Chapter 1

Introduction
Chapter 1

“The secret to creativity is knowing how to hide your sources.”

Albert Einstein

1.1 General Introduction

A molecular sensor or chemosensor is a molecule that interacts with an analyte to produce a detectable change. Molecular sensors usually combine molecular recognition unit with a reporter to show the presence of a guest analyte. The earlier examples of molecular sensors include crown ethers which show a high affinity for sodium ions in comparison to potassium etc. Some other receptors are shown to be sensitive to a molecular compound class but not to a specific class. One example is a grouped analysis of several tannic acids which accumulate in ageing. Some other similar receptors have been used to analyze tartrates in wine. Various ionic and neutral species have been found to show widespread use in medical diagnostics, catalysis, physiology and environmental chemistry.\textsuperscript{1,2} For example, Ag\textsuperscript{+} is useful in radiotherapy and photography technology. Cobalt is an essential element, is cardiotoxic and may cause lung cancer etc. Over the past couple of decades anion sensing has attracted considerable attention because of the role played by anions in biological, chemical and environmental processes.\textsuperscript{3–10} Biochemical reactions involve the passage and recognition of anions;\textsuperscript{11} most of enzyme substrates and co-factors are anionic in nature;\textsuperscript{12} fertilizers used in agricultural activities add nitrates, phosphates etc. cause eutrophication of water;\textsuperscript{13,14} rain water possesses sulfate and nitrate making it acidic; the malfunctioning of the processes of natural anion regulation causes cystic fibrosis and Alzheimer’s diseases\textsuperscript{15,16} and nuclear fuel processing produces radioactive pertechnetate which is also an alarming environmental concern.\textsuperscript{17}

Though the designing of anion-selective receptors research is booming, but it is still less developed than that of the reports for cations. This is because of the reason of some basic differences between the cations and the anions which include:

**Ionic size:** The size of anions is generally large than the cations and thus need some large binding sites. e.g, the ionic radius of 0.167 nm for Cl\textsuperscript{−} is much larger than 0.133 nm ionic radius of K\textsuperscript{+} which is almost same as that of the smallest F\textsuperscript{−} ion.
Shapes: Anions have different geometrical shapes e.g. linear (CN\(^-)\), spherical (Cl\(^-)\), tetrahedral (SO\(_4^{2-}\)), trigonal planar (NO\(_3^-\)) etc.

pH range: the range of pH for the existence of anions is much narrower than the that of cations as in phosphate. Also there are variable ionization states, e.g, carbonate and bicarbonate.

Hydration: Anions are usually more hydrated than the cations of comparable size.

The importance of the anion sensing is reflected from the scores of simple sensing probes which have been designed and developed in the last couple of decades.\(^{18-20}\) Only designing a selective probe for anion sensing is not desirable, but it should provide an instantaneous (high sensitivity) optical response on binding a very less amount of analyte (low detection limit) so as to be visually detectable. One such type of sensing probes is optical fluorescent probe. The fluorescent sensing technology has proved to be an indispensable tool being continuously used in biotechnology, molecular biology and biochemistry, clinical diagnostics, materials and environmental sciences.\(^{21,22}\) Unlike other methods like electrochemical detections, nuclear magnetic resonance (NMR), chemo-absorptive detections, radioactive tracing etc., features of fluorescent sensing make it highly distinguishable to be known as one of the unique transduction mechanisms for signaling the chemical recognition events especially inside living cells.\(^{23}\) For example, analytes don’t get consumed or destroyed during fluorescence phenomenon which make this non-invasive measurement ideal for bio-systems. Ultra-high selectivity, specificity and sensitivity of fluorescence sensing make possible the detection of even a single molecule/ion of analyte.\(^{24}\) The diversity in the emission wavelengths and the swift response time (10\(^{-8}\) to 10\(^{-9}\) s) make fluorescence sensing to provide the simultaneous real-time detection of various analytes.\(^{22}\) Furthermore, fluorescence sensing need no physical guide or reference which make the process economical as far as technical, instrumental and measurement costs are concerned. Hence, the fluorescence sensing technology has proved to be one of the important methods for future screenings and detections.\(^{25}\)

Fluorescent probes are treated as a crucial component in the technology of fluorescent sensing due to their ability of converting chemical events (conformational changes, binding, reactions etc.) to fluorescence signals which are visually detectable and have direct correlation with sensitivity, response range, specificity and other aspects of fluorescent sensing. Though several natural products possessing intrinsic fluorescence are used widely as fluorescent probes but they are limited as far as their
number is concerned. Therefore, synthetic fluorescent probe development has become very indispensable as it provides a diversity of fluorescent probes and widen the fluorescent technology areas.

A typical optical fluorescent sensing probe is made of three essential parts: first the selectively anion binding receptor, second the indicator which transform this binding event into an optical signal and third the spacer also called as linker. This conception is demonstrated in Figure 1.1.

![Illustration of a typical fluorescent probe](image)

Figure 1.1: An illustration of a typical fluorescent probe

1.1.1 The Receptor Moiety

The analyte recognition site is called receptor which is also known as chelator, ligand or binding site. Anion receptor designing has been found to be more challenging than cation ones due to several reasons. Firstly, anions possess lower charge density due to their larger size than their isoelectronic cation counterparts. This indicates anions will show weaker electrostatic interactions. Secondly, due to wide range of anionic geometries (for example, the spherical halide ions, the trigonal planar nitrate, and the tetrahedral phosphate etc.), the receptor has to be complementary to the target anion for high selectivity. Thirdly, pH sensitive anions demand the receptors to be functional within the whole pH window of anions. Furthermore, the binding of anions to receptors can be largely affected by solvent environment, for example protic solvent molecules can competitively bind the receptors.

The selectivity of a receptor for an anion depends on many factors. For example, the geometry and basicity of target anion, solvent effects, preorganization and complementarity of the receptor are important factors to be taken care of in the design of a receptor. The process of anion binding may involve several types of non-covalent interactions: electrostatic interactions between an anionic guest and the cationic receptor; hydrogen bonding interactions, which are directional in nature and
permit the design of receptors having specific shapes; metal or Lewis-acid coordination, in which an electron deficient Lewis acidic centre and an anionic guest interact; anion-π type of interactions capable of enhancing binding affinity.28–31

Several anion receptors having various functional groups (either charged or neutral) have been reported.32–34 The non-metallic charged anion receptors possess cationic centers like ammonium, imidazolium and guanidinium, which can exploit both electrostatic interactions and hydrogen bonding for anion complexation. On the contrary, most neutral systems with functional groups like pyrrole, amide, thioamide, urea and thiourea make use of only the hydrogen bonding interactions for anion binding.3,6,12,32 As a consequence, binding ability of neutral receptors is weaker. However, the disadvantages related to charged receptors are: competitive counter-ion binding and pH dependency on its environment. There is another class of anion receptors which is based on metals35–37 or Lewis acid centers with atoms like boron and mercury.38,39

1.1.2 The Sensing Moiety

In order to translate a binding event between the analyte and the receptor into an optical signal, either a fluorophore or a chromophore must be linked with a receptor to give a fluorescence emission or visible color change respectively.6,40,41 The most simple way to incorporate the colorimetric or fluorescent indicator is by covalently linking it to the receptor. This method is depicted in Figure 1.1. Upon anion guest binding with the receptor, a change induced in the receptor gets transformed to an optical signal in the indicator either by fluorescence “switched on or off” in case of fluorophore or a shift in the UV absorption wavelength in case of chromophore.

Besides, covalently linkage method, an indicator can be incorporated non-covalently to a receptor, known as indicator-displacement assay (IDA).42,43 Here, the competitive binding between anion guest and the indicator can be either fluorogenic or chromogenic. The added guest after displacing the indicator from the receptor induces a change in indicator resulting in the production of an optical signal. Figure 1.2 depicts this mechanism. 4-nitrophenolate,44 Brooker’s merocyanine,45 methyl red and resorufin46 are some of the examples of chromogenic indicators which are used in IDA. The use of different indicators with the same receptor and the adaptability to different receptors are the advantages of this mechanism.

In addition to covalent linkage between the receptor and indictor or displacem-
ent of indicators, the other technique of selective reactivity between the receptor and anion can be used for the design of an anion sensor. The reaction between these anion selective chemodosimeters and the target anion(s) form new molecule having different color or fluorescent properties from the initial reactants.47,48

![Diagram of indicator-displacement assay]

Figure 1.2: Illustration of indicator-displacement assay

1.1.3 The Spacer Moiety

The fluorophore or chromophore is linked to receptor through a spacer (also known as linker). The spacer may have variable length which depends on the mechanism involved in fluorescence modulation. For certain cases, these spacers are as short as to single bond.

The versatility of the sensing mechanism in fluorescent probes can be understood by the wide possibilities in monitoring the fluorescent output signals through absorption and emission spectra, fluorescent life times, fluorescence intensities, quantum yields and also in the form of anisotropies.49 Besides the developments in the advanced instrumentations which include fluorescence microscopes of several types, the development and design of unique fluorescent probes having high specificities, sensitivities, capability of spatial sampling etc. has become equally and particularly important. This research area is attracting the attention of scientific community and continues to be one of the exciting and multidisciplinary topics. However, still the current fluorescent probes are unable to detect numerous molecules, ions etc. especially in living cells because of too low concentrations of these molecules or they are hard to recognize. Hence, the development of novel artificial fluorescent probes are highly required.50
1.2 Criteria for designing fluorescent probes

The dependency of fluorescence sensing on the fluorescent probes makes the design of novel and appropriate probes having desired properties particularly important. In this direction, the criteria which need to be fulfilled is discussed below:

1) **Selective Response**: The selective and specific response to the analyte nullifying the effect of interferents is the first and foremost condition for the design of a fluorescent probe. For example, designing a chelation pocket or incorporating an electron deficient atom in receptor moiety shall allow the specific metal cation to bind over other cations or a particular anion over other anions respectively.

2) **Fluorescence brightness**: The high brightness of fluorescence is highly preferred which means the high quantum yield and large extinction coefficient are important and desired properties of the fluorescent probe in general and fluorophore in particular.

3) **Fluorescent probe robustness**: The probe must be stable against chemicals and light. There are many fluorescent probes falling in various categories which suffer from photobleaching effect i.e. an irreversible destruction of the fluorophore with high-intensity illumination, thus limiting the fluorescence detectability of these probes. Hence the properties of chemostability and photostability are highly desired to be shown by a fluorescent probe.

4) **Switch-on fluorescence**: In a fluorescence signal, the turn-on response (enhancement of fluorescence) is favored over turn-off signal. It is because the signal to noise ratio (SNR or S/N) is significantly elevated in case of switch-on condition which makes the visualization of fluorescence easier.

It is easier to damage the living organisms than the regular analytical specimens due to their complicated nature. So besides the above basic criteria, there are some more requirements which are to be considered for the fluorescent probes being applied in the living tissues or cells.

I. **Membrane permeability and water solubility**: The water present inside the plasma and cytosol of tissues and cells makes the water solubility of fluorescent probes highly desired as far as their applications in biological systems are concerned particularly for *in vivo* measurements. If the probe is
highly hydrophobic or lipophilic, it will lead to the easy aggregation and accumulation inside the membrane of the cells. This accumulation leads to the underperformance of the probe in terms of target localization, sensitivity and quantum yield. However, the adequate lipophilicity is necessary for the easy permeability of fluorescent probes through the membrane to enter into the cells or cellular organelles as particular targets.\textsuperscript{51} Hence the lipophilic-hydrophilic balance of a fluorescent probe is a critical factor to regulate its performance.\textsuperscript{52}

II. \textbf{Lower energy absorption and emission:} In case of biological applications the long-wavelength absorption/emission by a fluorescent probe is desired in comparison to the fluorescence in shorter wavelength ultraviolet (UV, $< 100$ nm) or short wavelength visible region ($< 500$ nm). This is because of the less photodamage caused to cells and tissues by the low energy fluorescence and lesser interference produced by auto-fluorescence and absorptions of biomolecules. In particular, the near infrared region (abbreviated as NIR, 650 nm - 900 nm) of the electromagnetic spectrum is treated as a golden range for fluorescent probes with bio-applicability. This is due to the reason that besides reducing the photodamage, near infrared wavelengths minimize the light scattering (Raman and Rayleigh) and absorbing interferences. Furthermore, NIR penetrates deep (up to 1-2 cm) in biological specimens facilitating \textit{in vivo} imaging for the molecular processes.\textsuperscript{53–55} Hence this NIR region is called “biological window”.\textsuperscript{56,57}

III. \textbf{Ratiometric responses in fluorescence:} The simultaneous change of fluorescence at two different wavelengths is called ratiometric response. The advantage of this two-channel fluorescence response is that it provides high SNR. Ratiometric response is helpful in intracellular measurements because of the elimination of fluorescence change due to the effects not related to the analytes under consideration like loading of dye unequally, varying thickness of cell, dye leakage, instrumental efficiency, photobleaching etc.

So far, no fluorescent probe is reported which fulfills all the criteria perfectly but to satisfy partially for a particular use will be helpful.
1.3 Fluorescence origin

The origin of term luminescence has been found to be from a Latin root (lumen = light). The concept was first presented by a science historian and a German physicist Eilhard Wiedemann in 1888 as luminescenz, who related it for all the light phenomena not affected by any change in temperature i.e. incandescence. Presently luminescence is being defined as “a spontaneous emission of radiation from an electronically excited species (or from a vibrationally excited species) not in thermal equilibrium with its environment”.\textsuperscript{58}

Luminescence has been classified in various types on the basis of excitation mode. Particularly, photoluminescence is a light emission which arises “from direct photoexcitation of the emitting species”.\textsuperscript{58} The well known forms of photoluminescence are- fluorescence, phosphorescence and delayed fluorescence. The other kinds of luminescence differ in the excitation mode and these include- chemiluminescence, thermoluminescence, radio luminescence, cathodoluminescence, bioluminescence, electroluminescence, triboluminescence, sonoluminescence.

The first report of fluorescence phenomenon dates back to 1845, when Sir John Frederick William Herschel put forward an observation after preparing an acidic solution of quinine sulfate and then stated,\textsuperscript{59}

\begin{quote}
“Though perfectly transparent and colorless
when held between the eye and the light, it yet
exhibits in certain aspects, and under certain
incidences of the light, an extremely vivid and
beautiful celestial blue color.”
\end{quote}

As the color he saw was superficial, thus he believed this to be hitherto unidentified phenomenon, “a case of superficial color presented by a homogeneous liquid, internally colorless.”\textsuperscript{60} He called the whole phenomenon as epiploic dispersion, originated from the Greek: επιπολή = surface.\textsuperscript{60}

In 1852, Sir George Gabriel Stokes in his detailed experimental studies identified a very common phenomenon which he called dispersive reflection: here the wavelength of the original light is always lesser than the wavelength of dispersed light.\textsuperscript{61} In his another experiment he formed a solar spectrum by the help of a prism. On moving a filled test tube containing quinine solution through visible portion of the spectrum, the solution retained its transparency. But the solution shined with a blue
color beyond the violet part i.e. invisible zone of ultraviolet region of the spectrum (Figure 1.3). Stokes wrote,61

“It was certainly a curious sight to see the tube instantaneously light up when plunged into the invisible rays: it was literally darkness visible. Altogether the phenomenon had something of an unearthly appearance.”

Figure 1.3: Blue emission of a quinine solution after ultraviolet irradiation.62,63

This observed phenomenon was called as dispersive reflection or true internal dispersion by Stokes and he then wrote,

“I confess I do not like this term. I am almost inclined to coin a word, and call the appearance fluorescence, from fluorspar, as the analogous term opalescence is derived from the name of a mineral.”

Then Stokes resolved to the use of word fluorescence in his second paper in 1853.64 After that people are continuously using modern physics and innovations in spectrofluorometer to reveal, comprehend and finally develop fluorescence and fluorescence technology.65

In earlier days after G. G. Stokes coined the word fluorescence, the fluorescence and phosphorescence were differentiated based on the duration of emission once the excitation process ends: fluorescence was treated as simultaneous disappearance of light emission and end of the excitation, whereas phosphorescence was thought as persisting light emission once the excitation ends.61 Though this
conception was followed for some time but the criterion was found insufficient as the short-lived phosphorescences (e.g., zinc sulfide’s violet luminescence) and long-lived fluorescences (e.g., divalent salts of europium) were discovered which have comparable durations (several hundred nanoseconds). So Francis Perrin in 1929 stated that the condition of phosphorescence is passage of excited species through a different intermediate state before undergoing emission. To be more precise, fluorescence involves the retention of spin multiplicity whereas in phosphorescence there is change of spin multiplicity, from singlet to triplet or vice-versa. Those molecules which possess the property of fluorescence are known as fluorophores and the most common example of fluorophores is aromatic compounds. Under the influence of radiations of light, the π-electrons in the lower energy orbitals (ground state of molecule) get excited to higher energy orbitals (excited state of molecule). The electrons excited to orbitals with higher energy (excited state) are unstable which rapidly return to their lower energy orbitals (ground state) by releasing energy in the form of emission photons.

1.4 Fluorescence overview

The standard way of illustrating the processes of absorption and emission in fluorescence is through the Jablonski diagram. The diagram got its name after Prof. Alexander Jablonski, who is treated as the father of fluorescence spectroscopy due to his contributions towards this field of science. A typical Jablonski diagram is displayed in Figure 1.4. The S₀, S₁, S₂ represent the singlet ground, singlet first excited and singlet second excited electronic states respectively. The fluorophores may exist in many vibrational energy levels at these electronic states. On getting irradiated with light, the fluorophore gets excited to the different vibrational levels of S₁, S₂ or higher singlet excited states (Sₙ) depending on the energy of irradiation (10⁻¹⁵ sec). Once excited, the fluorophore gets relaxed to its lower vibrational level of first singlet excited (S₁) via internal conversion (IC) and vibrational relaxation which take place within 10⁻¹⁴ to 10⁻¹¹ sec. This transition time being several orders of magnitude smaller in comparison to the magnitude of fluorescence emission rate (10⁻⁹ to 10⁻⁷ sec) gets completed earlier to the fluorescence and will not provide any radiative emission i.e. it is a non-radiative decay process. The fluorophore releases the energy as photons by relaxing to the different vibrational levels of S₀ from the lowest energy
vibrational state of $S_1$, a thermally equilibrated excited state. This radiative decay process is called as fluorescence. From the lowest energy vibrational state of $S_1$, the fluorophore may undergo an electron spin inversion through the process of intersystem crossing (ISC) to get the spin changed to its triplet excited state ($T_1$). The fluorophore in the $T_1$ state may either undergo a transition to $S_0$ state or goes back to $S_1$ by thermal activation. The former transition is called phosphorescence and the second transition results in fluorescence called as delayed fluorescence. Phosphorescence is a longer wavelength emission in comparison to fluorescence because of the lower energy of $T_1$ than $S_1$. As per the principles of quantum mechanics, the $T_1$ to $S_0$ transition is forbidden due to the similar spin orientation of electrons in $T_1$ and $S_0$. Hence, because of this reason the phosphorescence emission rate ($10^{-3}$ to $10^{-2}$ sec) is smaller than the fluorescence rate by several orders of magnitude.

It is reflected by the Jablonski diagram that energy of absorption is almost always greater than energy of fluorescence emission. This red shift in emission wavelength in comparison to the absorption wavelength was discovered by Sir G. G. Stokes in 1852 and is thus named as “Stoke’s shift”. A typical electronic spectrum comprises of both absorption and emission curves in a single plot. Usually the emission spectra are broader than the absorption spectra of $S_0$–$S_1$ transitions and are their mirror images. The intensity and shape of fluorescence emission spectra are governed by the structure of fluorophore, solvent medium etc.

The fluorescent molecules are characterized by parameters which include: maximum wavelength of absorption ($\lambda_{\text{abs}}$), maximum wavelength of emission ($\lambda_{\text{emi}}$), fluorescent quantum yield ($\Phi_f$), molar extinction coefficient ($\varepsilon$) and fluorescent lifetime ($\tau$). The maximum absorption and emission wavelengths denote the respective wavelengths related to the highest peaks of the spectra for absorption and emission. Molar extinction coefficient ($\varepsilon$), a constant in Lambert-Beer’s law denotes the ability for absorption of light of fluorescent molecules at a particular wavelength. Fluorescence quantum yield refers to the ratio of photons absorbed by fluorophore and the emitted photons via fluorescence. Fluorescence quantum yield in other terms provide the probability of excited state deactivation via fluorescence emission than the non-radiative transitions. The quantum yield in higher range is always desired for designing the fluorescent probes and some other technologies involving fluorescence. Fluorescence lifetime ($\tau$) is the average time which a fluorophore spends in excited
state before it relaxes back to ground state via fluorescence emission. The average fluorescence lifetime is of several nanoseconds.

Figure 1.4: Jablonski energy diagram

1.5 Common fluorophores for sensing

Most of the fluorescent molecules possess unique properties of absorption and emission which make them best for the labelling applications as well as for environmental and biological sensing. The sensing and labeling technique based on fluorescence is more informative, non-invasive, sensitive in comparison to radioactive labeling, absorbance based dye labeling and electrochemical sensing which make it feasible. Though the presence of naturally occurring biomolecules like NADH (reduced form of Nicotinamide adenine dinucleotide), green fluorescent proteins (GFP), tryptophan etc. show intrinsic fluorescence but it is the synthetically prepared fluorophores which extend the diversity of fluorescent sensing probes. Currently, there are thousands of fluorophores developed and most of them usually show their absorbance and emission in visible to near-infrared region. Among this diverse list of fluorophores, the π-conjugated materials based on dyes that fluoresce at various
wavelengths are of great interest in fluorescent sensing.\textsuperscript{67–75} It is because of the reason that these π-conjugated molecules can have both acceptor and donor groups on an aromatic ring. This will lead to charge transfer found responsible for emission behavior which is environment sensitive.\textsuperscript{76–78} For such systems, the fluorescence quantum yield is showing a decrease with the increasing polarity and large bathochromic shifts in the emission spectra.\textsuperscript{79} This unique behavior of π-conjugated molecules is utilized for detection of various molecules/ions fluorometrically.\textsuperscript{80} Out of the π-conjugated type of fluorophores, the most well-known and most studied are the fluorophore families viz rhodamines, fluoresceins, cyanines, boron-dipyrromethenes (BODIPYs), aza-boron-dipyrromethenes (aza-BODIPYs) and triarylboranes (TABs) (see Figure 1.5)

Figure 1.5: Commonly used families of fluorophores
1.5.1 Rhodamine
Rhodamine is a type of dye in xanthene class. Most of the rhodamine dyes possess strong emissions and absorptions around 500 nm and have higher quantum yields. The optical properties of Rhodamine dyes are least sensitive to pH and their photostability is very high.\textsuperscript{81} Rhodamine 6G, Rhodamine 101, Rhodamine B etc. are the examples of rhodamine dyes which have been widely used for fluorescent lifetime measurements, labeling agents and environmental examinations, fluorescence standards etc. The fluorescence modulations are possible in rhodamine due to the unique spirolactam ring open-close mechanism. The spiro-cyclization due to the amidation of rhodamine forms a five-membered spirolactam ring which shows no fluorescence because of the disrupted conjugation. The change in pH or adding metal ions or other analytes lead to rebuilding the conjugation by opening up the spirolactam recovering the fluorescence. Following this strategy, number of rhodamine based functionalized derivatives have been developed which possess variable optical and sensing properties and are used for ion-sensing, subcellular imaging, bio-labelling applications.\textsuperscript{82–85} Though much success has been achieved in the area of sensing but the rhodamine based fluorophores still show lower water solubility, less chances of modifications on its core and emission wavelengths in the shorter zone which limit their applications.

1.5.2 Fluorescein
Fluorescein is also a type of xanthene class of dyes. The maximum absorption and emission wavelengths in water for fluorescein are found at 490 nm and 512 nm respectively. The fluorescein possesses higher extinction coefficient, higher quantum yield, and good aqueous solubility which make it among the most used fluorophore for clinical diagnostic usage as in ophthalmology, optometry and bio-labeling applications.\textsuperscript{86} However, the disadvantages of fluorescein like its high rate of photo-bleaching, dependency of its emission and absorption spectra on pH and its relatively short emission wavelengths limit its widespread applications.

1.5.3 Cyanine
Cyanine, a family of synthetic dyes, belongs to polymethine group. A typical cyanine is composed of a polymethine chain with double bonds in conjugation connecting two heterocyclic rings. Cyanine dye is among the long wavelength absorbing and emitting
fluorophore. The wavelength of its absorption and emission (usually ranging from 600 nm to 900 nm) is decided by the double bonds present. As the longer emission and absorption wavelengths of cyanine dyes fall in the ideal ‘biological window’ (700-900 nm), which make them to be suitable as fluorescent probes both in industry as well as in biological area. The concentration dependency in aqueous solution and strong aggregation effect reduce the aqueous solubility, brightness of fluorescence and sensitivity of cyanine dyes significantly which hamper their use in biological sensing. Furthermore, the other demerits of cyanine dyes include poor photostability, less number of methods of functionalization and rotation/photoisomerization because of non-rigid structures which ultimately lead to their non-radiative decays.87–89

1.5.4 Borondipyrromethene (BODIPY)

1.5.4.1 Fundamentals and Merits of BODIPY dyes

The popular and concise name of ‘BODIPY’ comes from the abbreviation of borondipyrromethene. As per International Union of Pure and Applied Chemistry (IUPAC) nomenclature, BODIPY is named as 4,4-Difluoro-4-bora-3a,4a-diaza-s-indacene. The composition of a basic BODIPY core is dipyrromethene which is attached to a disubstituted boron atom, usually a BF₂ unit. The dipyrromethene ligand forms a N-B-N bridge due to the complexation of BF₂ providing a fixed planarity to π-electron system through which the positive charge gets delocalized via resonance between two nitrogen atoms (Figure 1.6). The X-ray structure of BODIPY shows that the π-electrons delocalize within the twelve-membered framework, a fused planar system.90 The fluorine atoms attached are not coplanar with the framework, instead they are perpendicular to it.90,91 Though the BODIPY dyes are not obeying the Huckel’s rule of aromaticity but their properties are like that of the aromatic π-systems. This is because the coordination of boron atom introduces rigidity in the overall structure. According to the IUPAC numbering system, there are eight functional positions in a BODIPY core. In the conventional numbering, α-, β- and meso- positions are also used.

As compared to fluorophores like rhodamine and fluorescein, BODIPY dyes have been the focus of fluorescent sensing research because these dyes possess some remarkable spectroscopic and chemical properties which are very much ideal for designing the next generation molecular fluorescent probes. For example, Borondipyrromethenes (BODIPYs) display excellent photophysical properties,92,93
like long absorption and fluorescence wavelengths in visible-NIR region, narrow absorption and emission bands with high extinction coefficients (usually > 8000 M$^{-1}$ cm$^{-1}$) and fluorescence quantum yields (leading to high brightness).$^{58}$ Longer singlet state lifetimes and enhanced photostability.$^{94,95}$ Furthermore, BODIPY dyes show chemical and physicochemical robustness with moderate redox potential, negligible sensitivity to solvents, pH and temperatures.$^{96}$ The absorption and emission bands of a typical BODIPY dye ranges from 480-550 nm having high quantum yields and extinction coefficients in organic solvents ($\varepsilon > 8000$ M$^{-1}$ cm$^{-1}$; $\Phi_f > 0.6$). The emission and absorption spectra of an unsubstituted boron dipyrromethene dye shows an absorption band at 500 nm which is ascribed to $S_0 \rightarrow S_1$ ($\pi-\pi^*$) transition while as the shoulder peak at 480 nm is assigned to 0-1 vibrational transition.$^{90,91}$

![Figure 1.6: Structure of BODIPY core and its numbering](image)

The basic BODIPY core does not show NIR fluorescence or long wavelength emission but the absorption and fluorescence bands of the BODIPY dye can be shifted at will towards red, deep red and even NIR regions by incorporating the appropriate substituents as these dyes are highly amenable to structural modifications at all the eight positions around the core where functionalization is possible.$^{97,98}$ The level of possibility of the structural modifications in BODIPY core can be thought by the fact though the synthesis of BODIPY dyes started late back in 1968 by Treibs and Kreuzer but the first report of synthesis of unsubstituted BODIPY core came in 2009.$^{90,99}$ The reason for this delay in BODIPY core synthesis is the difficulty which resulted from the possible reactions at the unblocked sites at pyrrole rings which undergo electrophilic attack.$^{60,90,100}$ Different types of reactions possible include electrophilic substitutions at 2,6-positions, condensation reactions at 3,5- or 2,6-positions, nucleophilic substitutions at 3,5- or 4,8-positions, cross coupling reactions
like Sonogashira and Suzuki at all the positions except 4-position catalyzed by palladium etc.\textsuperscript{91} All the above synthetic pathways increase diversity of possible BODIPY derivatives with different functionalities.

The emission and absorption bands red-shift by about 10-50 nm in the substituted BODIPY dyes which possess the electron donating groups like amino and alkyl, heavy atoms like iodine and bromine. Moreover, substitution at the electrophilic positions in the pyrrole rings of a BODIPY core with the conjugated aromatic rings, double and triple bonds leads to the red-shift in the emission and absorption wavelengths by 50-300 nm depending on the degree of $\pi$-extension. These excellent properties of actual BODIPY core or the derivatized BODIPY dyes have inspired researchers across the globe to develop and design BODIPY-based fluorescent probes to be used for photodynamic therapy and photosynthetic model systems with the property of harvesting light energy in general and for biological and environmental imaging and sensing applications in particular.\textsuperscript{101,102} Furthermore, BODIPYs being the excellent fluorophores have widely been used for sensing the metal cations but a limited number of reports are there for BODIPY based anion sensors.\textsuperscript{93,103}

### 1.5.4.2 Demerits and challenges of current BODIPY-based fluorescent probes

The application of BODIPY dyes in fluorescence anion sensing is still facing challenges especially in the fields of biosensing and imaging due to some undesirable features of BODIPY dyes. For example, in biological conditions, the efficient sensing of various analytes like transition metal ions, toxic species, intracellular pH etc. with higher sensitivities (i.e. low detection limits) by fluorescent probes is still lacking though some good BODIPY-based fluorescent probes have been reported. This signals that there is need of improvement as far as the specificities and sensitivities of BODIPY-based fluorescent probes for the above analytes is concerned. Furthermore, the hydrophobic nature of BODIPY core and its derivatives make the application of these fluorescent probes too difficult in aqueous conditions like biological systems. Moreover, BODIPY-based fluorescent probes usually absorb and emit short wavelengths leading to significant photodamage to cells and tissues. Till now, there is a report of a very few NIR emissive BODIPY dyes. All these issues and challenges limit the \textit{in vivo} and \textit{in vitro} labelling and biosensing applications of BODIPY-based fluorescent probes.
1.5.5 Aza-borondipyromethene (Aza-BODIPYs)

Aza-BODIPYs are the derivatives of BODIPY formed by replacing the meso-carbon by a nitrogen atom of imine type (Figure 1.7). The emission and absorption spectra of aza-BODIPYs are red-shifted relative to BODIPYs and their other excellent unmodified properties include higher molar extinction coefficients, higher fluorescence quantum yield, narrow spectral bands and good stability.\(^{104}\) The absorption and emission wavelengths of a conventional 1,3,5,7-tetraaryl aza-BODIPY are at 650 nm and 673 nm respectively which may be shifted towards red region by introducing the various groups.\(^{105}\) For example, addition of electron donating groups at the 3,5-phenyl ring\(^{106,107}\) and replacing the phenyl rings by the thiophene rings\(^{108,109}\) lead to the bathochromic shift for both absorption and emission upto 30-80 nm and 20-70 nm respectively. Similarly, the alkylnyl group(s) attachments at the α-phenyl substituents or the substitution at β-sites further red shift the emission and absorption wavelengths towards NIR region.\(^{109}\) In recent years the significance of NIR fluorescent dyes has been recognized widely in the biological applications.\(^{107,108,110–112}\) It is because of the reason that as compared to the visible light, NIR light penetrates deeper and noninvasively which is a favorable factor in intracellular fluorescent labelling,\(^{110,113–115}\) in vivo and in vitro chemosensing of ions,\(^{116–120}\) pH indicators,\(^{121}\) photodynamic therapy,\(^{102,107,122,123}\) etc.

Figure 1.7: Structure of aza-BODIPY core and its numbering

1.5.6 Triarylboranes

The tricoordinate boron species being electron deficient has an intrinsic propensity to reach its desired octet configuration. This is reflected through its i) pronounced Lewis acidity ii) trigonal planar geometry and iii) the resulting orbital interaction via the π-π\(^*\) conjugation between the vacant p orbital on boron and the π\(^*\) orbital of adjacent π-conjugated framework. Hence to overcome the challenge of sensitivity of
tricoordinate boron species against nucleophiles, moisture and oxygen is a key issue in the construction of new trivalent organoboranes.\textsuperscript{124} In accord with these characteristics, triarylboranes (TABs) and derivatives possess some intriguing electronic and photophysical properties due to which a variety of boron-based functional materials have been realized till date\textsuperscript{125,126} in a wide range of applications like anion sensing,\textsuperscript{127} two-photon absorption,\textsuperscript{128} photosensitization\textsuperscript{129} etc. For showing these applications, maintaining the Lewis acidity (electron accepting nature) of the boryl group is necessary. This can be achieved by avoiding the formation of Lewis acid-Lewis base complexes of tetracoordinate organoborate. The common and simple approach for stabilizing the reactive electron-deficient center of boron is attaching the sterically-protected groups like mesityl (2,4,6-trimethylphenyl),\textsuperscript{130} duryl (2,3,5,6-tetramethylphenyl),\textsuperscript{127,129} 2,4,6-tri-tert-butylphenyl\textsuperscript{131} etc. Though these protecting groups primarily shield the sensitive boron center from undergoing the undesired reactions but they must assure the desirable enhancement in the electronic communication among the vacant p-orbital on boron and the $\pi^*$ orbital of adjacent $\pi$-conjugated systems. One such example is Yamaguchi \textit{et al.} who presented a novel type of extended tri-9-anthrylboranes.\textsuperscript{132} This electron deficient dimesitylboryl-substituted compound showed stability towards air and moisture due to the steric crowding of mesityl and anthryl moieties. Though the dihedral angles between borane plane and anthracene planes were found to be 53° yet the presence of certain degree of electronic communication between the central boron atom and the substituents via $\pi$-conjugation was revealed by the electrochemical and spectroscopic data.

1.6 Signal mechanisms

Though the synthesis of fluorescent chemosensors and predicting the mechanism behind sensing has remained a focus for most of the studies, but detailed mechanisms of sensing are still lacking. Nevertheless, understanding the mechanism of sensing is vital for fluorescent sensors and a detailed study of their mechanism is significant not just to comprehend the working mechanism but for their design and application in environmental protection and human health. To design a new fluorescent chemosensor, it is of utmost importance to explore the sensing mechanisms involved in the interaction of recognition (receptor) and signal (sensing) reporting units and is gaining attention. Up to now, a number of signaling mechanisms developed on the basis of some photophysical processes are extensively used for the recognition and
detection of different species. Herein the simple classification of all the mechanisms is outlined as; (i) Electron transfer (ET): out of all the developed signaling mechanisms, this mechanism primarily via photoinduced electron transfer (PET) is being most widely utilized mechanism involved in fluorescent chemosensors. Because of the PET process, the fluorescence gets quenched in a fluorophore which may be recovered by inhibiting this process involving guest molecules like anions, cations etc.\textsuperscript{6,133–138} (ii) Charge transfer (CT): the processes included in CT are- intramolecular charge transfer (ICT), twisted intramolecular charge transfer (TICT) and metal to ligand charge transfer (MLCT). In ICT based chemosensors, there is a blue or red shift in their emission spectra either due to suppression or enhancement of ICT process which results in a ratiometric signal.\textsuperscript{6,134–138} In case of MLCT, the CT occurs from a ligand to metal usually transition metal cation. The MLCT is mostly observed in transition metal complexes like rhenium, ruthenium, iridium etc.\textsuperscript{6,134,139} TICT, a strong intramolecular CT occurs in excited states which involves relaxation of solvent around the molecule leading to the electron donor and electron acceptor continuing rotation till it gets twisted by about 90°.\textsuperscript{140,141} Due to the necessity of polar solvent relaxation for the occurrence of charge separation and intramolecular rotation in TICT state, the fluorescence behavior shows sensitivity towards steric hindrance and/or micropolarity for the molecular rotation.\textsuperscript{134,142,143} (iii) Energy transfer (ET): Electronic energy transfer (EET) and fluorescence resonance energy transfer (FRET) are two classes of ET on the basis of energy donor and acceptor interaction distance. The ET based chemosensors show the distance dependency. For example, for EET (also called as Dexter electron transfer) to be an efficient signaling mechanism, the donor and acceptor distance should be within 10 Å.\textsuperscript{6,134,135} While as FRET requires a significant spectral overlap between the absorption spectrum of acceptor and the emission spectrum of the donor. To be an efficient signaling mechanism, the distance between the acceptor and donor should be from 10-100 Å in FRET.\textsuperscript{144,145} (iv) Excimer/exciplex: the complex formed by the interaction of a fluorophore in the ground state and the fluorophore of the same structure in the excited state is called an excimer. But if a fluorophore in the ground state is different than that of fluorophore in excited state, the complex formed in such condition is called as exciplex. The emission spectrum of excimer/exciplex shows a red-shift in comparison to monomer. Also a simultaneous dual emission from excimer/exciplex and monomer is observed. Hence, the formation or deformation of excimer/exciplex on coming in contact with a
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guest species leads to sensing which can be achieved by excimer/exciplex band monitoring.\textsuperscript{6,134–136,138,146–149}

1.6.1 Photoinduced electron transfer (PET)

Photoinduced electron transfer based fluorescent probes make an important fluorophore and chemosensor family. In most of the cases, the probes following this mechanism are made of a fluorophore and a receptor. The receptor and fluorophore are linked through a spacer or orthogonally i.e., receptors and fluorophores are either isolated or very poorly conjugated in case of PET based probes (Figure 1.8). Usually PET, the electron transfer between receptor and fluorophore under excitation causes fluorescence quenching. On binding of analyte to receptor forbids the PET thus recovering the fluorescence. Hence, for the design of “off-on” response type of fluorescent probes, PET mechanism shows a significant role.

In PET, the receptor either functions as an electron donor or an electron acceptor. For electron donor case, the electron rich receptor gets oxidized whereas the fluorophore gets reduced, such type of PET is called as reductive-PET. In electron acceptor case, the fluorophore is oxidized whereas electron deficient receptor gets reduced, this is called oxidized-PET. Very less number of reports about oxidized-PET based fluorescent probes have been there in literature which is because of the difficult designing and synthesis of the electron acceptor receptors. Most of the PET based BODIPY, \textit{aza}-BODIPY and TAB fluorescent probes usually show reductive-PET process. Thus, the reductive-PET process is discussed here.

The molecular frontier orbitals theory can be used to illustrate the reductive-PET process, which is a tool to represent the picture of the mechanism of “off-on” fluorescence switching of probes (Figure 1.8). The analyte-free receptors are some groups (like para-amino-phenyl groups, tertiary amine groups etc.) which are electron rich relative to the fluorophores in these probes. The highest occupied molecular orbital (HOMO) of these analyte-free receptors lies in between HOMO and the lowest unoccupied molecular orbital (LUMO) of the fluorophore. One of the electrons of the fluorophore HOMO gets promoted to its LUMO under light excitation. Consequently, the HOMO orbital vacancy of fluorophore gets filled by the rapid transference of one of the receptor HOMO electron, which leads to stoppage of the radiative decay of the fluorophore’s excited electron and ultimately to very weak fluorescence or the complete fluorescence quenching. The receptor coordination or
chelation to the analyte leads to significant reduction of electron density on the receptor, thus lowering its HOMO to stop the transference of electron to the fluorophore completely. This situation leads to the ‘turn-on’ of the intrinsic fluorescence of the fluorophore.

Figure 1.8: Mechanism illustration of reductive-PET based fluorescent probes

1.6.2 Intramolecular charge transfer (ICT)

When the electron deficient fluorophore with π-conjugation gets integrated with an highly electron rich and electron donating receptors, then another process occur on photo excitation which is intramolecular charge transfer (ICT). As the process gets modulated with the changing capacity of the electron donating receptors, this proves to be appropriate mechanism for the designing of fluorescent probes.

The fluorescent probes based on ICT is usually composed of an electron rich donor which is conjugated with an electron-withdrawing acceptor moiety. Under photoexcitation the receptor (electron donating moiety) and fluorophore (electron-withdrawing acceptor moiety) undergo an intramolecular charge transfer thus increases the dipole moments. The proton-promoted state (a Franck-Condon (FC) state or locally excited state) is thermodynamically less stable in polar solvent medium and gets relaxed rapidly to intramolecular charge transfer state (CT), which
attains the minimum free energy due to the thermodynamic equilibrium of fluorophore and solvent cage. Thus the solvent polarity controls the fluorescence emission (fluorescence wavelength, intensity, lifetime and quantum yield) from CT to ground state. For example, main fluorescence spectra observe red-shifts on increasing the polarity of solvent and also the quantum yield (Φ) is low in case of polar solvents not in non-polar solvents.

The mechanism of fluorescent probes based on ICT is shown in Figure 1.9. The efficiency of ICT gets changed when the electron rich receptor binds with analyte like metal cation or a proton, which decreases the electron donating ability of the receptor. This binding event thus alters the properties of the fluorescent probe which include maximum absorption wavelength (λ_{abs}), maximum emission wavelength (λ_{em}), fluorescence quantum yield (Φ), fluorescence intensity, fluorescence lifetime (τ). In comparison to PET based probes, ICT based probes besides changing the fluorescence intensity also result in the shifts of emission and absorption spectra which is because of the different energy gaps before and after the event of analyte binding. Many BODIPY based fluorescent probes are in this category.\textsuperscript{105,117,150–155}

Figure 1.9: Mechanism illustration for ICT based fluorescent probes
1.7 Motivation and Objectives

The subject of anion sensing has attracted the attention in recent years which led to the increase in studies on various anion receptors including cyclic supramolecular receptors,\textsuperscript{156–160} acyclic receptors,\textsuperscript{161–164} heteroditopic cation-anion receptors,\textsuperscript{165–167} and chemosensors.\textsuperscript{18–20} Most of these studies have stressed on the binding energies and structural features of receptor-anion complexes with halide ions,\textsuperscript{18–20,156–161,165–167} while a range of various common anions are taken into account by some studies.\textsuperscript{162–164}

Furthermore, there is no clear understanding of the principles for selecting a right kind of moiety as a chromophore and a sensor. Also the synthesis of fluorescent chemosensors and predicting the mechanism behind sensing has remained a focus for some of the studies, but detailed mechanisms of sensing are still lacking.\textsuperscript{18–20,156,157,162,163,167} Nevertheless, understanding the mechanism of sensing is vital for fluorescent sensors and a detailed study related to their mechanism is highly significant not just to comprehend their working mechanism but also for their development, design and application in human health and environmental protection.

In such conditions, quantum mechanical based electronic structure calculations are expected to give valuable information regarding the structural features which could hence be used further for better understanding and enhanced appreciation of the factors responsible for such properties of anion sensing and exploring the detailed sensing mechanism. This is because, the development in density functional theory (DFT) and time-dependent density functional theory (TD-DFT), quantum chemical methods, has reached to a point where the properties calculated are thought to be of an equitable chemical accuracy. Thus, motivated by the anion sensing capabilities of boron based systems and advancements and efficiency of the computational methods, we are aimed at investigating the structural, spectroscopic, binding properties of some boron based anion sensing systems (with various common inorganic anions of varying size and geometry) and exploring the mechanism behind their anion sensing in detail using DFT and TD-DFT with the following specific research objectives:

- Compare the ground state optimized geometry of boron based chemical entities using density functional theory (DFT) with their X-ray determined geometry (wherever available).
- Assign electronic (absorption and emission) spectra of these systems using time-dependent density functional theory (TD-DFT).
Study the effect of various solvents like dimethyl sulphoxide (DMSO), acetonitrile (CH$_3$CN) etc. on the electronic absorption spectra of these chemical entities.

Designate the photophysical effect related to electronic spectra through which anion sensing will occur.

Assign the mechanism of anion sensing that is whether they follow covalent linking, non-covalent linking or selective reactivity.

Develop DFT/TD-DFT based approach as a guiding tool for designing molecules with specific application in mind.
References

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