Chapter – 1

In this chapter a brief introduction of Curcumin and Chlorin \( p_6 \) are provided with a particular emphasis on some of their biological properties which are relevant to the work presented in this thesis. The chapter is concluded by providing the scope and motivation of the present work.

1.1. Curcumin

Curcumin is a naturally occurring yellow-orange pigment derived from the rhizomes of Curcuma longa (turmeric) [1]. Turmeric, cultivated in several tropical parts of Asia, is used as a spice in Indian cooking, a cosmetic agent for skin care and a traditional medicine. The medicinal potential of turmeric has been demonstrated against various diseases such as common cold, fever, skin disease, stomachache, liver disease etc. In addition it also has beneficial effects against wounds and chronic inflammations. Additional interest in the spice was created when epidemiological studies showed 10–50\% lower incidence in certain types of cancer among those who consumed the spice regularly [2-3]. About 2–8\% of turmeric by weight consists of a naturally occurring yellow-orange pigment known as Curcumin. The biological properties of Curcumin have been reported as early as in 1970s [4-6]. The ability of Curcumin to act as an anticancer agent was demonstrated in mid 1990 [7] and thereafter interest in the biological activity of the pigment has increased significantly.
1.1.1. Chemical structure and keto–enol tautomerism

The chemical structure of Curcumin (Scheme 1.1) consists of two o-methoxyphenolic moieties which are attached symmetrically through two π-conjugated ethylene linkers (α, β-unsaturated β-diketone). Various spectroscopic techniques have been employed to study the structural, photophysical, photobiological and biophysical properties of Curcumin and its derivatives in solution phase [8–22, 28-38, 43-50]. Although several possible isomeric structures of Curcumin are possible, only three of them can be considered as candidates for the ground state of the pigment (Scheme 1.1). Ab-initio computations showed that the trans-diketo form (II) has minimum energy compared to the cis-diketo form (I) [22] making this as the stable isomer of Curcumin. The driving force for the cis to trans isomerization was attributed to the negative charges on the carbonyl groups, which resulted in a strong electrostatic repulsion between the two carbonyl groups. However, NMR and IR spectroscopic investigation of Curcumin in chlorobenzene revealed that the cis-keto structure undergoes a keto-enol rearrangement by transfer of a proton from the CH$_2$ group to form the enol form (III) which is essentially the major conformer of Curcumin present in a variety of solvents [21-22, 28-32]. This was also supported by theoretical studies which showed that the cis-enol structure is the most stable form of the ground state of Curcumin both in the gas phase and in aqueous solution [33-38]. The large dipole moment (7.7 and 10.8 Debye in the gas and solution phases, respectively) of the cis-enol form leads to the formation of a strong intramolecular hydrogen bond, as well as the extended conjugation of the molecular backbone which makes it more favorable than the diketo form [33-34]. NMR spectroscopic analysis showed that in solution the cis-enol form coexist as two symmetric keto-enol tautomers (III & IV, Scheme 1.1) whose interconversion involves a rapid intramolecular hydrogen atom transfer (IHT) process [22].
Scheme 1.1: Chemical structures of the cis-diketo, trans-diketo and keto-enol tautomeric forms of Curcumin.
1.1.2. Excited state photophysical properties

Studies have shown that the anticarcinogenic and antibacterial effect of Curcumin is enhanced when combined with light and oxygen [23-27]. Therefore spectroscopic characterization of Curcumin excited state is a subject of current research interest.

The excited state properties of Curcumin have been investigated in several studies and the obtained results point to the occurrence of two fundamental photophysical processes: excited-state intramolecular hydrogen atom (or proton) transfer, ESIHT (or ESIPT) process, as well as dipolar solvation [9–20]. Photoexcitation of Curcumin from the ground electronic (S0) state to the first excited singlet (S1) state is accompanied by a significant change in the dipole moment (Δμ ∼ 6.1 D) [9] and therefore solvation is expected to play an important role in the excited-state relaxation dynamics of Curcumin. Also, several studies [9, 14-15, 17] have predicted that the ESIHT process in the hydrogen-bonded chelate ring of the cis-enol form (Structures III & IV, Scheme 1.1) plays an important role in the efficient nonradiative deactivation process of the excited state. It is pertinent to note that earlier studies on the ESIPT process occurring in molecules having an asymmetric intramolecular hydrogen-bonded chelate center have shown that this process is ultrafast (in the range of a few hundred femtosecond) [39–42]. Curcumin being a symmetric molecule (with respect to the hydrogen-bonded chelate center, structures III and IV, Scheme 1.1), it is therefore unlikely that the ESIHT process will lead to any change in the excited state absorption and emission profile thereby enabling to monitor the process spectroscopically. Adhikary et al. studied the excited-state photophysics of Curcumin in alcoholic solutions as well as in surfactant micelles using subpicosecond fluorescence upconversion spectroscopy [17-18]. They reported the presence of two decay components in the excited-state kinetics with the lifetimes of about 12-20 and 100 ps in methanol and ethylene
glycol (EG). Deuteration of the acidic hydrogens of Curcumin had no effect on the shorter lifetime component but significantly affected the longer lifetime component. Therefore they attributed the shorter lifetime component associated to solvation of the S\textsubscript{1} state and longer lifetime to the ESIHT process. Ghosh et al. has also studied the dynamics of the S\textsubscript{1} state in different solvents using time-resolved absorption and fluorescence spectroscopic techniques with subpicosecond time resolution [43]. They have observed that solvation is the major process contributing to the relaxation dynamics of the S\textsubscript{1} state. Solvation dynamics is also influenced by specific hydrogen-bonding interaction between Curcumin and the solvent. In nonpolar solvents nonradiative deactivation of the S\textsubscript{1} state occurs via ultrafast excited-state intramolecular hydrogen transfer (ESIHT) reaction in the six-membered hydrogen-bonded chelate ring of Curcumin. In polar solvents, nonradiative deactivation of the S\textsubscript{1} state occurs as a result of stretching vibration in the intermolecular hydrogen bonds formed in the hydrogen-bonding (both donating and accepting) solvents.

Erez et al. have studied the temperature dependence of nonradiative process of Curcumin in ethanol and 1-propanol by steady-state and time-resolved spectroscopy [44]. They have observed that the trends in nonradiative rate constants (temperature range: 175-250 K) are similar as the dielectric relaxation times of both neat solvents. They attribute the nonradiative process of the pigment to solvent-controlled proton transfer. They have also observed a kinetic isotope effect on the nonradiative process. Based on their results they propose that excited-state proton transfer breaks the hydrogen-bonded planar hexagonal chelate ring of the cis-enol form which, in turn, enhances the nonradiative process driven by the twist angle between the two phenol moieties.
They have also studied the ESIHT reaction occurring between excited Curcumin, a photoacid and acetate, a mild base, by monitoring the fluorescence property of Curcumin in methanol and ethanol solutions [45]. They observed that the steady-state emission intensity as well as the average fluorescence decay time is reduced by a factor of 5 in presence of 1.8 M acetate ions. This large reduction in the fluorescence is attributed to excited-state proton transfer from the acidic groups of Curcumin to the acetate anion.

1.1.3. Biological effects

It has been demonstrated that Curcumin exhibits potential therapeutic application against several chronic diseases including cancer [51-63]. Currently, there are several phase I and phase II ongoing clinical trials on Curcumin for the treatment of variety of cancers and also for Alzheimer’s disease [3]. Recent research has shown that Curcumin modulates a number of targets including growth factors, growth factor receptors, transcription factors, cytokines, enzymes and genes regulating cell proliferation and apoptosis [51-63]. Curcumin shows phototoxicity to bacterial systems, which is mediated through the excited states of Curcumin and their subsequent reactions with oxygen [64].

1.1.4. Interaction with biological membranes

Curcumin has a great affinity for biological membranes and has been shown to alter their properties [64-73]. Following an earlier study [64] it has been proposed that Curcumin can regulate the action of membrane proteins indirectly by changing the physical properties of the membrane rather than direct binding to the protein. Subsequently it has been demonstrated that
Curcumin is capable of altering the properties of model and cell membranes [65-68]. Jarugaa et al. have observed that rat thymocyte cells treated with Curcumin exhibit increased membrane permeability [64]. They have also observed that Curcumin significantly affects the bilayer properties in erythrocyte membranes [65].

Subsequently studies have been carried out to investigate the effect of Curcumin on the properties of several model bilayers [66-69]. The interaction of Curcumin with a model bilayer consisting of 1, 2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) lipid has been studied by isothermal titration calorimetry and X-ray lamellar diffraction [66]. Results obtained suggest that Curcumin causes thinning of the bilayer as well as weaken its elastic moduli. Solid-state NMR and differential scanning calorimetry experiments revealed that Curcumin has a strong effect on model membrane structure consisting of DMPC, DHPC and DPPC lipids. Results obtained suggest that the drug inserts deep into the membrane in a trans-bilayer orientation, anchored by hydrogen bonding to the phosphate group of lipids. In addition NMR results also suggested that Curcumin forms higher order oligomeric structures in the membrane that span and likely thin the bilayer [67]. In another study the effect of Curcumin on model multilamellar DPPC and DEPE membranes were studied by a variety of experimental techniques [68]. On the basis of the obtained results it has been suggested that the drug is oriented in the bilayer with its main axis parallel to the acyl chains and interacts with the polar head groups of the lipid in such a way that favors negative curvature of the membrane. Sun et. al., have investigated Curcumin induced changes in the membrane thickness and membrane area of giant unilamellar vesicles made from DOPC. They have observed that the drug binding to the bilayer is a two-step process. Initially Curcumin binds to the interface and then at higher concentrations gradually partitions to the
hydrocarbon region. The presence of Curcumin alters the physical properties of the lipid bilayer, including a decrease of the hydrocarbon thickness and softening of its elastic rigidity [69].

Kunwar et al. have investigated the fraction of Curcumin localized in different sites of phosphatidylcholine (PC) liposomes by monitoring the quenching of Curcumin fluorescence by potassium iodide and acrylamide. The results obtained showed that the distribution of Curcumin in the liposome is pH-dependent. The neutral form of Curcumin predominant at pH 5.0 is preferentially located in the hydrophobic phase, whiles the anionic form of Curcumin predominant at pH 9, is mostly located in the liposomal surface. At pH 7, the drug is non-uniformly distributed into different compartments of the liposomal bilayer [71].

1.2. Chlorin \textit{p}_6

Photosensitizers which are derived from the plant pigment Chlorophyll-a have received considerable attention because they possess significantly higher absorption in the longer wavelength region (660-800 nm) which is a desirable feature for achieving higher depth of treatment in photodynamic therapy [74]. Various Chlorophyll-a derivatives have been synthesized and evaluated for their photo-therapeutic efficacy. Chlorophyll-a derivatives, on the basis of their chemical nature can be categorized in two classes: hydrophobic such as pheophorbide and its derivatives and amphiphilic such as Chlorin-\textit{e}_6 and Chlorin \textit{p}_6 (\textit{Cp}_6) and their derivatives [75-78]. \textit{Cp}_6 is an amphiphilic photosensitizer having three ionizable carboxyl groups in the lower periphery of the molecule (Scheme 1.2). The molecule has a strong absorption around 400 nm (called the soret band) and another prominent absorption band around 660 nm (called \textit{q}-band) and good singlet oxygen generation capacity (singlet oxygen quantum yield \textasciitilde 0.60 in ethanol) [74]. The synthesis and phototoxic property of \textit{Cp}_6 was reported as early
as in 1986 by Hoober et al [76]. Several studies have been carried out at RRCAT and elsewhere on photophysical, photochemical and photo biological characterization of Cp₆ [76-98]. In-vivo studies carried out in hamster cheek pouch model on efficacy of Cp₆ for photodynamic treatment of tumors led to promising result. Cp₆ showed preferential accumulation in small size tumors (dia < 5 mm), rapid clearance from skin and complete tumor regression after PDT [87, 93]. However, for relatively large tumors its uptake was poor which compromised the PDT efficacy.

1.2.1. Photophysical and photobiological properties: Effect of pH

Cp₆ has three ionizable carboxylic acid groups and consequently neutral and several ionic forms (structures A-D, Scheme 1.2) of Cp₆ are expected to be present depending upon the pH of

\[ \text{Scheme 1.2: Different structures of Chlorin } p_{6} \text{ present in the pH range of 3-8.} \]
the solution. Steady state and time-resolved fluorescence properties of \( C_{p6} \), have been investigated as functions of pH [77]. It has been observed that a decrease in the pH of the medium causes protonation of the ionizable carboxylic acid side chain, leading to an increase in hydrophobicity and consequent aggregation. The pK\(_a\) for inter-conversion between structures C and D has been reported to be around 7.0 which is similar to the value observed for the pK\(_a\) of the side chain carboxylic groups of other porphyrins [96-97]. This would suggest that an increase in hydrophobicity at low pH would have significant influence on the cellular uptake of \( C_{p6} \). The effect of reducing the extracellular pH from 7.4 to 6.0 on the uptake of \( C_{p6} \), has therefore been investigated in two mammalian cell lines, human colon (Colo-205) and breast (MCF-7) adenocarcinoma cells [78]. In Colo-205 cells, the uptake of \( C_{p6} \) was observed to increase as the pH of the incubation medium decreased. In contrast, no significant variation in uptake was observed for MCF-7 cells. A possible explanation offered to explain this effect was mechanism for drug uptake is cell line dependent. It was proposed that drug uptake in Colo-205 cells occurs mainly through endocytosis whereas in MCF-7 cells the uptake is controlled by diffusion.

An understanding of the photobleaching of the photosensitizer and the resulting photoproducts is essential since these have significant influence on the photodynamic efficacy of the photosensitizer. Therefore the photobleaching process of the drug was studied in neat phosphate buffer, phosphatidylcholine liposomes and in 10% fetal bovine serum [84]. The rate of photobleaching of Chlorin \( p_6 \) in neat buffer and liposomes were comparable, but it was three times faster in the serum media. Additional studies indicate that the photobleaching of the drug in these environments may proceed via a type II mechanism.

Subsequently the effect of pH on the binding properties of the drug with various macromolecules has been studied [77, 80-82, 85-86, 90]. The effect of pH on the formation of
complexes of $Cp_6$ with surfactants has been studied [77, 81, 85]. Ionic surfactants are found to cause aggregation of the drug only at submicellar concentrations. A significant pH effect is observed in the ionic surfactant induced aggregation process as the charge on the drug is controlled by the pH of the medium. The neutral Tx-100 was also observed to induce aggregation of the drug at low surfactant concentrations. The obtained results are rationalized by the interplay of electrostatic and hydrophobic effects between the drug and the surfactants where pH plays a major role. The effect of pH on the binding of $Cp_6$ with phosphatidylcholine liposomes was also investigated by absorption and emission spectroscopy [80]. Substantial changes in the absorption and emission spectra of the drug were observed when liposomes were added at acidic pH. At higher pH these changes became progressively smaller. Fluorescence quenching studies suggest that at acidic pH (5.0) the drug localizes in the central hydrophobic region of lipid bilayer and for neutral pH (7.0) the drug binds closer to the charged liposome interface. The influence of pH on the binding of the drug with Cremophor EL, a potential drug delivery vehicle has been monitored by fluorescence spectroscopy [82]. Interestingly it has been observed that the hydrophilic species of the drug bound preferentially with the hydrophobic delivery medium. The binding of the drug with serum albumins and lipoprotein have also been studied and it was concluded that hydrophobic interactions are responsible for the binding [86, 90].

In a recent study the effect of silica nanoparticles (SiNPs) having positively charged amino groups on the acid-base ionization equilibrium of $Cp_6$ in aqueous medium is significantly affected as a result of strong electrostatic binding between the negatively charged drug and SiNP [92]. At neutral pH the spectroscopic signature of the drug bound to SiNPs suggests that the trianionic form of the drug remains bound to the positively charged SiNPs. It was concluded that
the interplay of hydrophobic and electrostatic forces in the drug-nanoparticle binding process might affect the relative uptake and photodynamic efficacy of the free drug and the drug-nanoparticle complex in cancer cells. Subsequently studies were carried out on cellular uptake and phototoxicity of the free drug and its complex with SiNP in colon (Colo-205) and oral cancer (Nt8e) cell lines [98]. Phototoxicity measurements showed that the C$_{p6}$-SiNP complex was more effective as compared to free C$_{p6}$. The observed increase in photodynamic activity of C$_{p6}$-SiNP complex was attributed to the enhanced photostability of the C$_{p6}$-SiNP complex.

The binding of the drug with positively charged gold nanorods have also been studied at neutral pH conditions [95]. Absorption, emission and photostability of the drug-nanorod complex were observed to depend critically on the nature of the nanorod coating material. Therefore before exploring the efficacy of the drug-nanorod complex for combined hyperthermia and photodynamic therapy applications a judicious choice of the nanorod coating material is essential.

1.3. **Objective and scope of the present thesis**

The excited state properties of Curcumin are a subject of current research interest. Solvation dynamics and excited state proton transfer of the pigment are shown to be the major processes occurring in the excited state of the pigment. In nonpolar solvents the major process is the ultrafast excited-state intramolecular hydrogen transfer (ESIHT) reaction occurring in the six-membered hydrogen-bonded chelate ring of Curcumin. Whereas in polar solvents both solvation and excited state proton transfer can occur. Additionally it has been shown that in polar solvents the dynamics of solvation is also influenced by specific hydrogen-bonding interaction between Curcumin and the solvent [38]. In this thesis a significant portion has been devoted to
elucidate the role of polarity and hydrogen bonding properties of polar solvents on the excited state properties of the pigment.

The Chlorophyll a derivative, $Cp_6$ has three carboxylic acid groups whose protonation and deprotonation plays an important role in controlling the hydrophobic and hydrophilic species of the drug at different pH. The pH dependent acid-base equilibrium of the drug is believed to play an important role in the higher uptake of $Cp_6$ in tumors where extra-cellular pH can be slightly acidic. Thus, knowledge of the factors governing the dynamics of the diffusion of $Cp_6$ across membranes may be useful for a better understanding of the cellular uptake of the drug. In addition drug-liposome interactions may also change the properties of a lipid bilayer [96]. Both Curcumin and $Cp_6$ have great affinity for biological membranes and therefore the effect of these two drugs on bilayer properties is a subject of potential interest. The second part of the thesis work is therefore devoted to investigate the diffusion properties of $Cp_6$ and the effect of liposomal Curcumin and $Cp_6$ on the diffusion properties of two organic cations across a lipid bilayer.
The organization of the remaining chapters of this thesis is as follows:

**Chapter 2** describes in detail about the spectroscopic characterization techniques used with a particular emphasis on the Second Harmonic spectroscopic technique.

**Chapter 3** describes the photophysical studies used to characterize the excited state processes of the medicinal pigment Curcumin in polar-nonpolar solvent mixtures using steady state and picosecond time resolved fluorescence spectroscopic techniques.

**Chapter 4** describes the diffusion characteristic of the photosensitizer $Cp_6$ across an egg lecithin membrane investigated by Second Harmonic spectroscopy.

**Chapter 5** describes the effect of Curcumin and $Cp_6$ on the transport kinetics of two organic cations across a negatively charged lipid bilayer investigated by the SH spectroscopy.

Finally in **Chapter 6** a summary of the major observations are presented. A brief outline of future investigations that may evolve from the thesis is also provided.
1.4. References


