CHAPTER 4

SYNTHESIS AND PHOTOPHYSICAL STUDIES INCLUDING SINGLET OXYGEN GENERATION EFFICIENCIES OF HEAVY ATOM SUBSTITUTED SQUARaine DYES

4.1. Abstract

The synthesis and photophysical studies, including measurements of quantum yields of triplet excited states and singlet oxygen generation of bis (3,5-dibromo-2,4,6-trihydroxyphenyl)squaraine (7) and bis (3,5-diiodo-2,4,6-trihydroxyphenyl)squaraine (8) have been described. Compounds 7 and 8 were prepared by the bromination and iodination, respectively, of the parent bis (2,4,6-trihydroxyphenyl)squaraine (6). These dyes exist in solution in the protonated, neutral, singly and doubly deprotonated forms, depending on pH. The pK_a values of these dyes were estimated by absorption spectrophotometry and were found to be relatively lower than those of the parent dye 6. Only the single deprotonated forms (Sq-) of 7 and 8 showed measurable fluorescence. In microheterogeneous media such as in the presence of β-cyclodextrin (β-CD), poly(4-vinylpyrrolidone) (PVP) and cetyltrimethylammonium bromide (CTAB), bathochromic shifts in the absorption and emission spectra of Sq- forms were observed with a substantial enhancement in their fluorescence yields. The increase in emission quantum yields has been maximum with CTAB. Triplet excited states are the main transient intermediates obtained upon 532 nm laser excitation of all the forms of 7 and 8 in methanol. These triplets have lifetimes in the range from 0.061 to 132 μs. The triplet quantum yields of both the protonated and doubly deprotonated forms are low (Φ_T = < 0.01), whereas, the neutral and Sq- forms of 7 (Φ_T = 0.12 and 0.22) and 8 (Φ_T = 0.24 and 0.5), respectively, exhibited significant triplet
yields. Quantum yields of singlet oxygen generation \([\Phi(1^1O_2)]\) by \(\text{Sq}^-\) forms of 7 and 8 were determined by using 1,3-diphenylisobenzofuran as scavenger. The estimated quantum yields for 7 \((\Phi(1^1O_2) = 0.13 \text{ and } 0.25)\) and 8 \((\Phi(1^1O_2) = 0.47 \text{ and } 0.67)\) in methanol and in presence of PVP, respectively, were found to be quantitative and are in good agreement with the triplet yields obtained in these systems.

4.2. Introduction

Photodynamic therapy (PDT) is fast developing as a viable method for the treatment of various diseases and disorders of tissues (tumors).\(^1\)-\(^4\) It involves the administration of photoreactive drugs (photosensitizers) that are absorbed exclusively by the diseased tissues followed by light exposure. Upon exposure with a light source that can penetrate deeply into the tumor tissues, produces highly reactive species that cause cell death. Figure 4.1 shows the schematic representation of the various steps involved in PDT treatment. PDT is

![Figure 4.1. Schematic representation of photodynamic therapy.](image_url)
relatively a safe treatment since the induction of cytotoxicity by PDT ceases when light is switched off, contrary to the conventional chemotherapy and radiotherapy. Moreover, the advent of lasers, fibre optics, endoscopy and laparoscopy have made it possible by PDT to alter only the irradiated area, with minimal systemic toxicity. This has improved to extend the clinical applications of PDT to a variety of tissues. The idea of combining light and a drug in this way is not new, but it is only in the past 10 years or so that this approach has become clinically feasible. Recently, PDT has been approved in Canada, Japan, Holland and the United States for patients with certain types of cancer involving the lungs, bladder and oesophagus.

4.2.1. Mechanism of photodynamic therapy

In PDT, the sensitizer is a foreign molecule, a xenobiotic, and as with any other drug its metabolism is likely to follow a multiplicity of pathways. Absorption of photon by the sensitizer molecule leads to singlet and triplet excited states, and activated species derived from them, which inevitably react with a variety of biomolecules in the cell. As shown in Figure 4.2, the singlet excited state of

![Image of Jablonski diagram]

Figure 4.2. The Jablonski diagram for the generation of excited sensitizer (porphyrin) states and reactive oxygen species.
the sensitizer can abstract an electron from the substrate to form free radicals which react in a number of ways with the biomolecules (Type I mechanism). Alternatively, the singlet excited state can undergo intersystem crossing to yield the triplet excited state, which in turn can react with the substrates (Type I mechanism) or can react with molecular oxygen to produce singlet oxygen, a key agent of cellular damage (Type II mechanism). In most of the sensitizers that have hitherto been tested for application in PDT, it is the triplet excited state that has been observed to mediate the therapeutic effect.\textsuperscript{1-4} Thus, the principal cause of photodamage in PDT is regarded as involving the following processes shown in Scheme 4.1, where, P = photosensitizer, S\(_0\) and S\(_1\) are the ground and first excited singlet states, T\(_0\) and T\(_1\) are the ground and first excited triplet states, isc = intersystem crossing, \(3\text{O}_2\) = ground state triplet molecular oxygen and \(1\text{O}_2\) = first excited singlet state molecular oxygen.

\[
P(S_0) \xrightarrow{\text{hv}} \text{P}(S_1) \xrightarrow{\text{isc}} \text{P}(T_1) \\
P(T_1) + 3\text{O}_2 \rightarrow \text{P}(S_0) + 1\text{O}_2
\]

Scheme 4.1

Evidence for the involvement of singlet oxygen was obtained from the oxygen monitoring during PDT, using singlet oxygen scavengers and also by spectroscopy. Experiments on porphyrins adsorbed on the cell surface\textsuperscript{5} exhibited the singlet oxygen emission (\(\lambda_{\text{em}} \sim 1270\) nm), whereas, the \textit{in vitro} studies in the presence of 1,3-diphenylisobenzofuran (singlet oxygen scavenger)\textsuperscript{6} resulted in a significant protection against the photodynamic effect.
4.2.2. First generation photosensitizers: Haematoporphyrin derivative

Haematoporphyrin derivative (HpD), together with its commercial variants Photofrin, Photosan, Photogem and Photocarcinorin, holds an important place in the development of PDT. These are the first generation photosensitizers\(^1\text{-}^4,^7\text{-}^12\) on which first observations of activity were made and the first regulatory authorizations for the clinical use were obtained.\(^1,^13\text{-}^21\) HpD was described by Lipson and his colleagues\(^13\) in 1961 as a diagnostic tool and its tumor-killing potential was demonstrated by Diamond\(^22\) et al. in 1972. The preparation of HpD involves two stages. Treatment of haematoporphyrin (1a) (Chart 4.1) or its hydrochloride with 5% sulfuric acid in acetic acid at room temperature for 30 min gives a purple solid (HpD Stage 1) which contains about ten principal components, the major one being haematoporphyrin diacetate (1b).\(^23\) The HpD stage 2 involves the preparation of a solution for injection. The purple solid thus obtained in the first stage is treated with aqueous base and then brought back to neutrality. This causes hydrolysis and elimination of the 2-acetoxyethyl functions, to give back

\[
\begin{align*}
(a) & \quad R_1 = R_2 = CH(OH)Me \\
(b) & \quad R_1 = R_2 = CH(OAc)Me \\
(c) & \quad R_1 (R_2) = CH(OH)Me \\
& \quad R_1 (R_2) = CH=CH_2 \\
(d) & \quad R_1 = R_2 = CH=CH_2
\end{align*}
\]

Chart 4.1
haematoporphyrin and to generate 3(8)-hydroxyethyl-8(3)-vinyldeuteroporphyrin (1c) and protoporphyrin (1d) (Chart 4.1). However, in vivo bioassay showed that the photonecrotic activity of HpD stage 2 resides not with these components, but with higher molecular weight material, which have been postulated to be a mixture of porphyrin dimers and oligomers involving ether, ester, and carbon-carbon interporphyrin linkages.

Although, HpD and its commercial variants have been used extensively in experimental clinical work and approved for treatment of a variety of cancers in a number of countries, there are some disadvantages with the use of these first generation photosensitizers. HpD is a mixture of, at least nine components and its preparation is highly sensitive to the experimental conditions. Therefore, the elucidation of mechanism by which HpD acts, has been hampered by the complexity of its composition of matter. HpD is slowly cleared from the body and causes cutaneous photosensitivity and immunosuppression. More importantly, it has only a weak absorption in the red region of the spectrum, and hence the difficulty in delivering light to some tumor sites and also incomplete light penetration for larger tumors.

4.2.3. Design of second generation photosensitizers

Since the clinical results of PDT by employing complex porphyrin mixtures (HpD) were very positive, the search for effective second generation photosensitizers and creation and testing of new instrumentation have become important in recent years. Ideally, the photosensitizer must be a single substance, should be localized in or around tumor mass and non-toxic to normal tissues. It should have high triplet yields and high efficiency of singlet oxygen generation, since singlet oxygen is the main phototoxic agent of cellular damage. Because the transmission of light increases with increasing wavelength, the photosensitizer should have high absorption coefficient in the red to infrared region. Above 600
nm, the biological tissue is relatively transparent to light but at wavelengths below 600 nm, the depth of non-thermal penetration of light is limited to a few millimeters due to competitive absorption of light by biological molecules. The photosensitizer, following production of a lethal amount of the cytotoxic agent, should ideally be bleached during PDT, or be converted into a form that no longer absorbs the activating wavelength of light. It should have an adequate shelf-life with amphiphilic properties so that it can be injected as a solution without any carrier.

Among the second generation sensitizers, chlorins,\textsuperscript{25,26} glycoconjugated porphyrins,\textsuperscript{27} porphycenes,\textsuperscript{28,29} phthalocyanins,\textsuperscript{30-32} purpurins,\textsuperscript{33} aminolevulinic acid-mediated porphyrins and monoclonal antibody-dye conjugates\textsuperscript{27} have been extensively studied. Structures of some of these second generation sensitizers that are being developed by different companies are shown in Chart 4.2. These sensitizers are under evaluation at various clinical phases of PDT. Favourable optical properties and biodistribution patterns are possessed by purpurins, but require solubilizing or emulsifying agents such as cremophore, liposomes, or lipoproteins for their application in PDT. Chlorins have strong absorption in the red and infrared regions of the spectrum and compete favourably \[(\Phi(1O_2) = 0.43)^{26}\] with Photofrin. However, skin photosensitivity is a major problem with them. Phthalocyanins and metallophthalocyanins have been found to have strong absorption in the 600-700 nm, but details of the extent of sulfonation vs the activity are not clear.

4.2.4. Objectives of the present investigation

Squaraines form a class of dyes possessing sharp and intense absorption bands \((\epsilon \sim 5 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1})\) in the red to infrared region (6, Chart 4.3).\textsuperscript{34,35} The photochemical and photophysical aspects of these dyes have been studied extensively,\textsuperscript{34-38} since their absorption and photochemical characteristics make
Benzoporphyrin derivative (2)
(Quadra Logic Technologies, Vancouver)

Porphycene (3)
(Cytopharm, California)

Bacteriochlorine (4)
(Scotia Pharmaceuticals, England)

Zinc (II) phthalocyanine (5)
(Ciba Geigy - Basel, QLT - Vancouver)

Chart 4.2

them highly suitable for a number of industrial applications such as in xerographic photoreceptors, solar cells and optical recording.\textsuperscript{39,40} Due to the very low intersystem crossing efficiency\textsuperscript{36-38} of these dyes, their potential as photosensitizers
in PDT have not yet been explored. However, recently the biological applications of squaraine-N-hydroxysuccinimide esters as long wavelength fluorescent labels have been reported in the literature.\textsuperscript{41} The objective of the present study has been to design squaraine dyes which could function as efficient photosensitizers for their use in PDT. In this context, it was felt that the heavy atom substituted derivatives of 6 could be potential photosensitizers, since they would also be sufficiently water soluble as 6, to be useful for biological applications. The heavy atom substitution would ensure enhanced intersystem crossing efficiency\textsuperscript{42,43} and better triplet yields. This chapter deals with the results of investigations on the photophysical properties of halogenated squaraine dyes 7 and 8 (Chart 4.3) in homogeneous and microheterogeneous media, lifetimes and quantum yields of their triplet excited states and also studies with respect to their abilities to generate singlet oxygen.\textsuperscript{44}

4.3. Results and Discussion

4.3.1. Absorption and emission properties

Squaraine dyes 7 and 8 exist in solution in the protonated, neutral, singly deprotonated or doubly deprotonated forms, depending on pH of the solution. Figure
4.3 shows the absorption spectra of the four forms of 7 at different pH in solutions of 20% (vol/vol) methanol in water. By following the absorption characteristics of

![Absorption spectra of various forms of 7 in 20% (vol/vol) methanol-water solutions.](image)

**Figure 4.3.** Absorption spectra of various forms of 7 in 20% (vol/vol) methanol-water solutions; (a) pH 3.5, Sq; (b) pH 8.0, Sq^2-; (c) pH 0.5, SqH^+, in 50% (vol/vol) methanol-water solution; (d) pH 6.3, Sq^-; (e) pH 0.5, aggregate of SqH^+.

7 with the change in pH of the solution gave three isosbestic points at 622 nm, 532 nm and 578 nm, indicating that 7 exists in four different species in protonation equilibria. Similar observations were made with the iodo derivative 8, with the change in pH of the solution. The protonation equilibrium between different ionic forms of 7 and 8 is shown in Scheme 4.2.

Of all the different forms, only the protonated species of 7 and 8 tend to form aggregates and these aggregates have absorption around 700 nm and are formed even at 1 μM concentrations in 20% methanol-water solution at 25 °C. The absorption characteristics of various forms of 7 and 8 in different solvent mixtures are summarized in Table 4.1. Data for the parent squaraine dye 6,
reported earlier\textsuperscript{37} are also included for comparison. The neutral forms of 7 and 8 which exist predominantly in the 2.7-4.0 pH range, exhibited broad absorption bands with maxima at 506 and 508 nm, respectively. In contrast, the singly deprotonated forms of 7 and 8 showed relatively intense sharp bands at 610 and 616 nm, respectively. The absorption maxima for both the protonated and neutral forms of 7 and 8 are relatively unchanged from that of 6, whereas the singly deprotonated and doubly deprotonated forms have their absorption maxima red shifted by about 12-30 nm, relative to that of the corresponding species of the parent dye 6.\textsuperscript{37} The iodo derivative shows a larger bathochromic shifts than the bromo derivative.

The reduction of the HOMO-LUMO gap\textsuperscript{43,45,46} upon halogenation in the cases of 7 and 8 may explain the bathochromic shifts observed for the singly and doubly deprotonated forms. Interestingly, it is evident from Table 4.1 that marginal
blue shifts (2-20 nm) and significantly large hypsochromic shifts were exhibited by all forms of 7 and 8 in 20% (vol/vol) methanol in water, when compared to that

Table 4.1. Absorption characteristics of various forms of 7 and 8 in different solvent mixtures

<table>
<thead>
<tr>
<th>Compound</th>
<th>Solvent</th>
<th>$\lambda_{\text{max}}$ nm (e, $10^5$ M$^{-1}$ cm$^{-1}$)</th>
<th>$\text{SqH}^+$</th>
<th>$\text{Sq}$</th>
<th>$\text{Sq}^-$</th>
<th>$\text{Sq}^{2-}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>$\text{SqH}^+$</td>
<td>$\text{Sq}$</td>
<td>$\text{Sq}^-$</td>
<td>$\text{Sq}^{2-}$</td>
</tr>
<tr>
<td>7</td>
<td>20% methanol-water</td>
<td>576 (0.25) 506 (0.30) 610 (0.47) 555 (0.31)</td>
<td>Methanol</td>
<td>--</td>
<td>518 (0.80) 612 (2.10) 569 (0.93)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>20% methanol-water</td>
<td>563 (0.98) 508 (0.45) 617 (0.63) 562 (0.27)</td>
<td>Methanol</td>
<td>--</td>
<td>528 (0.88) 620 (2.49) 593 (0.98)</td>
<td></td>
</tr>
<tr>
<td>6$^*$</td>
<td>30% methanol-water</td>
<td>561 (1.32) 506 (0.49) 588 (1.47) 543 (0.52)</td>
<td>Methanol</td>
<td>--</td>
<td>510 (0.53) 588 (1.40)  ---</td>
<td></td>
</tr>
</tbody>
</table>

*The protonated species of both 7 and 8 form aggregates at 1 µM concentrations in 20% (vol/vol) methanol in water. Hence, the absorption spectra of these forms were recorded in 50% and 30% (vol/vol) methanol-water solution, respectively.

**In 30% (vol/vol) methanol-water solution, taken from ref. 37 for comparison.
in methanol. These absorption changes with solvent polarity confirms the fact that these species exist in solution in the quinoid form with donor-acceptor-donor (D-A-D) character, as reported earlier for several squaraine derivatives based on semiempirical calculations\textsuperscript{47} and X-ray crystallographic studies.\textsuperscript{48-51} In squaraines, the $S_0$-$S_1$ electronic excitation involves a charge-transfer process from oxygen atoms to the cyclobutane ring and this transition along with the extended conjugated $\pi$-electron donor network, leads to the intense bands in the visible region.\textsuperscript{47} Therefore, the observation of large hypsochromic shifts with the increase in solvent polarity in the case of 7 and 8 could be due to the involvement of an electronic transition state, which is charge-transfer in character.

The differences in the absorption characteristics of the various forms of 7 and 8 were used to determine the pK$_a$ values of the equilibria shown in Scheme 4.2. For example, Figure 4.4 shows the change in absorbance vs pH in 20% (vol/vol) methanol in water, indicating the equilibrium between the neutral and singly deprotonated forms of 7. The increase in pH led to a decrease in absorption at 506 nm (corresponding to the neutral form) and an increase in the absorption at 610 nm (singly deprotonated form). Similarly, Figure 4.5 shows the equilibrium between the neutral (monitored at 508 nm) and singly deprotonated forms (monitored at 617 nm) of the iodo derivative 8. The pK$_a$ values obtained for various equilibria are summarized in Table 4.2. As the protonated species forms aggregates even at 1 $\mu$M concentrations in 20% (vol/vol) methanol-water solution, the pK$_a$ values based on the equilibria between the neutral and protonated forms of 7 and 8 were determined in 50% and 30% (vol/vol) methanol-water solutions, respectively.

Comparison of the pK$_a$ values of halogenated squaraine dyes 7 and 8 with that of the parent dye 6 (Table 4.2), shows that 7 and 8 are more acidic than the parent dye. This can be attributed to the electron-withdrawing nature of the bromo
Figure 4.4. Plots of absorbance vs pH for 7 (1.68 x 10^{-5} M) in 20% (vol/vol) methanol-water solution. (a) 506 nm and (b) 610 nm (pK_a = 5.1).

Figure 4.5. Plots of absorbance vs pH for 8 (1.77 x 10^{-5} M) in 20% (vol/vol) methanol-water solution. (a) 500 nm and (b) 617 nm (pK_a = 5.4).
Table 4.2. pKₐ values of various ionic species of 7 and 8 in 20% (vol/vol) methanol in water

<table>
<thead>
<tr>
<th>Equilibrium</th>
<th>pKₐ</th>
</tr>
</thead>
<tbody>
<tr>
<td>6⁺ → Sq⁻</td>
<td>3.5</td>
</tr>
<tr>
<td>7</td>
<td>2.1</td>
</tr>
<tr>
<td>8</td>
<td>2.3</td>
</tr>
<tr>
<td>Sq⁻ → Sq²⁻</td>
<td>7.0</td>
</tr>
<tr>
<td>7</td>
<td>5.1</td>
</tr>
<tr>
<td>8</td>
<td>5.4</td>
</tr>
<tr>
<td>Sq⁻ → Sq⁻</td>
<td>9.5</td>
</tr>
<tr>
<td>7</td>
<td>6.6</td>
</tr>
<tr>
<td>8</td>
<td>6.6</td>
</tr>
</tbody>
</table>

*In 30% (vol/vol) methanol-water solution, taken from the ref. 37 for comparison.

and iodo substituents in 7 and 8, respectively. The bromo derivative 7 is more acidic than the iodo derivative 8, which is understandable, as bromine is more electronegative than iodine. Similar effects have been reported on halogenation of other systems. Thus, for example, the pKₐ value of 5-bromodeoxyuridine (pKₐ = 8.1) was found to be less than that of deoxyuridine (pKₐ = 9.3).⁵² Similarly, lower pKₐ values for the halogenated thiazine and oxazine derivatives were reported when compared to the corresponding parent dyes.⁴⁵,⁴⁷

Of all the forms, only the singly deprotonated forms of 7 and 8 have measurable fluorescence. Figure 4.6 shows the emission spectra of Sq⁻ forms of 7 and 8 in 2% (vol/vol) methanol in water. The emission maximum of the iodo derivative 8, showed a bathochromic shift of 9 nm, as that of 7 in 2% (vol/vol) methanol-water solution. The λₘₐₓ and fluorescence quantum yields of singly deprotonated forms of 7 and 8 are summarized in Table 4.3. The quantum yields
Figure 4.6. Fluorescence emission spectra of Sq forms of (a) 7 (3.3 $\times$ 10$^{-6}$ M) and (b) 8 (2.8 $\times$ 10$^{-6}$ M) in 2% (vol/vol) methanol-water solution. Excitation wavelength, 575 nm.

Table 4.3. Emission properties of singly deprotonated forms of 7 and 8 in different solvent mixtures

<table>
<thead>
<tr>
<th>Compound</th>
<th>Solvent</th>
<th>$\lambda_{\text{max}}$ nm</th>
<th>$\Phi_t \times 10^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>Methanol</td>
<td>627</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>20% methanol-Water</td>
<td>625</td>
<td>0.170</td>
</tr>
<tr>
<td>8</td>
<td>Methanol</td>
<td>634</td>
<td>0.600</td>
</tr>
<tr>
<td></td>
<td>20% methanol-Water</td>
<td>632</td>
<td>0.115</td>
</tr>
</tbody>
</table>

*Excitation wavelength, 575 nm.
of fluorescence ($\Phi_f$) in methanol were found to be 0.01 for 7 and 0.006 for 8, which are lower than that of the parent dye 6 ($\Phi_f = 0.02$).\textsuperscript{37} Further, $\Phi_f$ of both 7 and 8 are very low in water compared to methanol. The low quantum yields in the case of both the halogenated derivatives 7 and 8, when compared to 6, could be attributed to the enhanced intersystem crossing efficiency due to the heavy atom effect in these systems.\textsuperscript{42-46,53,54}

4.3.2. Effect of additives on absorption and emission properties

It has been established that the mechanism of PDT works primarily through the vascular disruption, thereby, depriving tumor cells of their oxygen supply and causing cell death. Therefore, the photosensitizer stability under biological conditions and the influence of cellular components on photophysical properties of the sensitizer become important. In order to have a fair idea about how the halogenated squaraine dyes react to the various organized media, the absorption and emission properties of singly deprotonated forms of 7 and 8 were examined in the presence of microheterogeneous media such as $\beta$-cyclodextrin ($\beta$-CD), poly(4-vinylpyrrolidone) (PVP) and cetyltrimethylammonium bromide (CTAB). These media are unique in their properties, since $\beta$-CD forms inclusion complexes with the guest molecules,\textsuperscript{54} PVP forms hydrophobic microcages around the guest molecules and CTAB forms micellar structure thereby provides in them both the hydrophobic and hydrophilic environment.\textsuperscript{55}

4.3.2.1. Effect of $\beta$-cyclodextrin ($\beta$-CD)

Cyclodextrins (CD, Figure 4.7) are cyclic oligosaccharides that have a central cavity capable of accommodating guest molecules in aqueous solution.\textsuperscript{54} These molecules, containing six ($\alpha$-CD), seven ($\beta$-CD) and eight ($\gamma$-CD) glucose units, each having a different cavity diameter of approximately 4.5, 6.5 and 8.5 Å,
respectively. The primary hydroxyl groups are located on the narrower side of the torus, whereas the secondary hydroxyl groups occupy the broader side. β-CD has an internal diameter of ≈ 6.5 Å and a height of 7.9 Å. The interiors of the cavities encircled by ether oxygens provide a hydrophobic microenvironment in an aqueous solution. The guest molecules that are accommodated in these cavities are relatively isolated from the bulk water environment and often have enforced and constrained conformation. With a view to examine the effects of hydrophobic environment as well as organized media, the photophysical properties of 7 and 8 were examined in the presence of β-CD.

Thus, for example, addition of β-CD to the solution of the singly deprotonated form of 7 brings about a red-shift in the absorption maximum to 614 nm from 606 nm. Figure 4.8 shows the effect of β-CD concentration on the absorption spectrum of singly deprotonated form of 7 in 2% (vol/vol) methanol-water solution. Increase in the concentration of β-CD, led to a gradual increase in the red-shift of the absorption maximum and reached saturation at 0.16 mM of β-CD. No shift in the absorption maximum was observed on further
addition of β-CD. Similar results were obtained with the singly deprotonated form of 8, where the absorption maximum shifts from 614 to 623 nm, upon addition of β-CD. The absorption properties of Sq⁻ forms of 7 and 8 in the presence of various additives are summarized in Table 4.4.

Similar to the absorption properties, the fluorescence emission spectra of both the halogenated derivatives were found to be sensitive to the presence of β-CD. Thus, for example, addition of β-CD to the solution of the singly deprotonated form of 7 brings about a bathochromic shift in the emission maximum from 621 nm to 640 nm. This is also accompanied by a marginal enhancement in the fluorescence yield. At the highest concentration of β-CD studied, the emission yield of Sq⁻ of 7 is nearly 5-fold of that observed in 2% (vol/vol) methanol-water solution (Table 4.4). Figures 4.9 and 4.10 show the
Table 4.4. Absorption and emission properties of singly deprotonated forms of 7 and 8 in the presence of β-cyclodextrin (β-CD), poly(4-vinylpyrrolidone) (PVP) and cetyltrimethylammonium bromide (CTAB) in 2% (vol/vol) methanol-water solution

<table>
<thead>
<tr>
<th>Additive</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\lambda_{\text{max}}$ nm</td>
<td>$\Phi_f$</td>
</tr>
<tr>
<td></td>
<td>abs</td>
<td>em</td>
</tr>
<tr>
<td>*</td>
<td>606</td>
<td>621</td>
</tr>
<tr>
<td>β-CD (1 mM)</td>
<td>614</td>
<td>640</td>
</tr>
<tr>
<td>PVP (6 mM)</td>
<td>626</td>
<td>653</td>
</tr>
<tr>
<td>CTAB (6 mM)</td>
<td>618</td>
<td>647</td>
</tr>
<tr>
<td>CH$_3$OH</td>
<td>612</td>
<td>627</td>
</tr>
</tbody>
</table>

*In 2% (vol/vol) methanol-water solution without an additive.

effect of concentration of β-CD on the fluorescence intensity of 7 and 8, respectively, whereas, Figure 4.11 shows the dependence of quantum yield of fluorescence of 7 and 8 on β-CD concentration. It is evident from Figures 4.9 and 4.10 that as the β-CD concentration increases, the $\lambda_{\text{max}}$ of emission shifts (around 20 nm) to higher wavelengths and reaches a maximum. Also, the quantum yield of
Figure 4.9. Effect of β-cyclodextrin (β-CD) concentration on the emission spectrum of 7 (Sq', 2.8 x 10^{-6} M) in 2% (vol/vol) methanol-water solution. [β-CD] (a) 0; (b) 0.04; (c) 0.1; (d) 0.4 and (e) 1.0 mM. Excitation wavelength, 575 nm.

Figure 4.10. Effect of β-cyclodextrin (β-CD) concentration on the emission spectrum of 8 (Sq', 2.7 x 10^{-6} M) in 2% (vol/vol) methanol-water solution. [β-CD] (a) 0.0; (b) 0.04; (c) 0.16; (d) 0.32 and (e) 2.68 mM. Excitation wavelength, 575 nm.
Figure 4.11. Plots of quantum yield of fluorescence ($\Phi_f$) vs $\beta$-CD concentration in 2% (vol/vol) methanol-water solution. (a) Sq$^-$ of 7 ($3.1 \times 10^{-6}$ M); (b) Sq$^-$ of 8 ($2.1 \times 10^{-6}$ M).

Fluorescence of 7 and 8, increases with $\beta$-CD concentration and reaches a saturation point at 1 mM of $\beta$-CD (Figure 4.11), indicating thereby that Sq$^-$ forms of both 7 and 8 attain an equilibrium with $\beta$-CD cavities beyond 1 mM of host concentration.

The significant bathochromic shifts in the absorption and fluorescence properties in the presence of $\beta$-CD can be attributed to the formation of inclusion complexes between $\beta$-CD and Sq$^-$ forms of 7 and 8. In order to have a better understanding of these inclusion complexes, the fluorescence behaviour of these systems was analysed by Benesi-Hildebrand equation 1, for a 2:1 complex formation (Chart 4.4) between $\beta$-CD and Sq$^-$ forms of 7 and 8,

\[
\frac{I}{(\Phi_f - \Phi_{ob})} = \frac{I}{(\Phi_f - \Phi_{fc})} + \frac{I}{K (\Phi_f - \Phi_{fc}) [\beta$-CD]$^2}
\]
where, $K$ is the association constant, $\Phi_f$ is the quantum yield of emission of free $\text{Sq}^-$, $\Phi_{ob}$ is the observed quantum yield and $\Phi_{jc}$ is the quantum of emission of $\text{Sq}^-$ - $\beta$-CD complex. A plot of $1/(\Phi_f - \Phi_{ob})$ vs $1/(\beta-CD)^2$ was found to be linear for both 7 (Figure 4.12) and 8, indicating thereby the formation of a 2:1 complex between $\beta$-CD and $\text{Sq}^-$ forms as shown in Chart 4.4. Similar 2:1 complex

![Chart 4.4](image)

**Figure 4.12.** Plot of $1/(\Phi_f - \Phi_{ob})$ vs $1/(\beta-CD)^2$ for the fluorescence quantum yield enhancement of $\text{Sq}^-$ of 7 ($2.1 \times 10^{-6}$ M) in 2% (vol/vol) methanol-water solution.
formation was proposed earlier\textsuperscript{37} for the parent dye 6. Based on Eq. 1, association constants for these inclusion complexes were calculated and are found to be $9.0 \times 10^6 \text{M}^2$ for 7 and $1.3 \times 10^7 \text{M}^2$ for 8 in 2\% methanol-water solution.

The singly deprotonated forms of these dyes exhibit hydrogen bonding with solvent water molecules. Because the cavity of $\beta$-CD is hydrophobic, encapsulation of the dyes by $\beta$-CD is likely to prevent the intermolecular hydrogen bonding with the surrounding water molecules (Chart 4.4). Hence, the enhancement of the fluorescence yields of 7 and 8 can be attributed to the decrease in rotational freedom of the encapsulated molecule and elimination of quencher water molecules from the immediate surroundings. However, the fluorescence enhancement was only 5-fold in the case of 7 and 8, when compared to the 90-fold enhancement\textsuperscript{38} in the case of the parent dye 6, thereby suggesting that either the encapsulation by $\beta$-CD is incomplete or the presence of heavy atoms enhances the intersystem crossing efficiency in the former cases.

4.3.2.2. Effect of poly(4-vinylpyrrolidone) (PVP)

Poly(4-vinylpyrrolidone) is a water soluble polymer and forms microcages\textsuperscript{55} around the guest molecules, when dissolved in aqueous medium. These microcages are hydrophobic in nature, hence, displaces water molecules from the sphere. Figure 4.13 shows the structure of poly(4-vinylpyrrolidone).

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{structure.png}
\caption{Structure of poly(4-vinylpyrrolidone).}
\end{figure}
Microencapsulation of the guest molecules in these microcages leads to break up of the hydrogen bonding between the solvent molecules and the guest molecules. Also, because of the microencapsulation, rotational freedom of the guest molecule gets reduced and thereby affects the photophysical properties of included molecules. The fluorescence and absorption properties of singly deprotonated forms of 7 and 8 were examined in the presence of PVP (molecular mass = 44,000), with a view to understand the effects of polymer microcages on photophysical properties of these dyes.

Thus, for example, addition of PVP to the solutions of 7 and 8, stabilizes the anionic forms and also brings about bathochromic shifts in the absorption maxima (~ 20 nm). Figure 4.14 shows the effect of PVP concentration on the absorption spectrum of singly deprotonated form of 7 in 2% (vol/vol) methanol-water solution. Similar observations were made in the case of 8. The absorption
maximum is shifted from 607 nm to 626 nm in the case of the bromo derivative 7, whereas, in the case of the iodo derivative 8, the shift was from 614 nm to 635 nm (Table 4.4).

Both bathochromic shifts in the emission maxima and enhancement in fluorescence yields of 7 and 8 were more pronounced with PVP than β-CD. Thus, for example, addition of PVP to the solution of the singly deprotonated form of 7 brings about a bathochromic shift in the emission maximum from 621 nm to 653 nm in 2% (vol/vol) methanol-water solution (Figure 4.15). This is accompanied by a 62-fold enhancement in the fluorescence yield, at the highest concentration of β-CD studied (Table 4.4). Similarly, a large bathochromic shift of 34 nm in emission maximum was observed in the case of the iodo derivative 8 (Figure 4.16). However, the fluorescence quantum yield enhancement is marginal (32-fold) in the case of 8, when compared to the bromo derivative 7. Figure 4.17 shows the dependence of quantum yields of fluorescence of 7 and 8 on PVP concentration in 2% (vol/vol) methanol-water solution.

![Figure 4.15](image)

**Figure 4.15.** Effect of polyvinylpyrrolidone (PVP) on the emission spectrum of 7 ([SQ'], 6.7 x 10^{-6} M) in 2% (vol/vol) methanol-water solution. [PVP] (a) 0.0; (b) 1.0; (c) 3.0 and (d) 6.0 mM. Excitation wavelength, 575 nm.
Figure 4.16. Effect of polyvinylpyrrolidone (PVP) on the emission spectrum of \(8\) (Sq\(^-\), \(3.7 \times 10^{-6}\) M) in 2% (vol/vol) methanol-water solution. [PVP] (a) 0.0; (b) 0.7; (c) 12.0 and (d) 20.0 mM. Excitation wavelength, 575 nm.

Figure 4.17. Plots of quantum yield of fluorescence \(\Phi_f\) vs [PVP] in 2% (vol/vol) methanol-water solution. (a) Sq\(^-\) of 7 (3.1 \times 10^{-6}\) M) and (b) Sq\(^-\) of 8 (2.1 \times 10^{-6}\) M).
The observed bathochromic shifts in the absorption spectra (marginal) and emission maxima (pronounced) of 7 and 8, support the fact that these dyes are entrapped by the polymer microcages. Since the interior of these microcages is hydrophobic in nature, the microencapsulation of the dye molecules by the polymer prevents the intermolecular hydrogen bonding between the dye and the solvent water molecules. Further, existence of intramolecular hydrogen bonding of the included dye molecules within the microcage could decrease the rotational freedom and also the rates of non-radiative decay of the excited singlet states of 7 and 8 in the polymer media.

4.3.2.3. Effect of cetyltrimethylammonium bromide (CTAB)

Above a certain concentration range, surfactant molecules aggregate in aqueous solution to form particles of colloidal dimensions called micelles. The micelle formation takes place over a narrow range of surfactant concentrations, around the critical micelle concentration (CMC). Figure 4.18 shows the structure of CTAB and a micellar structure. In the micelle, the alkyl chains of the surfactant form the interior hydrophobic core, whereas the polar heads

\[
\begin{align*}
\text{Cetyltrimethylammonium bromide} \\
\text{(CMC} = 9.2 \times 10^{-4} \text{ M)}
\end{align*}
\]

Figure 4.18. Structure of a micelle.
Pointing towards the bulk aqueous medium. Therefore, the unique micellar structure confers in them both hydrophobic and hydrophilic environments and allows the partition of the guest/dye either in the bulk aqueous medium or in the micellar structure. In order to evaluate the preferential interaction of squaraine dyes with the hydrophobic and hydrophilic environments, the absorption and fluorescence properties of singly deprotonated forms of 7 and 8 were examined in the presence of various concentrations of CTAB.

Addition of CTAB to a solution of the singly deprotonated forms of 7 and 8 exhibited greater stabilization as well as red shifts in the absorption maxima. Figure 4.19 shows the effect of CTAB concentration on the absorption spectrum of 7 in 2% (vol/vol) methanol-water solution. In the presence of CTAB, the absorption maxima of 7 was shifted from 606 nm to 618 nm (Table 4.4). Similarly,

![Figure 4.19. Effect of cetyltrimethylammonium bromide (CTAB) concentration on the absorption spectrum of 7 (Sq, 3.7 x 10^{-6} M) in 2% (vol/vol) methanol-water solution. [CTAB] (a) 0.0; (b) 2; (c) 4 and (d) 6 mM.](image-url)
a bathochromic shift of about 12 nm was observed in the absorption maximum of 8 in the presence of CTAB. It is evident from Figure 4.19 that as the concentration of CTAB increases, the $\lambda_{\text{max}}$ of absorption shifts to higher wavelengths with a slight decrease in extinction coefficient ($\varepsilon_{\text{max}}$). Further addition of CTAB (6 mM), gave an increased $\varepsilon_{\text{max}}$ and a saturated value for the absorption maximum.

Addition of CTAB to the singly deprotonated forms of 7 (Figure 4.20) and 8 in 2% (vol/vol) methanol-water solution, exhibited red shifts in the emission maxima and also a significant enhancement in their fluorescence quantum yields (117-fold for 7 and 75-fold for 8) (Table 4.4). Figure 4.21 shows the dependence of quantum yields of 7 and 8 on CTAB concentration. The quantum yields of 7 and 8 increase with increase in concentration of CTAB and reaches saturation level at 6 mM of CTAB.

![Figure 4.20](image-url)
Figure 4.21. Plots of quantum yield of fluorescence ($\Phi_f$) vs [CTAB] in 2% (vol/vol) methanol-water solution. (a) Sq of 7 ($3.7 \times 10^{-6}$ M) and (b) Sq of 8 ($1.4 \times 10^{-6}$ M).

The effect of CTAB as in the case of PVP and $\beta$-CD, could be attributed to the microencapsulation of the singly deprotonated forms of 7 and 8, leading to a breakup of the hydrogen bonding between solvent molecules and the dye and also due to the restriction of the rotational freedom of the guest molecules. The significant enhancement in the fluorescence yields (116-fold for 7 and 75-fold for 8) in the presence of CTAB could be due to the fact that CTAB micelles enable elimination of water molecules more effectively and restrict the rotational freedom of the guest molecules, thereby decreasing the rate of non-radiative decay.

4.3.3. Laser flash photolysis studies

High triplet excited state quantum yields and efficient generation of singlet oxygen are the prerequisite properties of a molecule to be used as a sensitizer in PDT applications. Therefore, the major excited states involved in the case of squaraine dyes 7 and 8 were characterized by means of nanosecond laser flash
photolysis technique. All the forms of 7 and 8 have sufficient absorption around 532 nm making it possible to directly excite them with the second harmonic of the Nd:YAG laser. Excitation of various forms of 7 and 8 by 532 nm laser pulses (10 ns, 50 mJ/pulse) led to the formation of a transient absorption with a bleach in the region corresponding to the ground state absorption spectra. For example, Figures 4.22 and 4.23 show the transient absorption spectra of singly deprotonated forms of 7 and 8, respectively. As can be seen from the Figures 4.22 and 4.23, these transients have fairly strong absorption in the short as well as long wavelength regions of the bleach. Similar transient absorption spectra were obtained for neutral, protonated and doubly deprotonated forms of 7 and 8. The transient absorption, which is formed within the laser pulse decays by a first order process and leads to the recovery of the ground state absorption (insets of Figures 4.22 and 4.23), indicating negligible formation of any permanent products.

The transient absorption is readily quenched by dissolved oxygen, suggesting that the absorption may be due to the triplet excited state formation. Further, this was confirmed by quenching of the transients by β-carotene. Addition of β-carotene, which possesses a low energy level triplet\(^3\) led to a quenching of the transient absorption which was accompanied by a growth of the β-carotene triplet. Intersystem crossing efficiency in β-carotene is negligible and hence the formation of the β-carotene triplet in these systems clearly confirms that the transient absorptions obtained upon laser excitation of various forms of 7 and 8 are due to the triplet excited states of these forms. The triplet yields (\(\Phi_T\)) and extinction coefficients (\(e_{TT}\)) of the various forms were measured employing the method of energy transfer to β-carotene using tris(bipyridyl)ruthenium (II) complex as the reference. The data obtained for the different forms of 7 and 8 are summarized in Table 4.5. It may be mentioned that the laser excitation of various forms of the parent dye 6 did not give any transient absorptions under similar conditions.
Figure 4.22. Transient absorption spectrum, recorded immediately following the laser pulse (532 nm) excitation of Sq$^-$ of 7 ($4 \times 10^{-4}$ mM) in methanol. Inset shows the decay of the transient at 480 nm.

Figure 4.23. Transient absorption spectrum, recorded immediately following the laser pulse (532 nm) excitation of Sq$^-$ of 8 ($7 \times 10^{-4}$ mM) in methanol. Inset shows the decay of the transient at 500 nm.
Table 4.5. Photophysical properties of triplet excited states of various forms of 7 and 8 in methanol

<table>
<thead>
<tr>
<th>Compound</th>
<th>Ionic form</th>
<th>$\lambda_{\text{max}}$ (nm)</th>
<th>$\varepsilon$ (M$^{-1}$cm$^{-1}$)</th>
<th>$\tau$ (µs)</th>
<th>$\Phi_T$</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>Sq</td>
<td>445</td>
<td>6400</td>
<td>4.2</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>SqH$^+$</td>
<td>450</td>
<td>-</td>
<td>0.38</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td></td>
<td>Sq$^-$</td>
<td>525</td>
<td>9900</td>
<td>132</td>
<td>0.22 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>Sq$^{2-}$</td>
<td>425</td>
<td>-</td>
<td>0.31</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>8</td>
<td>Sq</td>
<td>450</td>
<td>8100</td>
<td>1.5</td>
<td>0.24 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>SqH$^+$</td>
<td>465</td>
<td>-</td>
<td>0.49</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td></td>
<td>Sq$^-$</td>
<td>530</td>
<td>11000</td>
<td>36</td>
<td>0.5 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>Sq$^{2-}$</td>
<td>460</td>
<td>-</td>
<td>0.061</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>

Comparison of the lifetimes of the triplet excited states of the various forms of 7 and 8 (Table 4.5) indicates that the singly deprotonated species are long lived and the lifetimes decrease in the order $\text{Sq}^{-} \gg \text{Sq} > \text{SqH}^{+} > \text{Sq}^{2-}$ for both 7 and 8. Further, triplet excited states of various forms of the iodo derivative 8 are relatively short lived when compared to the corresponding species of the bromo derivative 7. The quantum yields of triplet excited states of protonated and doubly deprotonated forms of 7 and 8 are found to be very small ($< 0.01$), whereas, the neutral and singly deprotonated forms of 7 and 8 have significant yields in the order $\text{Sq}^{-} > \text{Sq}$. The triplet yield ($\Phi_T$) of the singly deprotonated form of the iodo derivative 8 was found to be 0.5, which is much higher than the corresponding
quantum yield for the formation of triplet of the bromo derivative 7 ($\Phi_T = 0.22$) (Table 4.5).

The intersystem crossing efficiencies of most of the earlier reported squaraine dyes are very low.$^{36-38,57}$ The triplet excited states of most of these dyes were studied by triplet-triplet energy transfer methods using the excited state triplets of 9,10-dibromoanthracene$^{58}$ and 1-pyrenecarboxaldehyde$^{59}$ as donors. In the present study, although, the triplet quantum yields of the protonated and doubly deprotonated forms of 7 and 8 are very low, whereas, the neutral and singly deprotonated forms have substantial triplet yields. The low triplet yields of the protonated as well as the doubly deprotonated forms suggest that the non-radiative processes are far more efficient in these cases. The short lifetimes of the triplet states of these forms compared to those of the singly deprotonated and neutral forms are indicative of this. Similarly, the lack of fluorescence for most of these forms, other than the singly deprotonated form, is indicative of efficient non-radiative processes from the excited singlet state. The non-radiative decay processes in squaraines can involve rotational relaxation around the C-C bond between the phenyl ring and the cyclobutane ring of the excited states.$^{36-38}$ In the singly deprotonated form, probably an ideal situation arises involving intramolecular hydrogen bonding between the hydroxyl group of the phenyl ring and the oxygen atom of the cyclobutane ring. Such an interaction can impart some rigidity to the excited state leading to a reduction in the rate of the non-radiative processes. The observation of emission from the excited singlet state as well as the significantly large triplet lifetimes of singly deprotonated forms of 7 and 8 as compared to the other forms support this view.

4.3.4. Photosensitized generation of singlet oxygen

Because triplet excited states are the major transient intermediates obtained upon 532 nm laser flash photolysis studies of 7 and 8 (Table 4.5), the efficiency
of photosensitized singlet oxygen generation by these systems have been examined, since singlet oxygen is the main cytotoxic agent of the type II reactions in PDT.\textsuperscript{60} Singlet oxygen is approximately 24 Kcal/mol higher in energy than the ground state oxygen. The energy of the $T_1$ state of the dye should be close to or higher than this energy relative to the energy of the $S_0$ state for an efficient production of singlet oxygen.\textsuperscript{19,42}

In the present study, only the singly deprotonated forms of 7 and 8 have been chosen because of the fact that they have significant triplet yields of 0.22 and 0.5 and have long lifetimes of 132 and 36 $\mu$s, respectively (Table 4.5). Moreover, under the biological pH conditions ($p$H = 6 to 7), the singly deprotonated forms of 7 and 8 are expected to be the predominant species. Quantum yields for singlet oxygen generation in air saturated methanol were determined by monitoring the dye sensitized photooxidation of DPBF. Quantum yields were calculated by plotting the depletion in the absorbance of DPBF at 410 nm against the irradiation time (Figure 4.24) and comparing with those of standards such as MB or RB.

![Figure 4.24. Plots of absorbance change of DPBF at various time intervals in the presence of $S\mathsf{q}^-$ of 7 ($S\mathsf{q}^-$, 2.17 x $10^{-5}$ M). Excitation Wavelength, 600 nm.](image-url)
Figure 4.25. Plots of change of concentration of 1,3-diphenylisobenzofuran (DPBF) at 410 nm vs irradiation time. (a) 7 (Sq, \(2.17 \times 10^{-5} \) M); (b) 8 (Sq, \(2.20 \times 10^{-5} \) M): Results with methylene blue (c) are also shown as standard. Excitation wavelength, 600 nm.

sensitized photooxidation of DPBF. The plots obeyed linearity as shown in Figure 4.25. Values of \(\Phi(1\text{O}_2)\) for singly deprotonated forms of 7 and 8 are summarized in Table 4.6.

Table 4.6. Singlet oxygen quantum yields [\(\Phi(1\text{O}_2)\)] of singly deprotonated forms of 7 and 8 in the presence and absence of poly(4-vinyl)pyrrolidone (PVP) in methanol

<table>
<thead>
<tr>
<th>Compound</th>
<th>PVP (mM)</th>
<th>(\Phi(1\text{O}_2))</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>0</td>
<td>0.13 ± 0.005</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>0.29 ± 0.001</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>0.47 ± 0.017</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>0.62 ± 0.007</td>
</tr>
</tbody>
</table>
It is evident from Table 4.6 that the singlet oxygen quantum yield increased from 0.13 for the bromo derivative 7 to 0.47 for the iodo derivative 8. These values are in good agreement with the results obtained by laser flash photolysis studies of these systems. Since organized media were found to stabilize the singly deprotonated forms of 7 and 8 (Table 4.4), the quantum yields of singlet oxygen generation by these systems were determined in the presence of PVP. Figure 4.26 shows the plots of depletion in the absorbance of DBPF at 410 nm against the irradiation time in the presence of 14 mM of PVP. The photooxidation of DBPF by 7 and 8 was found to be more efficient in the presence of PVP, when compared to methanol. The quantum yields of singlet oxygen generation increased from 0.13 to 0.29 for the bromo derivative 7 and from 0.47 to 0.62 for the iodo derivative 8 (Table 4.6).

Figure 4.26. Change in absorbance of 1,3-diphenylisobenzofuran (DPBF) at 410 nm vs irradiation time in the presence of polyvinylpyrrolidone (14 mM) in methanol. (a) 7 (S^-, 0.85 x 10^-5 M) and (b) 8 (S^-, 1.17 x 10^-5 M) in the presence of PVP. Results with methylene blue (c) are also shown as standard.
Laser flash photolysis studies and studies of singlet oxygen generation with 7 and 8 suggest that the substitution of the parent dye 6 with heavy atoms such as bromine and iodine, enhances the intersystem crossing efficiency of 7 and 8 considerably. Intersystem crossing from the S₁ state to the T₁ state can be promoted by increased spin-orbit coupling between an attached heavy atom and the molecular orbitals of the dye. Higher triplet and singlet oxygen yields, observed (Table 4.5) in the case of the iodo derivative 8 are in accordance with the fact that as the size of heavy atoms increases, the spin-orbit coupling should increase thereby increasing the intersystem crossing and triplet yields. A similar trend has been reported in the case of heavy atom substituted pyrylium dyes, where an increase in intersystem crossing processes for the tellurapyrylium dye resulted in an increase in singlet oxygen generation yields from 0.0004 to 0.12. The significant enhancement of the quantum yields for the generation of singlet oxygen by 7 and 8 in the presence of organized media (PVP) could be attributed to the increased triplet excited state lifetimes of these systems.

4.4. Conclusions

The results obtained in this study have confirmed that halogenation of squaraines 7 and 8 has increased intersystem crossing efficiency when compared to the parent squaraine 6. The triplet excited states of 7 and 8 interact with molecular oxygen, generating singlet oxygen in good yields. Halogenation also resulted in an increase in water solubility and decrease in pKₐ values of the various forms of 7 and 8. The large bathochromic shifts in the absorption spectra and stabilization of singly deprotonated forms of 7 and 8 in the presence of β-CD, PVP and CTAB make them useful in biological applications. The iodo derivative 8, in particular, appears to have great potential as a new type of second generation photosensitizer for its use in photodynamic therapy. The singly deprotonated form of 8, which is expected to be present predominantly under the biological pH
conditions has an absorption which extends well into the near infrared region. Reasonable lifetime ($\tau_T = 36 \mu s$) and significant quantum yield of the triplet excited state ($\Phi_T = 0.5$) and quantitative generation of singlet oxygen ($\Phi(\cdot O_2) = 0.47$) by the singly deprotonated form of 8, makes it a promising compound for photodynamic therapy.

4.5. Experimental Section

All melting points are uncorrected and were determined using a Mel-Temp-II melting point apparatus. The IR spectra were recorded on a Perkin Elmer model 882 Infrared spectrometer. The electronic absorption spectra were recorded on a GBC double beam UV-Visible spectrophotometer. The mass spectra were recorded on a JEOL JMS AX 500 HA mass spectrometer. Quantum yields of fluorescence were measured by the relative methods using optically dilute solutions. Cresyl violet ($\Phi_f = 0.52$) and the squaraine dye 6 ($\Phi_f = 0.02$) in methanol were used as standards. An Elico pH meter was used for the pH measurements. NaOH, NH$_4$OH or HCl were used to vary the pH of the solutions. Solvents used were purified before use. All experiments were carried out at room temperature ($25 \pm 1^\circ C$).

4.5.1. Chemicals

1,3-Diphenylisobenzofuran (DPBF), obtained from Aldrich was recrystallized from a mixture (1:3) of methanol and acetone. β-Carotene (Aldrich) was recrystallized from a mixture (1:1) of ethanol and chloroform. Methylene blue (MB), rose bengal (RB), β-cyclodextrin (β-CD), cetyltrimethylammonium bromide (cetrimide), poly(4-vinylpyrrolidone) (PVP) and tris(bipyridyl)ruthenium (II) chloride (Ru(bpy)$_3^{2+}$) (all from Aldrich) were used without further purification. Commercially available Reinecke's salt (NH$_4$Cr(NH$_3$)$_2$(NCS)$_4$) was converted to
its potassium salt by dissolving the salt in warm water and adding excess of solid potassium nitrate. The solution was cooled and the precipitate was recrystallized from warm water. Bis(2,4,6-trihydroxyphenyl)squaraine (6) was synthesized from squaric acid and 1,3,5-trihydroxybenzene by a reported procedure. 64

4.5.2. Synthesis of bis(3,5-dibromo-2,4,6-trihydroxyphenyl)squaraine (7)

The squaraine dye 6 (100 mg, 0.3 mmol) was dissolved in glacial acetic acid (85 mL) by stirring the solution at 50 °C for 90 min. After cooling the solution to room temperature, bromine (215 mg, 1.3 mmol) in glacial acetic acid (15 mL) was added dropwise, over a period of 1 h. The reaction mixture was kept in the refrigerator for 4 h to yield 160 mg (80%) of 7, mp 315 °C (d), which was recrystallized from a mixture (4:1) of water and isopropanol. IR (KBr) $\nu_{\text{max}}$ 3413, 1622, 726 and 519 cm$^{-1}$; UV [20% (vol/vol) methanol-water] $\lambda_{\text{max}}$ 497 nm ($\varepsilon$, 30000 M$^{-1}$cm$^{-1}$) and 610 ($\varepsilon$, 47000 M$^{-1}$cm$^{-1}$). Molecular weight calculated for $C_{16}H_{18}O_{9}Br_4$: 642.6874. Found: 642.6879 (high resolution mass spectrometry).

4.5.3. Synthesis of bis(3,5-diiodo-2,4,6-trihydroxyphenyl)squaraine (8)

The squaraine dye 6 (100 mg, 0.3 mmol) was dissolved in glacial acetic acid (85 mL) by stirring the solution at 50 °C for 90 min. After cooling the solution, iodine monochloride (218 mg, 1.34 mmol) in glacial acetic acid (15 mL) was added dropwise, over a period of 1 h. The reaction mixture was stirred for 1 h more. Water (15 mL) was added to the reaction mixture and kept in the refrigerator for 5 h. The solid precipitate was filtered to give 180 mg (71%) of 8, mp 270 °C (d), which was recrystallized from a mixture (4:1) of methanol and isopropanol. IR (KBr) $\nu_{\text{max}}$ 3383, 1603, 726 and 508 cm$^{-1}$; UV [20% (vol/vol) methanol-water] $\lambda_{\text{max}}$ 509 nm ($\varepsilon$, 45000 M$^{-1}$cm$^{-1}$) and 617 ($\varepsilon$, 63000 M$^{-1}$cm$^{-1}$). Molecular weight
calculated for C₁₆H₆O₈I₄: 834.6320. Found: 834.8360 (high resolution mass spectrometry).

4.5.4. Laser flash photolysis

Nanosecond laser flash photolysis experiments were carried out by employing an Applied Photophysics Model LKS-20 Laser Kinetic Spectrometer using GCR-12 Series Quanta Ray Nd:YAG laser. The analyzing and laser beams were fixed at right angles to each other. The laser energy was 50 mJ at 532 nm. The triplet yields (Φₜ) and extinction coefficients (εₜₜ) of the various forms were measured employing the method of energy transfer to β-carotene⁶⁵ using tris(bipyridyl)ruthenium (II) complex as the reference.⁶⁶ For these experiments, Ru(bpy)₃²⁺ and dyes 7 and 8, optically matched at 532 nm, were mixed with a known volume of β-carotene solution (end concentration of β-carotene was 2.0 × 10⁻⁴ M). The transient absorbance (ΔA) of the β-carotene triplet, formed by the energy transfer from Ru(bpy)₃²⁺ or the squaraine dye triplet, was monitored at 515 nm. Comparison of plateau absorbances (ΔAₓ) following the completion of sensitised triplet formation, properly corrected for the decay of the donor triplets in competition with energy transfer to β-carotene, enabled to estimate Φₜ of the neutral and anionic forms of 7 and 8 based on equation 2,

\[
Φ_T^{sq} = \frac{Φ_T^{ref} \ ΔA^{sq}}{ΔA^{ref}} \frac{k_{obs}^{sq}}{k_{obs}^{ref} - k_o} \frac{k_{obs}^{ref} - k_o}{k_{obs}^{ref}}
\]

(2)

where, superscripts sq and ref designate different forms of squaraine dyes and Ru(bpy)₃²⁺, respectively, k_{obs} is the pseudo-first order rate constant for the growth of the β-carotene triplet and k_o is the rate constant for the decay of the donor triplets, in the absence of β-carotene, observed in solutions containing Ru(bpy)₃²⁺.
or a squaraine dye at the same optical density as those used for sensitization. Under the experimental conditions, the direct excitation of \( \beta \)-carotene did not result in any significant triplet formation because of negligible triplet yield. This was checked by direct laser flash photolysis of solutions containing \( \beta \)-carotene only. \( \Phi_T \) in methanol was taken to be unity.\(^6\) The \( \Phi_T \) data obtained in this manner are reliable to the extent to which the assumption regarding 100% efficiency of energy transfer to \( \beta \)-carotene is valid.

The extinction coefficients (\( \varepsilon_{TT} \)) of T-T absorption were measured by comparing end-of-pulse absorbances (\( \Delta A_o \)) observed at the respective maxima in the course of the direct laser flash photolysis (532 nm) of methanol solutions of Ru(bpy)_3\(^{2+} \) (reference) and the squaraine dye (optically matched at the laser wavelength) using the following equation 3,

\[
\frac{\varepsilon_{sq}}{\varepsilon_{TT}} = \frac{\Delta A_o^{sq}}{\Delta A_o^{ref}} \frac{\Phi_T^{ref}}{\Phi_T^{sq}}
\]

where, superscripts \( sq \) and \( ref \) designate squaraine dyes and Ru(bpy)_3\(^{2+} \), respectively. \( \varepsilon_{TT} \) of the reference in methanol was taken to be 13500 M\(^{-1} \) cm\(^{-1} \) at 370 nm.\(^6\)

4.5.5. Quantum yields for singlet oxygen generation

Photolysis was carried out with a light source of 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). Reinecke's salt \([\text{NH}_4\text{Cr(NH}_3)_2\text{(NCS)}_4]\) (converted into its potassium
salt) actinometry\textsuperscript{67} was used as a calibration check for determining the intensity of light source.

Fresh solutions of the salt were prepared and irradiated at 545 nm in a 2.5 cm cell for different time intervals. The extent of photochemical equation of KCr(NH\textsubscript{3})\textsubscript{2}(NCS)\textsubscript{4} was determined by analysing the free thiocyanate ion released during photolysis in the following manner. At the end of irradiation, 2 mL of the solution was diluted exactly to 10 mL with a reagent of 0.1 M Fe(NO\textsubscript{3})\textsubscript{3}.9H\textsubscript{2}O in 0.1M HClO\textsubscript{4}. The absorbance of the resulting iron thiocyanate complex was measured at 450 nm (ε, 4.3 x 10\textsuperscript{3} M\textsuperscript{-1} cm\textsuperscript{-1}). From the known quantum yield of thiocyanate released during photolysis, the intensity of the light source (I) was calculated using equation 4,

\begin{equation}
I = \frac{A V_2 V_3}{1000 V_1 \varepsilon \Phi t}
\end{equation}

where, A = absorbance at 450 nm, V\textsubscript{1} = volume of the irradiated solution taken for measurement (2 mL), V\textsubscript{2} = volume of the solution after dilution with reagent (10 mL), V\textsubscript{3} = volume of the solution taken for irradiation (8 mL), \Phi = quantum yield of thiocyanate released (0.282 at 545 nm), ε = extinction coefficient (4.3 x 10\textsuperscript{3} M\textsuperscript{-1} cm\textsuperscript{-1} at 450 nm).

The intensity of the light calculated in the above manner was found to be 1.48 x 10\textsuperscript{-8} Einstein/sec. The experiment was repeated with 5 cm cell and the light intensity was found to be 1.7 x 10\textsuperscript{-8} Einstein/sec. The light source was also calibrated at 590 nm and the intensity of light at this wavelength was calculated to be 7.5 x 10\textsuperscript{-9} Einstein/sec.

Quantum yields of singlet oxygen generation in air saturated methanol were determined by monitoring the photooxidation of DPBF, sensitized by the Sq\textsuperscript{-} of 7 and 8. DPBF is a convenient acceptor since it absorbs in a region of dye tranparency and rapidly scavanges singlet oxygen to give colorless products. The
reaction occurs with little or no physical quenching. Singlet oxygen quantum yields were measured at low dye concentrations (optical density is 0.2-0.3 at the irradiation wavelengths 590 and or 635 nm) to minimise the possibility of singlet oxygen quenching by the dyes. Irradiations were carried out at low conversion (≤ 10%) of DPBF such that its concentration may be assumed to be fixed at the initial value. The quantum yields of singlet oxygen generation were calculated by a relative method using optically matched solutions and comparing the quantum yield of photooxidation of DPBF sensitized by the dye of interest to the quantum yield of methylene blue (Φ(′O₂) = 0.52) sensitized DPBF photooxidation as the reference. The following equation 5 was used,

\[ \Phi(′O₂)^{sq} = \Phi(′O₂)^{MB} \frac{m^{sq}}{m^{MB}} \frac{F^{MB}}{F^{sq}} \]  

where, superscripts \( sq \) and \( MB \) designate singly deprotonated forms of either 7 and 8 and methylene blue respectively, \( Φ(′O₂) \) is the quantum yield of singlet oxygen, \( m \) is the slope of a plot of change in absorbance of DPBF (at 410 nm) with the irradiation time and \( F \) is the absorption correction factor, which is given by \( F = 1 - 10^{OD} \) where, \( OD \) is the optical density at the irradiation wavelength.
4.6. References


