Chapter – 3: Fluorescence Studies on Lumichrome

3.1. Introduction

Lumichrome (7,8-dimethylalloxazine, LC, structure I in figure 3.1) represents a group of heterocyclic compounds related to lumazines and biologically important flavins. Although isoalloxazines (structure II), especially flavins, are very closely related compounds to alloxazines, the spectral and photophysical properties of these two groups of compounds are distinctly different. Particularly, the fluorescence intensity and excited state lifetime for flavins are substantially higher than the corresponding alloxazines due to the presence of yellow chromophore characteristics of flavoproteins – enzymes occurring widely in animals and plants. The ground and excited state properties of flavins and its representatives like riboflavin, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) are well characterized [52-56]; however, alloxazines have received relatively little attention. The interest in LC and other substituted alloxazines have intensified recently due to their important role in different biological systems [57-61]. For example, LC is known as a triplet photosensitizer to generate singlet oxygen (\(^{1}O_2\)), which initiates the oxidation of many biological substrates like enzyme, proteins, nucleic acids and hormones etc [62-63]. Alloxazine group of ligands are also found to be very effective in binding to several proteins and nucleic acids [64-67]. Furthermore, LC is known to inhibit the flavin reductase in living E-Coli cells, the riboflavin uptake by human-derived liver cells Hep G2, colonic epithelial NCM460 cells, and also Caco-2 human intestinal epithelial cells [58-59]. The application of LC has been reported in photodegradation of polyamidehydroxyurethane polymers in aqueous solution [63], polymerization of 2-hydroxyethyl methacrylate [68,69], and also as an optical transistor device [70] etc.

Alloxazine nucleosides, for which the hydrogen bonding characteristics resemble with thymidine, can further be used as fluorescence probe [71]. It has already been reported that hydrogen bonding with acetic acid or pyridine derivatives promote the tautomerization in alloxazine scaffold to produce isoalloxazine type of structure [72,73]. It was also suggested in literature that solvent hydrogen bonding plays an important role on the photophysics of LC [74 (a)]. More particularly, both the absorption and emission maxima show red shift, emission quantum yield increases, and also the fluorescence state becomes more stable in polar protic
environments when compared with the aprotic medium. However, to the best of our knowledge, so far no systematic study of LC solvatochromism with solvent hydrogen bonding ability is available in the literature.

![Figure 3.1: Tautomerization of lumichrome, LC (I) and isoalloxazine (II).](image)

The nature of the interaction of a solute molecule with amphiprotic solvents like water, alcohol etc. can have far-reaching consequences in terms of solubility as well as chemical properties of the solute. In some cases, the solvents can function as mere supporting matrices with its physical parameters like dielectric constant, viscosity, refractive index and polarizibility etc. However, in many cases, the interactions of heterocyclic organic compounds with amphiprotic solvents involve creation of new molecular complexes through hydrogen bond formation. The chemical nature of the solute is mostly controlled by the properties of the hydrogen bonded complex. Hydrogen bonding can occur in different modes, depending on the structure of the solute and solvent. The situation becomes more complicated when a solute molecule, like LC, possesses multiple hydrogen bonding site and the solvent molecule, like water and/or acetic acid, can act both as a proton donor as well as a proton acceptor. Under this condition, the competition among different molecular species resulting from hydrogen bonding interaction between the solute and the solvent molecules becomes inevitable. LC provides an important example to study the hydrogen bonding effect because the molecule itself can exist in more than one prototropic species having multiple hydrogen bonding sites (figure 3.2). The most stable structure and associated spectroscopic properties will depend strongly on the relative abundance of several species, as well as their hydrogen bonding mode with the solvent. Furthermore, the efficacy of hydrogen bond formation in the excited state and consequently the tautomerization process may change due to charge redistribution after excitation.

In this chapter, we will discuss the steady state and picosecond time-resolved fluorescence spectral properties in a series of pure solvents with varying polarity as well as hydrogen bond donor and acceptor abilities to find quantitative information about their relative contribution on LC solvatochromism. Furthermore, solution phase spectral properties of LC were
theoretically modeled by using density functional approach to gather the information on molecular origin of specific solvent effect and driving force towards the excited state tautomerization process.

Figure 3.2 Possible isomeric structures of lumichrome, LC (I).

3.2. Spectral Properties in Homogeneous Solvents

3.2.1. Steady state spectral properties in pure solvents

Figure 3.3 shows some representative absorption and emission spectra of LC in different solvents. It is observed that LC shows two absorption peaks; the first one is within the spectral range of 280 nm ~ 350 nm with a maxima at ~330 nm, whereas, the second one is relatively structured and within the spectral range of 350 nm ~ 420 nm with a peak position at around ~375 nm. Fluorescence emission spectra obtained by exciting at 330 nm shows good mirror image relationship with the absorption profile having a structured emission in 350–400 nm range with a clean broad emission peak at 450 nm. Interestingly, in aqueous media, the structured profile is lost in both the absorption and emission spectra. Excitation at the absorption peak (~340 nm) as well as at the shoulder (~380 nm) gives similar structureless and broad emission at 475 nm (figure 3.3, top panel). The excitation spectra obtained by monitoring the broad low-energy emission peak nicely resembles with the absorption spectra. Multiple peaks in the absorption spectra and structured fluorescence indicate that the excited states of LC are vibronically coupled even in polar solvents. However, in aqueous media, there exists a
strong specific interaction of the excited states with the solvent resulting broad and structureless profile both in absorption and emission spectra. From the relatively large absorption coefficient ($\epsilon_{\text{max}} \sim 5 \times 10^4$ dm$^3$ mol$^{-1}$ cm$^{-1}$) of the low energy absorption band, it can be concluded that this transition is $S_1(\pi) \leftarrow S_0(\pi)$ in nature. The assignment of the absorption band as well as the origin of the structured absorption/fluorescence band is further confirmed from theoretical calculations described in the following sections.

![Figure 3.3](image)

**Figure 3.3** Steady state absorption (left panel) and fluorescence emission (right panel) spectra of $\sim 6.0 \times 10^{-6}$ mol dm$^{-3}$ LC solution in 1,4-dioxane (a), acetonitrile (b) and water (c). The excitation wavelengths are 330 nm (solid line) and 380 nm (open circles) for both (a) and (b); whereas, for water, the high energy excitation was done at 350 nm.

3.2.2. Solvatochromism of LC photophysics: Importance of hydrogen bond acidity of the solvent

The spectroscopic behavior of LC was studied in a series of solvents with varying polarity and hydrogen bonding parameters given in table 3.1. Table 3.2 summarizes the steady state spectral behavior of LC in these solvents. Although, the results do not show any regular variation
of steady state spectral properties, careful observation reveal several interesting trends. For example, fluorescence maxima ($\lambda_{em}$) shows appreciable shift in protic solvents along with almost two fold increase in fluorescence quantum yield ($\phi_f$), when compared with their aprotic counterpart. Similar observations were also reported in an earlier report, although the number of solvents taken was relatively few [74 (a)].

Table 3.1 Solvent parameters

<table>
<thead>
<tr>
<th>No.</th>
<th>Solvents</th>
<th>$\Delta f(\varepsilon,n)^a$</th>
<th>$E_t(30)^b$</th>
<th>Kamlet-Taft solvent parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$\alpha$</td>
</tr>
<tr>
<td>1</td>
<td>Benzene</td>
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<tr>
<td>2</td>
<td>Toluene</td>
<td>0.02</td>
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<td>0.0</td>
</tr>
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<td>1,4-dioxane</td>
<td>0.03</td>
<td>36</td>
<td>0.0</td>
</tr>
<tr>
<td>4</td>
<td>Ethyl acetate</td>
<td>0.19</td>
<td>38.1</td>
<td>0.0</td>
</tr>
<tr>
<td>5</td>
<td>Tetrahydrofuran</td>
<td>0.21</td>
<td>37.4</td>
<td>0.0</td>
</tr>
<tr>
<td>6</td>
<td>Dichloromethane</td>
<td>0.22</td>
<td>40.7</td>
<td>0.0</td>
</tr>
<tr>
<td>7</td>
<td>1-pentyl alcohol</td>
<td>0.25</td>
<td>49.1</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>1-butanol</td>
<td>0.26</td>
<td>49.7</td>
<td>0.84</td>
</tr>
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<td>DMSO</td>
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</tr>
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<td>1-propanol</td>
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</tr>
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<td>Isopropanol</td>
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<td>48.4</td>
<td>0.76</td>
</tr>
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<td>Acetone</td>
<td>0.28</td>
<td>42.2</td>
<td>0.08</td>
</tr>
<tr>
<td>14</td>
<td>Acetonitrile</td>
<td>0.3</td>
<td>45.6</td>
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</tr>
<tr>
<td>15</td>
<td>Methanol</td>
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<td>55.4</td>
<td>0.98</td>
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<td>Water</td>
<td>0.32</td>
<td>63.1</td>
<td>1.17</td>
</tr>
</tbody>
</table>

$^a$ Polarity parameter; $^b$ Reichardt solvent parameter.

To verify the effect of solvent polarity, several steady state spectral parameters (P) like emission maxima ($\nu_{em}$) and Stokes-shift ($\Delta \nu_{ss}$) of LC in a variety of solvents mentioned in Table 3.1 were plotted against the solvent polarity parameter $\Delta f(\varepsilon,n)$ with the general form of Lippert-Mataga (LM) equation given below [75,4a].

$$P = P^o + a \times \Delta f(\varepsilon,n)$$  \hspace{1cm} (3.1)

where, the orientation polarizibility, $\Delta f(\varepsilon,n)$ is related to the solvent dielectric constant ($\varepsilon$) and refractive index (n) with the relation:

$$\Delta f(\varepsilon,n) = \frac{\varepsilon - 1}{2\varepsilon + 1} - \frac{n^2 - 1}{2n^2 + 1}$$  \hspace{1cm} (3.2)
$P^0$ is the measured property in gas phase or in non-interacting solvents like hexane and the slope of the equation (3.1) represents a term containing the difference in dipole moment between the ground and excited states. From the results given in figure 3.4 (lower panel), it is clear that the spectroscopic properties of LC do not show any regular solvatochromism behavior on the solvent polarity parameter. This observation points to the existence of specific solute-solvent interactions.

Table 3.2 Steady state spectral properties LC in homogeneous solvents$^a$.

<table>
<thead>
<tr>
<th>Solvents$^b$</th>
<th>$\nu_{\text{abs}}$ /cm$^{-1}$</th>
<th>$\nu_{\text{em}}$ /cm$^{-1}$</th>
<th>$\Delta \nu_{\text{ss}}$ /cm$^{-1}$</th>
<th>$\phi_f$ /10$^{-2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>26316</td>
<td>23419</td>
<td>2897</td>
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</tr>
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<td>26316</td>
<td>23474</td>
<td>2842</td>
<td>2.3</td>
</tr>
<tr>
<td>3</td>
<td>26316</td>
<td>23255</td>
<td>3060</td>
<td>1.9</td>
</tr>
<tr>
<td>4</td>
<td>26316</td>
<td>23474</td>
<td>2842</td>
<td>5.9</td>
</tr>
<tr>
<td>5</td>
<td>26247</td>
<td>23474</td>
<td>2773</td>
<td>4.4</td>
</tr>
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<td>26247</td>
<td>23529</td>
<td>2717</td>
<td>11.3</td>
</tr>
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<td>25974</td>
<td>22471</td>
<td>3502</td>
<td>8.2</td>
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<td>8</td>
<td>25974</td>
<td>22421</td>
<td>3553</td>
<td>9.3</td>
</tr>
<tr>
<td>9</td>
<td>26042</td>
<td>22779</td>
<td>3263</td>
<td>1.5</td>
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<td>10</td>
<td>26110</td>
<td>22988</td>
<td>3121</td>
<td>4.8</td>
</tr>
<tr>
<td>11</td>
<td>25974</td>
<td>22522</td>
<td>3452</td>
<td>8.8</td>
</tr>
<tr>
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<td>26042</td>
<td>22573</td>
<td>3468</td>
<td>5.9</td>
</tr>
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<td>23310</td>
<td>3006</td>
<td>3.8</td>
</tr>
<tr>
<td>14</td>
<td>26247</td>
<td>22779</td>
<td>3468</td>
<td>3.5</td>
</tr>
<tr>
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<td>26042</td>
<td>22371</td>
<td>3670</td>
<td>6.8</td>
</tr>
<tr>
<td>16</td>
<td>25974</td>
<td>21551</td>
<td>4422</td>
<td>9.3</td>
</tr>
</tbody>
</table>

$^a$Abbreviations used: $\nu$ = absorption and emission energy; $\Delta \nu_{\text{ss}}$ = Stokes shift; $\phi_f$ = fluorescence quantum yield; $^b$The name of the solvents are listed in table 3.1.

As a first trial, the empirical solvent polarity scale, $E_T(30)$, built with a betaine dye, was used to correlate the solvent dependence of the steady state spectral properties of LC. The uniparametric scale depends on both the solvent dielectric properties and hydrogen bonding acidity, but it does not take care of solvent hydrogen bonding acceptor basicity [76]. The specificity of Lewis acid base interactions in $E_T(30)$ parameter arise from the negative charge localized on the phenolic oxygen of betaine molecule. As it is seen in figure 3.4 (middle panel) again, a relatively better correlation (with correlation coefficient $R \approx 0.95$) is obtained for all the
spectroscopic parameters like fluorescence maxima ($\nu_{em}$) and Stokes shift ($\Delta \nu_{ss}$). This clearly indicates that apart from solvent polarity, LC solvatochromism is mostly modulated by solvent hydrogen bond donor acidity ($\alpha$); whereas, solvent hydrogen bond acceptor basicity ($\beta$) is relatively less important. To confirm this prediction further, the steady state spectral properties of LC are interpreted by means of the linear solvation energy relationship (LSER) concept using Kamlet-Taft equation [77],

$$P = P^0 + s\pi^* + a\alpha + b\beta$$  \hspace{1cm} (3.3)

where, $P$ is the value of the solvent dependent property to be modeled, $P^0$, $s$, $a$ and $b$ are the coefficients determined from the LSER analysis. The term $\pi^*$ indicates the measure of solvent dipolarity/polarizibility [78], whereas, $\alpha$ and $\beta$ is the scale of hydrogen bond donation acidity and acceptance basicity of the solvent, respectively [79]. The corresponding parameters for fourteen solvents are taken from literature [80, 81] and given in Table 3.1.

Regression analysis of fluorescence maxima ($\nu_{em}$) and Stokes shift ($\Delta \nu_{ss}$) with solvent properties results the following set of correlation equations:

$$\nu_{em}(\text{expt}, cm^{-1}) = 24161.66 - 1090.3\pi^* - 1048.96\alpha - 303.3\beta$$ \hspace{1cm} (3.4)

$$\Delta \nu_{ss}(\text{expt}, cm^{-1}) = 2337.47 + 880.78\pi^* + 874.12\alpha + 42.16\beta$$ \hspace{1cm} (3.5)

The correlation diagrams of the experimental values with those calculated from the above equations are shown in uppermost panel of figure 3.4. Reasonably acceptable correlation coefficient (R) and the value of the slope of linear plot close to unity indicate a very good correlation of the experimentally observed and theoretically calculated parameters. A close look into the above equations reveal several interesting feature for LC solvatochromism: (i) in general, the contributions from $\alpha$ parameter is always more significant than $b$ beside the $s$ parameter, indicating the importance of solvent hydrogen bonding acidity in LC spectroscopy, as indeed pointed out in the discussion above; (ii) the importance of hydrogen bonding acidity in LC spectroscopy points towards an efficient charge localization in LC upon excitation (see DFT calculation results in section 3.3 below); finally, (iii) the large emission spectral shift in water is due to the negative value of a parameter and its corresponding larger contribution. In summary, the solvatochromic analysis reveals that in polar protic solvents like water, for example, several
spectroscopic species may be present due to the hydrogen bonded donor and acceptor properties of the solvent in the ground state; however, in the excited state, the main fluorescing species is originated due to the hydrogen bonded complex formation of LC through the solvent hydrogen bonding acidity behavior. Theoretical calculation results (see below) indeed confirms that the extensive charge localization on N10 (see figure 3.1, for atom numbering) in the excited state plays a key role towards the formation of hydrogen bond in protic medium like water and/or acetic acid with LC, which subsequently initiates the tautomerization leading to the formation of isoalloxazinic structure through a six membered cyclic transition state. This prediction is supported by a “two-step” mechanism for acid catalyzed proton transfer in LC proposed earlier [82].

![Figure 3.4 Plot of fluorescence maxima (left panel, open squares) and Stokes shift (right panel, shaded squares) against polarity parameter, Δf(ε,n), using Lippert – Mataga plot (a), solvent E_t(30) parameter (b) and theoretically calculated values using Kamlet-Taft equation (c) of LC in different solvents.](image)
3.2.3. Time-resolved fluorescence measurements in homogeneous environments

The fluorescence decay times (τ) of LC are measured in different solvents at room temperature using time-correlated single photon counting method. Some of the representative decay traces are shown in figure 3.5 and the results are provided in the inset table. All decays could be reproduced with single exponential decay function. Fluorescence decay times measured in this study are in very good agreement with some of the data available in literature [74 (a,b)]. In general, the fluorescence decay time decreases with decrease in solvent hydrogen bonding acidity (for example, 0.24 ns in DMSO compared to 2.69 ns in aqueous buffer medium). This indicates that specific interaction through solvent hydrogen bond donation stabilizes the fluorescing states of LC. The increase in τ values i.e., the stabilization of fluorescing state in presence of hydrogen bond donor is quite obvious from the discussion made above as well as the theoretical calculation discussed below.

![Figure 3.5](image.png)

**Figure 3.5** Time-resolved fluorescence decay profile (open circles) and simulated data (solid line) of \( \sim 6.0 \times 10^{-6} \) mol dm\(^{-3} \) LC solution in different solvents. IRF indicates the instrument response function. The magnitude of τ in different solvent is tabulated in the inset. Solvent numbers are same as mentioned in table 3.1.
3.3. Theoretical Calculation Using Density Functional Theory

3.3.1. Energetic of different conformers in the ground state

Full geometry optimization of different conformers of LC (structures are given in figure 3.2) in isolated condition as well as in different solvent with SCRF/PCM model was done using B3LYP/6-311++G(d,p) methodology. The fully optimized structures alongwith all the geometrical parameters are shown in figure 3.6. The corresponding energies are listed in Table 3.3.

It is clear that the structure I is the most stable conformer in the isolated condition as well as in different solvent systems. Therefore, the ground state population of LC is mainly contributed by structure I and we will confine our discussion of spectral properties based on this structure only. To understand the absorption spectral behavior, we have carried out TD-DFT calculation of conformer I in different solvents for the first ten excited singlet states. The contributions of different orbitals in the corresponding excited states within the experimentally observed spectral range (280–420 nm) are given in Table 3.4. The calculated excitation wavelengths are in very good agreement with the absorption spectra shown in figure 3.3. From the table, it is clear that the excited state of LC is mainly contributed from the HOMO (H) → LUMO (L) transition with little contribution from H-1 and L+1 orbitals also. Careful analysis of electron density distribution in all these orbitals (see below) confirms the $\pi^* \leftarrow \pi$ nature of transition involved in optical absorption spectroscopy of LC.

<table>
<thead>
<tr>
<th>Conformer</th>
<th>Gas Phase</th>
<th>1,4-dioxane</th>
<th>Acetonitrile</th>
<th>Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>-833.0499109</td>
<td>-833.076708227</td>
<td>-833.082797005</td>
<td>-833.084065647</td>
</tr>
<tr>
<td>Ia</td>
<td>-833.0132127</td>
<td>-833.040351540</td>
<td>-833.046950983</td>
<td>-833.048484606</td>
</tr>
<tr>
<td>Ib</td>
<td>-833.0275746</td>
<td>-833.055739194</td>
<td>-833.062265591</td>
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</tr>
<tr>
<td>Ic</td>
<td>-833.0249842</td>
<td>-833.054456108</td>
<td>-833.061430244</td>
<td>-833.063097388</td>
</tr>
</tbody>
</table>

* Structures are given in figure 3.2.
3.3.2. Analysis of frontier orbitals: Charge localization in the excited state

All the four MOs, mainly involved in electronic transition of LC, are depicted in figure 3.7. From the discussion made in the previous section and also the results given in Table 3.4, it is clear that the major contribution for the electronic absorption arises from the transition to the lowest unoccupied molecular orbital (LUMO). Careful analysis of this orbital and comparing with the occupied molecular orbitals reveals that extensive charge redistribution of LC occurs in the excited state. It is indeed observed that the charge localization to a greater extent occurs on the N10 atom whereas N1 hydrogen becomes strongly acidic on excitation. Therefore, it is highly probable that excited state photophysics of LC will be strongly modulated by hydrogen bond donation of the solvent with N10 atom; on the other hand, hydrogen bond accepting solvents like DMSO mainly acts through proton abstraction from the N1 atom. Analysis of solvent
dependence of LC fluorescence discussed earlier has shown that the fluorescence properties show good correlation even with solvent $E_{r}(30)$ parameter, that mainly takes care of solvent hydrogen bond donation ability (acidity). Consequently, it is reasonable to believe that in an amphoteric medium like water or acetic acid, the key step involves the formation of hydrogen bond through solvent hydrogen bond acidity at N10 atom. Once this hydrogen bond formation occurs, geometric rearrangement of the solvent occurs to form a cyclic six membered structure through solvent hydrogen bond basicity with N1 proton of LC.

![Figure 3.7 Frontier orbital diagram of different MOs for LC (I). The block arrows in LUMO indicate charge accumulation (filled arrow) and removal (empty arrow) on N10 and N1, respectively on electronic excitation.](image)

A similar mechanism was proposed earlier by Sikorska et. al. for proton transfer in LC-acetic acid complex [82]. The authors termed the second step as the formation of an appropriate structure to account for the slow proton transfer following the very fast initial hydrogen bond donated complex at N10 position of LC with acetic acid. However, in contrary to hypothesis proposed by the authors, we believe that the first hydrogen bonded complex is not available in the ground state [74 (b)]. Charge localization in the excited state is the origin of this specific hydrogen bond to occur at N10 of LC which serves as the catalytic pathway for the
excited state proton transfer towards the formation of isoalloxazine (II) and the mechanism of the proton transfer is believed to be following the scheme given in figure 3.8(a).

![Figure 3.8](image)

**Figure 3.8** (a) Mechanism of water-assisted proton transfer of LC (I) to form isoalloxazine (II).

**Table 3.4** The energy of absorption (nm), its oscillator strength (f) and contribution of different orbitals in the transition to the corresponding excited states (ES) of LC (structure I) in gas phase as well as in some representative solvents obtained by TD-DFT calculation at B3LYP/6-311++G(d,p) level of calculation. The highest occupied and lowest unoccupied MOs are represented by numbers 63 and 64, respectively.

<table>
<thead>
<tr>
<th>ES</th>
<th>Gas Phase</th>
<th>1,4-dioxane</th>
<th>Acetonitrile</th>
<th>Water</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>365.8 nm((f = 0.0626))</td>
<td>381 nm((f = 0.0626))</td>
<td>383.2 nm((f = 0.0626))</td>
<td>383.5 nm((f = 0.0626))</td>
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<tr>
<td></td>
<td>62→64</td>
<td>0.1279</td>
<td>62→64</td>
<td>62→64</td>
</tr>
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<td>62→65</td>
<td>62→65</td>
</tr>
<tr>
<td></td>
<td>63→64</td>
<td>0.6475</td>
<td>63→64</td>
<td>63→64</td>
</tr>
<tr>
<td>2</td>
<td>362.3 nm((f = 0.0014))</td>
<td>353.6 nm((f = 0.0018))</td>
<td>351.6 nm((f = 0.0016))</td>
<td>351 nm((f = 0.0019))</td>
</tr>
<tr>
<td></td>
<td>60→64</td>
<td>0.1169</td>
<td>60→64</td>
<td>60→64</td>
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<tr>
<td></td>
<td>61→64</td>
<td>0.6748</td>
<td>61→64</td>
<td>61→64</td>
</tr>
<tr>
<td>3</td>
<td>321.4 nm((f = 0.2173))</td>
<td>333.7 nm((f = 0.3472))</td>
<td>334.8 nm((f = 0.3407))</td>
<td>335.0 nm((f = 0.3398))</td>
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<tr>
<td></td>
<td>62→64</td>
<td>0.1169</td>
<td>62→64</td>
<td>62→64</td>
</tr>
<tr>
<td></td>
<td>63→65</td>
<td>-0.2230</td>
<td>63→65</td>
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</tr>
<tr>
<td>4</td>
<td>310.2 nm((f = 0.0002))</td>
<td>299.4 nm((f = 0.0001))</td>
<td>292.2 nm((f = 0.0001))</td>
<td>296.7 nm((f = 0.0001))</td>
</tr>
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<td>57→64</td>
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</tr>
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<td></td>
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</tr>
</tbody>
</table>
3.3.3. Water assisted proton transfer in LC: Analysis of transition state

The ground state energy parameters of the alloxazine (I) and isoalloxazine (II) structures in gas phase as well as in different solvents calculated using SCRF/PCM model at B3LYP/6-311++G(d,p) level are listed in Table 3.5. It is found that although structure II is less stable by 54.06 kJ mol⁻¹ energy relative to that of structure I in the gas phase, the energy difference decreases substantially in presence of solvents (for example, ~26.27 kJ mol⁻¹ in presence of water and ~26.30 kJ mol⁻¹ in presence of acetonitrile). In contrast, full geometry optimization with specific hydrogen bonded structures of I and II with water molecules predict an energy difference of ~34.0 kJ mol⁻¹ between them. These results indicate the importance of specific hydrogen bond formation in the proton transfer process and confirm that only solvent physical parameters like polarity, as imposed by SCRF/PCM model, is not sufficient to catalyze the process. Rather than acting merely as a matrix with dielectric continuum, the solvent directly takes part in the chemical transformation through hydrogen bonding interaction. The optimized structures along with the geometrical and energy parameters for these two conformers, specifically hydrogen bonded with water molecule, are given in Figure 3.8(b). It is to be noted here that only primary hydrogen bonded complex of I and II with water was considered to give different complexes. It is obvious that additional solvent molecules will combine to give secondary water cluster and the actual hydrated structure is far more complex than what is considered for these calculations. However, we restrict ourselves only in the first hydration layer, as the effect of internal hydrogen bonding (IHB) is expected to diminish very fast from the center of origin. Consequently, it is reasonable to believe that any further addition of water structure will have insignificant contribution toward the relative energy parameters.

Table 3.5 Energy parameters (in Hartree) of fully optimized structures of I and II in gas phase as well as in some representative solvents calculated by B3LYP/6-311++G(d,p) method. Solution phase calculations were done using SCRF/PCM methodology.

<table>
<thead>
<tr>
<th>Conformer</th>
<th>Gas Phase</th>
<th>1,4-dioxane</th>
<th>Acetonitrile</th>
<th>Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>-833.0499109</td>
<td>-833.0767082</td>
<td>-833.0827970</td>
<td>-833.0840656</td>
</tr>
<tr>
<td>II</td>
<td>-833.0293184</td>
<td>-833.0641662</td>
<td>-833.0723984</td>
<td>-833.0740615</td>
</tr>
</tbody>
</table>

*Structures are given in figure 3.1.*
Figure 3.8 (b) Fully optimized ground state geometrical parameters (bond lengths and bond angles are given in angstrom and degree, respectively) of hydrogen bonded water complex with I and II calculated by B3LYP/6-311++G(d,p) method. The corresponding energies are also indicated.

The calculated energy difference between I and II in the ground state is \( \sim 34.0 \text{ kJ mol}^{-1} \). The potential energy surface (PES) (Figure 3.9), constructed by intrinsic reaction coordinate (IRC) calculation from the transition state (TS), indicates that the water assisted conversion of I to II is associated with a large activation barrier of \( \sim 89.88 \text{ kJ mol}^{-1} \). So, in ground state, the possibility of proton transfer is very less, both from kinetic and thermodynamic point of view. However,
TD-DFT calculation results show that, the relative energy difference between I and II is very small in the first excited state (~1.8 kJ mol\(^{-1}\)) and barrier height for the proton transfer also reduces substantially to ~68.5 kJ mol\(^{-1}\). As a result, the water catalyzed proton transfer process becomes highly feasible in the excited state. The TS structure (Figure 3.10) is confirmed by the presence of an imaginary frequency (~1590.35 cm\(^{-1}\)) which is active towards the proton translocation within N1 and N10 atoms of alloxazine moiety.

![Figure 3.10](image)

**Figure 3.10** Ground state structure of the transition state (TS) for water catalyzed I→II conversion obtained by IRC calculation using B3LYP/6-311++G(d,p) method. The bond lengths are given in angstrom and the angles are in degree.

### 3.4. Photophysical Properties in Heterogeneous Media

#### 3.4.1. General review of alloxazine photophysics in heterogeneous environment

Recently, interest in alloxazines has intensified because of their important role in a wide range of biological systems [57]. Lumichrome (7,8-dimethylalloxazine), for example, was found to inhibit flavin reductase in living Escherichia coli cells [59]. Said et al. [58, 83-84] reported that the riboflavin uptake by human-derived liver cells Hep G2, colonic epithelial NCM460 cells and Caco-2 human intestinal epithelial cells is inhibited by lumichrome. Another interesting application is in an optical transistor device with a thin film of lumichrome on conductive SnO\(_2\).
glass [85]. A further point of interest is the possibility of using alloxazines to sensitize the photo-oxidation of substituted phenols in water [86, 87]. The lumichrome-sensitized photodecomposition of substituted phenols has been found to be very efficient, with photolysis rates that may be ordered as p-methoxyphenol > p-chlorophenol > phenol > p-nitrophenol [86]. The singlet oxygen producing capacity of lumichrome in water was examined under UVA [88].

LC is a nontoxic molecule and has some natural biological functions [83,89-92], but in contrast to flavins, it does not show catalytic activity as a cofactor compound. Some flavoproteins have the flavin moiety covalently bonded to the apoprotein. However, the majority of them contain the flavin noncovalently bound [93]. Recently, the binding of lumichrome to dodecins, a small flavoprotein, has been studied by fluorescence spectroscopy and X-ray crystallography [66]. The quaternary structure of dodecin consists of a dodecameric complex making a hollow sphere-like shape, where complexation of flavins occurs with 1:1 stoichiometry. The study shows that LC has a high affinity to dodecin. Therefore, we decided to study the non-covalent interaction of LC with several heterogeneous media like surfactants and cyclodextrins which are known to provide the nano-cavities to host small organic molecules. Some of the literature reports the interaction of LC with different heterogeneous media [94-98]; however, to the best of our knowledge, detail study of LC photophysical behavior in surfactants as well as solution phase complexation with β-cyclodextrin is not available so far. In this section we describe the results of fluorimetric titration results of LC with different surfactants like SDS and CTAB as well as in presence of CD.

### 3.4.2. Splitting of alloxazine fluorescence into component spectra

The emission spectra of lumichrome show broad band with maxima at 472 nm, with a shoulder on the red side at ca. 520 nm. This shoulder could be attributed to the emission of isoalloxazinic tautomer (II); whereas, the 472 nm band is due to the alloxazine (I) [99]. To understand the effect of heterogeneous media on the fluorescence of different components, it is essential to split the total fluorescence band into the component Gaussian spectra [100]. In order to isolate the relative change in fluorescence property of individual species, the whole fluorescence in aqueous buffer as well as in presence of different concentrations of heterogeneous system were analyzed with two Gaussian functions [equation (3.6)].
\[ F = F_0 + \sum_{n=1}^{2} \frac{A_n}{w_n} \left( \frac{e}{w_n} \right) e^{-\frac{2(x-x_0)^2}{w_n}} \]  

(3.6)

Where, \( A, w \) and \( x^0 \) are the area, width and position of the component spectra. The fluorescence behavior (intensity and position) of individual components were recovered from the component spectra and analyzed further. The acceptability of the component splitting was judged by visual inspection as well the correlation coefficient (R) value. A typical example of spectral splitting is shown in figure 3.11. It has been found that component I does not show any regular trend with increasing concentration of the heterogeneous media. Therefore, in the following sections, we mainly discuss the results of component II (isoalloxazine) in presence of surfactants and CD.

![Figure 3.11](image)

**Figure 3.11**  *Splitting of LC fluorescence spectra in aqueous media into the two individual component spectrum using equation (3.6).*

3.4.3. Variation of isoalloxazine fluorescence in presence of SDS and CTAB

Figure 3.12 shows the variation of isoalloxazine fluorescence with increasing concentration of surfactants in aqueous buffer solution of pH = 7.4. It is seen that in presence of both the surfactants, the fluorescence intensity increases with little spectral shift towards the higher energy (~ 200 cm\(^{-1}\)). The variation in spectral intensity as well as the shift indicates that the environment around the probe changes with addition of surfactants and the fluorophore experiences a different microenvironment. Also, it is seen from figure 3.12(a) that the
fluorescence intensity variation curve with increasing surfactant concentration shows a break at ~ 8 mM, which is very close to the critical micelle concentration (cmc) of SDS.

Figure 3.12  Variation of component II fluorescence spectra of LC with increasing concentration of SDS (a) and CTAB (b).

The binding of a probe to micelles can be described by the following equilibrium [101]:

\[
S_a + D_m \leftrightarrow S_m
\]  

(3.7)

where, \(S_a\) and \(S_m\) denote the substrate concentrations expressed as molarities in terms of total volume of solution in the aqueous phase and in the micellar pseudophase.

The equilibrium constant for reaction (3.7), often termed as binding constant, is given by

\[
K_S = \frac{[S_m]}{[S_a][D_m]}
\]  

(3.8)

Considering the aggregation number of the micelle to be constant, the total substrate concentration (\(S_t\)) and total detergent concentration (\(D_t\)) can be written as \([S_a]+[S_m]\) and \([S_m]+[D_m]+cmc\), respectively. \(cmc\) indicates the critical micelle concentration.

The fraction of micellar associated substrate is defined as:

\[
f = \frac{[S_m]}{[S_t]}
\]  

(3.9)

Then, from equation (3.8), one obtains

\[
\frac{f}{1-f} = K_s \left\{[D_t] - [S_t] \times f \right\} - K_s \times cmc
\]  

(3.10)
Under the condition of \([S_m][D_t]\) and \([D_t]\) \(\text{cmc}\), the above equation can be approximated as

\[
\frac{f}{1-f} = K_S \times [D_t]
\]  

(3.11)

Experimentally, \(f\) can be calculated by

\[
f = \frac{A - A_0}{A_m - A_0}
\]  

(3.12)

where, \(A\), \(A_0\) and \(A_m\) are the area under the whole absorption or fluorescence emission spectra of the probe in surfactant, water and in fully micellized condition, respectively. Substituting the value of \(f\) in equation 3.11, one can write:

\[
\frac{A - A_0}{A_m - A_0} = K_S \times [D_t]
\]  

(3.13)

From the above equations of straight lines, the binding constant \((K_S)\) can be calculated with the respective plots.

The binding constant \((K_S)\) values obtained for SDS are quite small \((86.4\pm4.1 \text{ M}^{-1})\) in comparison to that for CTAB \((486.9\pm40.2 \text{ M}^{-1})\). Since the binding constants differ by about an order of magnitude, one can conclude that LC binds more preferentially to cationic CTAB compared to the anionic SDS.

**Figure 3.13** Schematic representation of distribution of excited fluorophore in the interfacial layer of anionic (left) and cationic (right) surfactant.
A possible explanation for the difference in binding constant values can be put forward with the model described in figure 3.13. In presence of surfactant concentration above cmc, the organic fluorophores are normally believed to be residing at the interfacial layer. Also, due to preferential charge accumulation in the excited state, the fluorophore can be assumed to be anionic in nature. Due to the presence of anionic head groups (in SDS), the fluorophore assumes a repulsive force and consequently provides a weak binding with the micelle. However, the cationic head groups in CTAB provide additional electrostatic force of attraction along with the hydrophobic force. As a result, the fluorophore penetrates more into the micelle core and gives higher binding constant values.

3.4.4. Fluorescence behavior in cyclodextrin

Complexation behavior of LC was monitored with β - cyclodextrins (CD) in buffer solution of pH = 7.4. The fluorescence characteristic of LC (component II) in aqueous buffer solution is seen to undergo drastic changes in presence of CD [figure 3.14(a)]. The fluorescence intensity decreases with increase in CD concentration and then reaches a saturation limit 4~6 mM β-CD concentration. In addition, the fluorescence peak position also shifts to the lower energy side of the spectrum. All these results also point towards the formation of a fluorophore-CD complex.

![Figure 3.14 Decrease in component II fluorescence spectral intensity of LC with increasing concentration of β-CD (a). B-H plot for 1:1 and 2:1 stoichiometry is also shown (b).](image)

The stoichiometric ratio and apparent binding constant for a fluorophore complexed with CD can be determined by analyzing the changes in fluorescence emission intensity with CD
concentration. The equilibrium reaction for 1:1 binding between the probe and CD can be written as:

\[
Probe + CD \rightleftharpoons Probe:CD
\]  
(3.14)

The equilibrium constant for the above reaction is

\[
K_C = \frac{[Probe:CD]_{eq}}{[Probe]_{eq}[CD]_{eq}}
\]  
(3.15)

If the initial concentration of the probe is represented by \([Probe]_0\) and in the condition of \([CD] >> [Probe]_0\), the above equation can be reduced to:

\[
K_C = \frac{[Probe:CD]_{eq}}{([Probe]_0 - [Probe:CD]_{eq})[CD]}
\]  
(3.16)

At any instance, the observed fluorescence intensity \((F)\) is the sum of the fluorescence intensities from the free and bound Probe, respectively. Under this condition, one can write

\[
F = F_0 \frac{[Probe]_{eq}}{[Probe]_0} + F_\alpha \frac{[Probe:CD]_{eq}}{[Probe]_0}
\]  
(3.17)

where, \(F_0\) and \(F_\alpha\) are the fluorescence intensities of the free and fully complexed Probe, respectively. Since, \([Probe] = [Probe]_{eq} + [Probe:CD]_{eq}\), from equation (3.17) we can further write

\[
\frac{[Probe:CD]_{eq}}{[Probe]_0} = \frac{F - F_0}{F_\alpha - F_0}
\]  
(3.18)

From equations (3.17) and (3.18), the modified form of Benesi-Hilderbrand (BH) relation [102] can be written as:

\[
\frac{1}{F - F_0} = \frac{1}{F_\alpha - F_0} + \frac{1}{K_C(F_\alpha - F_0)} \times \frac{1}{[CD]}
\]  
(3.19)

A similar treatment of 2:1 host-guest complex gives the expression for BH relation as follows:

\[
\frac{1}{F - F_0} = \frac{1}{F_\alpha - F_0} + \frac{1}{K_C(F_\alpha - F_0)} \times \frac{1}{[CD]^2}
\]  
(3.20)

Therefore, for an 1:1 complex formation, the double reciprocal plot of \(1/(F-F_0)\) against \(1/[CD]\) should give a straight line. On the other hand, for a 2:1 complex, \(1/(F-F_0)\) versus \(1/[CD]^2\)
gives a straight line. In both cases, from the slope and intercept, the equilibrium constant ($K_c$) can be calculated.

Figure 3.14(b) shows the BH plot both for 1:1 and 2:1 cases. It is seen that, a linear plot is obtained only for a stoichiometric combination of two CD molecules to host one LC. The high binding constant value ($\sim 7.1 \times 10^5 \text{ M}^{-2}$) indicates a very tight complex formation between LC and CDs. The decrease in fluorescence intensity with increasing [CD] can be assumed to the due to relatively less polar hydrophobic core as well as absence of water molecule to catalyze the excited state alloxazine $\leftrightarrow$ isoalloxazine inter-conversion.