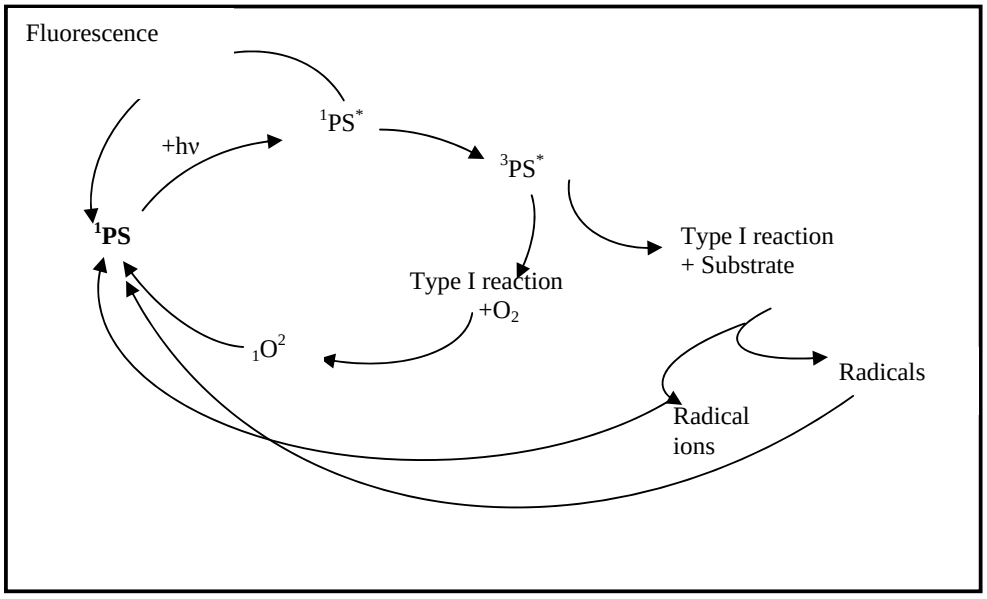

Figure 1.1: The Mechanism of PDT: Type I and Type II Reactions.

Firstly: It returns to the lowest vibrational and rotational level within the excited state by internal conversion. Then the molecule returns to its ground state by emitting fluorescence light.

Secondly: When the electron is in its lowest rotational and vibrational level within the excited state, it can transfer to the triplet excited state by inter-system crossing. No loss of energy occurs during this transferring since the electron spin cannot be changed. Another internal conversion to the lowest level within the triplet state is expressed. Then the molecule can decay to the ground state by an emission called phosphorescence. Figure 1.2 represents the transition between levels by Jablonski Diagram.

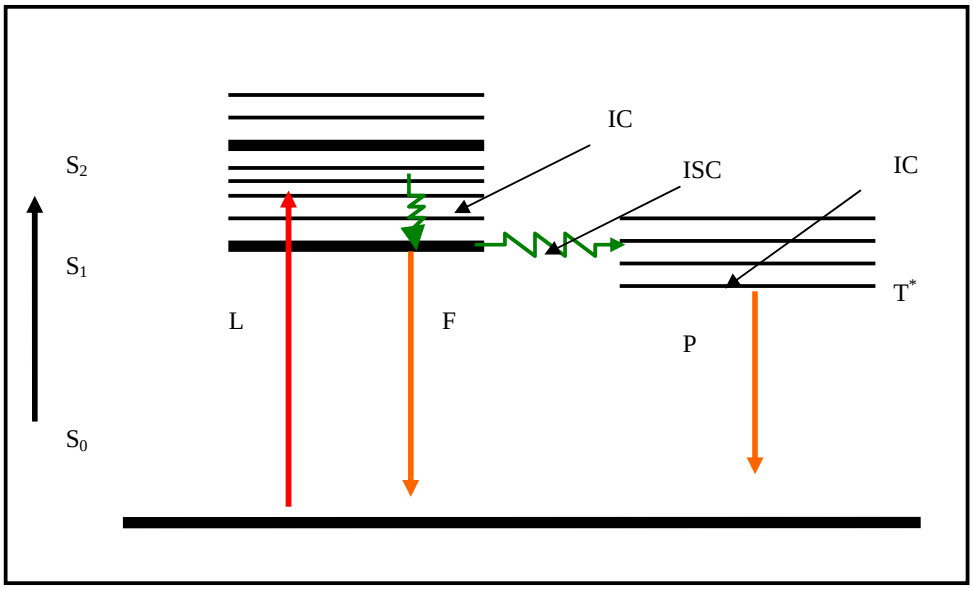

Figure 1.2: Jablonski Diagram explains excitation and decaying of the molecule by fluorescence or phosphorescence.

Where: S: singlet state, T: triplet state, L: laser, F: fluorescence, P: phosphorescence, IC: internal conversion, ISC: inter-system crossing.

1.5 Photosensitizers

Hematoporphyrin and HpD were the first photosensitizers used in PDT. HpD was found to have selective tumour localization compared with hematoporphyrin. Dougherty produced a widely used photosensitizer named photofrin in 1980s during purifying of HpD (Dougherty 1987).

The slow clearance of photofrin from the body causes skin sensitivity to light for a long time. Several photosensitizers had been produced in clinical trials. Third generation photosensitizers were produced to absorb wavelengths further than 660 nm. Some of them are already used in clinical trials. The search continues in the vision to generate new photosensitizers of more activation, longer light wavelength absorption, faster uptake by tissue, and faster clearance of the body.

The ideal photosensitizer has some properties such as: High quantum yield of reactive oxygen species, stability, preferential uptake by tumour, rapid clearance, maximum light absorption at wavelengths more than 630 nm, and non-toxic in absence of light.

1.5.1 Selective Tumour Localization of Photosensitizers

The identification of target is necessary in PDT to facilitate drug development. Since the new photosensitizers tend to be pure compounds, the site of localization can often be identified. Mitochondria, lysosomes, nuclei, plasma membrane of tumour cells, and tumour vasculature are estimated as PDT targets. The vasculature damage is an important aspect of PDT (Juarranz *et al.* 2008). Drug localization can be determined by fluorescence microscopy, because most photosensitizers emit fluorescence (Peng 1996). Since the migration of cytotoxic singlet oxygen $^1O_2$ is less than 0.02 μm after its formation, the sites of photodamage can reflect the localization of the photosensitizer at the time of irradiation (Leung *et al.* 2002).

1.5.2 Subcellular Damage

The most highly selective photosensitizers are the monocationic porphyrin for membranes and the chlorin p6 for lysosomes (Kessel *et al.* 1995), the porphycene for the mitochondria (Kessel and Luo 1998), 5-aminolevulic acid (ALA) – induced PpIX produced in mitochondria (Dougherty *et al.* 1998), Foscan® for endoplasmic reticulum and Golgi apparatus (Teiten *et al.* 2003). Photosensitizers localized in mitochondria are likely to induce apoptosis (Cantatore and Kriegel 2004), and that

localized in the plasma membrane are likely to induce necrosis (Ricci and Wei-Xing 2006).

There are lipophilic and hydrophilic photosensitizers. The lipophilic has an affinity to the plasma membrane, while the hydrophilic has an affinity to mitochondria (Moan *et al.* 1980). The plasma membrane damage can be observed within minutes after exposure to light and appears as swelling and blebs formation. Shedding of vesicles containing cytosolic and lysosomal enzymes, reduction of active transport, depolarization of the plasma membrane, increased uptake of the photosensitizer, increased permeability to chromate, and cytosolic enzymes (Nowis *et al.* 2005, Dougherty *et al.* 1998), up and down regulation of surface antigens (Davies 1986). This type of damage causes cell death by necrosis.

Cell death by apoptosis was reported for the first time in 1991 as a response to PDT (Agarwal *et al.* 1991) . The mechanism of apoptosis is normally part of the genetic apparatus. It ends with fragmentation of DNA and dissociation of cell into particles bounded by membrane that are engulfed by macrophages without any harmful materials around. The time required for initiation of apoptosis varies widely and results in death of more than 80% of tumour cells in 1-3 days (Dougherty *et al.* 1998). Since most PDT photosensitizers do not aggregate in cell nuclei, PDT has a low potential of damaging DNA, mutations, and carcinogenesis (Moan and Sommer 1983).

1.6 Objectives of the Study

In this study a new photosensitizer of porphyrin type with Sn(IV) metal in the center of the carboxyl group which expected to have more stable properties than free base porphyrin photosensitizers. The principal objectives of this thesis can be

summarized in the following points: firstly, to investigate the pH dependence and photobleaching of the Sn (IV) chlorin e6 photosensitizer in different environments. Secondly, to compare the pH dependence of cellular drug uptake by MCF7 breast cancer cell lines with and without foetal bovine serum as well as to determine the selective drug uptake by tumours using cancer and normal cells. The final purpose of this research is to investigate the effect of the photosensitizer in PDT using MCF7, T47D and HepG2 cancer cell lines.

1.7 Outline of Thesis

This thesis contains eight chapters having the common theme, the characterization of Sn (IV) chlorin e6 as a new photosensitizer and its effect in photodynamic therapy. The first chapter provides background information on the historical improvement of photodynamic therapy, its principles, mechanisms, and the objectives of this research.

A literature review on the porphyrin and their characterization is discussed in the second chapter of the thesis. This chapter is categorized into four main subjects: i) Acid base properties of porphyrin type photosensitizers, ii) Cellular uptake of porphyrins, iii) Photodynamic effect of porphyrins, iv) Photobleaching of porphyrins. Chapter three is a description of the materials, chemicals and instruments that were used throughout this research. Chapter four presents the study of Sn (IV) chlorin e6 in different environments which are analytical buffered solution, DMSO, methanol and PBS. The pH dependence of the photosensitizer in analytical buffered solution with and without the presence of foetal bovine serum is also mentioned in this chapter. The photobleaching and photoproducts of the photosensitizer upon

irradiation with red laser beam for different times is described in chapter five. Studying the main factor responsible for photobleaching is considered in this chapter.

Chapter six discusses the cellular drug uptake of the photosensitizer by different cancer cell lines and its dependence on extracellular pH value. The cellular drug uptake was found to be pH dependent where it increased with the decreasing of the pH value. Chapter seven presents the effect of this photosensitizer on MCF7, T47D and HepG2 cancer cell lines. The photosensitizer was found to cause photodamage for about 60% of cancer cells. Finally, the last chapter represents the conclusion of this research and provides a summary of the results and potential avenues for future research.

CHAPTER 2

LITERATUE REVIEW

2.1 Porphyrins

The basic porphyrin skeleton consists of an inner 16-member aromatic (18 π electron) ring containing four nitrogen atoms directed toward the center. This conjugated π-system is the electronic "heart" of the macrocycle and is responsible for the intensity, colour and optical properties of porphyrins. Substitution at any of the positions in the inner or outer atoms changes the photophysical properties of the macrocycle.

2.2 Acid Base Properties of Porphyrin Type Photosensitizers

Fluorescence excitation and absorption spectra efficiency are very important for photodynamic diagnosis (PDD). Fluorescence is an illumination phenomenon which occurs in some compounds when they are exposed to light, x-rays or radioactive particles. Fluorescence spectroscopy is considered a primary research tool in biochemistry, biophysics and the medical fields.

Due to the fact that there is a difference in pH between normal and tumor tissue, it is necessary to assess the impact of pH on the fluorescence emission intensity and absorption of any photosensitizer. Zimmermann *et al* (2002) investigated the pH dependence of absorption and fluorescence properties of the photosensitizer mTHPC. In vitro fluorescence measurements showed a significant decrease in the fluorescence intensity at pH values below 6. In the range of pH 6.5 – 7.2, no pH dependence was observed. Changes in the spectral shape of the absorption spectra for pH less than 6 were observed.

The pH dependence of absorption and fluorescence spectral properties of four porphyrin type photosensitizers relevant to photodynamic therapy Hp(IX), TPPS2a, mTHPP and mTHPC has been investigated in phosphate buffer saline (PBS) solvent. Results showed pH dependent modification in the physiological pH range of 6 - 9 only in the case of Hp(IX). This modification is probably related to the protonation of the carboxylic groups. Spectral changes were observed at pH less than 5 for all photosensitizers (inflection points of titration curves were at 5.1, 3.8, and 2.4 for TPPS2a, mTHPP and mTHPC, respectively), were likely due to the protonation of imino nitrogens (Cunderlikova *et al.* 2001).

The absorption, fluorescence excitation and fluorescence emission spectra of chlorin e6 were recorded as functions of pH in PBS solution with and without FCS. For neat PBS, different values of the pH of the solution resulted in a shift of both the absorption and the fluorescence spectra as well as in a decrease in fluorescence intensity. An aggregate formation at low pH values (pH less than 5) was observed. The presence of 5% FCS resulted in a shift of the titration curve, from an inflection point at about 6.5 to one at about 7.6. Spectral changes of the fluorescence emission spectra of serum bound chlorin e6 were also observed (Cunderlikova *et al.* 1999).

2.3 Photobleaching

Photobleaching can be defined as loss of absorption or emission intensity of the photosensitizer caused by the light during PDT treatment. Photobleaching sometimes can be referred to as photofading or the absence of light fastness (Bonnett 1999). When tissues and cells containing a photosensitizer are exposed to light with relevant wavelength during PDT treatment, changes in the photosensitizer fluorescence spectra as well as decay in fluorescence intensity can be observed.

These changes are due to the photodestruction of the photosensitizer macrocycles, resulting in a loss of absorbance and fluorescence. The concentration and photobleaching rates determine PDT damage to normal tissue surrounding the tumor which contains less concentrations of the photosensitizer (Das *et al.* 2005).

Photobleaching is considered an important factor in PDT dosimetry. Rapid photobleaching of photosensitizers decreases the efficiency of PDT. Photobleaching had been observed both in vitro and in vivo using the fluorescence observation. There are two types of irreversible photobleaching process leading to chemical changes in the chromophore :

Photomodification: A loss of absorbance or fluorescence occurs at some wavelength, but the chromophore is retained in a modified form.

True photobleaching: A deep-seated chemical change occurs, which results in small fragments that no longer have appreciable absorption in the visible region. The fluorescence is very sensitive to various quenching effects therefore the loss of fluorescence does not usually parallel to the loss of absorption. It is important to know which mode of observation is being employed. In this research the loss of absorption of Sn(IV) chlorin dichloride trisodium salt as new photosensitizer has been studied with various parameters as: environment, pH value, light dose, and with or without FBS and BSA in phosphate buffered saline (Bonnett and Martinez 2001). The photobleaching of porphyrin type derivatives and relative compounds has been extremely studied in kinetic terms but very little is known about the structures of the photoproducts.

In 1988, Rotomskis and co-workers studied the photobleaching of hematoporphyrin derivatives in PBS irradiated with argon laser – ion laser beam of wavelength 514 nm. It was found that the decrease of absorption throughout the spectrum simultaneous with the appearance of new band at 640 nm. Photobleaching was observed without new photoproduct when ethanol was used as a solvent. The researchers supposed that the photoproduct has been resulted from the aggregated porphyrins without oxygen involvement (Bonnett and Martinez 2001).

The photobleaching process of chlorin p6 in neat phosphate buffer, bovine serum albumin, and foetal bovine serum was studied by Das *et al.* (2005). It was found that FWHM of soret band increases in the presence of serum when compared to that in neat buffer. This increasing was due to the binding of the photosensitizer to lipid bilayer of proteins, which leads to aggregation. Changes in the absorption spectra were observed upon photoillumination which indicated that photomodification and photoproduct formation occurred. The photobleaching rate of chlorin p6 with 10% serum was three times faster than that in neat buffer. This observation was due to the reduction of diffusional motion of the drug when bound to serum proteins. A new non fluorescent band was observed at 730 nm after illumination. Singlet oxygen was assumed to have the main responsibility for photobleaching.

The photobleaching of meta-tetra (hydroxyphenyl) chlorin mTHPC in protein containing solutions was examined by Belitchenkol *et al.* (1999). Fluorescence intensity was reduced at excitation wavelength 423 nm and emission wavelength 655 nm. Also, a decrease in absorption throughout the spectrum and new absorption band appeared in region 325-450 nm. The rate of photobleaching was much lower in

absorption than that in fluorescence attributed to the assumption of the photobleaching of monomeric and fluorescing species of mTHPC.

Streckyte and Rotomskis (1993) studied phototransformations of porphyrins in aqueous solutions and micellar media. The formation of the photoproduct at 660 nm in micellar hematoporphyrin (Hp) and photosan-3 (PS) solutions and its formation in small amounts in PBS are related to the presence of covalently linked porphyrin structures and/or interaction with surfactant molecules.

Spikes (1992) studied the photobleaching of porphyrins in neutral PBS and concluded that low oxygen concentration (2 μM) significantly reduced the photobleaching yields and the singlet oxygen quencher, azide, had no effect, even at 0.1M. Inhibition and/or accelerating photobleaching rate depend on the compound, the porphyrin and the reaction conditions.

Photobleaching and formation of photoproducts of mTHPBC in PBS supplemented with human serum albumin (HSA) during irradiation with laser were studied by means of absorption and steady-state fluorescence spectroscopy. The rates of photobleaching obtained by fluorescence measurements were higher than in the absorption measurements. Two photobleaching rates probably reflect differences in the photosensitivity of monomer (bound to proteins) and aggregated (non-bound) forms. Irradiation of the mTHPBC solution led to phototransformation into mTHPC, a clinically used second generation photosensitizer (Lassalle *et al.* 2004).

Previous studies for the photobleaching of some porphyrin photosensitizers showed that, mTHPP yielded novel quinonoid porphyrins upon illumination in aqueous methanol. True photobleaching has been observed for mTHPC and mTHPBC under the same conditions. Also, several fragmentation products were recognized (Bonnett and Martinez 2002).

Rotomskis *et al.* (1996) found that, the illumination of hematoporphyrin, meso-tetraphenylporphyrin tetrasulphonate and hematoporphyrin derivatives in aqueous solutions caused photodegradation and formation of stable photoproducts absorbing in the red spectral region. A decrease in the absorption spectrum as well as fluorescence intensity upon illumination was accessed during the processing. The photodegradation was due to the opening of the porphyrin ring, leading to an increase in light absorption in the UV region. On the other hand, the formation of photoproducts at 640 nm was related to the aggregation state of the porphyrins. The spectroscopic features of the photoproducts of hematoporphyrin and hematoporphyrin derivative, with absorption bands in the visible region are similar to those of chlorin and/or porphyrin-chlorin linked systems.

The photobleaching of compounds of the porphyrins series (mTHPP, mTHPC, and mTHPBC) has been studied in methanol and methanol–water (3:2, v/v) using an argon - ion laser at 514 nm by observing the reduction of spectrum with time. True photobleaching occurred only for mTHPC and mTHPBC, while photomodification was the major process for mTHPP. The rates for the photobleaching of mTHPC and mTHPBC were presented in different solvents. The photobleaching rate of the mTHPBC is found to be 90 times higher than that of the mTHPC in methanol–water (3:2, v/v). Singlet oxygen appeared to be the main factor for the photobleaching of mTHPC and mTHPBC and the photomodification of mTHPP (Bonnett *et al.* 1999).

Comparative spectroscopic studies of hematoporphyrin like photosensitizers in PBS solvent have been performed. The photosensitizers studied are not photostable and are bleached during illumination. Formation of red-absorbing photoproducts was observed for hematoporphyrin like sensitizers simultaneous with

photobleaching. The efficiency of photobleaching and the formation of photoproducts in aqueous solution were conditioned by the aggregation state and chemical structure of the photosensitizer (Rotomskis *et al.* 1997).

2.4 Cellular Uptake

The difference between extracellular pH in malignant and normal tissues has been suggested to be responsible for selective uptake of photosensitizers in tumors. Accordingly, studying the cellular uptake of the photosensitizer by cancer cells and its dependency on pH value is very important. Investigating the dependence of drug cellular uptake on the presence of serum is necessary, since proteins are the main responsible for the drug distribution into the tissues. Several previous studies have been performed on porphyrin type photosensitizers.

A study by Hilf *et al.* (1983) examined the effect of the presence of serum on the cellular uptake by R3230AC mammary adenocarcinoma. An increase in the amount of serum in the medium gradually reduced the amount of HpD taken up by the cells at a level of 10% serum, uptake of HpD was reduced to less than 5%.

The effect of pH and the presence of serum on the cellular uptake of chlorin e6 by T47D cancer cell lines were examined by Cunderlikova *et al* (1999). It was found that the decreasing of pH resulted in increasing in the cellular uptake of chlorin e6. Similarly, a decreasing in cellular uptake was occurred in the presence of serum.

The uptake of photosan in vitro by bladder carcinoma cells and normal cells was investigated at different incubation times. The concentration of photosan, measured in $\mu g/10^6$ cells, showed a good correlation to the incubation time. At all

incubation times, control cells showed a lower uptake when compared with tumor cells (Miller *et al.* 1991).

The pH dependence of cellular uptake of some porphyrin type photosensitizers Hp(IX), $TPPS_{2a}$, mTHPP, and mTHPC by T47D cancer cell lines in different pH values of the environments have also been investigated. The experiments were performed at pH range of 6.5 - 8. The results showed that the highest drug uptake was at low pH value in the case of Hp(IX). On the other hand, there was no pH influence on the drug cellular uptake of mTHPP, mTHPC, and $TPPS_{2a}$ (Friberga, E *et al* 2003). In other studies on cellular drug uptake of $TPPS_{2a}$ and mTHPC by WiDr and THX cancer cells did not exhibit any dependence on the pH value. In the same study, the cellular uptake of Hp(IX) was found to be lower in the presence of serum (Cunderlikova *et al.* 2005).

Another study to examine the uptake of HpD by malignant cells by Bohmer and Morstyn (1985) found that, major determinant of HpD uptake was the concentration of serum in the medium. Here, increasing concentrations of FCS or BSA resulted in a reduction in the amount of HpD taken up by the cells. pH of the medium was a further factor affecting the uptake of the photosensitizer. At low pH the rate of HpD incorporation was much higher than at pH 7.4. Acidic pH and the differences in extracellular serum concentrations of malignant tumor tissue may play an important role in the selective uptake of HpD by malignant tumors.

Cellular uptake of chlorin e6 in the presence of serum in various pH values was performed by Cunderlikova *et al.* (1999). This uptake was significantly higher at pH 6.7 as compared with that at 7.3 and 7.6. It was concluded that the change of the pH value of the medium resulted in a change in the lipophilicity of chlorin e6.

The effect of pH on cellular uptake of chlorin p6 by human colon (Colo-205) and breast adenocarcinoma (MCF7) cell lines were studied by Sharma *et al.* (2004). Reducing the extracellular pH from 7.4 to 6.0 affected the cellular drug uptake of the photosensitizer by Colo-205 cells. The uptake of the drug increased with the decreasing of pH of the environment. This uptake took place mainly through endocytosis. On the other hand, no significant difference was observed in the uptake of the chlorin p6 by MCF7. It was suggested that, chlorin p6 is taken up by MCF7 cells through diffusion rather than endocytosis.

The cellular uptake of merocyanine 540 (MC540) was investigated by Cunderlikova *et al.* (2002) at two pH values of 6.8 and 7.4. No difference was observed in the spectral properties of the drug between the two pH values in the presence of either human blood plasma or FCS of concentrations of 0 – 2%. The cellular drug uptake by WiDr cells was significantly higher at pH 6.8, with and without FCS. Accordingly, the presence of serum and the alteration of pH value are important factors that affect the cellular drug uptake.

Variations of pH within the physiological range are not expected to influence the MC540/membrane interactions directly through modification of chemical structure of the drug or protonization of membrane-bound charge in the main region of MC540/membrane interaction which is the hydrophobic lipid region and not membrane protein one. Nevertheless, this might lead to changes in screening of negative surface charges and perhaps in membrane polarization. Such effects will certainly influence the response of potential sensitive probes such as MC540.

2.3 Photodynamic effect

Ding *et al.* (2004) applied ALA photosensitizer at human glioma cells and found that, the lowest viability was 24.26% ±2.76% with light dose of 25 J/cm^2 and 15.14%+ 3.60% with light dose of 100 J/cm^2 at the same concentration of photosensitizer 4 µM. The dark toxicity for ALA and the light toxicity without the presence of photosensitizer were very low.

The effect of photofrin on U87 and U521 cancer cells was applied by Jiang *et al.* (2003). The viability of the cells was studied at fixed laser dose of 100 mJ/cm^2 and different photofrin concentrations. The lowest viability was 78% and 50% at concentration of 5 ng/ml for U87 and U521 respectively.

Tetrakis (2-chloro-3-sulfophenyl) porphyrin $TCPPSO_3H$ was found to be non-toxic in the dark and caused a significant photodynamic effect against MCF7 cancer cell lines irradiated with red light at low light doses. The lowest viability was 7% at light dose 8 J/cm^2 and concentration of 2×10^{-5} M (Dabrowski *et al.* 2007). Dark toxicity of Chlorin e6 in MCF7 cancer cell lines was examined by Cavanaugh (2002). Lack of dark toxicity and an acceptable phototoxicity effect were found in this study.

In vitro phototoxicity measurement on G361 cell lines using ZnTPPS4 porphyrin type photosensitizer was performed by Kolarova *et al.* (2005). Photodamage effect on cancer cell lines was observed with the visible light. After 24 hours incubation of cell cultures with 10 µM ZnTPPS4, the cells were irradiated for 7.5 minutes at total irradiation dose of 12.5 J/ cm^2.

The effect of TAPP-4Val porphyrin type photosensitizer on MCF7 cancer cell lines was performed by Wang *et al.* (2008). Various concentrations of $1 - 6 \times 10^{-6}$ M of the photosensitizer resulted in lack of dark toxicity for the photosensitizer. For

the same concentration with light intensity of 1.4 W/cm^2 for 1 hour resulted in gradual decrease in the number of survival cells where the lowest viability was at concentration of 6×10^{-6} M.

Sharma *et al.* (2004) studied the effect of chlorin p6 on MCF7 cancer cell lines with the presence of light. The lowest viability at neutral pH was observed to be 27% using light dose of 18 kJ/m^2 where the cells were incubated with chlorin p6 (10 μM) in growth medium for 3 hours in dark.

CHAPTER 3

MATERIALS AND INSTRUMENTS

3.1 Cell Lines

3.1.1 Breast Cancer Cell Lines MCF7

Human breast adenocarcinoma cancer cells derived from epithelial mammary gland are shown in Fig. 3.1. The base medium for these cell lines is Eagle's Minimal Essential Medium (EMEM). To make a complete growth medium, the following components were added to 500 ml of base medium: 0.5 ml bovine insulin, 5 ml L-Glutamine, 5 ml sodium pyruvate, 5 ml non-essential amino acid, 10 ml sodium bicarbonate, and 10% of FBS to the final concentration.

3.1.2 Breast Cancer Cell Lines T47D

Human breast ductal carcinoma derived from mammary gland duct tissue is shown in Fig. 3.2. The base medium for these cell lines is RPMI 1640 medium. The same components in 3.1.1 were added to the medium to get a complete growth medium.

3.1.3 Liver Cancer Cell Lines HepG2

Human liver hepatocellular carcinoma cell lines are shown in Fig. 3.3. The base medium for these cell lines is EMEM. The same procedure was used to get a complete growth medium as described in 3.1.1 with no addition of bovine insulin.

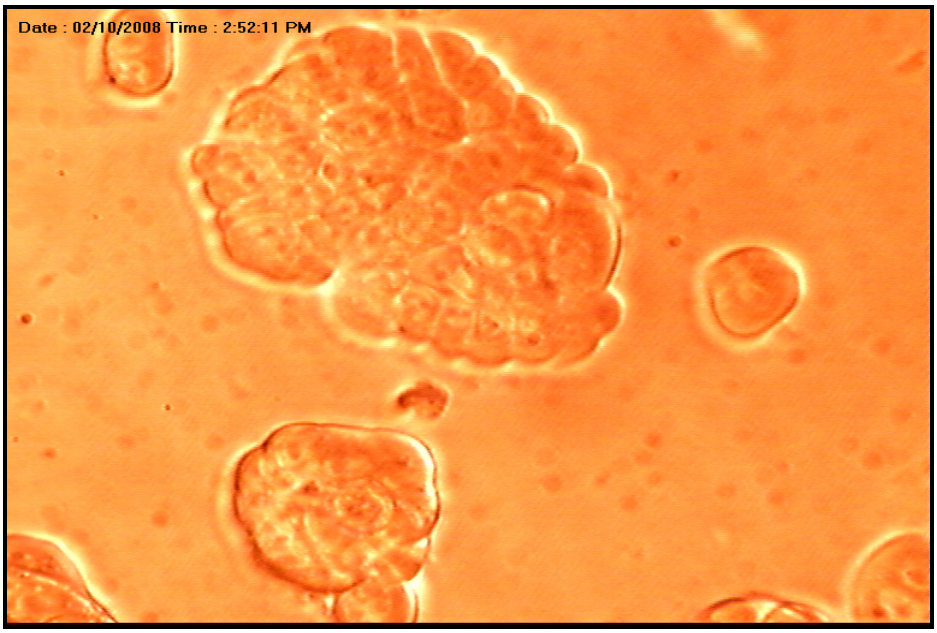

Figure 3.1: MCF7 breast cancer cells

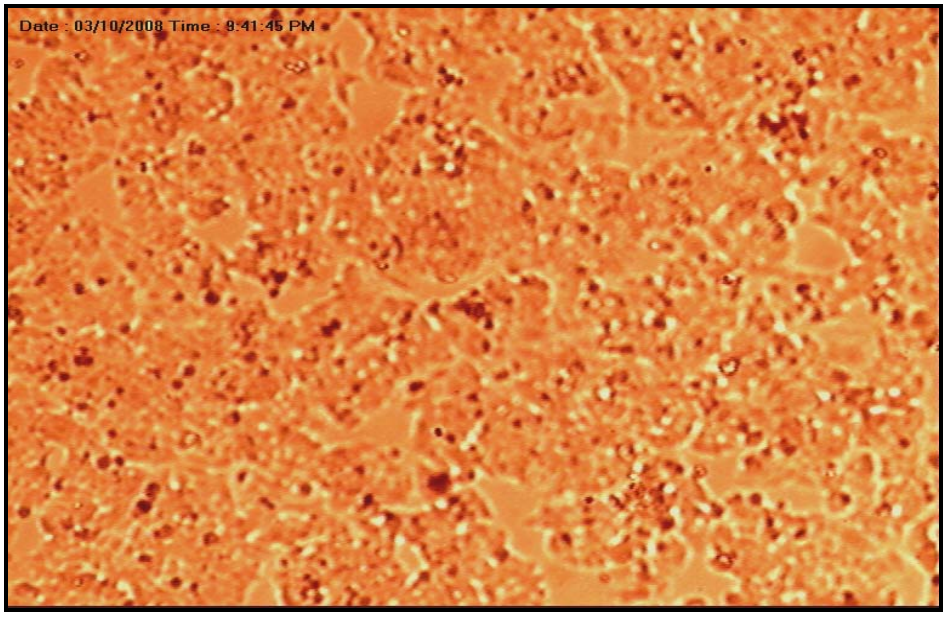

Figure 3.2: T47D breast cancer cells