Chapter 1: Introduction

1.1. Fluorescence Spectroscopy

Fluorescence is the most sensitive molecular property that is used for studying the structure and dynamics of the photoexcited state. Any electronically excited molecule comes back to its ground state either by radiative or nonradiative mechanism. In the radiative mechanism, the chromophores emit photons and the process is called luminescence emission.

The mechanisms by which electronically excited molecules come to ground state are given by the Jablonski diagram as shown in figure 1.1 and summarized the table 1.1. The absorption of a photon takes the molecule from ground singlet state ($S_0$) to either first or second excited singlet states (represented by $S_1$ or $S_2$, respectively). From this Franck-Condon (FC) excited state, the molecule relaxes to the lowest vibronic level of the $S_1$ state through internal conversion (IC). Further relaxation from this state to $S_0$ can occur via three mechanisms. First, by radiative emission of a photon and the process is termed as fluorescence. The other two nonradiative channels involve either direct relaxation to $S_0$ state (internal conversion) or passage to the triplet state ($T_1$) by intersystem crossing (ISC). The radiative transition from $T_1$ to $S_0$ state is quantum mechanically forbidden and hence is a very slow process relative to fluorescence and is called phosphorescence emission.

![Figure 1.1: Schematic representation of Jablonski diagram.](image-url)
The fluorescent photons have the information about energy, time, polarization and intensity at a given wavelength. Each of the above parameters of fluorescence gives information about the local environment surrounding the fluorophore under investigation. So, fluorescence intensity, spectrum, polarization and their dependence are important parameters that one can use for the characterization [1].

The maximum of phosphorescence spectrum is generally shifted to longer wavelength relative to the fluorescence. Also, the $S_1$ state can be deactivated by a quenching mechanism, in which a quencher Q quenches the singlet ($S_1$) state of the fluorophore through an excited state reaction. Reverse intersystem crossing from $T_1$ to $S_1$ can occur when the energy between $S_1$ and $T_1$ is small and when the lifetime of $T_1$ is long enough. This results in emission with the same spectral distribution as normal fluorescence but with a much longer decay time constant because the molecules stay in the triplet state before emitting from $S_1$. This fluorescence emission is thermally activated; consequently, its efficiency increases with temperature and is called delayed fluorescence of E-type. Again, in concentrated solutions, a collision between two molecules in the $T_1$ state can provide enough energy to allow one of them to return to the $S_1$ state. Such triplet-triplet annihilation thus leads to a delayed fluorescence emission, and termed as delayed fluorescence of P-type.

**Table 1.1:** Different photophysical processes, associated transition and other relevant parameters

<table>
<thead>
<tr>
<th>Process</th>
<th>Transition</th>
<th>Rate constant</th>
<th>Timescale (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Absorption (Excitation)</td>
<td>$S_0 \rightarrow S_1 \vee S_n$</td>
<td>Instantaneous</td>
<td>(\approx 10^{-15})</td>
</tr>
<tr>
<td>(b) Internal Conversion</td>
<td>$S_n \rightarrow S_1$</td>
<td>(\Delta IC)</td>
<td>(10^{-14} \approx 10^{-10})</td>
</tr>
<tr>
<td>(c) Vibrational Relaxation</td>
<td>$S_1 \rightarrow S_1$</td>
<td>(\Delta vr)</td>
<td>(10^{-12} \approx 10^{-10})</td>
</tr>
<tr>
<td>(d) Fluorescence</td>
<td>$S_1 \rightarrow S_0$</td>
<td>(\Delta f)</td>
<td>(10^{-9} \approx 10^{-7})</td>
</tr>
<tr>
<td>(e) Intersystem Crossing</td>
<td>$S_1 \rightarrow T_1$</td>
<td>(\Delta ISC)</td>
<td>(10^{-10} \approx 10^{-8})</td>
</tr>
<tr>
<td>(f) Non-radiative Relaxation, Quenching</td>
<td>$S_1 \rightarrow S_0$</td>
<td>(\Delta nr, \Delta q)</td>
<td>(10^{-7} \approx 10^{-5})</td>
</tr>
<tr>
<td>(g) Phosphorescence</td>
<td>$T_1 \rightarrow S_0$</td>
<td>(\Delta p)</td>
<td>(10^{-3} \approx 10^{-2})</td>
</tr>
<tr>
<td>(h) Non-radiative Relaxation, Quenching</td>
<td>$T_1 \rightarrow S_0$</td>
<td>(\Delta nr, \Delta q)</td>
<td>(10^{-3} \approx 10^{-2})</td>
</tr>
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</table>
1.1.1. Fluorescence Intensity and Spectra

Following are the different processes those can occur during a photo-excitation and de-excitation process.

\[ S_0 + h\nu_a \rightarrow S_1 \text{ Absorption} \]

\[ S_1 \rightarrow S_0 + h\text{heat} \quad \text{Internal Conversion (IC)} \]

\[ S_1 \rightarrow T_1 + h\text{heat} \quad \text{Intersystem Crossing (ISC)} \]

\[ S_1 \rightarrow S_0 + h\nu_f \quad \text{Fluorescence} \]

\[ S_1 + Q \rightarrow \text{Products} \]

\[ T_1 \rightarrow S_0 + h\nu_p \quad \text{Phosphorescence} \]

The amount of fluorescent photons emitted per unit time and per unit volume is called the steady state fluorescence intensity (I). The steady state fluorescence intensity per absorbed photon as a function of the wavelength of the emitted photons represents the fluorescence spectrum or emission spectrum which reflects the distribution of the probability of the various transitions from the lowest vibrational level of \( S_1 \) to the various vibrational levels of \( S_0 \).

The intensity versus wavelength plot (fluorescence emission spectrum) is characteristic of a fluorophore and sensitive to its local surrounding environment and consequently used to probe the structure of the local environment. Generally, the wavelength of maximum fluorescence intensity is shifted to longer wavelength relative to the wavelength of its absorption maximum. The difference between these two wavelengths is known as Stokes’ shift \((\Delta\nu_{ss})\). The Stokes’ shift arises because of the relaxation from the initially excited state to the ground vibronic level of the \( S_1 \) which
involves the loss of energy. Further loss of energy occurs due to the transitions from $S_1$ to higher vibrational levels of the $S_0$ state. This important parameter provides information on the excited state. As an example, when the dipole moment of a fluorescent molecule is higher in the $S_1$ state than in $S_0$ state, the magnitude of $\Delta\nu_{SS}$ increases with increase in solvent polarity (figure 1.2).

The fluorescence emission spectrum is generally independent of excitation wavelength. This is because of the rapid relaxation to the lowest vibrational level of $S_1$ prior to emission; irrespective of excitation to any higher electronic and vibrational levels. However, excitation on the extreme red edge of the absorption spectrum frequently results in a red-shifted emission. This shift occurs because red edge excitation (REE) selects only the fraction of the fluorophores which are most strongly interacting with the solvent (solvation dynamics) [2]. The fluorescence excitation spectrum observed for a given emission wavelength differs from that of the absorption spectrum for many systems. The large spectral width of the emission spectrum compared to absorption spectral width is mostly due to the multiple species in the excited state. The fluorescence emission spectrum is generally a mirror image of the absorption spectrum. This symmetric nature is lost when the structure or the geometry of the excited state is different from that of the ground state.

Fluorescence intensity is a measure of the probe’s ability to decay through radiative mechanism. Fluorescence quantum yields ($\Phi_f$) are the probability with which an excited fluorophore emits a photon and is related to the radiative rate and nonradiative rate of the deactivation of the excited state.

![Jablonski diagram for fluorescence with solvent relaxation](image)

**Figure 1.2:** (a) Jablonski diagram for fluorescence with solvent relaxation. (b) Effects of the refractive index ($n$) and dielectric constant ($\varepsilon$) on absorption and emission energies.
1.2. Fluorescence and Biology

In the current trend of modern science, chemistry is not only a science of synthesis and structural manipulations of molecules but it has gradually undertaken the more challenging task of biology-oriented field. Modern science craves to a deeper understanding of life phenomena particularly molecular network in living systems. Fast, sensitive, reliable, and reproducible detection of bimolecular interaction among various molecular or ionic species requires for investigation of many fundamental processes in biology. One of the best and popular methods to cover these all areas is the use of fluorescence techniques. Fluorescence detection one of the leading detection methods in the field of biology and photochemistry due to several advantages, including the following [3]: (a) Rapid signal acquisition that each individual fluorescent label can offer approximate $10^7$–$10^8$ photons per second for determination, (b) multi fluorescence dyes can be used for multiplexed assays, (c) high detection sensitivity (such as single molecule detection), (d) the luminescent signal is localized (different from some enzyme-linked based amplification strategies), and (e) The labeling procedure can be straightforward provided that suitable functional groups are available on the target analyte.

Understanding interface between chemistry and biology has great role in development of fluorescent probes. Nature is full of smart materials which has inbuilt ability to sense and react to changes in environment. Nature’s fact can be used to fantasies and generate fluorescence probes which represent ideal candidates for photonic photosensitivity. Surrounding environment determines the fluorescence of organic molecules. Fluorescent dyes are used to determine micro-environmental parameters, such as the polarity of media, as well as their relocation and distribution dynamics in micro-heterogeneous systems such as membranes, micelles, and cellular media as well as interfaces, polymers, and discrete supramolecular systems. Fluorescent probes are
attractive and versatile tools because of their high sensitivity, fast response time, and technical simplicity.

Heterocyclic rings are one of the fundamental components in the skeleton of the biologically active compounds produced by nature [4 (a)]. A new family of heterocyclic systems opens the possibility of finding further types of biologically active units for medicinal uses and generates potential functional materials to construct molecular devices. Therefore, developing new methodologies to increase the structural complexity in heterocyclic systems is a main area of research.

Figure 1.3: Advancement of fluorescence and bioluminescence imaging. Visualization of a specific molecule is achieved by two steps: development of fluorescent and bioluminescent materials (A), design and creating of the probes (B) (adapted from [4(b)]).
In a recent review, two different approaches were described for the synthesis of fluorescence probes [5]. The first combinatorial approaches appeared with fluorescent libraries that derivatized molecular recognition elements (also known as receptors) and included a fluorescent readout to develop new probes for particular targets. These libraries are called target-oriented fluorescent libraries (figure 1.4). Although the combinatorial derivatization of receptors has rendered a number of successful probes over the past few years, these approaches are limited to a few targets (e.g., metals, saccharides, anions, small peptides, or gases).
Figure 1.4: General structures of conventional fluorescent scaffolds for diversity-oriented libraries. Arrows point at the approximate fluorescence emission wavelengths of the fluorophores (Source adapted from [5]).
In an effort to increase the speed and scope of probe development, combinatorial approaches have been recently complemented with a significant growth in diversity-oriented fluorescent libraries (figure 1.5). Diversity-oriented fluorescent libraries consist of combinatorially derived fluorescent molecules that are directly evaluated for multipurpose sensing applications. Fluorescent libraries with broad chemical diversity are synthesized and screened in a high-throughput manner to discover new probes for targets that might not be accessible with known receptors.

**Figure 1.5:** Target-oriented fluorescent nucleic acid libraries. (a) The catalytic action of DNAzymes is exploited to prepare sensors that emit fluorescence only after analyte binding and concomitant RNA cleavage. SELEX strategies improve the characteristics of the nucleic acid probes with several rounds of mutation and positive and negative selection to amplify the DNA sequences with affinity for the analyte; (b) label-free aptamer probes include a fluorophore-binding sequence that quenches its fluorescence in the absence of the target. (Source adopted from [5]).
1.2.1. Biologically relevant heterocyclic systems used in this study

Several reviews have described the performance of fluorescent probes with specific applications [6-8]. Here in this study we identified some of the important biologically relevant probes or their derivatives (1-5) (figure 1.6) having specific applications to study the basic photo physics of this compounds in homogeneous as well as in micro heterogeneous media. Understanding of molecular recognition of these probes in the supramolecular assemblies on an atomic level is crucial to biological function and of significant, practical importance in the discovery of new drugs and in phototherapy. The weak, noncovalent interactions (hydrophobic, electrostatic, van der Waals and hydrogen bonding) govern the ligand-binding process during complexation. Elucidating the role of these interactions and the time scales involved provides insights into the mechanism of molecular recognition and the role of binding cooperativity. These confined geometries, ranging from “spheres” (micelles) and “cones” (cyclodextrins) to the more complex, three-dimensional networks of structures (proteins), provide a unique

![Figure 1.6: Structure of the compounds used in this study.](image-url)
(i) Ethidium bromide (1). Ethidium bromide is basically a deoxyribonucleic acid DNA intercalator. DNA has a central role in life process, as it contains all of the genetic information for the development and functioning of living organisms. Studies of biologically relevant small molecules with DNA are the main cellular target for carcinogens, steroids, and several classes of drugs [9]. There is growing interest in exploring the binding of biologically relevant small molecules with DNA for the rational design and construction of new and more efficient drugs targeted to DNA, to serve as sensitive molecular reporters for monitoring nucleic acid structure, and so on [10]. There are three types for binding of small molecules with double-helix DNA in a noncovalent way:

(i) electrostatic interaction, which is the electrostatic attractions with the anionic DNA phosphate backbone and the cationic end of the polar drug;
(ii) groove binding, where hydrogen bonding or van der Waals interaction with the nucleic acid bases in major or minor groove of DNA double helix; and
(iii) Intercalation, where the drug insert itself within the base pairs of a nucleic acid [11].
The electrostatic binding can take place out of the groove but intercalation and groove binding can take place only in the grooves of the DNA double helix. The most effective mode of the drugs targeted to DNA is intercalative binding, which is related to the antitumor activity of the compound [12]. Therefore the detail photophysics of these intercalators is a growing field of interest now-a-days.

The term intercalation was first introduced in 1961 by Leonard Lerman to describe the insertion an appropriate size and chemical nature planar, aromatic or hetero-aromatic compounds between adjacent nucleotide base pairs of DNA double stranded [13]. Intercalative binding occurs according to the “neighbor exclusion principle” where every second site along the helix remains unoccupied [14]. The double helix DNA unwinds itself dynamically to open a space between its base pairs to fit intercalator which disturbs its biological function. The degree of unwinding depends on the intercalator. The unwinding angle of daunomycin was 12 ± 2 degrees, ethidium bromide was 15 ± 3 degrees and that of aclacinomycin with the DNA double helix is an angle of 8 ± 2 degrees [15]. This unwinding causes structural changes to the DNA strand like lengthening of the DNA strand or twisting of the base pairs. This structural perturbation in the double helix structure can lead to the inhibition of transcription, replication and DNA repair processes, making the intercalators as potent mutagen. Intensively studied DNA intercalators include berberine [16], ethidium bromide and propidium iodide [13b], daunomycin [17], proflavine etc. Ethidium bromide and proflavine were first known intercalators among them.

(ii) *Porphyrin derivatives (2A-B).* The most well-known members of the porphyrin family are bacteriochlorophylls in phototrophic bacteria, chlorophylls in plants, and heme in red blood cells in mammals (figure 1.7). The reddish-orange fluorescence of porphyrins is the longest wavelength among all naturally occurring fluorophores. The majority of the porphyrins emit strongly around 600-800 nm. One exception is heme, whose fluorescence is completely quenched by the coordinated iron. The fluorescence of porphyrins in vivo (in plants and bacteria) is markedly lower than when they are free in solution and the efficiency of emission strongly depends on the light conditions. At low light levels, the fluorescence is almost absent and only appears at high irradiation.
In natural photosynthesis chlorophyll plays the major role. Light is absorbed by an antenna system of a few hundred chlorophyll molecules which are arranged in a protein environment [14]. For efficient energy transformation, the anisotropic arrangement of the chlorophyll molecules is significant because it prevents the formation of quenching aggregates by shielding the molecules in polypeptide cages.

Porphyrin derivatives are extensively used in photodynamic therapy (PDT). PDT is a promising approach for the treatment of superficially localized tumors. PDT is a treatment that uses a drug, called a photosensitizer or photosensitizing agent, and a particular type of light. When photosensitizers are exposed to a specific wavelength of light, they produce a form of oxygen that kills nearby cells [18–20]. Each photosensitizer is activated by light of a specific wavelength [20, 21]. This wavelength determines how
far the light can travel into the body [20, 22]. Thus, doctors use specific photosensitizers and wavelengths of light to treat different areas of the body with PDT.

(iii) Pthalazine derivatives (3a-c). Pthalazine derivatives are well known drug used for hypertension. Hypertension is the most common cardiovascular disease which affects approximately billions of people all around the world. Hypertension represents the major risk factor for endothelial dysfunction, renal dysfunction, congestive heart failure, and stroke [23]. Relaxation of vascular smooth muscles is one of the strategies for the treatment of hypertension [24]. There are lots of drugs for these diseases but all are with some side effects. So, there is a continuous need to explore, search and develop new vasorelaxant agents with fewer side effects. The a1-adrenergic receptors (a1-ARs), a family of G-protein coupled seven-transmembrane helix receptors, are mainly involved in the cardiovascular and central nervous system [25]. In the last few years due to their importance in the treatment of hypertension and asthma the research for new selective a1-AR antagonists are increases more [26-29].

(iv) Polyhydroquinoline derivatives (4a-c). Polyhydroquinoline and 1,4-dihydropyridine(DHP) are well known calcium channel blockers. Calcium channel blockers are the drugs which used to lower blood pressure. They work by slowing the movement of calcium into the cells of the heart and blood vessel walls, which makes it easier for the heart to pump and widens blood vessels. As a result, blood pressure lowers. There are two types of calcium channel blockers such as L-type and N-type. 1, 4-DHPs and polyhydroquinolines are particularly well known in pharmacology as L-type calcium channel blockers. Some of the other compounds used in the treatment of hypertension are nifedipine, nicardipine, amlodipine, and felodipine. 1,4-DHPs and polyhydroquinolines are relatively vascular selective in their mechanism of action in lowering blood pressure, compared with certain other L-type calcium channel blockers like Verapamil of phenylalkylamine class. They can cure the disordered heart ratio as a chain-cutting agent of factor IV channel, possessing the calcium channel agonist–antagonist modulation activities [30].
(v) *Coumarin derivatives (5a-b).* Coumarins are widely occurring in nature. It was first isolated in 1820 from a specific variety of bean, and many other coumarin derivatives found in a wide range of plants [30]. Compounds of the coumarin series are fluorescent dyes and materials for dye lasers [31]. Coumarins exhibit interesting fluorescence properties, including a high degree of sensitivity to their local environment like polarity and viscosity. This sensitivity has led to their widespread application as sensitive fluorescent probes of a wide range of systems, including homogeneous solvents and mixtures, and heterogeneous materials [32]. Coumarin derivatives can be used for anticoagulant, antibacterial, antihelminitic, hypothermal properties and vasodilatatory action [33]. A recent study has shown that 7-hydroxycoumarin inhibits the release of Cyclin D1, which is over expressed in many types of cancer.