Chapter 2

Introduction

In recent decades, the examination of biomolecular dynamics has gained importance for elucidating the fundamental principles that regulate biological activity. Proteins, DNA, and their complexes exhibit structural flexibility, conformational changes, and dynamic interactions that are crucial for their biological function.[1-3] Comprehending these dynamic behaviours at the molecular level requires advanced techniques, with fluorescence spectroscopy and microscopy serving as powerful tools for examining these processes.[4-7] Employing fluorescence-based spectroscopy and smFRET facilitates the real-time analysis of biomolecular interactions and conformational changes, providing insights into mechanisms essential for biological function.[8] In this thesis we studied the functionality and dynamics of several biomolecular systems, focusing specifically on protein-ligand interactions and DNA conformational dynamics.

This chapter will describe the biomolecular systems we have investigated, emphasizing their biological importance and the use of fluorescence spectroscopy and microscopy to identify their structural and dynamic properties.

2.1 Protein-Drug Interaction

Proteins execute numerous works, such as enzyme catalysis, ion and molecule transport across organs, muscular contraction, structural support in tendons and cartilage, immune response through antibodies, and regulation of cellular and physiological processes.[9] The functional characteristics of proteins are based on their three-dimensional conformations. The structure and conformation of a protein can be confirmed experimentally by techniques such as electron microscopy,[10] X-ray crystallography,[11] as well as NMR spectroscopy.[12]
Drug-protein interactions are crucial in the drug discovery process and significantly influence therapeutic strategies.[13-14] The drug interacts with a specific receptor, which may be an enzyme or a membrane protein, to execute its therapeutic effect.[15] Upon introduction into the human circulatory system, a drug complexes with various proteins that transport it throughout the body and activate its effects.[16] The interaction of these medicines with carrier proteins is crucial for their pharmacological and pharmacokinetic properties. There are mainly three types of protein classification: fibrous, globular and membrane protein.[17] In this thesis, we mainly worked with globular protein bovine serum albumin and lysozyme.

2.1.1 Bovine Serum Albumin

Serum albumin is the class of plasma protein and is essential for the transport of drug ligands. The BSA comprises 583 amino acids and features three homologous domains with a molecular weight of 66.4 kDa exhibiting a spherical morphology like a heart.[18] The homologous I, II, and III domains are linked by disulfide connections, with each domain comprising two sub-domains (A and B).[19] The protein has two tryptophan (Trp) residues responsible for its intrinsic fluorescence. The first is Trp-134, situated on the surface of the first domain, and Trp-213, positioned within a hydrophobic binding pocket in the second domain.[20]

It is thought to have a significantly high binding affinity for a diverse array of small molecules. It is one of the most extensively investigated proteins due to the presence of charged amino acids on its surface.[21] They bind several relatively insoluble endogenous and foreign chemicals, as well as a diverse array of medications, which are carried in the circulation either

freely, dissolved in the aqueous phase of plasma, or bound in complex with proteins.[21] Numerous investigations have been conducted on the ligation of small molecules into biomacromolecules. BSA performs multiple tasks within the organism, including the movement of molecules, regulation of bodily tissues and organs, catalysis of biochemical reactions, and the dissolution of gases and foreign particles.[22]

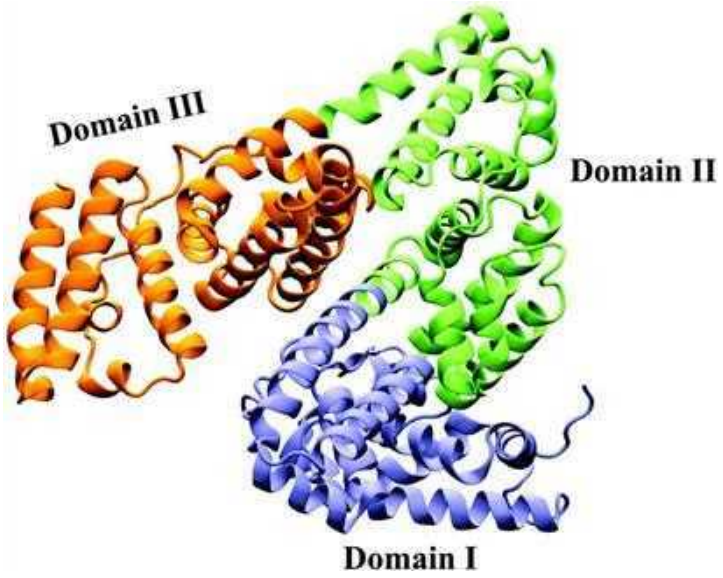


Figure 2.1 Crystal structure of globular protein Bovine Serum albumin (PDB ID:4F5S).

2.1.2 Lysozyme

The molecular interaction mechanism between proteins or other biological macromolecules with small drug molecules plays a significant role in drug design and applications.[13-14, 23-26] Lysozyme is a globular protein (M.W. 14.3 kDa) with 11 anionic and 19 cationic residues among 129 total residues, and it has four intramolecular disulfide bridges (Cys6-Cys127, Cys30-Cys115, Cys64-Cys80, Cys76-Cys94).[27-30] Lysozyme consists of six tryptophan (Trp) residues, namely Trp-28, Trp-62, Trp-63, Trp-108, Trp-111, and Trp-123, among them in α domain Trp-28, Trp-62, Trp-63, Trp-123 are present and the rest two Trp group present in the hinge region of α and β-domains.[27-30] The surface of lysozyme is mostly polar, whereas its interior is hydrophobic; steady-state data reveals that 80% of the intrinsic fluorescence of this protein is from hydrophobic Trp-62 and Trp-108 residues.[29-30] It is found mainly in cells and secretions, including tears, mucus, saliva, and egg white protein.[28] This protein has several applications including anti-cancer, antiviral, and anti-inflammatory, and is used as a food preserver.[31-35] The main function of lysozyme is its antibacterial property; it damages gram-positive bacteria's peptidoglycan cell membrane by hydrolyzing the glycosidic bond.[36] Lysozyme is very useful in delivery systems as previously reported; it binds reversibly to many exogenous and endogenous ligands and acts as a drug carrier.[28, 37-39] These outstanding properties of lysozyme make it a centre of attraction for studying the mechanism of metabolism and transportation of molecules by biochemical and photophysical methods.

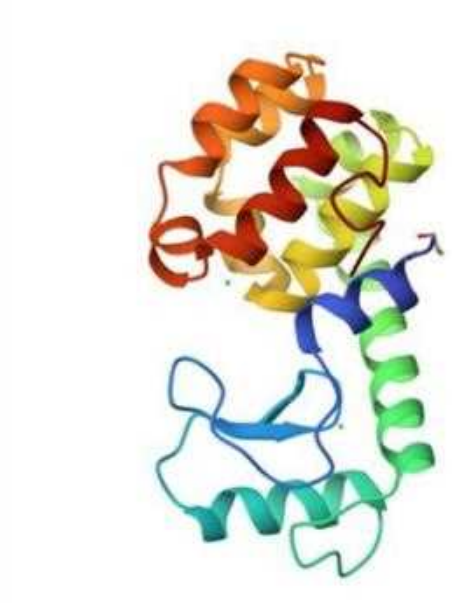


Figure 2.2 Crystal structure of Lysozyme (PDB ID: 253L).

2.2 Carriers for Hydrophobic Drugs

2.2.1 Micelle

A variety of drug carriers and stabilization techniques utilized in recent years, including liposomes, polymeric nanoparticles, polymers, and micelles.[40-44] Micelles have been extensively utilized as drug delivery vehicles for various compounds, including low molecular weight water-insoluble drugs.[45-46] A micelle generally possesses a hydrophobic core, constituted by the nonpolar tails, and a hydrophilic shell, comprised of the polar head groups that engage with the surrounding water.

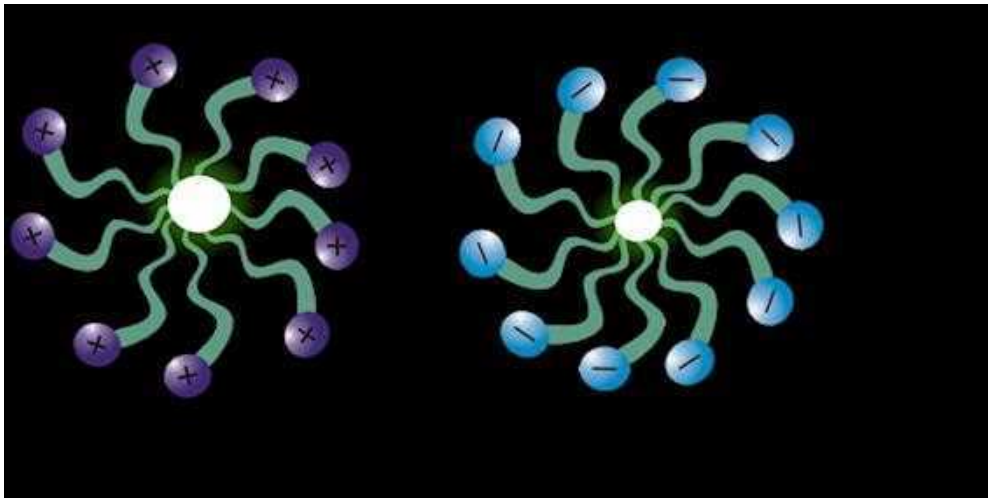


Figure 2.3 Structure of cationic and anionic micelle.

In comparison with alternative drug delivery methods, micelles possess two unparalleled benefits. The hydrodynamic diameters of micelles generated by self-assembled surfactants in an aqueous solution are typically smaller than 50 nm,[47] preferred for blood circulation and cellular absorption.[48] The second advantage of micelles is that their molecular arrangement and assembly are well-defined, allowing for large-scale production.[49] Notwithstanding their benefits, a primary

constraint of micelles as drug delivery vehicles is their diminished stability in response to physiological environmental changes. Also, a minimal surfactant concentration, known as the Critical Micelle concentration (CMC), is essential for preserving micelle structure. The traditional approach to determining the CMC relies on observing the characteristics of micelles through measurements of electrical conductivity,[50] surface tension,[51] chemical shifts via NMR,[51] AFM,[52] SANS,[53] SAXS[53] and fluorescence intensity.[50] TEM[52] and DLS[54] are frequently employed to elucidate the dimensions and morphology of micelles.

Besides drug delivery, micelles have been investigated for the co-delivery as combined therapies utilizing various medications with different mechanisms of action can improve therapeutic efficacy and prevent the development of drug resistance.[55]

2.2.2 Cyclodextrin

Cyclodextrins (CDs) are biocompatible and biodegradable substances generated by the enzymatic breakdown of starch. They comprise six to twelve (α-1,4)-linked α-D glucopyranose units. The glucopyranose moiety exhibits a truncated cone-shaped structure, characterized by a hydrophobic core cavity formed by the skeletal carbon atoms of glucose and a hydrophilic perimeter constituted by the hydroxyl groups of glucose.[56-59]. This nature enhances the solubility and stability of hydrophobic medicinal molecules, rendering them particularly valuable in drug delivery systems. The interior of the cyclodextrin cavity is lined with hydrogens from the C3 and C5 atoms, imparting a hydrophobic characteristic. Still, the secondary hydroxyl groups at C2 and C3, along with the primary hydroxyl group at C6, render the outer surface hydrophilic. The dimensions of the cavity are contingent upon the specific type of cyclodextrin: α-CD possesses a cavity diameter of roughly 4.7–5.3 Å, β-CD around 6.0–6.5 Å, and γ-CD approximately 7.5–8.3 Å. These dimensions enable CDs to enclose diverse guest molecules according to their size and polarity, resulting in non-covalent inclusion complexes. These complexes protect the drugs against degradation by the environment, decrease drug crystallinity, and enhance water solubility.

Additionally, CDs can serve as stabilizing agents by inhibiting medication deterioration induced by light, oxygen, or moisture. For instance, β-CD has been utilized in the formulations of hydrophobic pharmaceuticals such as diflunisal, where it improves solubility and stabilizes the drug within biological systems. Furthermore, γ-CD is employed for larger or more intricate medicinal compounds.

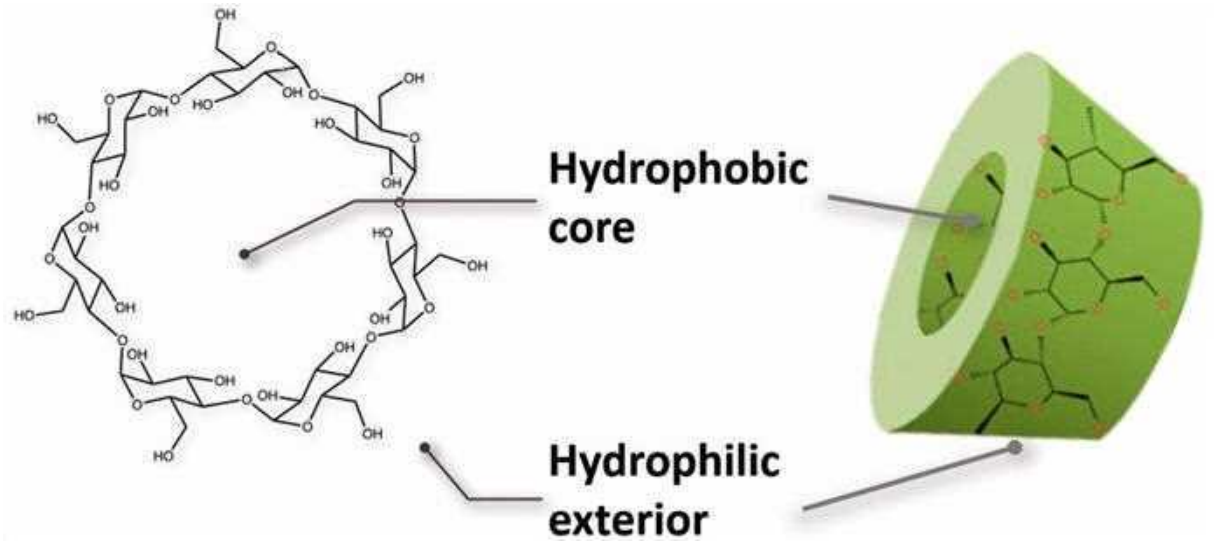


Figure 2.4 Cyclodextrin structure with the hydrophobic and hydrophilic interior. This figure is reproduced with permission Giuseppina Ioele et al 2017.[60]

CDs are often used as pharmaceutical excipients that have gained attention as host due to their nature to enhance the permeability of poorly water-soluble drugs and their complexation capacity to increase drug delivery therapeutically through biological membranes.[61-67] The natural CD complex forms aggregates in an aqueous medium by self-assembly depending upon their solubility. There is a dynamic equilibrium between the free drug molecule and the drug molecule bound within CDs in an aqueous solution. No covalent bond formation occurs during the encapsulation period.[68] The internal topology is a crucial factor of the cyclodextrins that the ability to form a host-guest inclusion complex by a fit-and-find detector with various ligands.[69-70] Hydroxypropyl β-cyclodextrin has recently gained attention as the modified class of β-CD due to its safety profile, higher hydrophilicity, and more water solubility.[71-72] HP-β-CD is formed by replacing the hydroxyl group of β-cyclodextrin with the hydroxypropyl group. Still, the two cyclodextrin molecules resemble each other in terms of hydrophobic cavity and surface interactions.

In drug delivery, β-CD is predominantly utilized owing to its optimal cavity dimensions, accommodating several small-molecule pharmaceuticals. Nonetheless, native cyclodextrins such as β-CD exhibit restricted solubility in water. Consequently, HP-β-CD is also utilized to enhance solubility and biocompatibility.

2.3 Hydrophobic Drug: A Focus on Curcumin and Diflunisal

Curcumin:

There are many ongoing clinical trials for understanding the therapeutic effect of curcumin against life-threatening diseases, including skin cancer, inflammation, respiratory metabolic disorder, cardiovascular disorder, etc.[73-76] The overall clinical highlighted data reported that curcumin has a major potential to be a natural medicine, specifically highly promising effects against malignant disorders, replacing synthetic chemical drugs. The biological effect of curcumin has been reported extensively – (1) antioxidant effect, (2) inhibitory effect on cytokine release, (3) anti-amyloidogenic effect, (4) chemo-preventive effect, (5) chemotherapeutic effect, (6) radioprotective effect, (7) regulatory effect on gut bacteria, (8) protective effect against bone disorders and many more.[74] In addition, the regulatory effect of cell signalling pathways have also been reported recently, e.g. protein kinase B, transforming growth factor-beta, increasing apoptosis by inhibition of pAKT,[77] and insulin growth factor downregulation of TGF- β, etc.[78-79] Curcumin can act as a scavenger for reactive oxygen and nitrogen free radicals, and is soluble in lipid membranes.[80-81] A lipophilic compound with a conjugated double bond can inhibit or bind to some regulatory enzymes and proteins. However, despite its effect as a promising therapeutic agent, the application of curcumin as a drug is low because of its low solubility in water, which limits its absorption and stability inside the human body under neutral and basic pH conditions.[82]

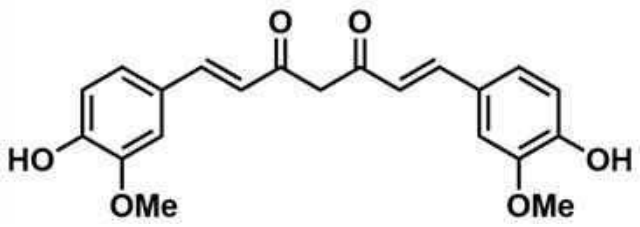


Figure 2.5 Chemical structure of curcumin.

Diflunisal:

Diflunisal is a diflurophenyl derivative of 5-(2′, 4′-difluorophenyl) salicylic acid, classified in NSAID-approved drugs with anti-inflammatory, analgesic, and antipyretic activity.[83-84] The major challenge with this drug is poor aqueous solubility, which affects oral bioavailability and

gastrointestinal toxicities during long-time therapy.[84] Several polyamidoamine dendrimers and hydrophilic polymers have been used to increase the water-solubility of diflunisal.[85-86]

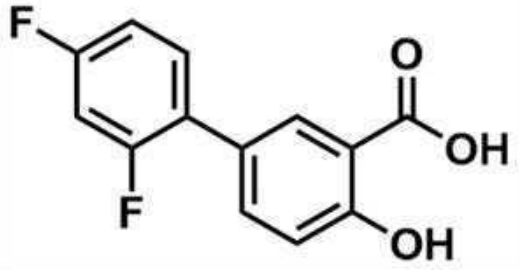


Figure 2.6 **Chemical structure of diflunisal.**

2.4 DNA Holliday Junction

Helical poly-nucleotide junctions are essential components of bio-molecular regulation, playing a critical role in maintaining functionality and diversity of genetic materials.[87-91] These junctions are involved in various fundamental biological processes, including DNA repair, homologous or non-homologous recombination and replication. By facilitating the correct alignment and interaction of DNA strands, helical polynucleotide junction ensures accurate genetic information transfer and prevents harmful mutations. Their dynamic nature allows them to adapt to different cellular conditions, making them versatile elements in the orchestration of complex cellular mechanisms. Understanding their structure and behaviour is pivotal for the advancement of genetic engineering, molecular biology and therapeutic interventions targeting genetic disorders. Among such examples, DNA Holliday Junctions (HJ) are the crucial intermediates in genetic information processing through homologous recombination.[92-99] HJs form a junction point where duplex poly-nucleotide segments intersect with discontinuities. [100] Once formed, HJs undergo spontaneous or protein-facilitated movement, which involves stepwise exchange of base-pairing partners to locate sequence homology, a process known as branch migration that stabilizes the intermediate junction phase.[100-102] Finally, as a result of homologous recombination, strands are exchanged between intersecting DNA duplexes to facilitate cross-over and non-crossover products.[103-104]

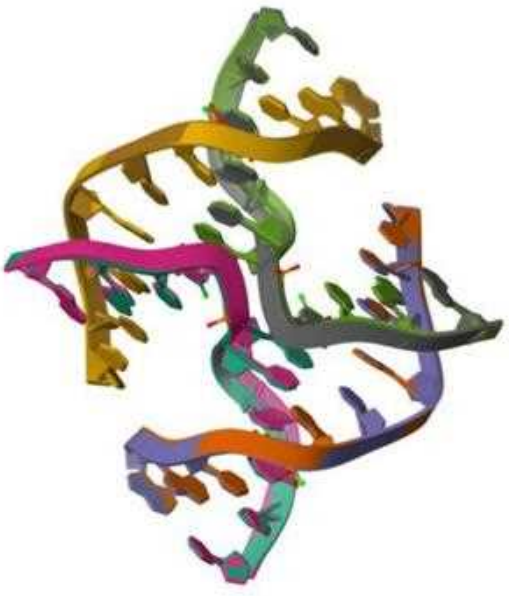


Figure 2.7 Crystal structure of DNA Holliday Junction (PDB ID:4GSG).

The structure of HJ, initially proposed by Professor Robin Holliday, is remarkably intriguing.[94] It can be best described as a stacked form where four DNA duplexes create two pairs, each pair stacked coaxially, with two strands crossing between the stacked pairs aligned parallelly in a 5′-3′ orientation.[105] However, these junctions are dynamic, as observed in previous single molecule studies, and are influenced by various proteins during later steps of recombination.[106-107] Previous studies suggest that the dynamic nature of HJ is effectively utilized for resolution by junction-resolving enzymes.[108-111] Resolvases, for instance, tend to bias HJ toward open-X conformation, accelerating the genetic recombination process by dictating the orientation and resolution of the junction.[110] Interconversion between the isomeric forms of DNA holidays junction occurs through an open-X planar structure.[112-113]

Junction-resolving enzyme GEN1 (human) in its monomeric form binds to an intact HJ, leading to an undesirable cleavage at the junction point before dimer formation through a partial dissociative step.[106, 109] Tetrameric motor proteins also bind to the open planar structure of HJ with some degree of sequence specificity.[112, 114] Bacterial resolvases like RuvC first bind to the stacked X isomers of HJ and then undergo a transition to an open planar structure in their dimer form.[115] It has been reported that the proteins such as RecU, RuvC, and CCE1, which dimerize, bind to an X-stacked isomer and induce structural changes in HJ by opening the junction centre to make a stabilized transition state, allowing the junction to migrate.[108, 116] Structural dynamics of HJ in the presence of enzyme occur either by conformation exchange between two isomers or branch migration with a partial dissociation state. [110, 117-118]; it has also been found the monovalent or divalent cation concentration increases the frequency of the partial dissociation state.[117] In the absence of protein or enzyme, the junction is crystallized in a stacked X conformation. At the same time, in the presence of an enzyme-HJ complex, it can exist as an open conformation or other hybrid structures.[112, 119] Crystal structure investigations showed phosphate groups at the point of strand exchange are influential contributors to electrostatic interaction.[120] Methylation in DNA HJ strands leads toward significant deformability, as observed in the neutralized phase.[121]

The stability of HJ also depends upon the sequence and the length of the base pair. It has been observed that the 24-base pair long DNA HJ can be formed at low ion concentration (0.1 M NaCl at 5 μM strand concentration), but 18 base pair long sequence is less stable, and 12 base pair long sequence can't be formed using the same ionic concentration. Further study investigated that if the core region of HJ is homologous, then the structure becomes more resistant to thermal denaturation. The divalent cation also increased the melting temp of DNA HJ.

DNA supercoiling is a critical factor for the structural bias in cruciform as observed in atomic force microscopy experiments, which is a general model for the DNA Holliday junction. It was shown the cruciform structure can adopt a parallel configuration even in the absence of any divalent cation, due to the supercoiling effect.[122] A specific trinucleotide core at the junction ACC has a pivotal role in the stability of DNA HJ in the solution phase; the general rule of the sequence is the core must have pupyC nucleotides, where pu refers the purine and py refer to pyridine moiety proven by the atomic force microscopy study. The inter-duplex angle of the junction having the core is ~ 30° to ~ 40°, while without this core, the structure has a ~ 60°-degree inter-duplex angle, which suggests that the ACC core pulls the arms of HJ closer to each other.[122]

Further investigation suggested crossing strands are often antiparallelly oriented and topologically locks the HJ.[123] In order to possess branch migration ability, HJ must adopt an open-X conformation in which the four strands are distant from each other. At sufficiently low concentrations of ion, HJ adopts the open conformation in order to reduce the inter-strand repulsions due to unshielded phosphate charges.[124] While branch migration imparts stability to the stacked conformation, all HJs experience occasional switching behaviours toward an open X state, even at high ion concentrations. At sufficiently low concentrations of ion, HJ adopts the open conformation in order to reduce the inter-strand repulsions due to unshielded phosphate charges.[124] While branch migration imparts stability to the stacked conformation, all HJs experience an occasional switching behaviour toward an open X state, even at high ion concentrations.

The Osmium tetraoxide (OsO_4) assays with the nucleotide thymine proved that the binding of divalent cation occurs at the junction point of DNA HJ.[125] Uranyl photo probing study reflects the binding of the ions in the junction point, which influences the conformational heterogeneity. As ion concentration increases, the inter-strand repulsions are reduced by masking phosphates with counter-ion condensation and the junction conformations are easily inter-convertible. The conformational heterogeneity of the junction is highly influenced by sequence at or near the junction point and specific base-pairing.[97, 126-127]

2.5 Fluorescence Spectroscopy and Microscopy to Study Biomolecules

Fluorescence spectroscopy and microscopy are crucial methods for investigating biomolecules, providing ample details about their structure, behaviour, and interactions at both ensemble and single-molecule scales.[128-129] Fluorescence spectroscopy works by exciting biomolecules with light at specific wavelengths, resulting in the emission of light at higher wavelengths. The emitted light conveys essential information regarding the surroundings of the fluorophore, molecular dynamics, and possible interactions. The exceptional sensitivity of fluorescence spectroscopy facilitates the identification of very small quantities of biomolecules, rendering it a preferred technique for investigating proteins, nucleic acids, and other biological molecules in solution or within cells.[130] It enables researchers to investigate features such as binding constants, quantum yields, and lifetimes, which can disclose insights into molecular conformational alterations, folding-unfolding dynamics, and intermolecular interactions. Time-resolved fluorescence spectroscopy elucidates the dynamic behaviour of molecules, offering insights into molecular surroundings and excited-state lifetimes.[131]

Fluorescence microscopy enhances spectroscopy by offering spatial resolution, facilitating the observation of biomolecules within intricate biological systems, including living cells or tissues.[132] Fluorescence microscopy can monitor molecular location, mobility, and interactions in real-time by tagging biomolecules with fluorescent dyes or proteins such as GFP.[133] Conventional fluorescence microscopy techniques, including epifluorescence and wide-field microscopy, have evolved into sophisticated approaches like as confocal microscopy[134] and TIRF[135] microscopy. Confocal microscope employs pinhole optics to enhance spatial resolution and eliminate out-of-focus light, facilitating 3D reconstruction of cells and tissues with sub-micron accuracy. TIRF microscopy enables the selective illumination of fluorophores within several hundred nanometers of the surface, rendering it optimal for investigating membrane-bound proteins, receptor-ligand interactions, and other surface phenomena.[136]