**Chapter-I: Introduction**

1.1 Luminescence

Luminescence is the emission of light from any substance and occurs from electrically excited state. The term “Luminescence” was introduced in to the literature by Wiedemann (1888). He also offered the first, although not entirely accurate, definition of luminescence as the excess emission over and above the thermal emission background [1, 2]. This definition reflected an important property, but it did not distinguish luminescence from other type of glow that are also excess emission over and above thermal emission background, among which are reflected and refracted light. Vavilov (1951-52) suggested complimenting Wiedemann’s definition by addition the correction of duration and using the term luminescence for the excess emission over and above the thermal emission of a body. If this emission has a duration considerably exceeding period of light oscillations [1-3]. Luminescence is the phenomenon in which all or part of absorbed energy is re-emitted in the form of electromagnetic radiation in the visible or near visible region of the spectrum. The luminescence involves at least two steps, the excitation of the electronic system of the material and the subsequent emission of photons. An example of luminescence is the light or glow emitted by a luminous watch dial. Luminescence contrasts with incandescence which is the production of light by heated materials [1-5].

When certain material absorbs various kinds of energy, some of the energy may be emitted as light. This process involves two steps:

1. The incidental energy causes the electron of the atoms of the absorbing materials to become excited and jump from the inner orbits of the atoms to the outer orbits.

2. When the electrons fall to their original state, a photon of light is emitted. The interval between the steps may be short (less than 1/100, 00 of a sec.) or long (many hours). If the interval is short, the process is called fluorescence, if the interval is long, the process is called phosphorescence. In
either case the light produced is almost always of lesser energy and of longer wavelength than the exciting light [1, 3, 4].

1.1.3. Jablonski Diagram:

Processes which occur between the absorption and emission of light are usually illustrated by Jablonski diagram. A typically Jablonski diagram is shown in Figure 1. The ground, first and second electronic states are depicted by \( S_0 \), \( S_1 \) and \( S_2 \), respectively. At each electronic energy levels the fluorophores can exist in a number of vibrational energy level (denoted 0,1,2, etc). Transitions between states are depicted as vertical lines to illustrate the instantaneous nature of light absorption. Transitions occur in about \( 10^{-15} \) seconds, a time too short for significant displacement of nuclei. (Frank-Condon principal) [5]

1.1.1 Phosphorescence:

Phosphorescence is a emission of light from triplet-excited state, in which the electron in the excited orbital has the same spin orientation as the ground-state electron. Transition to the ground state are forbidden and the emission rate are slow \( (10^{-3}-100\text{s}^{-1}) \), so phosphorescence lifetimes are typically milliseconds to seconds. Phosphorescence is usually not seen in fluid solutions at room temperature, but there are many deactivation processes that compete with emission, such as nonradiative decay and quenching processes. [6]

1.1.2. Fluorescence:

Fluorescence is emission light from singlet-excited states, in which the electron in the excited orbital is period (of apposite sign) to the second electron the excited orbital. Return to the ground state is spin-allowed and occurs rapidly by emission of a photon [4, 7, 8]. Fluorescence is usually excited to some higher vibrational level of either \( S_1 \) or \( S_2 \). With a few rare exceptions, molecules in condensed phase rapidly relax to the lowest vibrational level of \( S_1 \). This process, called internal conversion, is nonradiative and take place in \( 10^{-12} \) seconds or less. Return to the ground state occurs to a higher excited vibrational ground-state level, which then quickly reaches thermal equilibrium. An interesting consequences of emission to a higher vibrational ground state is
that the emission spectrum is typically a mirror image of the absorption spectrum of the $S_0 \rightarrow S_1$. This emission rates of fluorescence are typically $10^8 \text{ s}^{-1}$, so that a typically fluorescence lifetime is near 10 ns. Fluorescence spectral data are generally presented as emission spectra. Emission spectra vary widely and are dependant upon the chemical structure of the fluorophore and the solvent in which it is dissolved.

### 1.2. Types of Fluorescence:

The fluorescence is observed as long as excitation is in process and is known as steady state or prompt fluorescence. When excitation is cut off, fluorescence ceases. However, in some case even after cutting of source of excitation, the emission of light persists as a glow and is known as delayed fluorescence.

1. Steady State Fluorescence:
2. Delayed Fluorescence

![Jablonski Diagram](image)

**Fig. 1.1.** A simplified Jablonski diagram with absorption, internal conversion, fluorescence, intersystem crossing, and phosphorescence.
1.3. Processes competing with Fluorescence:

1.3.1 Vibrational Relaxation (VR):

Emission spectra are typically independent of the excitation wavelength. Upon excitation to higher electronic and vibrational levels, the excess energy is quickly dissipated, leaving the fluorophore in the lowest vibrational levels of $S_1$. It is from this position that photon will be emit [Kasha’s Rule]. As a consequence, fluorescence form solution, when it occurs, always involves a transition from the lowest vibrational level of an excitation level of an excited state [4, 9]. A consequence of the efficiency of vibrational relaxation is that the fluorescence band for a given electronic transition is displaced toward lower frequencies or longer wavelengths from the absorption bands (the stock effect).

1.3.2 Internal conversion (IC):

Internal conversion is a non-radiative transition between two electronic states of the same spin multiplicity. In solution, this process is followed by a vibrational relaxation towards the lowest vibrational level of the final electronic state. The excess vibrational energy can be indeed transferred to the solvent during collisions of the excited molecule with the surrounding solvent molecules.

When a molecule is excited to an energy level higher than the lowest vibrational level of the first electronic state, vibrational relaxation (and internal conversion if the singlet excited state is higher than $S_1$) leads the excited molecule towards the 0 vibrational level of the $S_1$ singlet state with a time-scale of $10^{-13} - 10^{-11}$ s [10].

From $S_1$, internal conversion to $S_0$ is possible but is less efficient than conversion from $S_2$ to $S_1$, because of the much larger energy gap between $S_1$ and $S_0$. Therefore, internal conversion from $S_1$ to $S_0$ can compete with emission of photons (fluorescence) and intersystem crossing to the triplet state from which emission of photons (phosphorescence) can possibly be observed.

1.3.3 Intersystem Crossing (ISC):

Intersystem crossing is a non-radiative transition between two isoenergetic vibrational levels belonging to electronic states of different multiplicities. For
example, an excited molecule in the 0 vibrational level of the $S_1$ state can move to the isoenergetic vibrational level of the $T_n$ triplet state; then vibrational relaxation brings it in to the lowest vibrational level of $T_1$. Intersystem crossing may be fast enough ($10^{-7}–10^{-9}$ s) to compete with other pathways of de-excitation from $S_1$ (fluorescence and internal conversion $S_0\rightarrow S_1$). Crossing between states of different multiplicity is in principle forbidden, but spin–orbit coupling (i.e. coupling between the orbital magnetic moment and the spin magnetic moment) can be large enough to make it possible. The probability of intersystem crossing depends on the singlet and triplet states involved. If the transition $S_0\rightarrow S_1$ is of $n \rightarrow \pi^*$ type for instance, intersystem crossing is often efficient. It should also be noted that the presence of heavy atoms (i.e. whose atomic number is large, for example Br, Pb) increases spin–orbit coupling and thus favors intersystem crossing [11].

Once ISC has occurred, the molecule undergoes the usual IC process and falls to the lowest vibrational level of the first excited triplet state. Therefore, ISC can compete with fluorescence and thus it decreases the quantum efficiency of fluorescence. The population of triplet state has a significance in producing delayed fluorescence and phosphorescence which is a radiative decay of triplet state molecule to the ground state, are summarized in Fig. 1.2.

![Fig.1.2. De-Excitation pathway of excited Molecules](image-url)
1.4. **Experimental Observables:**

Following experimental observables are used to measure the properties of any luminescent system.

1. Absorption spectra  
2. Emission spectra  
3. Excitation spectra  
4. Quantum efficiency and yields

1.4.1 **Absorption spectra:**

The quantization condition for the absorption or emission of light by an atom or by molecule is given by Einstein relation

$$h\nu = \frac{hc}{\lambda} = E_2 - E_1 \quad \text{(1)}$$

where, $E_2$ and $E_1$ are the electronic energy levels, $h$ is Planck’s constant and $\nu$, $\lambda$ and $c$ are the frequency, wavelength and velocity of the incident photon.

The absorption of energy by a molecule is governed by the Beer-Lambert’s law. According to this relationship

$$\log_{10}\left(\frac{I_0}{I}\right) = \varepsilon \cdot c \cdot l \quad \text{(2)}$$

Where

- $I_0$ = intensity of incident light
- $I$ = intensity of transmitted light
- $\varepsilon$ = molecular extinction coefficient
- $c$ = concentration of the solution
- $l$ = path length of the absorbing system through which light passes

and

$$\log_{10}\left(\frac{I_0}{I}\right) = \text{optical density or absorbance of the material}$$

In practice, the absorption spectrum is plotted in terms of molecular extinction coefficient ($\varepsilon$) against frequency or wavelength.

Figure a typical absorption spectrum for a $S_0 \rightarrow S_1$ transition. The probability of the absorption depends upon the degree of overlap of the wave function of the lowest vibrational level of the ground state $S_{00}$ and the wave function of the vibrational levels of the first excited singlet state $S_{10} \rightarrow S_{1n}$. 
Fig. 1.3. Illustration of the vibrational bands in the absorption and fluorescence spectra of aromatic hydrocarbons.

The positions of the absorption peaks and its nature are of significance in the spectroscopic studies. The nature of the absorption spectrum indicates the monomeric or dimeric state of the molecular systems which is of importance to explore whether the ground state dimer or a monomer is excited in the absorption process [12]. In solution the broad absorption band is an indication of dimeric nature of molecules in the ground state while the structured spectrum indicates the existence of monomolecular species[13]. But in solids the absorption spectra are not as structured as in solution. The nature of absorption bands also gives an idea about the lattice structures of molecular systems under study and suggests the possibility of formation of dimeric species [14].
Extensive work has been carried out to establish exact relationship between absorption and emission properties of organic crystalline materials with a view to explore electronic and molecular structure of materials in condensed state [15-18].

The position of the absorption bands in the spectra of aromatic hydrocarbons depends upon the number of benzene rings and the way by which they are condensed. In linearly condensed molecules, longest wavelength band of absorption spectrum moves towards longer wavelength of the electromagnetic spectrum. This effect is mainly due to decrease in energy difference between lower excited and ground state of the molecule. As the number of condensed rings increases this energy difference becomes progressively less in the linear polynuclear aromatic hydrocarbons[19]. But in non-linear hydrocarbons the situation becomes reverse. The energy difference in general increases as the number of bends (angular condensation) in the molecule increases.

1.4.2. Emission Spectra:

The molecule is in the lowest vibrational level of the ground state at room temperature and on absorption of visible or ultraviolet radiation the molecule gets excited to the higher electronic energy level. This transition occurs too rapidly within $\sim 10^{-15}$ sec. The molecule now has the possibility to drop down to the ground state with the emission of light. The fluorescence always takes place from the lowest vibrational level of the first excited singlet state and the shape of the fluorescence emission spectrum is independent of the wavelength of the exciting light.

The fluorescence emission spectrum is obtained by irradiating the sample by a wavelength of maximum absorption as indicated by absorption spectrum of the sample. Figure 3. reveals the levels of the ground $S_0$ and excited state $S_1$ associated with the absorption and emission spectra. It is observed that the absorption spectra gives data about the vibrational levels of the excited state and the emission spectra yields data about the vibrational levels of the ground state. In most of the organic materials the emission
spectrum is the mirror image of the absorption spectrum. This relationship is an indication of the similarity of the respective vibrational wave functions in the excited and ground states.

Birks and Dyson (1963) [20] reported that this mirror image relationship is not observed in some molecular systems. In such systems there is some degree of hindrance to the normal relaxation processes in the excited or ground state.

1.4.3. **Excitation spectra:**

Excitation spectroscopy of the fluorescent samples gives a further way of gaining information about the excited states of organic molecules shown in Fig.3. An excitation spectrum is obtained by examining variation in the luminescence quantum intensity as a function of excitation wavelength. For most of the organic molecules the quantum yield is independent of excitation wavelength because of very efficient process of internal conversion from higher excited states to the lowest excited singlet states.

Even for those molecules for which quantum intensity is invariant with excitation wavelength, excitation spectra provide a powerful tool for measuring the absorption spectra of molecules, which are at too low a concentration for detection by absorption spectrometry.

The excitation spectrum will be identical to the absorption spectrum where $\varepsilon_{cd}<<1$. The measurement of quantum intensity is limited by the sensitivity of the spectrofluorometer and that depends upon the intensity of the excitation source. Parker (1968) [21] estimated that concentrations as low as $10^{-12}$ M can be detected by excitation spectroscopy compared with a minimum concentration of $10^{-8}$ M by absorption spectroscopy. Excitation spectroscopy is also used to determine the quantum efficiency of energy transfer between donor and acceptor molecules.
1.4.4 Fluorescence Quantum Yields:

Of the excited molecules into the higher excited states all will not return to the ground state by fluorescence or phosphorescence process because these processes competed by IC and ISC.

The quantum efficiency of fluorescence $\Phi_f$ is defined as the ratio of the number of fluorescence photons emitted to the number of photons absorbed. It is important to distinguish at same stage the difference between the term’s quantum efficiency and quantum yield. The term quantum yield is used to define the fraction of excited molecules, which may follow a given pathway either radiatively or non-radiatively. The methods of determination of absolute quantum efficiency need to compare the rate of absorption of excitation light with the total rate of emission of fluorescence at all wavelengths and in all direction. In principle this is simple but in practice it is a difficult experiment to perform with precision.

The most direct method for determination of fluorescence efficiency was reported by Vavilov [22], Waber and Teale [23]. They have devised an absolute method in which a standard fluorescent solution is not required and in addition to this the method is applicable to dilute solutions also. Determination of relative quantum efficiency at room temperature of the two substances is practically simple [24]. The measurement with modern light sensitive Spectrofluorometers involves negligible errors due to excessive absorption of incident light or due to self absorption. Under such conditions the rate of fluorescence emission is proportional to the product $I_0 \varepsilon c \mathcal{I} \Phi_f$.

The integrated area under the corrected fluorescence spectrum is proportional to the rate of emission of fluorescence and thus, if the fluorescence emission spectra of two solutions/ sytems are measured with the same experimental conditions like same instrumental geometry and at the same intensity of exciting light. The ratio of the two fluorescence intensities is given by
1.5. Structural factors on Fluorescence Measurements:

1.5.1. Electron-withdrawing substituents: (carbonyl and nitro compounds)

The fluorescence properties of aromatic carbonyl compounds are complex and often difficult to predict. Many aromatic aldehydes and ketones (e.g. benzophenone, anthrone, 1- and 2- naphthaldehyde) have a low-lying n–p excited state and thus exhibit low fluorescence quantum yields, as explained above. The dominant de-excitation pathway is intersystem crossing (whose efficiency has been found to be close to 1 for benzophenone).

Some aromatic carbonyl compounds have a low-lying \( \pi \rightarrow \pi^* \) excited state and thus have a reasonable quantum yield (e.g. 0.12 for fluorenone in ethanol at 77 K and 0.01 at room temperature). However, if a \( n \rightarrow \pi^* \) state lies only slightly higher in energy, the fluorescence quantum yield strongly depends on the polarity of the solvent (proximity effect). In fact, in some solvents, the energy of the \( n \rightarrow \pi^* \) state can become lower than that of the \( \pi \rightarrow \pi^* \) state. When the polarity and the hydrogen bonding power of the solvent increases, the \( n \rightarrow \pi^* \) state shifts to higher energy whereas the \( \pi \rightarrow \pi^* \) state shifts to lower energy. Therefore, intense fluorescence can be observed in polar solvents and weak fluorescence in nonpolar solvents (e.g. xanthone). When an aromatic molecule has a carboxylic group as a substituent, photophysical effects due to
conformational changes can be observed. For instance, anthracene-9-carboxylic acid exhibits a broad fluorescence spectrum deprived of apparent vibronic bands, in contrast to its absorption spectrum and to both absorption and fluorescence spectra of the conjugate base (similar to the anthracene spectrum). Such a difference between the fluorescence spectra of the acidic and basic forms can be explained in terms of conformation of the carboxylate group $\text{-COO}^-$, which should be almost perpendicular to the ring so that the p system of the anthracene ring is only slightly perturbed. On the contrary, the carboxylic group $\text{-COOH}$ may be in a position close to the coplanarity of the ring; the resulting interaction induces an intramolecular charge-transfer character to the $\pi \rightarrow \pi^*$ transition. Charge-transfer fluorescence bands are indeed usually broad and structureless. However, because the absorption spectrum of the acidic form exhibits vibronic bands, the rotation of the $\text{-COOH}$ is likely to be photoinduced. In general, the fluorescence of aromatic hydrocarbons possessing an $\text{-NO}_2$ substituent is not detectable. The existence of a low-lying $n \rightarrow \pi^*$ transition explains the efficient intersystem crossing process (e.g. for 2-nitronaphthalene, the quantum yield for intersystem crossing is 0.83 in benzene solution at room temperature). Many nitroaromatics are indeed phosphorescent. However, in some cases, the quantum yield for intersystem crossing is significantly less than 1. Therefore, the absence of detectable fluorescence is likely to be due to a high rate of $S_1 \rightarrow S_0$ internal conversion, which may be related to the considerable charge-transfer character of the excited state, as a result of the strong electron-withdrawing power of the $\text{-NO}_2$ group. It should be mentioned that many nitroaromatics undergo photodegradation. For instance, 9-nitroanthracene is transformed into anthraquinone upon illumination.

1.5.2. Electron-donating substituents: ($\text{-COH, -COR, -CNH}_2$, $\text{-CNHR, -CNR}_2$)

In general, substitution with electron-donating groups induces an increase in the molar absorption coefficient and a shift in both absorption and fluorescence spectra. Moreover, these spectra are broad and often structureless
compared to the parent aromatic hydrocarbons (e.g. 1- and 2-naphthol compared to aphthalene). The presence of lone pairs of electrons on the oxygen and nitrogen atoms does not change the $\pi \rightarrow \pi^*$ nature of the transitions of the parent molecule. These lone pairs are indeed involved directly in p bonding with the aromatic system, in contrast to the lone pairs of electrons of carbonyl substituents or heterocyclic nitrogen. To make the distinction between the two types of lone pairs, Kasha and Rawls suggested using the term l orbital for the lone pair orbital of aromatic amines and phenols. A significant intramolecular charge transfer character of the relevant transitions is expected for planar aromatic amines and phenol; this is confirmed by the fact that the fluorescence spectra are broad and structureless. If, for steric reasons, the $-\text{NH}_2$ group is twisted out of the plane of the aromatic ring, the degree of conjugation of the l orbitals is decreased, but the transitions corresponding to the promotion of an electron from an l orbital to a p orbital are still different (in particular more intense) to the $n \rightarrow \pi^*$ transitions involving the lone pairs of a carbonyl or nitro group. Departure from coplanarity with the aromatic ring is also pronounced with a -OR substituents, whereas an -OH group is nearly coplanar [25].

1.6. Factor Affecting on Fluorescence Spectra:

1.6.1 Solvent Effect:

Although the fluorescence of gases [26-28] and solids [29] have been measured the vast majority of fluorescence spectra have been measured in solution. As in the case of ultraviolet absorption spectra [30], solvent effects are observed in fluorescence measurements. These effects are manifested by wave length shifts and/or quenching of fluorescence. Fluorescence solvent effects are less well known than ultraviolet absorption solvent effects and the effects of pH on absorption and fluorescence spectra; nevertheless, several groups of compounds are known in which change of solvent brings about remarkable changes. These changes are in some instances accompanied by comparable changes in the fluorescence excitation spectra, and hence also in the ultraviolet absorption spectra; in other instances shifts in the position of the fluorescence
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emission maxima are not accompanied by comparable changes in the ultraviolet absorption spectra. Displacements in the ultraviolet and fluorescence emission spectra imply interaction with solvent in both the ground state and the excited state of the molecule. On the other hand, when the fluorescence emission spectra alone are affected by change of solvent, interaction between solvent and the excited state, but not with the ground state of the molecule, is indicated. An attempt will be made to discuss here the observed fluorescence solvent effects in the light of accompanying changes, or lack thereof, in the ultraviolet absorption spectra. As early as 1904 Kauffmann and Beisswenger [31] observed changes in the fluorescence color of 3-aminophthalimide, I, with change in solvent from violet in hydrocarbons to blue-green in ethanol. Wavelength shifts brought about by solvent have been observed in both absorption and fluorescence spectra of many compounds. The effect of the dielectric constant of the solvent was used in explaining some fluorescence solvent effects [32-35] and hydrogen bonding has been invoked in other instances [36-38]. However, the interpretation of solvent effects is often complicated because the observed changes may be the resultant of several quite different forces which may either augment or minimize each other and these effects have been discussed by various authors [39-43]. Bayliss and McRae [39,40] discussed the origin of solvent effects and point out that all spectra undergo a *polarization* shift to longer wave length. This shift results from the induced polarization of the solvent produced by the transition dipole of the solute. It is more clearly observed in nonpolar solvents and solute, i.e., a red shift occurs with increasing dielectric constant of the solvent. However, it is often obscured by associated effects, *e.g.*, in polar or hydrogen bonding compounds. When the molecule changes to the excited state by the absorption of energy this relationship is upset. An excited solute molecule in this situation is in the *Franck-Condon* state. This state prevails for a very brief period of time but the solvent molecules cannot reorient themselves during the transition from the ground to the excited state. It follows then from the Franck-Condon principle that there may be a difference in solvation energy of the ground and
excited states and this will be reflected in changes in absorption and emission spectra. The “blue shift,” i.e., hypsochromic shift observed in absorption spectra in solvents of high dielectric constant have been described [42,37] in the light of the Franck-Condon principle. The blue shift is associated with $n \rightarrow \pi^*$ a transition which refers to the excitation of a nonbonding electron to an antibonding orbital [43]. However, it was pointed out that $n \rightarrow \pi^*$ transitions are usually not observed in fluorescence spectra and when present are weak [44].

1.6.2. Effect of pH:

The basicity or acidity of a molecule is determined by its electronic structure and this may undergo detailed changes during excitation from the ground state by the absorption of light, i.e., the basicity or acidity of a compound may change during excitation. A difference in the basicity or acidity of the ground and excited states will be reflected in differences between the absorption and fluorescence spectra with change in pH. The nature of the changes will depend on whether the basicity is increased or decreased during ionization. Also, in order for the expected changes in pK to be observed, it is essential that the ionization equilibrium be established during the lifetime of the excited state. Weller [44] discussed the absorption and fluorescence spectral shifts in terms of the energy changes involved. A simplified energy diagram is presented in Fig.. This diagram represents the energy terms involved in the dissociation of an acid (HA) and the corresponding base (B). $\Delta H$ represents the enthalpy of the dissociation reaction in the ground state and $\Delta H^*$ the same for the excited state. When $\Delta H > \Delta H^*$ a red shift occurs in absorption and fluorescence spectra on acid dissociation; when $\Delta H < \Delta H^*$ a blue shift occurs on acid dissociation, and when $\Delta H = \Delta H^*$ there is no shift in either absorption or fluorescence spectra.
Fig. 1.4. Energy diagram for dissociation of an acid (HA) in the ground and excited state.

Since,  
\[ \Delta H - \Delta H^* = \Delta E_{HA} - \Delta E_A \]  

where  
\[ \Delta E_{HA} = \text{excitation energy for the 0-0 transition of the acid,} \]
\[ \Delta E_A = \text{the excitation energy for the 0-0 transition of the base,} \]

assuming that the entropy of the reaction of acid dissociation is the same for the ground and the excited states it is possible to calculate the difference in pK between the two states from equation 2.

\[ pK - pK^* = \frac{\Delta E_{HA} - \Delta E_A}{2.303 RT} \]

When  
\[ \Delta E_{HA} - \Delta E_A > 0 \] the excited state is more acidic than the ground state, e.g., oxy- and amino aromatic compounds; when  
\[ \Delta E_{HA} - \Delta E_A < 0 \] the excited state is less
acidic, e.g., acridine and acridone, and, of course, when $\Delta E_{HA^-} - \Delta E_A = 0$ there is no difference in pK between the ground and excited states, e.g., pyridine. From the position of the 0-0 transition for acridine and the acridinium cation, Weller, using the method described above, calculated the pK for the excited state of acridine to be 10.35 and that for the ground state, 5.45. On this basis then it is expected that in aqueous solution acridine will show a fluorescence change at pH 10 which is independent of the absorption change at pH 5.45. The green fluorescence of the cation should change to the blue fluorescence of the free base between pH 9 and 11. Neutral aqueous acridine solutions, however, fluoresce blue so that it has to be concluded that the required ionization equilibrium is not established during the lifetime of the excited state.

1.6.3. Effect of Concentration:
Increases in concentration of fluorescent solutions usually result in quenching at high concentrations and this is often accompanied by wavelength shifts. Concentration effects can be ascribed to various phenomena including reabsorption of emitted light, “true” concentration quenching, dimerization or aggregation, and miscellaneous experimental and instrumental factors. The examination of concentration effects is of importance because of the information obtained in this manner about solute-solute and solute-solvent interactions. Furthermore, the effects of concentration have to be taken into account in the measurement of quantum yields of fluorescence and relative fluorescence intensities [45].

1.7. Fluorescence Quenching Phenomenon:

1.7.1 Fluorescence quenching:
The phenomenon of decrease in intensity of fluorescent compound at its $\lambda_{max}$ is known as fluorescence quenching. When one compound diminishes or abolishes the fluorescence of another, it is said to quench the fluorescence. The quenching of fluorescence can be brought about in many ways. Further, the quenching of one compound by another is often unpredictable [46-19]
Quenching of fluorescence can occur by-

a) Inner filter effect
b) Energy degradation
c) Chemical change
d) Energy transfer
e) Electron transfer

1.7.1. Quenching by inner filter effects:

Inner filter effects are instrumental effects. They have no influence on the primary process of emission from molecule originally excited but simply reduce the observed intensity of luminescence within the material being tested [10,37,38]

The two kinds of inner filter effects are excessive absorption of the exciting light, and absorption of the luminescence emitted (solute quenching). Therefore, the fluorescence intensity of mostly dyes increases linearly with increasing concentration at relatively low dye concentrations. The measured intensity is usually proportional to the optical density (OD) of the dye solution only to an OD of about 0.05. Beyond these concentrations the fluorescence intensities can be approximately corrected for the inner filter effects as follows

$$F_{corr} \approx F_{abs} \cdot anti \cdot \log[(OD_{ex} + OD_{em})/2]$$

Many fluorescent dyes from dimmer or higher aggregates in solutions of higher concentrations particularly in non-polar solvents. Often the energy transfer [50, 51]

The second type of inner filter effect is that produced by the absorption of the fluorescence light either by absorption of an excessive concentration of the fluorescent dye itself (self absorption), or by other solutes. Self-absorption mainly affects the low wavelength side of the fluorescence emission band because it is in this region that overlap with the first absorption band occurs. Self-absorption reduces the fluorescence intensity. The presence of a second solute that absorbs -strongly in the region where the first solute fluoresces will also cause distortion in the emission spectrum of the fluorescing dye.
1.7.2. Quenching by energy degradation:

Such quenching process involves concentration of singlet excited state molecule into triplet state by energy or electron transfers. So the fluorophores no longer emits its energy as fluorescence. The quenching effects of oxygen certain iodo, bromo and nitro compounds and probably may be due to energy transfer. The quenching of fluorescent molecules by electron donating I-, Br-, SCN-, and $S_2O_3^-$ and electron accepting anions such as $IO_3^-$, $NO_3^-$ and $S_4O_6^-$ are cases of involving electron transfer between donor-acceptor. The certain for energy transfer should be that the triplet energy level; quencher should be lie below the excited singlet level of fluorophor which is probable in condensed system where donor-acceptor (quencher) are more closer than in solution [52,53]

1.8.3. Quenching by energy transfer:

The system of donor – acceptor pair is excited by proper radiation which selectively excited donor molecules. The donor-acceptor pairs are such that they satisfy the conditions required for efficient transfer of energy. The acceptor molecule accepts the energy from donor by which donor deactivation to its ground state and acceptor is raised to singlet excited level. The excited acceptors so formed de-excites to ground level either radiatively or non-radiatively. In non-radiatively process the fluorescence characteristics of acceptor molecule is exhibited. The systematic variation of acceptor concentration gradually quenches the fluorescence of donor by ET with simultaneous sensitization of acceptor fluorescence. Quenching by energy transfer can take place by two fundamentally different mechanisms [53-57]. In the electron-exchange mechanism, two single independent electron –transfer-one in each direction-result in formation of the sensitizer’s ground state and quencher’s excited state. Energy transfer by dipole-dipole mechanism operates by Coulombic resonance interaction (the “transmitter-antenna mechanism”), in which the oscillating electrons of an excited-state sensitizer are coupled with those of the quencher by an induced dipole interaction. Energy and electron transfer processes, quenching mechanism shown in Scheme – 1. The photo-
physical and photo-chemical aspects of such systems are understood by the kinetic studies of both fluorescence quenching and sensitization effect. The analytical reactions are established in such studies to develop methods for quantitative estimations of medicinal compounds such as vitamins, erythromycin etc. [58-60]. Photo physical steps of energy and electron transfer are shown in fig.

![Diagram of Photochemical steps of energy and electron transfer](image)

**Fig.1.5. Photochemical steps of energy and electron transfer**

**Energy and Electron transfer nomenclature**

\[
\begin{align*}
D^+ + A & \rightarrow D + A^+ \quad \text{Hole transfer} \\
D^- + A & \rightarrow D + A^- \quad \text{Electron transfer} \\
D^* + A & \rightarrow D + A^* \quad \text{Energy transfer}
\end{align*}
\]

**1.8. Fluorescence quenching:**

Quenching is any process that decreases the fluorescence intensity, and as such, may be the result of a variety of processes. Some of these processes are uninteresting, such as concentration changes or inner filter effects due to excessive concentration or due to light scattering. Photobleaching, in which high radiation intensity may damage the fluorophores, may also cause an
apparent quenching; the time-dependent nature of the process differentiates photobleaching from normal quenching effects. One experimentally useful type of quenching is due to collisions between quenching agents and fluorophores, and is called collisional or **dynamic quenching**. A second type of quenching, sometimes confused with dynamic quenching, is **static quenching**, in which the quenching agent forms a non-fluorescent complex with the quenching agent. A final type of quenching, discussed below, is resonance energy transfer. Static and dynamic quenching requires direct contact between the fluorophore and the quencher. For dynamic quenching, the result of this contact is loss of the fluorescence pathway for return to the ground state, although the mechanism can vary significantly between quenchers. Some quenchers act by inducing intersystem crossing (oxygen and iodide are thought to quench by this method). Others, such as aromatic amines, appear to donate electrons to the excited state. In the case of dynamic quenching, contact must occur while the fluorophore is in excited state. Dynamic quenching is exhibits a concentration-dependence that is described by the Stern-Volmer equation:[61]

\[
\frac{F_0}{F} = 1 + k_q \tau_0 [Q] = 1 + K_D [Q]
\]

where \( \tau_0 \) is the lifetime of the fluorescent state in the absence of the quenching agent. If the quenching is not known to be due to dynamic quenching, \( K_D \) is replaced by \( K_{SV} \). For dynamic quenching,

\[
\frac{F_0}{F} = \frac{\tau_0}{\tau}
\]

because the quenching agent decreases the lifetime of the excited state. Dynamic quenching increases with temperature, because temperature increases diffusion rates. The term \( k_q \) is the second order rate constant that describes the quenching process. It is proportional to the effectiveness of the quencher and the accessibility of the fluorophore to collisions with the quencher. The quenching rate constant is actually comprised of two terms:

\[
f_0 = f_0 k_0
\]
where \( f_Q \) is the fraction of collisions that result in quenching, and \( k_0 \) is the diffusion controlled bimolecular rate constant:

\[
k_0 = \frac{4\pi N_0 (r_f + r_q)(D_f + D_q)}{1000 \, \text{cm}^2 \, \text{L}}
\]

where \( N_0 \) is Avogadro’s number, \( r_f \) and \( r_q \) are the radii of the fluorophore and quencher, and \( D_f \) and \( D_q \) are the diffusion coefficients of the fluorophore and quencher. Typical values of \( k_0 \) for free fluorophores and free quenchers are \( \sim 10^{10} \, \text{M}^{-1} \, \text{sec}^{-1} \). If the fluorophore is bound to the surface of a protein, the \( k_0 \) will be roughly half of this value due available surface occupied by the protein. If the fluorophore is buried within the protein, the value of \( k_0 \) will be even smaller, depending on the accessibility of the fluorophore. Static quenching is the result of the formation of a non-fluorescent complex between the fluorophore and the quencher. The association constant for the quencher fluorophore complex describes the effectiveness of a static quencher:

\[
K_s = \frac{[FQ]}{[F][Q]}
\]

where \([FQ]\) is the complex concentration, and \([F]\) and \([Q]\) are the concentrations of free quencher and free fluorophore. Because the total fluorophore concentration, \([F]_0 = [F] + [FQ]\),

\[
K_s = \frac{[F]_0 - [F]}{[F][Q]}
\]

Which is rearrange

\[
K_s = \frac{[F]_0}{[F][Q]} - \frac{1}{[Q]}
\]

If you assume that the all of the decrease in observed fluorescence is due to complex formation, the equation becomes:

\[
\frac{F_0}{F} = 1 + K_s [Q]
\]

which is identical in form to the Stern-Volmer equation for dynamic quenching. Static and dynamic quenching can be distinguished by lifetime measurements because dynamic quenching reduces the apparent fluorescent
lifetime, while static quenching merely reduces the apparent concentration of the fluorophore. Alternatively, temperature effects can be used to distinguish the two forms of quenching. Diffusion rates, and therefore dynamic quenching rates, increase with higher temperature. In contrast, complex formation strength tends to be inversely proportional to temperature, and therefore static quenching tends to be higher at lower temperatures. In some cases, the effect of the quencher is due to a combination of static and dynamic quenching shown in Fig. 1.6. This results in a modified equation: [62]

\[
\frac{F_0}{F} = (1 + K_d [Q])(1 + K_s [Q]) = 1 + (K_d + K_s)[Q] + K_d K_s [Q]^2
\]

**Fig. 1.6. Distinction between dynamic and static quenching.**

At high quencher concentrations, a dynamic quencher will appear to exhibit combined quenching. This is thought to be due to the fact that, at high concentrations, a significant amount of the quencher molecules are already in close proximity to the fluorophore. Assuming that any quencher within a sphere surrounding the fluorophore will quench the fluorescence, a modified Stern-Volmer equation can be derived: [25]

\[
\frac{F_0}{F} = (1 + K_d [Q])e^{\frac{[Q]VN}{1000}}
\]

in which \(V\) is the volume of the sphere (\(V\) is usually slightly larger than would be predicted for the sum of the quencher and fluorophore radii). In proteins, more than one population of fluorophore may be present. This is especially true for tryptophan residues, where some may be readily solvent accessible, and others may be buried. Stern-Volmer plots for these proteins frequently curve downward, reflecting the quenching of the accessible fluorophore. Assuming the buried fluorophore is not quenched, and fluorescence will be:
where $K_a$ is the Stern-Volmer constant for the accessible quencher. In practice, it is likely that the buried fluorophore will exhibit some quenching also, and therefore the curve would be expected to be more complex than is described by this equation. In quenching experiments with proteins, the quenching agent may interact with the protein in ways that alter the protein structure or that affect the degree of quenching observed. One method for examining this is the use of several quenchers with different properties. Differential quenching by positively and negatively charged quenchers suggests a charged environment. Small $K_D$ values typically reflect steric hindrance of quencher-fluorophore collisions. Quenching studies may allow isolation of signals from different fluorophores. They may also allow characterization of conformational changes that alter the accessibility of the fluorophore to the quenching agent.

1.9. **Fluorescence Resonance Energy Transfer (FRET) Process:**

The use of Förster or fluorescence resonance energy transfer (FRET) as a spectroscopic technique has been in practice for over 50 years. A search of ISI Web of Science with just the acronym “FRET” returns more than 2300 citations from various areas such as structural elucidation of biological molecules and their interactions, in vitro assays, in vivo monitoring in cellular research, nucleic acid analysis, signal transduction, light harvesting and metallic nanomaterials. The advent of new classes of fluorophores including nanocrystals, nanoparticles, polymers, and genetically encoded proteins, in conjunction with ever more sophisticated equipment, has been vital in this development. This review gives a critical overview of the major classes of fluorophore materials that may act as donor, acceptor, or both in a FRET configuration. [63]

Fluorescence resonance energy transfer (FRET) is a nonradiative process whereby an excited state donor D (usually a fluorophore) transfers energy to a proximal ground state acceptor A through long-range dipole–dipole interactions (Figure 1.7 and 1.8) [63-65]. The acceptor must absorb energy at
the emission wavelength(s) of the donor, but does not necessarily have to remit
the energy fluorescently itself (i.e. dark quenching).

Fig.1.7 Mechanism of Energy Transfer in Donor and Acceptor
Fig. 1.8. Schematic of the FRET process: Upon excitation, the excited state donor molecule transfers energy nonradiatively to a proximal acceptor molecule located at distance $r$ from the donor. The acceptor releases the energy either through fluorescence or nonradiative channels. The spectra show the absorption (Abs) and emission (Em) (63).

The rate of energy transfer is highly dependent on many factors, such as the extent of spectral overlap, the relative orientation of the transition dipoles, and, most importantly, the distance between the donor and acceptor molecules [66]. FRET usually occurs over distances comparable to the dimensions of most biological macromolecules, that is, about 10 to 100 Å. Although
configurations in which multiple donors and acceptors interact are increasingly common, the following equations consider energy transfer between a single linked D/A pair separated by a fixed distance r and originate from the theoretical treatment of Förster. [66-68] The energy transfer rate \( k_T(r) \) between a single D/A pair is dependent on the distance r between D and A and can be expressed in terms of the Förster distance \( R_0 \). \( R_0 \) is the distance between D and A at which 50% of the excited D molecules decay by energy transfer, while the other half decay through other radiative or nonradiative channels. \( R_0 \) can be calculated from the spectral properties of the D and A species

\[
R_0^6 = \frac{9000 \ln 10 k^2 \Phi_D}{128 \pi^3 n^4 N} \int \frac{f(\lambda) \varepsilon(\lambda) d\lambda}{\nu^4} \quad \text{Å}
\]

The factor \( k^2 \) describes the D/A transition dipole orientation and can range in value from 0 (perpendicular) to 4 (collinear/parallel). There has been much debate about which dipole orientation value to assign for particular FRET formats. Only in a few cases can the crystal structure of the D/A molecules be determined; there is no other reliable experimental method to measure absolute or fixed \( k^2 \) values, which leads to potential uncertainties in subsequent calculations. [69-70] Fortunately, the accumulated evidence has shown that the mobility and statistical dynamics of the dye linker lead to a \( k^2 \) value of approximately 2/3 in almost all biological formats. This also sets an upper error limit of 35% on any calculated distance.[66] Excellent discussions of this issue are provided by dos Remedios and Moens[70] as well as Stryer.[71] The refractive index n of the medium is ascribed a value of 1.4 for biomolecules in aqueous solution. \( Q_D \) is the quantum yield (QY) of the donor in the absence of the acceptor and J (\( \lambda \)) is the overlap integral, which represents the degree of spectral overlap between the donor emission and the acceptor absorption. The values for J (\( \lambda \)) and \( R_0 \) increase with higher acceptor extinction coefficients and greater overlap between the donor emission spectrum and the acceptor absorption spectrum. Whether FRET will be effective at a particular distance r can be estimated by the “rule of thumb” \( R_0 \pm 50\% R_0 \) for the upper and lower limits of the distance.[69,70] The efficiency of the energy transfer
can be determined from either steady-state [Eq. (2)] or timeresolved [Eq. (3)]
measurements.

\[
E = 1 - \frac{F_{DA}}{F_D} \quad \text{-------------------} \quad (2)
\]

\[
E = 1 - \frac{\tau_{DA}}{\tau_D} \quad \text{-------------------} \quad (3)
\]

F is the relative donor fluorescence intensity in the absence (F_D) and
presence (F_{DA}) of the acceptor, and t is the fluorescent lifetime of the donor in
the absence (F_D) and presence (F_{DA}) of the acceptor. FRET is very appealing
for bioanalysis because of its intrinsic sensitivity to nanoscale changes in D/A
separation distance (proportional to r^6). This property is exploited in FRET
techniques ranging from the assay of interactions of an antigen with an
antibody in vitro to the real-time imaging of protein folding in vivo.[72,73] The
myriad FRET configurations and techniques currently in use are covered in
many reviews.[69,74,75] Herein, we focus primarily on the fluorophore
materials utilized in bioanalytical FRET rather than the process itself. The
materials can be divided into various classes: organic materials, which includes
“traditional” dye fluorophores, dark quenchers, and polymers; inorganic
materials such as metal chelates, and metal and semiconductor nanocrystals;
fluorophores of biological origin such as fluorescent proteins and amino acids;
and biological compounds that exhibit bioluminescence upon enzymatic
catalysis. These materials may function as either FRET donors, FRET
acceptors, or both, depending upon experimental design.

1.10. **Surfactant:**

Surfactants are characterized by a hydrophilic charged ‘head’ and a
hydrophobic hydrocarbon ‘tail’. The most outstanding property of surfactants is their
tendency to form aggregates, the micelles, at sharply defined critical micelle
concentration (CMC). The micelles formed by ionic amphiphilic molecules in
aqueous solutions are in dynamic association of surfactant molecules that achieve
segregation of their hydrophobic proteins from the solvent via self assembly [76].
The aggregation of single molecules of a certain type when dissolved in water, to form a particle of colloid dimension called a micelle which is shown in Fig.1.9. The single molecules or monomers which can take part in these processes are characterized by possessing two regions in their chemical structure. One is hydrocarbon chain, the hydrophobic region of the molecule, and other is an ionized group or water soluble group, the hydrophilic region of the molecules e.g. Sodium lauryl sulphate.

![Fig. 1.9. Structure of Micelle (76)](image)

The existence in one compound of two moieties, one of which has affinity for the solvent and other of which is antipathetic to it, has been called amphipathy by Hartlet [76]. This dual nature is responsible for the properties of micellization, surface activity, and solubilization. As a class, these substances, which include soaps and detergents, can be called associate in solution, forming particles of colloidal dimensions. Due to their tendency to become adsorbed at interfaces, they are often called surface-active agents or colloidal surfactants.
1.11. CLASSIFICATION OF SURFACTANTS [77]:

From the commercial point of view surfactants are often classified according to their use. However, this is not very useful because many surfactants have several uses, and confusions may arise from that. The most accepted and scientifically sound classification of surfactants is based on their dissociation in water. The figures in page 4 show a few typical examples of each class.

Anionic Surfactant:

1.11.1. Anionic Surfactants:

Anionic Surfactants are dissociated in water in an amphiphilic anion, and a cation, which is in general an alcaline metal (Na+, K+) or a quaternary ammonium. They are the most commonly used surfactants. They include alkylbenzene sulfonates (detergents), (fatty acid) soaps, lauryl sulfate (foaming agent), di-alkyl sulfosuccinate (wetting agent), lignosulfonates (dispersants) etc… Anionic surfactants account for about 50% of the world production.

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Name</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Potassium laurate</td>
<td>CH$_3$(CH$<em>2$)$</em>{10}$COO$^-$ K$^+$</td>
</tr>
<tr>
<td>2</td>
<td>Sodium dodecyl (lauryl) sulphate</td>
<td>CH$_3$(CH$<em>2$)$</em>{11}$SO$_4^-$ Na$^+$</td>
</tr>
<tr>
<td>3</td>
<td>Hexadecylsulphonic acid</td>
<td>CH$_3$(CH$<em>2$)$</em>{15}$SO$_3^-$ H$^+$</td>
</tr>
<tr>
<td>4</td>
<td>Sodium dioctylsulphosuccinate</td>
<td>C$<em>8$H$</em>{17}$OOCCH$_2$</td>
</tr>
</tbody>
</table>

1.11.2. Nonionic Surfactants:

Nonionic Surfactants come as a close second with about 45% of the overall industrial production. They do not ionize in aqueous solution, because their hydrophilic group is of a nondissociable type, such as alcohol, phenol, ether, ester, or amide. A large proportion of these nonionic surfactants are made hydrophilic by the presence of a polyethylene glycol chain, obtained by
the polycondensation of ethylene oxide. They are called polyethoxylated nonionics. In the past decade glucoside (sugar based) head groups, have been introduced in the market, because of their low toxicity. As far as the lipophilic group is concerned, it is often of the alkyl or alkylbenzene type, the former coming from fatty acids of natural origin. The polycondensation of propylene oxide produce a polyether which (in opposition to polyethylene oxide) is slightly hydrophobic.

<table>
<thead>
<tr>
<th>Sr. NO</th>
<th>Name of the surfactant</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Polyoxyethylene p-tertoctylphenyl ether</td>
<td>(\text{C}<em>8\text{H}</em>{17}\text{C}_6\text{H}_4\text{O} (\text{CH}_2\text{CH}<em>2\text{O})</em>{10}\text{H})</td>
</tr>
<tr>
<td>2</td>
<td>Polyoxyethylene monohexadecyl ether</td>
<td>(\text{CH}_3(\text{CH}<em>2)</em>{15}(\text{OCH}_2\text{CH}<em>2)</em>{21}\text{OH})</td>
</tr>
</tbody>
</table>

1.11. 3. Cationic Surfactants

Cationic Surfactants are dissociated in water into an amphiphilic cation and an anion, most often of the halogen type. A very large proportion of this class corresponds to nitrogen compounds such as fatty amine salts and quaternary ammoniums, with one or several long chain of the alkyl type, often coming from natural fatty acids. These surfactants are in general more expensive than anionics, because of the high pressure hydrogenation reaction to be carried out during their synthesis. As a consequence, they are only used in two cases in which there is no cheaper substitute, i.e. (1) as bactericide, (2) as positively charged substance which is able to adsorb on negatively charged substrates to produce antistatic and hydrophobant effect, often of great commercial importance such as in corrosion inhibition. When a single surfactant molecule exhibit both anionic and cationic dissociations it is called amphoteric or zwitterionic. This is the case of synthetic products like betaines or sulfobetaines and natural substances such as aminoacids and phospholipids.
1.11.4. Zweterionic or Ampholytic Surfactant:

This type can behave either as an ionic, non-ionic, or cationic species, depending on the pH of the solution.

<table>
<thead>
<tr>
<th>Name of surfactant</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-dodecyl-N:N-dimethyl betaine</td>
<td>$\text{C}<em>{12}\text{H}</em>{25}\text{N}^+\text{(CH}_3\text{)}_2\text{CH}_2\text{COO}^-$</td>
</tr>
</tbody>
</table>

1.12. The micellization process:

The most important property of amphipathic molecules or ions characterized by a polar hydrophilic head and non-polar hydrocarbon tail is their tendency to form large aggregates, the micelles, above a certain rather sharply defined concentration. So micelle is defined as “a colloidal particle together with its surrounding stabilizing agent” [76]. Surfactant molecules (e.g. CTAB, SDS, Triton X-100, Brij – 35 etc.) self-aggregate into supremolecular structure when dissolved in water or oil. The simplest aggregate of these surfactant molecules is called a micelle, and dispersion of the aggregates in water or oil is referred to as micellar solution [77-80]. A typical micelle has size of ~50 Å and is made of about 100 surfactant molecules. [81] The self association gives rise to rich variety of phase structure shown in fig.1.10. Aggregation is not, however host limited to aqueous solution; it is some time
observed in non-aqueous polar solvents such as ethylene glycol and non-polar solvents such as hexane. [82]

Fig. 1.3. Solubilization of Organic Compounds: Self association property of surfactants to form self-assembled aggregates, the micelles, in an aqueous medium has a profound effect on the solubility of some Organic substance (Additives), otherwise sparingly soluble in water [83-84]. Solubilization by micelles is of importance in many
industrial processes such as detergency, emulsion polymerization, oil recovery, etc. and in a verity of fundamental research oriented polymerization like micellar modeling of biological membrane. [85]

The solubilization can be ascribed to incorporation of hydrophobic substance in to micelle in solution of surfactant. Solubilization has been treated as partitioning of additive molecules (solubilizates) between a micellar phase and intermicellar bulk phase [86-89]. The partitioning behavior of solubilization between intermicelle bulk phase and micellar phase is an indication of the hydrophilic – lyophilic balance of the molecule. It has been discussed that the affinity of water for the solubilizates is important in partitioning [90] due to water dragging effect where the water is carried as a shell around the solubilize and water between the solubilize and micelles, and between water and micellar phase play a vital role in the partitioning process. The partition coefficient is dependent on the structure of solubilizate and the surfactant that constituent the micelles.

In addition to the solubilization equilibrium, the micro viscosity of the micellar interior and location of the solubilize within the micelles is also important in many applications of micellar solubilization. The physical behavior of the surfactant micelles can be visualization as the construction of model membrane to mimic a biological surface with the solubilizate, drug carrier and drug release [91, 92].

**1.13.1. Solubilization:**

Solubilization is believed to occur at a number of different sites in the micelle as shown in Scheme-2.

1. One is the surface of the micelle at the micelle solvents interface
2. Between the hydrophilic head group
3. In the so called palisade layer of the micelle between the hydrophilic ground and the first few carbon atoms of the hydrophobic groups that comprise the outer core of the micellar interior.
4. More deeply in the palisade layer
5. In the inner core of the micelle
Ionic micelles ordinarily have an extensive hydrophobic core region, which can interact strongly with hydrocarbon and halogenated hydrocarbons of solutes. Hydrophobic effects have often been considered to be dominating in determining the locus of solubilization [93-94]. Surfactant micelles can be pictured as having a highly nonpolar interior and a relatively polar interfacial region. Therefore nonpolarized or easily polarizable compounds are solubilized, in aqueous medium in the inner core of the micelle, between the ends of the hydrophobic groups of the surfactant molecules. Polarized hydrocarbons are solubilized by absorption at the micelle-water interface, replacing water molecules that may have penetrate into the core of the micelle close to the polar heads, but solubilization of additional material is either deep in the palisade layer as located in the inner core of micelle [84]. The polarizability of the \( \pi \) electron cloud of the aromatic nucleus and its consequent ability to interact with the positively charged groups at the micelle-water interface may account for the initial adsorption of these hydrocarbons in that location Scheme-2[95]
1.14. **Application:**

The precise location and nature of the interactions between specific molecular species in living cells is of major interest in many areas of biological research, but investigations are often hampered by the limited resolution of the instruments employed to examine these phenomena. Conventional widefield fluorescence microscopy enables localization of fluorescently labeled molecules within the optical spatial resolution limits defined by the Rayleigh criterion, approximately 200 nanometers (0.2 micrometer). However, in order to understand the physical interactions between protein partners involved in a typical biomolecular process, the relative proximity of the molecules must be determined more precisely than diffraction-limited traditional optical imaging methods permit. The technique of **Fluorescence Resonance Energy Transfer** (more commonly referred to by the acronym **FRET**), when applied to optical microscopy, permits determination of the approach between two molecules.

Biosensors designed on the principle of fluorescent resonance energy transfer (FRET) have been widely applied to visualize signaling cascades in live cells with high spatiotemporal resolution. The application of FRET biosensors in studying molecular events in live cells and analysis methods to explore complex biological information implicated in FRET images

**a) Determination of distance between donor and acceptor**

The Forster resonance energy transfer can be used as a spectroscopic ruler in the range of 10-100 Å. The distance between the donor and acceptor molecules should be constant during the donor lifetime, and greater than about 10 Å in order to avoid the effect of short range interactions. Some precautions must be taken to ensure correct use of the spectroscopic ruler as:

i) The critical distance $R_0$ should be determined under the same experimental conditions as those of the investigated system because $R_0$ involves the quantum yield of the donor and the overlap integral, which both depend on the nature of the microenvironment.

ii) As regards the orientation factor $k^2$, it is usually taken as 2/3, which is the isotropic dynamic average, i.e. under the assumptions that both
donor and acceptor transient moments randomize rapidly during the donor lifetime and sample all orientations.

b) Distributions of distances in donor-acceptor pairs

The donor-acceptor distance may not be unique, especially when the donor and acceptor are linked by a flexible chain (e.g. end-labeled oligomers of polymethylene oxides, polymer chains). The most commonly used method for the evaluation of a distribution of distances is based on the measurement of the donor fluorescence decay.

c) RET can be used to measure the extent of binding. The steady state measurements are often used to measure binding interactions. It has efficient application to study macromolecular systems when there is more than a single acceptor molecule near a donor molecule.

d) Qualitative and quantitative applications of RET

In addition to the determination of distances at a supramolecular level, RET can be used to demonstrate the mutual approach of a donor and an acceptor at a supramolecular level as a result of aggregation, association, conformational changes, etc. The donor and acceptor molecules generally covalently linked to molecular, macromolecular or supramolecular species that move toward each other or move away. Because of its simplicity, the steady state RET-based method has been used in many diverse situations as shown below.

i) In chemical sciences

- Polymers: interpenetration of polymer chains, phase separation, compatibility between polymers, interdiffusion of latex particles, interface thickness in blends of polymers, light-harvesting polymers
- Supramolecular systems: molecular devices, artificial photosynthesis, antenna effect, etc.
- Chemical sensors
- Scintillators
ii) In life sciences

- Ligand-receptor interactions
- Conformational changes of biomolecules
- Proteins: in vivo protein-protein interactions, protein folding kinetics, protein subunit exchange, enzyme activity assays, etc.
- Membranes and models: membrane organization (e.g. membrane domains, lipid distribution, peptide association, lipid order in vesicles, membrane fusion assays, etc.)
- Drug-protein interactions
- Immunoassays
1.15. Literature Survey on Energy transfer and quenching Mechanism:

Yavuz Onganer et al. studied the fluorescence resonance energy transfer from fluorescein to merocyanine 540 in aqueous sodium dodecyl sulfate, cetyltrimethylammonium bromide and Triton X-100 micellar solutions at room temperature using steady-state and time-resolved fluorescence spectroscopy techniques.[96].

Markus Sauer et al. studied the fluorescence resonance energy transfer (FRET) efficiency of different donor–acceptor labeled model DNA systems in aqueous solution from ensemble measurements and at the single molecule level. The donor dyes: tetramethylrhodamine (TMR); rhodamine 6G (R6G); and a carbocyanine dye (Cy3) were covalently attached to the 5′-end of a 40-mer model oligonucleotide. The acceptor dyes, a carbocyanine dye (Cy5), and a rhodamine derivative (JA133) were attached at modified thymidine bases in the complementary DNA strand with donor–acceptor distances of 5, 15, 25 and 35 DNA-bases, respectively. Anisotropy measurements demonstrate that none of the dyes can be observed as a free rotor; especially in the 5-bp constructs the dyes exhibit relatively high anisotropy values. [97].

F. G. Sánchez et al. reported the experimental study on the energy transfer process between perylene and fluorescein in cetyltrimethylammonium bromide (CTAB) micelles. The spectral behaviour and the dynamic and polarization data of dyes confirmed that perylene is solubilized in the inner hydrocarbon region of the micelle, while fluorescein micellized in the inner part of the Stern region. The results obtained from the quenching experiments demonstrated that a static mechanism operates for the perylene-fluorescein pair in CTAB micelles. The study of the influence of the acceptor concentration on the energy transfer efficiency suggested that only intramicellar energy transfer contributes to the overall process. Moreover, the influence of the addition of 0.01 M NaOH on CTAB micelles was investigated by the changes in both the critical micelle concentration (CMC) and the micellar aggregation number. CMC values were obtained from the pyrene 1:3 ratio method, and micellar
aggregation numbers were determined by using a procedure based on the energy transfer process. [98]

S. A. Stevenson and G.J. Blanchard have studied micelles comprised of cationic (CTAB) and anionic (SDS) surfactants through the interactions of solution phase anionic disodium fluorescein (DSF) and cationic rhodamine 110 (R110) dyes with perylene sequestered within the micelles. The efficiency of this process is mediated by the extent to which the ionic dyes interact with the micelle palisade layer, and their fluorescence lifetime data allow us to determine the association constants for acceptor–micelle interactions [99].

J. Hadjianestis and J. Nikokavouras studied the spectral parameters and quantum yield of luminal,3-aminophalate and fluorescein in cetyltrimethyl ammonium chloride at various concentration, together with the solubilization sites in the micelles. Chemiluminescence energy transfer to fluorescein results in at least a tenfold increase in the intensity in the micellar system compared with that in the absence of fluorescein. [100].

Kevin Toerne and Ray von Wandruszka shows that the Förster energy transfer from excited surfactant species was used to monitor their association with micellized perylene at different temperatures. Fluorescence from the perylene acceptor was obtained through sensitization by surfactant monomers and aggregates, the latter being identified as spectroscopic rather than physical. Surfactant monomer fluorescence was subject to a strong inner filter effect inside the micelles, indicating an abundance of spectroscopically monomeric species there. Temperature increases caused reductions in native surfactant fluorescence through thermal quenching, but sensitized fluorescence intensities generally increased with temperature. This was ascribed to a temperature-induced approach between the perylene acceptor and both monomeric and aggregated surfactant donors. Clouding of the solutions showed little evidence of organizational changes at the micellar level [101].

M Darbyshir et al report a new type of remote optical sensor of metal ions in solution based on fluorescence lifetime quenching due to dipole-dipole energy transfer across a polymer-water interface. Ion selectivity is provided by
spectral overlap between the sensor fluorescence and metal ion absorption. The sensor is demonstrated in the detection of cobalt ions in water [102].

**T. Salthammer** et al reported the fluorescence quenching of perylene by CoCl₂·6H₂O in small unilamellar DPPC vesicles via energy transfer. At the probe-to-lipid ratio of 1:200 and quencher to lipid ratios of ≥ 12.5:1, donor-donor energy transfer between clustered perylene molecules was observed as well as energy transfer from the perylene molecules to cobalt ions both above and below the main phase transition temperature of the lipid. The fluorescence quenching of perylene by CoCl₂·6H₂O in the lipid gel state is shown to be well described by Förster long-range energy transfer when both donor-donor and donor-acceptor energy transfer are considered. [103]

**O. S. Wolfbeis** et al described two new fluorescence resonance energy transfer (FRET) compatible labels, their covalent linkage to oligonucleotides, and their use as donor and acceptor, respectively, in FRET hybridization studies. The dyes belong to the cyanine dyes, and water solubility is imparted by a phosphonate which represents a new solubilizing group in DNA labels. They were linked to amino-modified synthetic oligonucleotides via oxysuccinimide (OSI) esters. [104]

**A. Periasamy** et al, have shown that the FRET is an ideal technique to estimate the distance between interacting protein molecules in live specimens using intensity-based microscopy. The spectral overlap of donor and acceptor—essential for FRET—also generates a contamination of the FRET signal [105]

**S A. Stevenson et al**, they are studied micelles comprised of cationic (CTAB) and anionic (SDS) surfactants through the interactions of solution phase anionic disodium fluorescein (DSF) and cationic rhodamine 110 (R110) dyes with perylene sequestered within the micelles. Fluorescence lifetime measurements monitor energy transfer between the nonpolar optical donor within the micelle and ionic probes in the surrounding solution. The efficiency of this process is mediated by the extent to which the ionic dyes interact with
the micelle palisade layer, and our fluorescence lifetime data allow us to determine the association constants for acceptor–micelle interactions [106].

Interactions between the cationic polymethine dyes 3,3'-diethyloxacarbocyanaine iodide (DiOC2), 3,3'-dioctadecyloxacarbocyanine iodide (DiOC18), and 1,1'-dioctadecyl-3,3',3',3'-tetramethylindocarbocyanine perchlorate (DiI) in the nano-scale volume of anionic sodium dodecylsulfate (SDS) micelles have been studied using optical spectroscopy techniques. The solubilization of pairs of dye molecules (DiOC18/DiI or DiOC2/DiI) within SDS micelles was monitored by Forster resonance energy transfer (FRET) between the dyes in each pair. The influence of the hydrophobicity of the dyes DiOC2 and DiOC18 on the efficiency of their binding to SDS micelles and, consequently, on the efficiency of FRET between DiOC18 and DiI or DiOC2 and DiI fluorophors has been analyzed. It has been shown that hydrophobic interactions in addition to electrostatic interactions are of key importance for the binding between the positively charged dyes and negatively charged surfactant micelles [107].

Ququan Wang et al. shows the fluorescence resonance energy transfer (FRET) model using two-photon excitable small organic molecule DMAHAS, \((trans-4-(N-2-hydroxyethyl-N-ethyl amino)-42-(dimethyl amino) stilbene)\) as energy donor has been constructed and tried in an assay for avidin. In the FRET model, biotin was conjugated to the FRET donor, and avidin was labeled with a dark quencher DABS-Cl (4-(Dimethylamino) azobenzene-42-sulfonyl chloride). Binding of DABS-Cl labeled avidin to biotinylated DMAHAS resulted in the quenching of fluorescence emission of the donor, based on which a competitive assay for free avidin was established.[108]

White-light emission is achieved from a single layer of diblock copolymer micelles containing green- and red-light-emitting dyes in the separate micellar cores and blue-light-emitting polymer around their periphery, in which fluorescence resonance energy transfer between fluorophores is inhibited due to micelle isolation, resulting in simultaneous emission of these three species [109].
Fluorescence resonance energy transfer (FRET) in a cascade scheme between three amphiphilic dyes 3,3′-dioctadecyloxacarbocyanine perchlorate (DiOC\(_{18}(3)\), donor), 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiIC\(_{18}(3)\), acceptor/donor) and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate (DiIC\(_{18}(5)\), acceptor) has been investigated at low dye concentration (10\(^{-5}\) mol/l) in water–micellar solutions due to a forced assembling of dyes in nanoscale volume. The experimental data have revealed that sodium dodecyl sulfate (SDS) micelles solubilize dye molecules such that their hydrophilic heads are in contact with water, while hydrophobic tails are embedded into the hydrocarbon core of the micelle. FRET efficiency has been found to depend on the concentration of dyes in micelles and the most effective when each SDS micelle. [110]

**Kumar Pal et. al.** studied structural and photophysical properties of aggregated CdS quantum dots (QDs) capped with 2-mercaptoethanol in aqueous medium. The hydrodynamic diameter of the nanostructures in aqueous solution was found to be ~160 nm with the dynamic light scattering (DLS) technique, which is in close agreement with atomic force microscopy (AFM) studies (diameter ~150 nm). However, the UVvis absorption spectroscopy, powder X-ray diffraction (XRD), and transmission electron microscopy (TEM) studies confirm the average particle size (QD) in the nanoaggregate. The steady-state and time-resolved photoluminescence studies on the QDs further confirm preservation of electronic band structure of the QDs in the nanoaggregate. To study the nature of the nanoaggregate we have used small fluorescent probes, which are widely used as biomolecular ligands (2, 6-p-toluidinonaphthalene sulfonate (TNS) and Oxazine 1), and found the pores of the aggregate to be hydrophobic in nature. The significantly large spectral overlap of the host quantum dots (donor) with that of the guest fluorescent probe Oxazine 1 (acceptor) allows us to carry out Förster resonance energy transfer (FRET) studies to estimate average donor-acceptor distance in the nanostructure, found to be ~ 25 Å. carry out Förster resonance energy transfer
(FRET) studies to estimate average donor-acceptor distance in the nanostructure, found to be ~25 Å.[111]

**Jesper Wengel** and co-workers have studied the detection of nucleic acid hybridization via fluorescence resonance energy transfer (FRET) using pyren-1-ylmethyl and perylen-3-ylmethyl N2′-functionalized 2′-amino-LNA nucleosides incorporated into oligonucleotides exhibited a clear distance dependence of the FRET efficiency, ranging from below 10% when the fluorophores were approximately 40 Å apart to approximately 90% when the fluorophores were in close proximity [112].

**Sarah A. Stevenson and G.J. Blanchard**, studied the micelles comprised of cationic (CTAB) and anionic (SDS) surfactants through the interactions of solution phase anionic disodium fluorescein (DSF) and cationic rhodamine 110 (R110) dyes with perylene sequestered within the micelles. Fluorescence lifetime measurements monitor energy transfer between the nonpolar optical donor within the micelle and ionic probes in the surrounding solution. The efficiency of this process is mediated by the extent to which the ionic dyes interact with the micelle palisade layer, and our fluorescence lifetime data allow us to determine the association constants for acceptor–micelle interactions.[113]

**J. C. Thomes**, co-workers, measured the relative quantum yield of fluorescence of proflavine bound to DNA as a function of the number of bound dyes per nucleotide and the ionic strength allow the determination of the binding constants and respective number of the two types of sites previously postulated. It is demonstrated that 2-3% of the base pairs form sites where the dye is strongly bound and fluoresces normally while in the other set of sites the binding constant is 3-4 times weaker and the fluorescence completely quenched. Comparison with complexes of Pro with double stranded [114].

**1.16. Present work:**

Fluorescence Resonance Energy transfers (FRET) is a nonradiative process where by an excited state donor (D) transfer energy to a ground state acceptor (A). FRET mainly occurs over distances comparable to most
biological macromolecules i.e. about 10 to 100 Å. The rate of energy transfer is highly dependent on the extent of spectral overlap between the relative orientation of the transition dipoles and distance between the donor and acceptor molecules. It is a powerful technique for studying conformational distribution and dynamics of biological molecules such as DNA, Protein etc, which play key role in maintaining human health. Many of the biomolecules and nutritional components of the diet need to be detected and analyzed. During scrutiny of literature we found that the earlier methods used for determination of biomolecule involved chromatographic separation followed by spectrophotometry and fluorimetry analysis. Fluorescent Polynuclear aromatic hydrocarbons such as perylene, anthracenes etc. are known as efficient donor of energy in organic solvents. These methods involve laborious, time consuming separations process prior to analysis. The work planned was to study the photophysical properties of some biomolecules which are of significant in human diet, Polynuclear aromatic hydrocarbons and dyes. The spectral data obtained would found useful in deciding the donor (fluorescent probe) and acceptor (biomolecules) pairs for FRET studies. One of the objectives was to develop a technique based on FRET for determination of biomolecules from nutrient or drugs without prior separation.

Many of the lotions and medicines such as vitamins. The effectiveness of medicines depends upon their intermolecular interaction between drug and biomolecules. The ground and excited state molecular interaction can be understood by absorption and fluorescence quenching spectroscopy involving energy transfer or charge transfer between drug and biomolecules. The attempts are made to study the interaction using suitable donor-acceptor pair of drug and biomolecule.

The quenching experiments were set systematically with a view to obtain the gradual quenching of donor fluorescence with successive additions of acceptor solution. The validity of stern-volmer equation for the observed biomolecular fluorescence quenching was tested and the quenching rate constants were determined to known efficiency of energy transfer process. The
selective excitation of donor and fluorescence quenching data helped to establish analytical relation between extent of quenching and acceptor concentration which is used further for determination of acceptor in medicinal tablets. The quenching studies were performed in micellar solution to provide close approaches between donor-acceptor pair and to enhance energy transfer process. The pharmokinetic studies performed in present work was given to information regarding critical energy transfer distance and distance of separation between centers of donor and acceptor molecules in the aqueous and micellar solution.
1.16. References:

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