

FLUORESCENCE SPECTRAL STUDIES ON THE INTERACTION OF TRYPSIN WITH 4- DICYANOMETHYLENE-2,6-DIMETHYL-4H-PYRAN (DDP) DYE IN AN AQUEOUS SOLUTION

THESIS

submitted in partial fulfilment of the requirements for the award of

MASTER OF SCIENCE IN CHEMISTRY

BY

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DEPARTMENT OF CHEMISTRY

SCHOOL OF SCIENCE AND HUMANITIES

SATHYABAMA

INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)

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MAY,2023



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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of N.C. APARAJITHA (41910002) who carried out the project entitled "FLUORESCENCE SPECTRAL STUDIES ON THE INTERACTION OF TRYPSIN WITH 4- DICYANOMETHYLENE-2,6-DIMETHYL-4H-PYRAN (DDP) DYE IN AN AQUEOUS SOLUTION" under my supervision from 04/01/2023 to 05/05/2023.

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ABSTRACT

This study investigated Fluorescence spectral studies on the interaction of trypsin (*guest molecule*) with 4- dicyanomethylene-2,6-dimethyl-4h-pyran (DDP) dye (*host molecule*) in an aqueous solution. The absorbance peak of the DDP dye decreased upon the addition of trypsin at different concentrations, while the absorbance at the ICT absorption maximum remained unchanged. Stern Volmer analysis identified the fluorescence quenching of the DDP dye, which was attributed to the tryptophan (Trp) and tyrosine (Tyr) residues in trypsin that are highly sensitive to their local environment. Additionally, the fluorescence decay of the DDP dye in the presence of trypsin exhibited a tri-exponential decay, with changes observed in the fluorescence lifetime components of the dye. Specifically, one component showed a significant decrease in fluorescence lifetime, while another component showed an increase, and the relative amplitude distribution remained unchanged. The multi-exponential lifetime components of the dye suggest the existence of various hydrogen-bonding arrangements of dye with water molecules in the presence of trypsin. The results suggest that the interaction between DDP dye and trypsin is largely hydrophobic, with the presence of hydrogen-bonding functional groups in trypsin influencing the excited-state spectral properties of the DDP dye. Further studies could help expand our understanding of the interaction between DDP dye and biomolecules, with potential applications in biosensors and other optical technologies.

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LIST OF ABBREVIATIONS AND SYMBOLS

ABBREVIATIONS

SL.NO	ABBREVIATIONS	DEFINITION
1	AO	Atomic Orbital
2	CT	Charge transfer
3	DCM	Dicyanomethylene-2-methyl-6-p-dimethylaminostyryl- 4H-pyran
4	DDP	Dicyanomethylene-2,6-dimethyl-4H-pyran
5	FRET	Fluorescence resonance energy transfer
6	fs	Femtoseconds
7	HOMO	Highest occupied molecular orbital
8	ICT	Intramolecular Charge Transfer
9	LUMO	Lowest occupied molecular orbital
10	MEP	Molecular electrostatic potential
11	MO	Molecular orbital
12	ns	Nanoseconds
13	ps	Picoseconds
14	TAC	Time to Amplitude Convertor
15	TCSPC	Time Correlated Single Photon Counting
16	TICT	Twisted Intramolecular Charge Transfer
17	TRP	Tryptophan
18	TRY	Trypsin
19	TYR	Tyrosine

20	UV-Vis	Ultraviolet-Visible
SYMBOLS		
SL.NO	SYMBOLS	DEFINITION
1	χ	Chi
2	C	Concentration
3	Q	Concentration of the Quencher
4	I	Fluorescence intensity of dye with hydrogen-bonding solutes
5	I_0	Fluorescence intensity of free dye
6	f	Frequency
7	ν	Frequency in cm ⁻¹
8	τ	Lifetime
9	mol L ⁻¹	Number of moles per litre
10	π	Pi
11	B	Relative amplitude
12	K_{SV}	Stern-Volmer constant
13	nm	Wavelength

CHAPTER 1

INTRODUCTION

Fluorescence spectral studies in micro-heterogeneous environments investigate the behaviour of molecules and materials confined or localised within micro-heterogeneous structures. These studies aim to comprehend how the microenvironment influences the photophysical properties of molecules or materials within this confined system, such as absorption, emission, energy transfer, and photochemistry. In micro-heterogeneous systems, charged interfaces in hydrophilic and hydrophobic domains explain the precise characteristics of more than one homogeneous environment. Species that form molecular aggregates and self-assemblies that are predominantly formed through hydrogen bonding are the two main types of micro-heterogeneous systems. The primary reasons for studying micro heterogeneous system assemblies are (i) photophysical and photochemical processes to probe the dynamic and static properties of these organised systems; and (ii) excited-state molecular processes in a novel microenvironment. The distribution of solutes within a solution or aggregate can be inhomogeneous. Solute may be able to access unique properties in hydrophobic and hydrophilic cavities/cages/pockets/pools or pores. Charged interfaces where electrostatic effects can have a significant impact on solute distribution and reactions. We need optically transparent (non-turbid) solutions that can be investigated photochemically using a steady state and pulsed photolysis techniques. As the system becomes more complex, no single technique can provide a solution or even clearly resolve various processes that can use a variety of physical and chemical methods.

It is known that, at theoretical and practical levels, the nature of interactions with relatively soluble solutes in aqueous solutions has become increasingly important. Two major interactions that govern and operate are hydrogen bonding and hydrophobic interactions. Among these interactions, hydrogen bonding plays an important role in biology, enabling the reversible formation of aggregates that are not covalently linked and the stability of multiple hydrogen bonding systems is dependent not only on the number of hydrogen bonds but also on the hydrogen bonding pattern with the solvent molecules.

Conversely, in a large number of physiological biochemical processes, hydrophobic interaction is important. Hydrophobic interactions are well known to be one of the major components of biology, and it is clear that water molecules around a non-polarized solute were involved in an increased order or molecular structure which led to their interaction (*Kalyanasundaram K, 1987; Lakowicz JR, 1999*). This study used spectroscopic techniques to investigate how DDP dye (*host molecule*) interacted with trypsin (*guest molecule*).

1.1. EXCITED STATE PROPERTIES:

Fluorescence is a type of luminescence that occurs when certain molecules absorb light at a particular wavelength and subsequently emit light at a longer wavelength after a short time delay, typically in the nanosecond range. This phenomenon is commonly observed in a wide range of natural and synthetic materials, including dyes, proteins, and minerals, and has important applications in fields such as analytical chemistry, biochemistry, and materials science. The fluorescence process involves the excitation of a molecule from its ground state to an excited electronic state upon absorption of a photon of light. The excited state is typically short-lived, with a lifetime in the range of picoseconds to nanoseconds, and can decay through several pathways, including fluorescence emission, non-radiative relaxation, and intersystem crossing. If the excited-state molecule decays through fluorescence emission, it emits light at a longer wavelength than the absorbed light, resulting in a characteristic fluorescence spectrum.

The fluorescence properties of a molecule can be affected by various factors, such as the molecular structure, solvent environment, and temperature. These properties can be characterised by various parameters, including the fluorescence quantum yield, fluorescence lifetime, and emission spectrum, which can provide valuable information about the molecular structure, dynamics, and environment. The process is governed by three important events, all of which occur on time scales separated by several orders of magnitude, as illustrated by the Jablonski energy diagram, a graphical representation of photophysical processes that includes the various energy levels that a molecule can occupy and the transitions between those levels and are often used as the starting point for discussing light absorption and emission.

Jablonski diagrams are used in a variety of forms, to illustrate various molecular processes that can occur in excited states. These diagrams are named after Professor Alexander Jablonski, who is regarded as the father of fluorescence spectroscopy. The Frank-Condon principle, on the other hand, is a concept that describes a molecule's vibrational relaxation during electronic transitions. Solvation is the process by which a molecule in a solution is surrounded by solvent molecules.

When a molecule or atom absorbs a photon of light, it is excited to a higher electronic state $S_0 \rightarrow S_1$. The time scale for absorption is typically on the order of femtoseconds (10^{-15} s), which is the time it takes for the photon to be absorbed. After absorbing a photon of light, the excited molecule or atom rapidly loses energy through collisions with its surroundings, such as other molecules or solvent molecules. This causes the molecule or atom to vibrate and rotate, eventually reaching the lowest vibrational level of the excited state. The time scale for vibrational relaxation is typically on the order of picoseconds (10^{-12} s).

In some cases, the excited molecule or atom may undergo a non-radiative transition to a lower electronic state, releasing energy as heat $S_2 \rightarrow S_1$. This process is called internal conversion, and it can occur on a time scale of nanoseconds (10^{-9} s) to microseconds (10^{-6} s), depending on the molecule or atom.

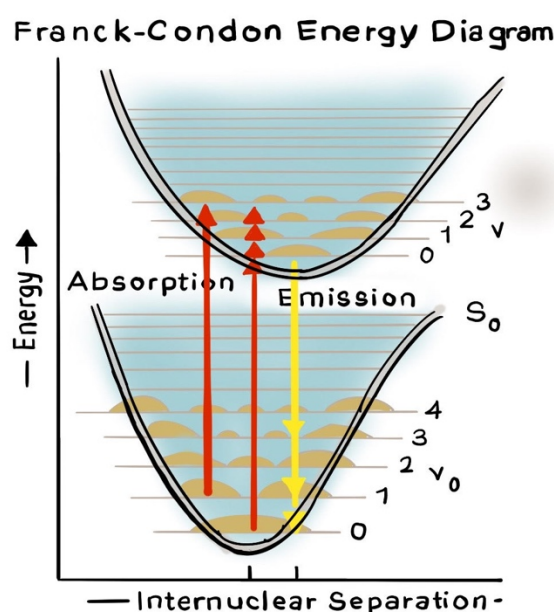


Fig.1.1.A. Franck-Condon Energy Diagram

When the excited molecule or atom returns to the ground state $S_1 \rightarrow S_0$, it can emit a photon of light as fluorescence. The time scale for fluorescence is typically on the order of nanoseconds to microseconds, depending on the molecule or atom. As a consequence of the rapid vibrational relaxation and internal conversion processes described above the fluorescence occurs, with some exceptions, from the lowest vibrational level of the first electronic excited singlet state to the singlet ground state. This energy loss prior to fluorescence is the physical origin of the famous Kasha's Rule which states that: "luminescence (fluorescence or phosphorescence) only occurs with appreciable yield from the lowest excited state of a given multiplicity". It is also responsible for the Stokes-Shift; where the fluorescence occurs at a longer wavelength than the absorption.

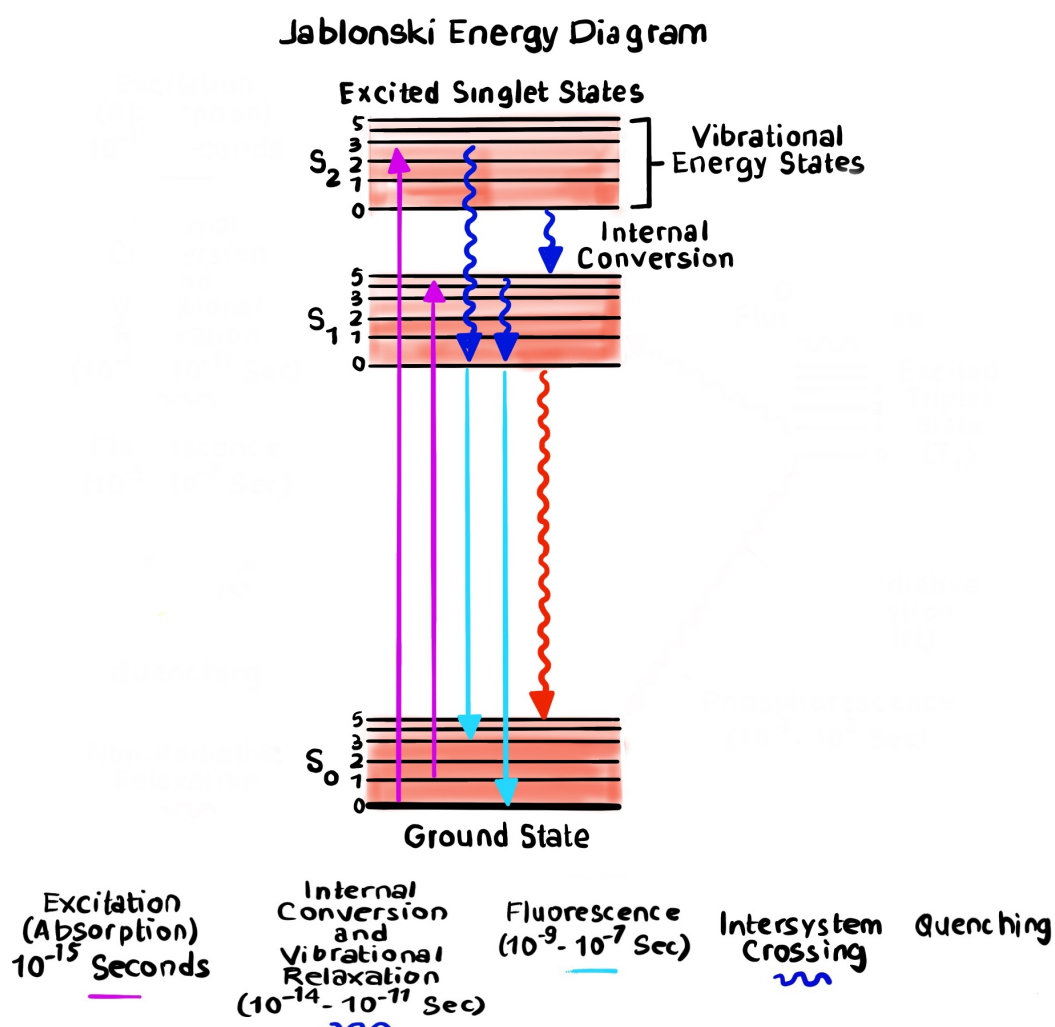


Fig.1.1.B. Jablonski Diagram

1.2. TRYPSIN:

In recent years, there has been a lot of focus on the interaction between small molecules and proteins. Trypsin a non-fluorescent protein is a class of serine proteases, which is widely found in the pancreas and is an important digestive protease in humans. Trypsin consists of 223 amino residues with a molecular mass of 23,300 Da (Yue Mu,2011). It contains two domains of nearly equal size, and each domain comprises a set of six antiparallel strands of polypeptide chain laced together into a β -sheet unit by a network of H-bonds. The residues His57, Asp102 and Ser195 located between the two domains are the catalytic active centre of trypsin, the three structures above form a catalytic triplet (Yingying Liu,2015). Trypsin has diverse biological functions such as immune responses, digestion and hemostasis (Hongcai Zhang,2019). Trypsin, the water-soluble globular protein, (Yingying Liu,2015) forms hydrogen bonds with water molecules which helps to solvate the protein and stabilize its structure in the aqueous environment.

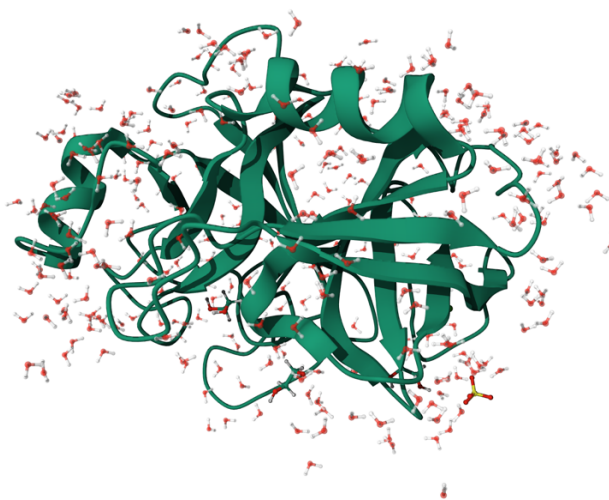


Fig.1.2. Structure of Trypsin

1.3. DDP DYE (4-Dicyanomethylene-2,6-dimethyl-4H-pyran):

DDP dye (Dicyanomethylene-2,6-dimethyl-4H-pyran) is a water-soluble fluorophore with electroluminescent properties. The structure of DDP dye is similar to that of DCM dye, a well-known ICT dye. There is no possibility of the Twisted Intramolecular Charge Transfer (TICT) phenomenon, unlike DCM dye such that the complexity of DCM dye is completely eliminated. DDP dye contains a strong acceptor

moiety (dicyanomethylene, $C(CN)_2$) in the 4th position and a substituted donor moiety (methyl) in the 2nd and 6th positions. DDP dye has a significant advantage over DCM dye in terms of water solubility, and its photophysical nature can be probed by the addition of hydrogen bonding to study the nature of host-guest interaction. DCM dye, on the other hand, is not soluble in water, so no such studies could be carried out in an aqueous solution. DDP dye exhibits excellent luminescence character in the presence of more electron-releasing moieties and acts as an ideal that is used as the host molecule. (Somasundaram Gayathri, 2018;2019)

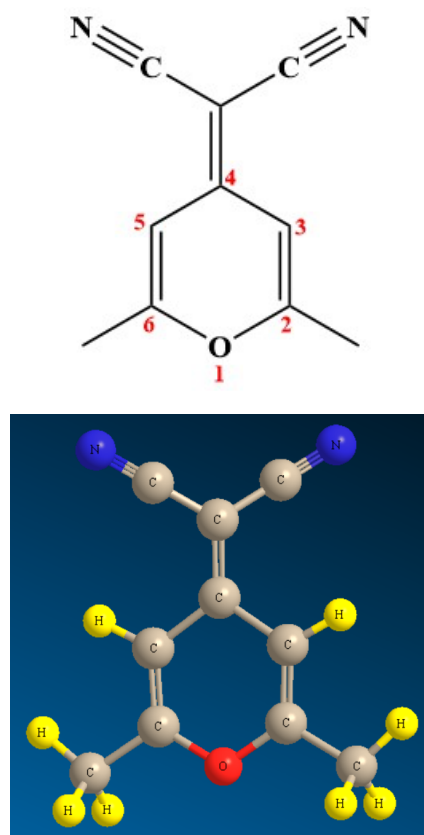


Fig.1.3. 4-Dicyanomethylene-2,6-dimethyl-4H-pyran (DDP dye)

1.4. PREPARATION OF DDP DYE:

The mixture of 2,6-dimethyl-4-pyrone (4: 1.25 g, 10.06 mmol) and malononitrile (0.783 g, 11.85 mmol) in 5 mL of acetic anhydride was stirred and heated to 140 °C for 1 h under nitrogen atmosphere. The acetic acid was aspirated off, and the residue was washed with 10 mL of boiling water and collected by filtration to give brown material (WoodsL L,1958).

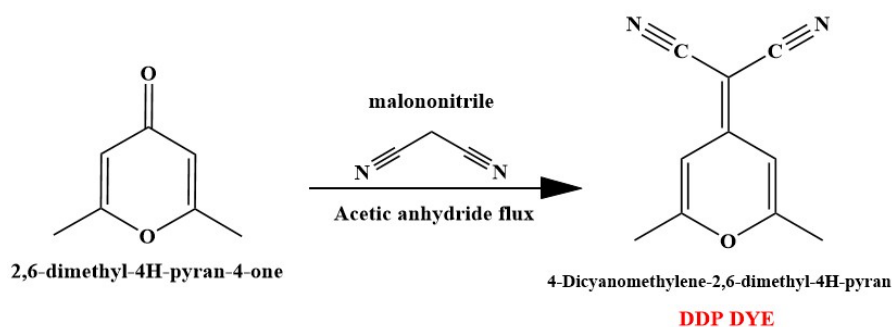


Fig.1.4. Preparation of DDP dye

1.5. INTRAMOLECULAR CHARGE TRANSFER (ICT) BEHAVIOUR OF DDP DYE:

DDP is an example of a donor-acceptor molecule. Its structure is comprised of an electron-donating methyl group and an electron-accepting di-cyano group, where it participates in intramolecular charge transfer. During this process, the excited free electron moves from the donor to the acceptor before relaxing back to a less polar ground state. The intramolecular charge transfer is mediated by a π molecular orbital (MO) that extends from the donor to the acceptor by overlapping the p-orbitals from the constituent carbon atoms or from lone pairs. In the ground state of a conjugated system, the highest occupied molecular orbital (HOMO) is fully occupied and electrons cannot move. However, if an electron from the donor group is excited into the lowest unoccupied molecular orbital state, it is free to migrate along the delocalised orbital.

The existence of an overlap of the HOMO and LUMO wave functions and transition cannot be attributed to pure charge transfer character. Electron density redistribution clearly suggests $\pi \rightarrow \pi^*$ transition character, except for the small fraction of $\sigma \rightarrow \pi^*$ coming from donor methyl groups for DDP dye. The position of the absorption maxima in the spectra of the DDP dye is because of the bands that formed with the participation of π -type Molecular Orbitals delocalized on the Atomic Orbitals (AO) of the pyran ring and with any one of the methylene group. The molecular electrostatic potential (MEP) of DCM dye shows that nitrogen atoms of the cyano group and oxygen of the pyran cycle are responsible for the proton-acceptor properties in the ground and excited states of the molecules which give strong evidence for DDP dye where charge-transfer to occur.

The donor group (methyl substituents) of DDP dye is expected to have orbitals that are in between pure sp^3 and sp^2 hybrid orbitals. This is due to resonance structures that involve the delocalisation of the lone pair to the pyran ring of the dye molecule. The dye molecule takes on a more planar conformation with respect to the ring because the orbitals are closer to sp^2 and the lone pair resides in a p-orbital. The overlap between the lone pair orbital and the π orbitals in the pyran ring determines the ability of the lone pair to delocalised; thus the sp^2 hybridisation is preferable for charge transfer to occur. The charge from the lone pair orbital is then free to migrate towards the acceptor cyano group on the pyran ring. This polar excited state is referred to as a charge transfer state. (*Taylor TR, 1986; Bourson J, 1989; Meyer M and Mialocq J, 1987*)

CHAPTER 2

LITERATURE SURVEY

2.1. Webster and W. McGolgin. Arylidene dye lasers, US patent 3,852,683, 1974:

DCM is a fluorescent dye developed by the Eastman-Kodak company, in the mid-1970s BY F. Webster and W. McGolgin. to extend the range of dye lasers into the red spectrum. Its spectral properties include a broad fluorescence band, long excited-state lifetime, and large solvent-dependent Stokes shift. DCM has been widely studied for its excited-state dynamics and molecular configuration. It is a donor- π -bridge-acceptor molecule, containing an electron-donating dimethylamino group and an electron-accepting dicyano group. Photoinduced intramolecular charge transfer is expected to occur in such systems, involving the migration of the excited free electron from the donor to the acceptor before relaxing back to the ground state. Research on DCM has focused on its energy, excited-state dynamics, and potential applications in sensing and imaging.

2.2. Absorption of DCM Dye in Ethanol: Experimental and Time Dependent Density Functional Study:

Seyed Hassan Nabavia, (2018) has studied Absorption of DCM Dye in Ethanol: Experimental and Time Dependent Density Functional Study in which he describes the synthesis of the DCM intermediate and DCM dye using a combination of malonitrile, 2,6-dimethyl-4H-pyran-4-one, (N, N-Dimethyl) benzaldehyde, toluene, piperidine, and acetic acid. The intermediate was obtained through refluxing, washing, and recrystallization, while the dye was produced by refluxing the intermediate under argon gas for 20 hours, followed by column chromatography for purification. The hydrogen NMR spectrum of the final DCM product is available in supporting information. A literature survey may explore the properties and uses of DCM dyes, the synthesis of pyran derivatives, and the potential applications of the intermediate in organic synthesis.

2.3. Fluorescence umpolung enables light-up sensing of N-acetyltransferases and nerve agents:

Chenxu Yan, (2021) discusses the recent advances in the development of Intramolecular Charge Transfer (ICT) based fluorophores along with tailoring their emission properties for high-fidelity bioimaging by investigating the use of high-performance donor- π -acceptor (D- π -A) fluorescent dyes, such as dicyanomethylene-4H-pyran (DCM), quinoline-malononitrile (QM), and so on, for biosensing and bioimaging applications. These fluorophores are known for their high sensitivity to electron disturbance and large Stokes shifts, making them promising platforms for developing fluorescent dyes and probes. However, the interaction of the donor receptor with electron-withdrawing targets (EWTs) can suppress the ICT pathway and quench the fluorescence, limiting their applicability. To address this issue, a molecular design strategy has been proposed wherein an indazole building block is inserted into the D- π -A motif to regulate intramolecular rotational driving energy. This strategy leads to a fluorescence umpolung effect, completely overturning the EWT-induced quenching mode into the light-up mode. The straightforward insertion of an indazole building block into the D- π -A fluorophores can substantially increase intramolecular rotation, resulting in a rotation-induced dark state, whereas the incorporation of EWTs can efficiently suppress rotation and enhance fluorescence. This strategy expands our understanding of ICT mechanisms and enables the design of ICT probes for light-up sensing of EWTs, such as N-acetyltransferases and nerve agents.

2.4. Photophysical and Electrochemical studies of 4-dicyanomethylene 2,6-dimethyl- 4H-pyran (DDP) dye with amides in water:

Somasundaram Gayathri, (2018) discussed that the DDP dye, also known as Dicyanomethylene-2,6-dimethyl-4H-pyran, which is an intermediate in the preparation of DCM dye was synthesized and studied the photophysical and electrochemical properties of DDP dye with formamide and alkyl-substituted amides in water were investigated. The addition of amides results in an isosbestic point and fluorescence enhancement of the DDP dye. The position of emission maxima of DDP dye shifted towards the blue and red regions with the addition of ACM and DMF, respectively. The fluorescence lifetime and relative amplitude of DDP dye vary significantly by the addition of amides in an aqueous solution, which is influenced by the amide water

hydrogen-bonding network and hydrophobic influences of the alkyl-substituted amides. The interaction between dye and amide is predominantly through hydrogen bonding. EIS results showed that there are at least three different microenvironments that support the existence of different fluorescence lifetimes of DDP dye. The fluorescence spectral technique is an efficient tool to elucidate the nature of the interaction of water-soluble probes with hydrogen-bonding solutes. Overall, the study provides insights into the photophysical and electrochemical properties of DDP dye and its interactions with different amides in aqueous solution.

2.5. Spectro electrochemical Investigation of 4-dicyanomethylene 2,6-dimethyl-4H-pyran (DDP) dye with Guanidine hydrochloride (GuHCl) in water:

Somasundaram Gayathri, (2018) investigates the interaction between guanidine hydrochloride (GuHCl) and 4-dicyanomethylene-2,6-dimethyl-4H-pyran (DDP) dye through photophysical and electrochemical techniques. The results indicate that the interaction between DDP dye and GuHCl is primarily through hydrogen bonding, although electrostatic interactions also play a role in the aqueous phase. The addition of amides to the aqueous solution affects the ground state and excited state properties of DDP dye, as evidenced by fluorescence enhancement and shift in the emission maximum. Hydrogen bonding and hydrophobicity of the amides are the main factors influencing this photophysical nature. The formation of a dye-water-amide hydrogen-bonding network along with dye-water and amide-water hydrogen-bonding assemblies is responsible for this effect.

2.6. Photophysical and Electrochemical Impedance Spectral studies on the interaction of 4-dicyanomethylene 2, 6-dimethyl- 4H-pyran (DDP) dye with urea derivatives in aqueous solution:

Somasundaram Gayathri, and Rajaraman Vasanthi, (2018) performed studied the interaction between urea derivatives and DDP dye using photophysical and electrochemical techniques. The addition of urea derivatives to DDP dye resulted in fluorescence enhancement and a shift in emission wavelength. Fluorescence lifetime studies showed the coexistence of three different components, indicating a heterogeneous microenvironment around the dye. The urea derivatives influenced the excited state characteristics of the dye, resulting in the formation of different

microenvironments. Electrochemical impedance spectral studies validated the existence of multiple environments attributed to urea-water structural behaviour. The urea derivatives' hydrophobicity and hydrogen-bonding properties influenced the dye's photophysical and electrochemical nature.

2.7. Characterization of the toxic mechanism of two fluoroquinolones to trypsin by spectroscopic and computational methods:

Yanxiu Guo, (2019) highlights the quenching of intrinsic fluorescence of trypsin by fluoroquinolones (FQs) ciprofloxacin (CPFX) and enrofloxacin (ENFX) through moderately strong complexes formed via van der Waals forces and hydrogen bonds. The binding of FQs caused conformational and micro-environmental changes in trypsin, altering its molecular activity, which was observed through multiple spectroscopic methods and functional tests.

2.8. Correlation of Tryptophan Fluorescence Spectral Shifts and Lifetimes Arising Directly from Heterogeneous Environment:

Chia-Pin Pan, (2011) investigated the use of tryptophan (Trp) fluorescence as a tool for studying protein conformational ensembles in solution. The interpretation of wavelength-dependent complex fluorescence decays of proteins is controversial due to multiple plausible origins of the typical multiple fluorescence lifetimes. The author demonstrates how multiple conformational populations can result in the same strong wavelength-lifetime correlation in cyclic hexapeptides containing a single Trp residue in this study. The quenching of fluorescence in these peptides is caused by electron transfer. The author calculated fluorescence wavelengths and lifetimes for hexapeptides using simulations and discovered that rotamers with blue-shifted emission spectra have a higher probability of quenching during fluctuations in the environment.

CHAPTER 3

AIM AND SCOPE

3.1. AIM:

To investigate fluorescence spectral studies on the interaction of trypsin with 4-dicyanomethylene-2,6-dimethyl-4h-pyran (DDP) dye in an aqueous solution.

3.2. SCOPE:

Our study focuses on Fluorescence spectral studies in a micro heterogeneous environment to investigate the behaviour of Host and Guest molecules. These studies aim to comprehend how the microenvironment influences the photophysical properties such as absorption, emission, and fluorescence lifetime of an Intramolecular charge transfer (ICT) based 4- Dicyanomethylene-2,6-dimethyl-4H-pyran (DDP) dye with the addition of trypsin. The presence of both hydrophilic and hydrophobic moieties in the solutes behaves as an ideal host molecule. The majority of the research in the literature on the probe–host interactions took place in non–aqueous and organic solvents, but in the present investigation, the photophysical studies of these probes are carried out in the water, which is considered of biological importance.

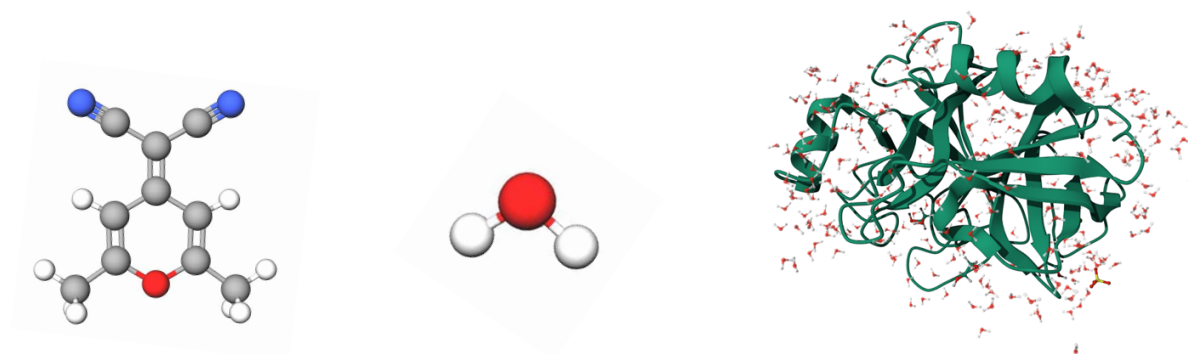


Fig.3.2. Interaction of Trypsin with DDP dye in water

3.2.1. The objective of the study:

Fluorescence spectral analyses of the interaction of trypsin with DDP dye in an aqueous solution have the following objectives:

- Absorption spectroscopy to ascertain the mode of interaction between trypsin and the DDP dye.
- Investigate how trypsin affects the DDP dye's micro-heterogeneous environment and learn more about the nature of the interaction through emission spectroscopy.
- Examine and analyse the quenching mechanism of the DDP dye by trypsin using the Stern-Volmer method.
- Using lifetime analysis, determine the fluorescence lifetime of the DDP dye in the presence and absence of trypsin and learn about the interaction mechanism.
- Provide a comprehensive understanding of the interaction between trypsin and the DDP dye in an aqueous solution using multiple fluorescence spectroscopic techniques, as well as develop and apply new methodologies and advancements for studying guest-host interactions.

CHAPTER 4

MATERIALS AND METHODS

4.1. MATERIALS:

2,6-dimethyl-4-pyrone; malononitrile; acetic anhydride and trypsin Powder, the porcine pancreas was purchased from Sigma-Aldrich are the chemicals used in the study. Ultrapure water was used throughout the experiments.

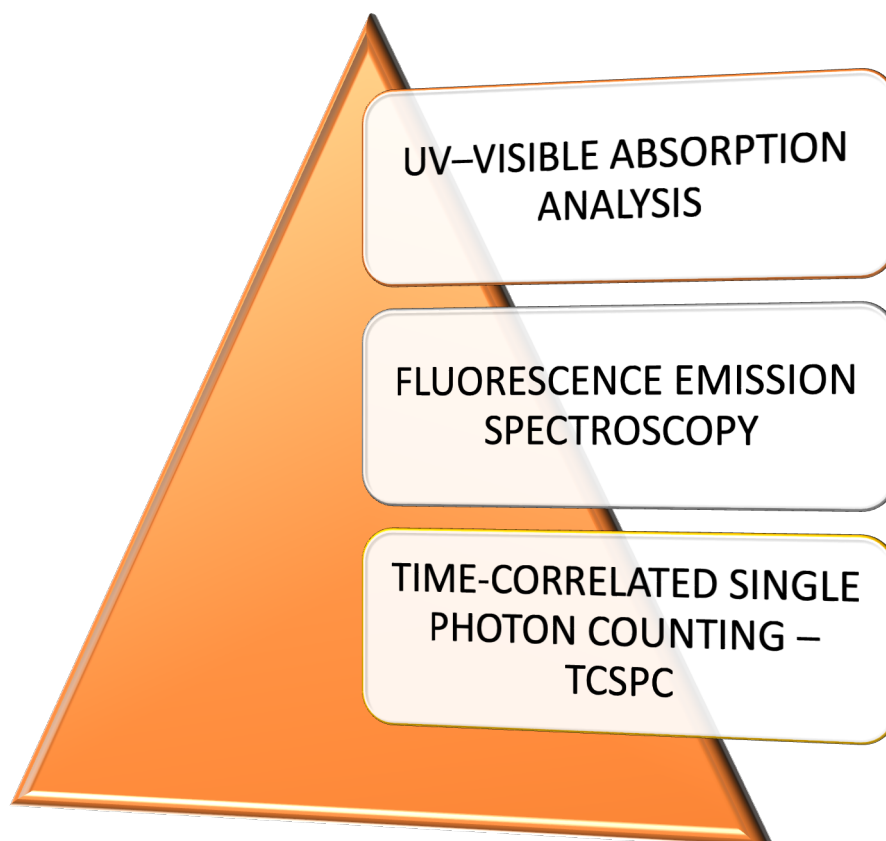
4.2. METHODS:

4.2.1.1 PREPARATION OF DDP DYE AND TRYPSIN SOLUTIONS FOR SPECTRAL STUDIES:

Trypsin was dissolved in ultra-pure water to form a 5×10^{-5} mol L⁻¹ solution and then preserved at -20°C; different concentrations of trypsin (2.0 M, 4.0 M, 6.0 M, and 8.0 M) were prepared where the concentration of DDP dye (8×10^{-5} mol L⁻¹) was fixed for steady-state absorption, emission and time-resolved fluorescence spectral studies.

4.2.2. INSTRUMENTAL TECHNIQUES:

The study employed spectroscopic methods to examine the interaction between DDP dye (the host molecule) and trypsin (the guest molecule).



4.2.2.1. UV-VISIBLE ABSORPTION ANALYSIS:

UV-VIS spectroscopy is based on the measurement of light absorbance across the ultraviolet (UV) and visible (VIS) regions of the electromagnetic spectrum with wavelength ranges of 200-800 nm. Incident light hits matter which can either be absorbed, reflected, or transmitted. The absorbance of radiation in the UV-Vis range causes atomic excitation, which refers to the transition of molecules from a low-energy ground state to an excited state.

The working principle of UV spectroscopy is based on the Beer-Lambert principle. When a monochromatic light beam passes through a solution of an absorbing material,

the rate at which the intensity of the radiation decreases with the thickness of the solution is proportional to both the incident radiation and the concentration of the solution.

$$A = \log(I_0/I) = \epsilon cl$$

A – Absorbance; I_0 - Intensity of incident light; I - Intensity of emission of light from the sample; ϵ – Molar extinction coefficient (measure of probability of electronic transition); c – Concentration of absorbing species; l – Distance covered by the light through the solution

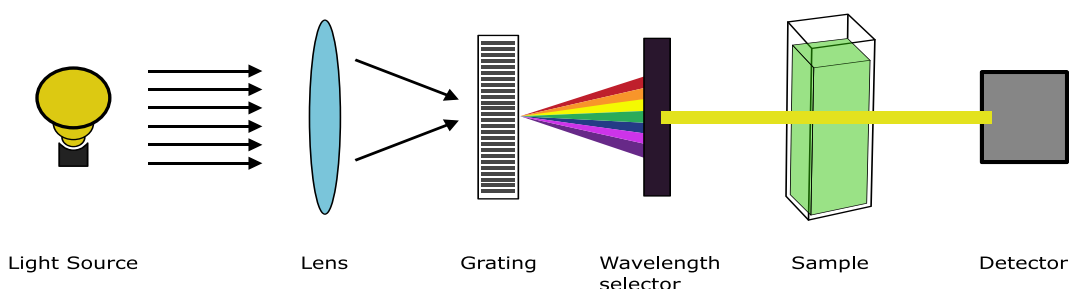


Fig.4.2.2.1.A. Components of UV Spectrophotometer

When a sample is placed in a spectrophotometer, it absorbs light at specific wavelengths determined by its chemical composition and structure. The detector measures the remaining light as it passes through the sample. The detector converts the intensity of the light into an electrical signal, which is then displayed on the screen as an absorbance versus wavelength spectrum.



Fig.4.2.2.1.B. UV-VIS-1900I Spectrophotometer

(Department of Chemistry, Sathyabama Institute of Science and Technology)

4.2.2.2. FLUORESCENCE EMISSION SPECTROSCOPY:

Fluorescence emission spectroscopy is a potent analytical method that's frequently used for investigating a sample's electronic and molecular characteristics. It is based on the fluorescence phenomenon, which is when a molecule emits light after absorbing light of a particular wavelength.

When electromagnetic radiation interacts with matter's atoms and molecules, the electrons contained within these atoms may absorb energy and jump to a higher energy state, losing their stability. The excited electron is unstable in this higher energy state and rapidly loses its energy by vibrating with the nearby molecules, a process known as vibrational relaxation. A photon of light with a longer wavelength than the absorbed light is released as a result of this energy loss, which causes the excited electron to transition to a lower energy state. This spectrum of radiation emitted by electrons in excited atoms or molecules is known as the emission spectrum.



Fig.4.2.2.2. F-7100 FL Spectrophotometer

(International Research Centre (IRC), Sathyabama Institute of Science and Technology)

4.2.2.3. TIME-CORRELATED SINGLE PHOTON COUNTING – TCSPC:

TCSPC stands for time-correlated single photon counting is a technique used to measure the time-of-flight of photons emitted by a sample. It is a method of using the timing of a pulsed excitation source, like a laser or LED, with the timing of the arrival of single photons on a detector to reconstruct the lifetime decay over many events (repetition of pulses and photons detected). This technique is based on the fact that the probability of detecting a single photon

at time, t after an exciting pulse is proportional to the fluorescence intensity at time t .

The repetition of a laser or LED pulsed at a relatively high repetition rate (10 kHz to 100 MHz) is synchronized with the time the next photon arrives at a detector (i.e. PMT). Timing electronics in the form of a time-to-digital converter or time-to-amplitude converter (TAC) are used to record these events in succession until sufficient statistics are collected to reconstruct the decay. The decay is then fit to an exponential function to model the lifetime decay (τ). TCSPC is typically used to measure fluorescence lifetimes from the picosecond to the microsecond time scale.



***Fig.4.2.2.3. Time-Related Single Photon Counting (TCSPC) [Fluorolog – 3]
(Department of Medical Physics, Anna University)***

CHAPTER 5

RESULTS AND DISCUSSION

5.1. UV–VISIBLE ABSORPTION ANALYSIS:

The UV–Visible absorption spectra of DDP dye with the addition of Trypsin at different concentrations were investigated by a UV–Vis-1900i spectrophotometer (Shimadzu, Japan) at the Department of Chemistry in Sathyabama Institute of Science and Technology, which was equipped with 1cm quartz and performed from 180nm to 500nm.

DDP dye exhibits absorption peaks at 248 ± 2 nm and 348 ± 2 nm accompanied by a shoulder around 360 nm in water as shown in Fig 5.1.1. The absorption peaks at 248 ± 2 nm and 348 ± 2 nm are attributed to $n \rightarrow \pi^*$ and charge transfer (CT) transitions respectively. (Fig.5.1.1).

The addition of Trypsin (10^{-5} M to 1.0 M) results in no significant change in the absorbance at the longest wavelength of DDP dye. The absorbance at the ICT absorption maximum remains unaltered even in the presence of more trypsin molecules surrounding the close vicinity of the dye but there is a decrease in the intensity of absorption peak upon the addition of trypsin. (Fig.5.1.2)

The results indicate that the addition of trypsin to DDP dye decreased the dye concentration but had no effect on the dye's absorption properties or ICT process. These experiments shed light on the interaction of trypsin and dyes, which could be useful in a variety of applications.

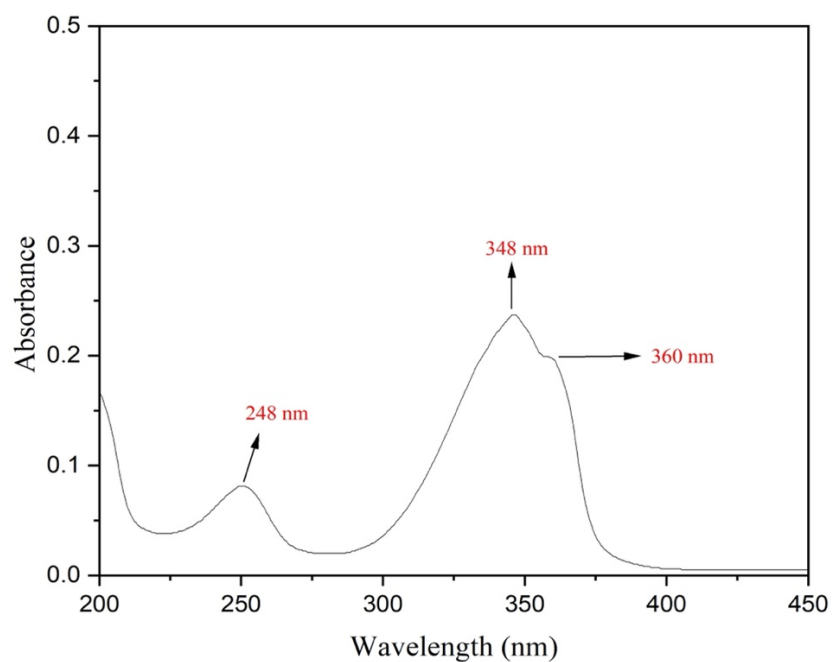


Fig.5.1.1. Absorption spectra of DCM dye (8×10^{-5} M) in water.

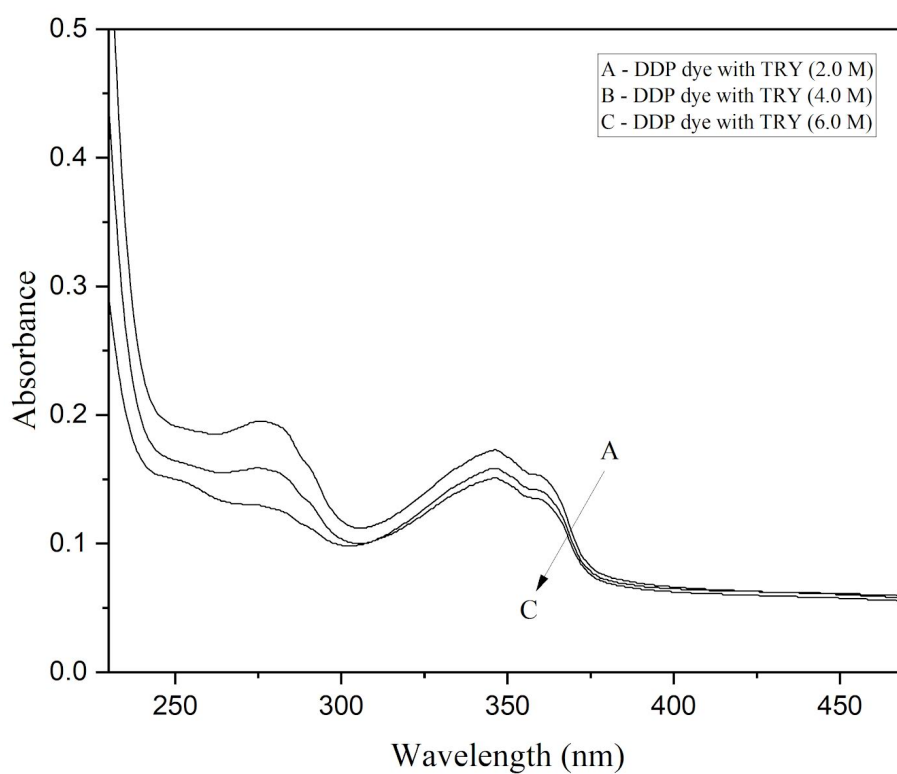


Fig.5.1.2. Absorption spectra of DDP dye (8×10^{-5} M) with varying concentrations of Trypsin in water. The concentration of Trypsin: 1) 2.0 M, 2) 4.0 M, 3) 6.0 M

The absorption spectrum could not completely resolve the authenticity of the nature of the interaction between dyes and trypsin. To determine the precise nature of the interaction between dye and trypsin, steady-state and time-resolved fluorescence spectral studies were used to characterise the excited state properties of dye in the absence and presence of Trypsin.

5.2. FLUORESCENCE SPECTRA ANALYSIS:

All fluorescence spectra were recorded on an F-7100 FL Spectrophotometer (Hitachi, Japan) at the International Research Centre (IRC) in Sathyabama Institute of Science and Technology, equipped with a 150W Xenon lamp source, and a 10mm quartz cell. The solution was moved to a quartz cell for analysis, and the widths of the emission and excitation slits were both set at 10.0 nm. The fluorescence emission spectra varied from 390 to 600nm, while the wavelength of excitation was chosen to be 370 nm.

The fluorescence spectrum of DDP dye in water exhibits a broad peak at approximately 436 ± 2 nm when excited at the longest wavelength absorption maximum, and this peak is assigned to the LE state emission. fig.5.2.A. Spectroscopy is an effective and efficient way to investigate the conformational changes in proteins, as it allows for non-intrusive measurements of substances at low concentrations under simulated physiological conditions. Trypsin has intrinsic fluorescence primarily due to the tryptophan (Trp) and tyrosine (Tyr) residues, which are highly sensitive to their local environment. Upon adding trypsin to DDP dye, the fluorescence intensity gradually decreases without any new emission band appearing, even at the highest concentration of trypsin (6×10^{-5} M), as shown in fig.5.2.B. This observation rules out the formation of an exciplex as an intermediate. It is presumed that trypsin orients between the donor and acceptor moiety of DDP dye, resulting in a large variation in the microenvironment around the dye's close vicinity, leading to a quenching of the fluorescence intensity.

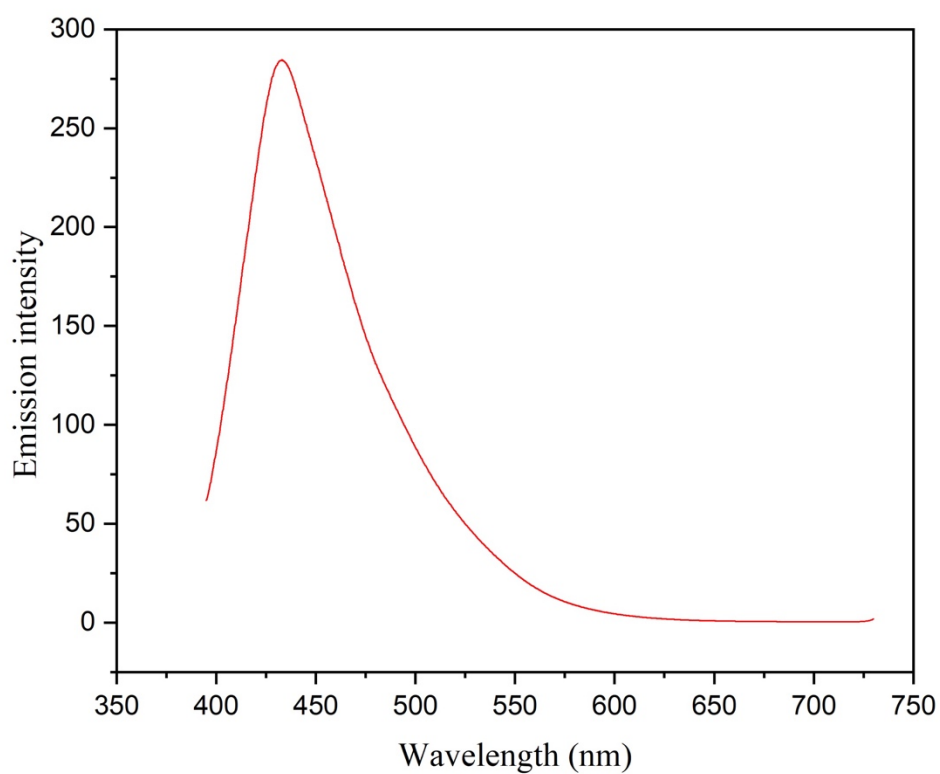


Fig.5.2.A. Emission spectra of DDP dye ($8 \times 10^{-5} \text{ M}$) in Water.

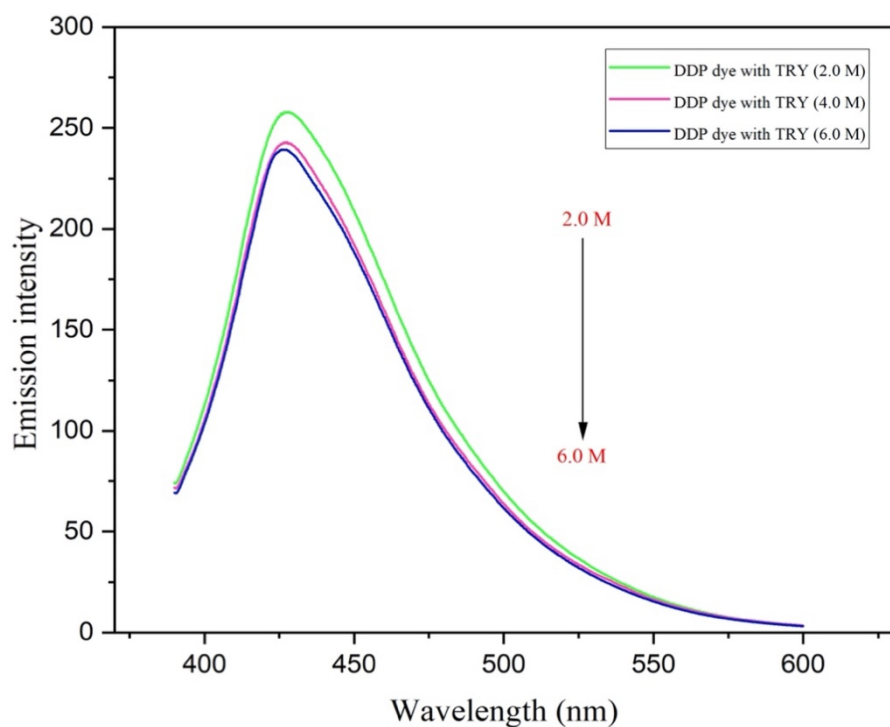


Fig.5.1.B. Emission spectra of DDP dye ($8 \times 10^{-5} \text{ M}$) with varying concentrations of Trypsin in water. The concentration of Trypsin: 1) 2.0 M, 2) 4.0 M, 3) 6.0 M

5.2.1. FLUORESCENCE QUENCHING MECHANISM:

Fluorescence quenching refers to the decrease in intensity of fluorescence emission from a fluorophore due to various processes. There are three main categories of quenching mechanisms: dynamic quenching caused by collisions with other molecules, static quenching resulting from the formation of ground-state complexes between the fluorophore and quencher, or a combination of both. Collisional quenching occurs when the excited-state fluorophore loses its energy through non-radiative pathways upon contact with other molecules in the solution. This process is not chemically altering the fluorophore or the quencher. Non-FRET quenching refers to the decrease in fluorescence emission that is not due to fluorescence resonance energy transfer (FRET), but rather to other mechanisms such as collisional quenching. For collisional quenching, the decrease in intensity is described by the well-known Stern-Volmer equation:

$$\frac{I_0}{I_n} = 1 + K_q \cdot \tau_0 \cdot [Q]$$

The diagram illustrates the Stern-Volmer equation with the following labels and arrows:

- Measured in absence of [Q]**: Points to I_0 in the numerator.
- Measured for different [Q]**: Points to I_n in the denominator.
- Lifetime of the excited state**: Points to τ_0 .
- Concentration of the quencher**: Points to $[Q]$.
- quencher rate coefficient**: Points to K_q .

The Stern-Volmer plot is a graphical representation of the Stern-Volmer equation, where the fluorescence intensity ratio I_0/I_n is plotted against the concentration of a quencher molecule. The plot usually forms a straight line, with the slope representing the Stern-Volmer constant (K_{SV}) and the y-intercept equal to or less than 1. By analysing the slope of the Stern-Volmer plot, I_0/I_n versus $[Q]$, it is possible to determine the quenching mechanism and obtain the Stern-Volmer quenching constant, which is a parameter used to describe the efficiency of fluorescence quenching by a quencher, such as a protein (trypsin), providing insight into the mechanism of quenching.

In the case of trypsin and DDP dye, non-FRET such as collisional quenching occurs due to the hydrolysis of the dye molecule by trypsin, leading to a change in its electronic structure, resulting in the loss of its fluorescence properties, and hence the decrease in fluorescence emission.

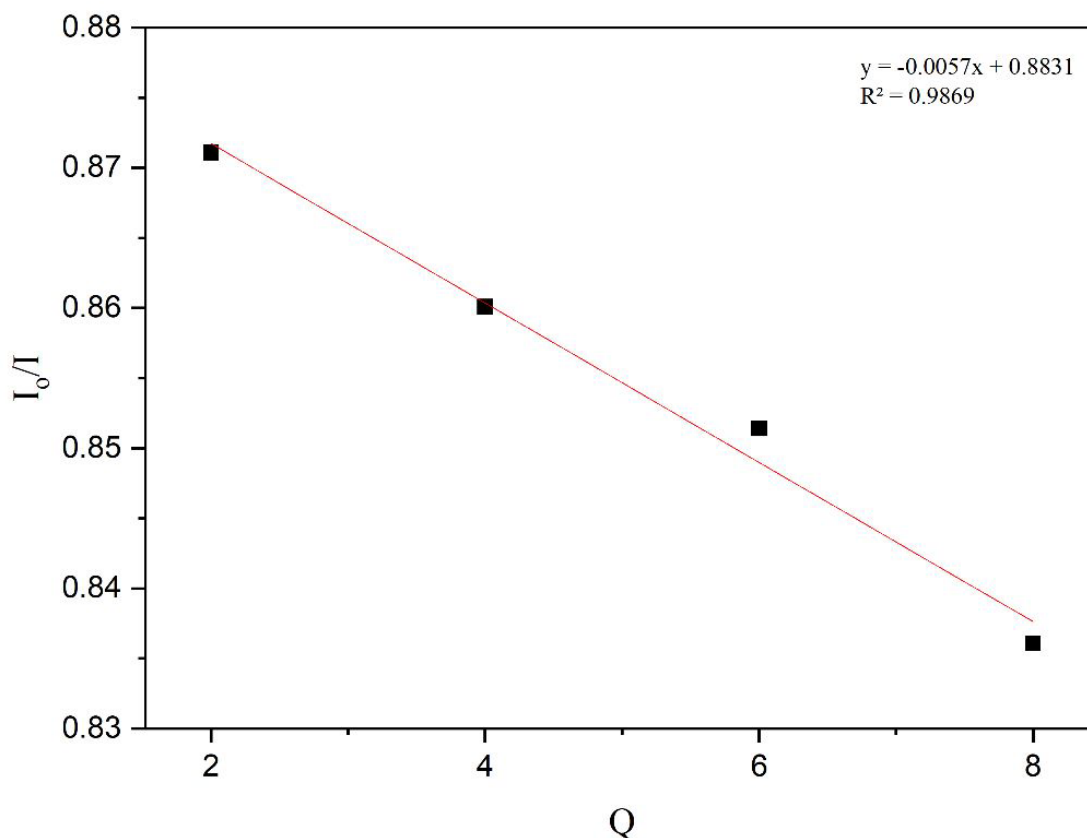


Fig. 5.2.1. Extent of fluorescence quenching of DDP dye on the addition of Trypsin.

5.3. TIME-RESOLVED FLUORESCENCE STUDIES:

Time-resolved fluorescence spectra of DDP dye in the presence and absence of trypsin at different concentrations were analysed by Fluorolog-3 at the *Department of Medical Physics at Anna University with λ_{ex} 370 nm and λ_{em} 436 nm.*

The fluorescence decay of DDP dye exhibits a triexponential lifetime in an aqueous solution. A short lifetime component of 0.3 ns accompanied by a relatively long lifetime component coexisting in an aqueous solution at any concentration of the trypsin is observed.

The addition of trypsin results in a decrease and increase in the three-lifetime components irrespective of the concentration of the trypsin used. The fluorescence decay DDP dye with trypsin is shown in Fig.5.3.B. and the fluorescence lifetime is provided in Table.5.3.

All the three-lifetime components coexist in the aqueous phase even in the absence of trypsin. On the addition of trypsin, there is no abrupt change in the relative amplitude distribution of these components but a significant decrease (6.75,4.01,3.34,1.04) in the fluorescence lifetime of one component and an increase (3.12,3.31,4.1,6.0) in the fluorescence lifetime of another component.

The fluorescence lifetime component of ≈ 0.3 ns of DDP dye exists in solution even in the presence of a high concentration of Trypsin. The existence of the small nanosecond component of DDP dye clearly reveals that the dye is predominantly surrounded by water molecules in its close proximity even in the presence of trypsin.

The various hydrogen-bonding arrangement of dye with water molecules in the presence of trypsin coexist in the aqueous phase with large variations in their distribution supporting the existence of the multi-exponential lifetime components of dye.

The decrease and increase in the fluorescence lifetime component of DDP dye are attributed to the hydrogen-bonding interaction of DDP dye by trypsin.

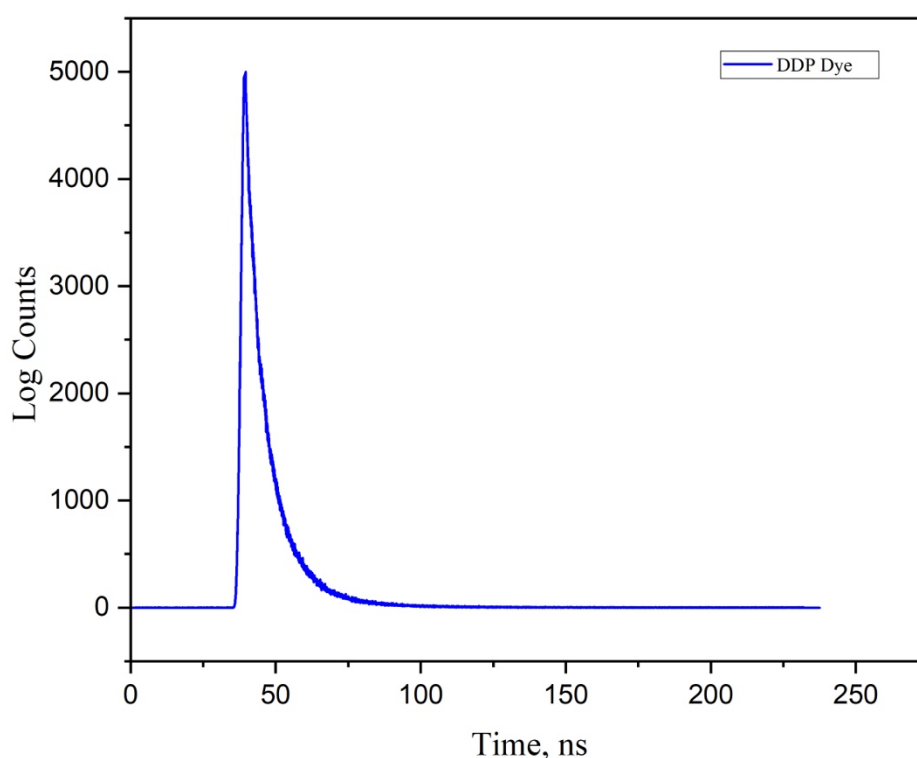


Fig.5.3. (A)

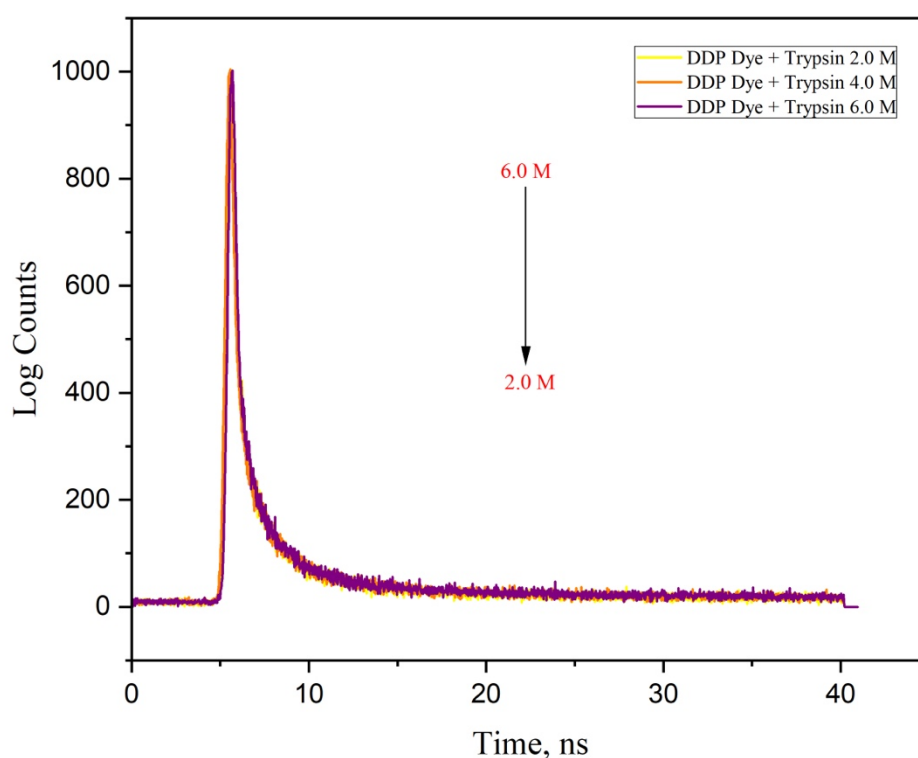


Fig.5.3. (B)

Fig.5.3. Fluorescence decay of DDP dye ($8 \times 10^{-5} \text{ M}$) as a function of TRYPSIN in water. λ_{ex} 370 nm and λ_{em} 436 nm. (A) DDP dye alone; (B) i) DDP dye + Trypsin 2.0M, ii) DDP dye+ Trypsin 4.0M, iii) DDP dye + Trypsin 6.0 M.

Table.5.3. Fluorescence lifetime decay analysis of DDP dye with Trypsin in water

S.NO	[C]	τ_1	τ_2	τ_3	B	B	B	χ^2
1	0M	0.31	3.12	6.75	6	64	30	1.16
2	2M	0.4	3.31	4.01	37	29	33	1.1
3	4M	0.3	4.1	3.34	8	68	24	1.6
4	6M	0.4	6.0	1.04	12	58	30	1.23

5.4. TRYPSIN DDP-DYE HYDROGEN BONDING IN THE PRESENCE OF WATER MOLECULES:

The Role of Hydrogen Bonding in the Interaction of Trypsin and DDP Dye in Aqueous Solutions in Different Phases:

In the shell phase, trypsin and DDP dye can form a well-organised hydrogen-bonding network with water molecules. The water molecules in the shell phase can surround the dye, and the hydrogen atoms of the water molecules can form hydrogen bonds with the electronegative atoms in the DDP dye and trypsin. In this phase, the microenvironment of the dye is mainly influenced by the surrounding water molecules.

In the intermediate phase, the dye is surrounded by fewer trypsin molecules in the aqueous solution. In this phase, hydrogen bonds between the dye and trypsin may form via trypsin's amino acid residues. The dye's microenvironment in this phase is influenced by both the surrounding water molecules and the trypsin residues.

In the bulk phase, the dye is surrounded by more trypsin molecules in the aqueous solution. The formation of hydrogen bonds between the trypsin molecules can influence the hydrogen bonding between the dye and trypsin during this phase. The dye's microenvironment in this phase is primarily influenced by the surrounding trypsin molecules, but the presence of water molecules can also contribute to the hydrogen bonding interactions between the dye and trypsin.

CHAPTER 6

SUMMARY AND CONCLUSION

The study investigated the interaction between trypsin and the DDP dye in an aqueous solution through fluorescence spectral analysis. The absorption peak for the DDP dye was found at 248 ± 2 nm, 348 ± 2 nm, and 360 nm, and on adding trypsin at different concentrations, the intensity peak decreased, while the absorbance at the ICT absorption maximum remained unaltered. This decrease was attributed to the promotion of an intramolecular charge transfer process between the donor and acceptor moieties of the dye ring structure.

Additionally, the fluorescence emission peak was found at 436 ± 2 nm, and the fluorescence quenching of the DDP dye was identified using Stern Volmer analysis. Furthermore, the fluorescence decay of the DDP dye in the presence of trypsin showed a tri-exponential decay, indicating the presence of varying microenvironments around the dye with different fluorescence lifetimes and distributions. Specifically, one component exhibited a significant decrease in fluorescence lifetime, while another showed an increase, and the relative amplitude distribution remained unaltered. This suggests that the dye is located in a heterogeneous environment that causes variations in fluorescence lifetime and distribution.

Overall, the interaction of the DDP dye with trypsin was largely hydrophobic, but the presence of hydrogen-bonding functional groups in trypsin influenced the excited-state spectral properties of the DDP dye.

6.1. FUTURE DIRECTIONS:

There are several potential future directions for fluorescence spectral studies on the interaction of trypsin with DDP dye in an aqueous solution. One possible direction is to perform molecular modelling and simulations to better understand the structural and dynamic aspects of the interaction between trypsin and DDP dye. This could involve using computational methods to explore how changes in the micro-environment.

Quantum chemical calculations could also be used to investigate the electronic structure and excited state properties of the DDP dye in the presence of trypsin. These

calculations could help to elucidate the underlying mechanisms of fluorescence quenching observed in the current study. Additionally, such calculations could assist in the design of new probes with improved photophysical properties for use in biosensors or laser applications.

Furthermore, it could be interesting to study the interaction between DDP dye and other proteins or enzymes to determine photophysical properties or are more broadly applicable. This could involve using fluorescence spectral techniques to monitor changes in the dye's emission spectra or fluorescence lifetime in the micro-environment presence of different molecular aggregates components.

Overall, further investigations using advanced computational methods and a broader range of biological targets could help to expand our understanding of the interaction between DDP dye and biomolecules, with potential applications in biosensors and other optical technologies.

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