Synthesis of Bis-coumarin Based Probe for the Detection of Serum Albumin

A

Dissertation report submitted

in partial fulfilment of the requirement of the degree of

MASTER OF SCIENCE

IN

CHEMISTRY

BY

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July, 2025

DECLARATION

I, hereby declare that the dissertation entitled "**Synthesis of Bis-coumarin Based Probe for the Detection of Serum Albumin"** is a research work done by me in the partial fulfilment of the requirement for the award of the degree of Masters of Science in Chemistry, submitted in the Department of Chemistry and Biochemistry, Thapar Institute of Engineering and Technology, Patiala, under the supervision of **Prof. Kamaldeep Paul** from Jan – July, 2025.

All the Ideas and sources have been properly acknowledged. Further, any work of this dissertation has not been submitted to any other University for the award of any other degree or diploma.

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CERTIFICATE

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This is to certify the above statement made by student cornered is correct and true to the best of my knowledge.

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Acknowledgement

Firstly, I would like to express my heartfelt gratitude towards the supreme almighty for keeping me in hollow of his hand, providing me nourishment and comfort during my research. I am thankful to **Dr. Manmohan Chhibber**, Professor and Head, Department of Chemistry and Biochemistry for giving me this opportunity to work and allowed me to use various facilities in the department.

I am highly gratified for the inspiration and guidance of **Prof. Kamaldeep Paul**. We had a pragmatic discussion over my interest and lab specification after that we decided the topic for my project work. From basic amenities to guidance, they were a great help throughout my research. I am beyond thankful for their guidance and support. Heart-warming thanks to **Palak Sharma**, for their diligent presence throughout my research. She listened to me and taught me how to work in lab in a very soothing way. I am pleased that he corrected me whenever I committed any mistake during this project work. His friendly nature helped me to complete my project smoothly. Our interaction has proved to be of great assistance.

Working with **Dr. Sourabh Gupta**, **Dr. Rekha Thakur, Dr. Rohini Gupta, Palak Sharma, Shifali, Sonia Wadhwa, Anmol Jain, Tanya, Priya Thakur, Sanchi Sharma, Sahil Rana Vikash Sharma,** and **Rahul Rana** have been a positive experience for me.

In the presence of this project, there has been an important role of my father **Mr. Jai Parkash**, my mother **Mrs. Anju.** I am thankful to them who have showered unconditional love on me, encouraged and supported in every aspect.

Abstract

Bis-coumarin based probe has been synthesized which provides insight into the differential response on interaction with different bioanalytes. Photophysical studies has been performed to detect the bioanalytes and it has been observed that probe **5** selectively detects the serum albumin i.e., BSA and HSA with more sensitivity towards HSA. On gradual addition of HSA (62 μM), 74% quenching has been obtained while in case of BSA (62 μM) 60% quenching was observed. Further, binding constants have been calculated to demonstrate the effective binding between the synthesized probe **5** and the serum albumins and found to be 7.4×10^5 M^{-1} in case of HSA while 6.7×10^5 M^{-1} for BSA. Moreover, site marker study has also been carried out to find out the specific binding site of the probe **5** in HSA which revealed the warfarin like binding of the probe **5** i.e., Suldow's site I.

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Chapter 1
Introduction and Review of Literature

1.1 Introduction

Heterocyclic moieties are among the most common structural units found in various natural and synthetic bioactive compounds.[1] Over the years, there has been significant growth in research focused on synthesizing a variety of heterocyclic molecules for different biological activities.[2] Numerous studies in biochemistry, pharmaceutical chemistry, and cancer therapy have been dedicated to exploring the interactions between heterocyclic molecules and proteins, such as serum albumins, DNA, RNA, and among others.

Serum albumins are proteins found in biological systems that serve various physiological roles. They act as carrier proteins for various endogenous and exogenous molecules, including hormones, fatty acids and several medications.[3] Albumins comprise more than 60% of the total plasma protein content, with an approximate concentration range of 500–700 μM.[4,5] Serum albumin has a secondary structure shaped like a helical heart, consisting of three domains (I, II, and III) that are divided into two subdomains (A and B), forming molecular binding pockets. Sudlow sites I and II are primary drug binding sites located in subdomains IIA and IIIA, respectively, with a recently discovered third site in subdomain IB.[6,7] Site I primarily binds drugs through hydrophobic interactions, while site II employs both electrostatic and hydrophobic interactions. The unique arrangement and hydrophobicity of these binding sites lead to a specific affinity for different ligands. Site II, prefers ibuprofen like candidates for binding, whereas site I prefers warfarin and indomethacin like bulky heterocyclic compounds.[8] Therefore, the binding between serum albumin and ligand molecules or drugs is crucial, as it significantly impacts the distribution and bioavailability of drug candidates. This interaction is influenced by pharmacokinetic properties of the drug, which include targeting specificity, efficacy, half-life in the bloodstream, and toxicity.[9,10]

Among albumins, bovine serum albumin (BSA) and human serum albumin (HSA) have structural similarities of about 76%. However, HSA is a predominant protein in human blood plasma, known for its versatile binding capabilities, making it an attractive option for delivering therapeutic agents to targeted areas within the body.[11-13] Additionally, HSA serves as an important biomarker for various diseases, such as cancer and rheumatoid arthritis, as well as several medical conditions like acute liver failure, shock, trauma, and hypoalbuminemia.[14] Understanding how drugs interact with HSA is essential for grasping their effects on the human

body. This knowledge helps explain the differences in affinity among ligands that target the same protein or bind to other proteins. Such insights are crucial for drug development and optimization, as they provide researchers with valuable information about the pharmacokinetics and pharmacodynamics of drugs.[15,16]

Coumarin core have recently been found to be an interesting area of research in synthetic and medicinal chemistry. Coumarin has been extensively studied due to its molecular structure, which plays a significant role in drug development and possess a wide range of pharmacological properties, including anticoagulant, antimicrobial, anti-inflammatory, antidiabetic, anticancer, anticonvulsant, and antiproliferative activities.[17] This molecule found useful in medicinal chemistry due to its efficient protein-ligand interaction. π-Extended heterocycle-fused coumarins have drawn interest because of their remarkable photophysical characteristics, which include high fluorescence quantum yield, substantial Stokes shift, and strong light absorption. Despite their biological importance, they are known for their luminescent properties owing to their intrinsic charge transfer properties.[18,19] It shows photophysical and pharmacokinetic characteristics when a heterocycle is added to a coumarin unit. They are therefore frequently utilized as parts of molecules, which consistently have excellent optical characteristics such as high fluorescence quantum yield, visible excitation, and emission wavelengths, and strong photostability.[20-22] Among the various heterocycle-fused coumarins, bis-coumarins have recently drawn significant interest due to their photophysical properties. Compared to traditional coumarins, these bis-coumarins feature a larger π-conjugated planar system, giving them several advantages such as red-shifted excitation and emission wavelengths, larger Stokes shifts, and excellent fluorescence.[23-25] These qualities make them effective candidates, with promising applications in environmental monitoring and biosensing. There has been limited research in this area, prompting us to develop coumarin-based components to aid in detection of serum albumins.[26-29]

1.2 Review of Literature

Most of the fluorescent probes containing bioactive heterocyclic moieties were reported over the past few years. These play essential role in biosensing, transportation, drug delivery and bioimaging**.** Numerous drugs have been developed and marketed for their various properties.

Gupta *et. al* developed a fluorescent "Turn-On" probe based on benzimidazole-malononitrile **1** that distinguish between two structurally similar proteins, BSA and HSA. Each protein to which the probe interacts showed a different fluorescence response; notably, the

probe's sensitivity and emission enhancement are higher in the presence of BSA. According to mechanistic analyses, including molecular docking, FT-IR, FE-SEM, DLS, and lifetime fluorescence measurements, the contact causes probe aggregates to disassemble and encapsulated within the serum albumin cavities. In identifying HSA in human urine samples, **1** exhibited significant binding affinity, selectivity, and practical applicability, highlighting its robust potential for biomedical and diagnostic applications, especially in the identification of albumin-related diseases.[30]

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Yang *et. al.* introduced probe **2** which is sensitive and specific to human serum albumin (HSA). Fluorescent probe tetraphenylethylene-indole (TPE-indo) **2** is a new and efficient fluorophore. Using the aggregation-induced emission (AIE) mechanism, TPE-indo bound in a 1:1 molar ratio with HSA and provided a strong "turn-on" fluorescence response. Because of the little interference from other biological molecules in this interaction, HSA might be accurately measured in complex matrices like serum and urine. With a linear detection range of 60 μg/mL and a low detection limit of 0.30 μg/mL, the fluorophore is appropriate for both clinical diagnostics and pharmaceutical quality control. TPE-indo exhibited significant selectivity for HSA over BSA, which was explained by their distinct binding interactions and allowed for the detection of BSA adulteration in HSA preparations. TPE-indo's application in clinical diagnostics was further highlighted as it permitted real-time monitoring of HSA degradation and proteinuria grading.[31]

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Cao *et. al.* developed Rho-A hydrogen bond-driven fluorescence sensor **3**, which was found sensitive and selective for detecting human serum albumin (HSA). After attaching to

HSA through a particular N-H···X hydrogen bonding inside HSA's cavity. Rho-A, having rhodanine fluorophore and *N,N*-dimethylaniline donor, demonstrated a disaggregation-induced emission (DIE) mechanism. Extensive analytical methods validated the binding mechanism and interaction. The sensor displayed high sensitivity (LOD: 0.452 mg/L), quick reaction time (10 seconds), high HSA selectivity as compared to BSA, stability in physiological settings, and resistance to ions and other biomolecules. Rho-A's potential for clinical diagnostics and bioimaging was demonstrated by its successful applications in detecting HSA in urine samples and fluorescence imaging in HepG-2 cancer cells.[32]

3 **4**

A new hydrazide Schiff base fluorescent probe **4**, was successfully synthesized by Wang *et. al.* The structural variation at FA1 binding site allowed this probe to self-assemble into nanoparticles in aqueous conditions and showed great selectivity and sensitivity for human serum albumin (HSA) in comparison to related proteins, including bovine serum albumin (BSA). The precise binding of **4** to HSA was confirmed by molecular docking studies and displacement experiments. Because it is non-toxic and appropriate for cellular imaging, probe **4** was successfully located HSA in mitochondria. Its pH-responsive fluorescence made it possible to distinguish between healthy and malignant cells. Additionally, the **4**-HSA complex might be used to measure hemin levels and is a cisplatin carrier, drug delivery. Therefore, probe **4** showed promising results in fluorescent labelling, imaging, and drug delivery method utilizing HSA, with important biomedical applications in both diagnostics and treatment.[33]

A bifunctional fluorescent probe, 5-(triphenylamine)rhodanine-3-acetic acid (mRA), was synthesized by Chinnappan *et al.* and employed as a sensing probe for human serum albumin (HSA). Upon binding to HSA, the molecule exhibited enhanced emission through a twisted intramolecular charge transfer (TICT) mechanism, resulting in a distinct and detectable fluorescence "turn-on" response. Molecular docking studies revealed that the probe interacted selectively with specific albumin-binding sites, which accounts for its high specificity. These computational insights corroborated the experimental fluorescence enhancement by elucidating the underlying molecular-level interaction mechanisms between

mRA and HSA. The binding induced conformational restriction in the mRA molecule, triggering its fluorescence activation. mRA exhibited a strong, dose-dependent fluorescence response to HSA across a wide concentration range (0.01–400 μg/mL). Remarkably, the system achieved a low detection limit of 10 ng/mL, making it suitable for detecting HSA levels even in complex biological fluids.[34]

Cao *et al.* designed and synthesized a highly selective and sensitive "turn-on" fluorescent probe, TPA-MBT$^+$ (probe **6**), which utilized aggregation-induced emission (AIE) signalling to specifically detect human serum albumin (HSA). Structural modifications of 2-methylbenzothiazole moiety were employed to investigate the relationship between the probe's molecular structure and its sensing performance. Incorporation of a hydrophilic benzothiazole salt group not only enhanced the probe's aqueous solubility but also imparted selective recognition capabilities toward HSA, resulting in a distinct "off–on" fluorescence response. Leveraging the AIE mechanism, TPA-MBT$^+$ exhibited a rapid and pronounced fluorescent response upon interaction with HSA, enabling highly sensitive and quantitative detection with an impressively low detection limit of 0.022 μg/mL. Moreover, the probe demonstrated strong potential for real-world applications, showing sufficient sensitivity to detect HSA even in synthetic urine samples.[35]

A near-infrared (NIR) fluorescent probe **7** was developed by Ding *et al.* for the sensitive and specific detection of human serum albumin (HSA). The probe featured a donor–π–acceptor (D–π–A) architecture based on a bridging triphenylamine (TPA) core. Probe **7** exhibited a large Stokes shift (~190 nm) and an impressive 50-fold fluorescence enhancement upon binding to HSA, demonstrating exceptional selectivity—even over closely related analytes such as bovine serum albumin (BSA). The probe offered high sensitivity, with a LOD of 0.12 μM and linear detection range of 0–20 μM. Job's plot analysis and displacement assays confirmed 1:1 binding stoichiometry between probe **7**

and HSA. In addition to its strong selectivity, probe **7** demonstrated excellent biocompatibility, minimal background fluorescence, and the ability to detect exogenous HSA accurately without the need for sample pretreatment. Probe **7** successfully detected HSA in untreated human urine samples in under 30 seconds, with high recovery rates of 81.7% to 92.9% in the 0-10 µM concentration range.[36]

7

1.3 Research Gaps

There are several literature reports demonstrating probes appended with various heterocyclic moieties for selective determination of serum albumins. But bis-coumarin based probes has not been much explored for the detection of serum albumins specifically human serum albumin. Therefore, bis-coumarin based derivative has been synthesized owing to its binding affinity and subsequent fluorescence changes. So, the probe **5** was synthesized which is employed further to detect various bio analytes that are relevant to both biology and environment.

1.4 Objectives

1. Synthesis of bis-coumarin based derivative and its characterization
2. To detect the interaction of synthesized derivative with various bioanalytes through photophysical properties

Chapter 2

Material and methods

2.1 Chemicals and characterization of compounds

Sigma-Aldrich and Spectrochem supplied all the required solvents and chemicals. Spectroscopic experiments were conducted to characterize the optical properties, confirmed the structure and assessed the suitability of probe for intended applications. The reaction was monitored through thin layer chromatography (TLC). To characterize and identify the synthesized compounds, the (HRMS) XEVO G2-XS QTOF mass spectrometer from Waters was used to analyse the molecular weight of compounds. ^{1}H NMR and ^{13}C NMR spectra were obtained using Jeol ECS 500 MHz NMR spectrometer, with TMS (trimethyl silane) acting as the internal reference. UV-visible spectroscopy experiments were conducted using the SHIMADZU-2600 instrument, while fluorescence experiments were performed using the Cary Eclipse Fluorescence Spectrophotometer. All the synthesized compounds were purified by column chromatography using silica gel mesh 60-120. Chloroform, ethyl acetate, hexane and their mixture were used in TLC and column chromatography.

2.2 Experimental section

Synthesis of 3-Acetyl-2*H*-chromen-2-one

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3-Acetyl-2*H*-chromen-2-one was synthesised according to reported literature method.[36] In 50 ml RBF, salicyaldehyde (1g, 8 mmol) and 1.5 eq. of ethylacetoacetate (1.56 g, 12 mmol) in 10 ml ethanol were taken. Following this, 2-3 drops of piperidine were added and then stirred for 15 minutes at room temperature. The reaction was monitored by TLC. Once the reaction was done, precipitates were collected, filtered with water, dried and again filtered with ethanol and dried. Yield; 90%, colour: white.

Synthesis of 3,3'-(4-methyl-6-oxo-1,4,5,6-tetrahydropyridine-2,4-diyl)bis(2*H*-chromen-2-one)

Compound was synthesized by reacting 3-acetyl-2*H*-chromen-2-one (500 mg, 3 mmol) with diethylmalonate (2.13 mg, 15 mmol) in the presence of ammonium acetate (307 mg, 4 mmol) and ethanol (10 ml) for 1h at 60 ºC to yield white solid compound. The synthesized compounds were well characterized by ^{1}H NMR, ^{13}C NMR and HRMS.

Spectral data of compound 5

White solid; Yield: 76%, R_f 0.4 (10% EtOAc : Chloroform); m.pt: 275 °C; ^{1}H NMR (DMSO-d_6, 500 MHz): δ (ppm) 14.54 (s, 1H, NH), 8.88 (s, 1H, ArH), 8.51 (s, 1H, ArH), 7.91(d, *J* = 88 Hz, 1H, ArH), 7.68 (t, *J* = 8.6 Hz, 1H, ArH), 7.48 (d, *J* = 8.3 Hz, 1H, ArH), 7.42 (t, *J* = 7.8 Hz, 1H, ArH), 7.22 (dd, *J* = 14.1, 7.9 Hz, 2H, ArH), 7.04 (d, *J* = 7.8 Hz, 1H, ArH), 6.96 (t, *J* = 7.7 Hz, 1H, ArH), 4.14 (s, 1H), 2.82 (d, *J* = 10.5 Hz, 1H), 2.26 (s, 3H), 2.09 (d, *J* = 10.0 Hz, 1H). ^{13}C NMR (DMSO-d_6, 125 MHz): δ (ppm) 171.17, 158.56, 153.86, 150.13, 140.97, 132.76, 128.87, 128.43, 128.14, 126.11, 124.83, 121.91,117.65,116.59, 101.52, 82.34, 30.89, 30.77, 18.66 ppm. HRMS (ESI) Calcd. for $C_{22}H_{17}NO_5$ $[M+H]^+$ 399.1107 Found 398.1001.

Chapter 3

Results and Discussion

3.1 Chemistry

The synthetic route adopted for the synthesis of biscoumarin is mentioned in **Scheme 1**. Commercially available salicylaldehyde (**1**) was reacted with ethyacetoacetate (**2**) in the presence of piperidine in ethanol at room temperature for 15 min to yield white compound **3** in 90% yield. The synthesized compound **3** was dried and further reacted with diethylmalonate in the presence of ammonium acetate as base in ethanol at 60 ºC for 1.5 hr. After the completion of the reaction checked through TLC, solvent was evaporated and then work up with water solid precipitates were obtained filtered and washed thoroughly with cold water which is further purified through column chromatography. The resulting white precipitates were dried

in an oven, yielding a product **5** with 76% yield. Also, the mechanistic pathway involved in the formation of compound **5** has been shown in **Scheme 2**.

Scheme 1: Synthesis of compound 5

Scheme 2: Mechanistic Pathway for the formation of compound 5.

^{1}H NMR spectrum showed 1H singlet at δ 14.54 ppm that corresponds to NH of amide. 1H singlet at δ 8.88 ppm, 1H singlet at δ 8.51 ppm, 1H doublet at δ 7.91 ppm, 1H triplet at 7.68 ppm, 1H doublet at δ 7.48 ppm, 1H triplet at 7.42 ppm, 2H doublet of doublet at δ 7.22 ppm, 1H doublet at δ 7.04 ppm, 1H triplet at δ 6.96 ppm due to CH's in aromatic region. 1H singlet at δ 4.14 ppm due to alkene CH, two 1H doublets at δ 2.82 and 2.09 ppm due to methylene group and 3H singlet at δ 2.26 ppm due to methyl group (**Figure 1)** which confirmed the formation of compound **5**.

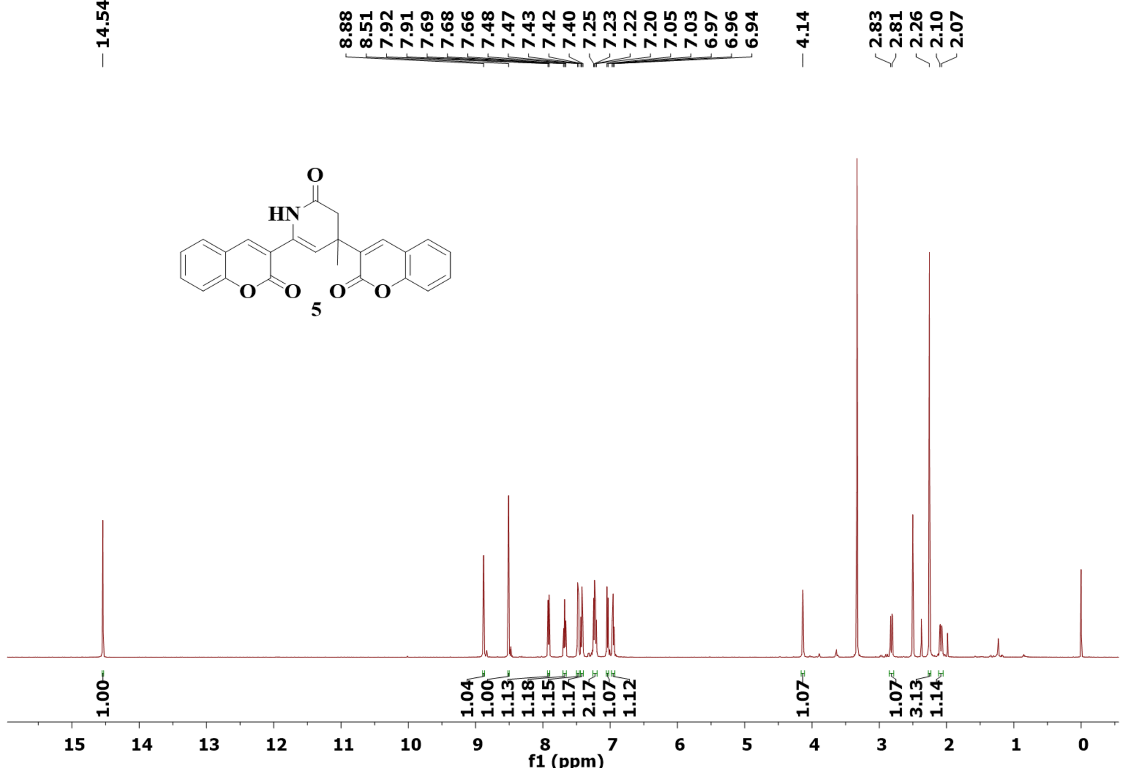

Figure 1: ^{1}H NMR spectrum of compound 5

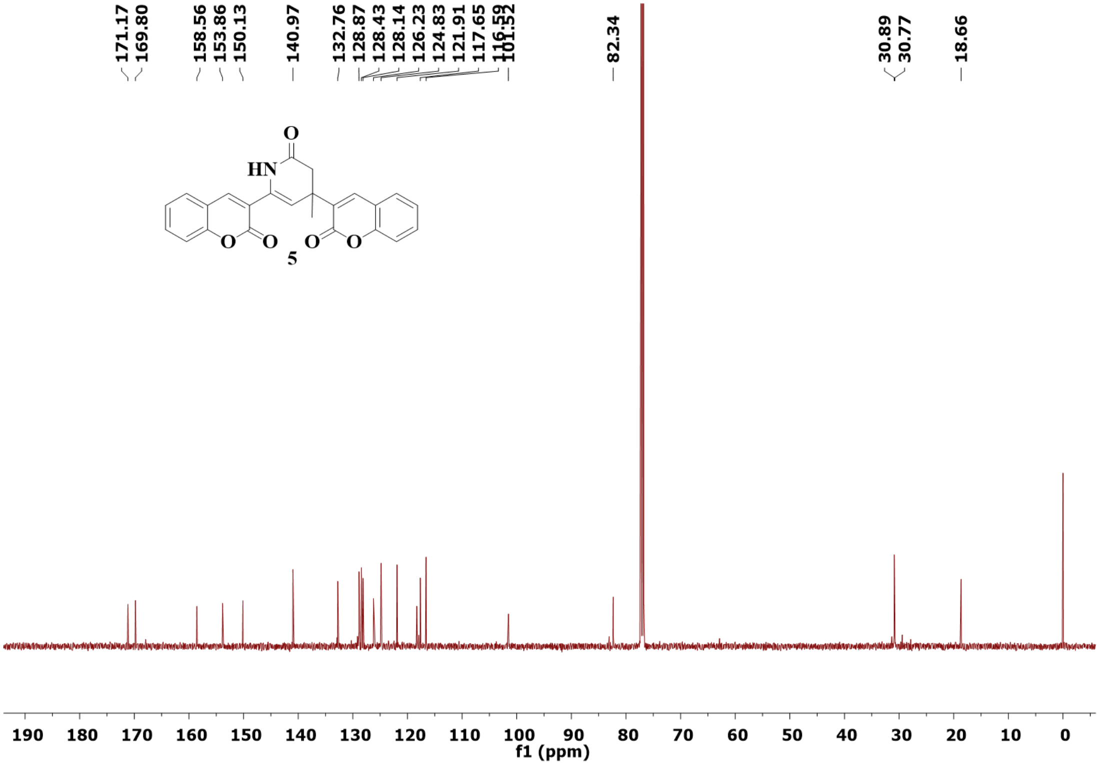

Figure 2. ^{13}C NMR spectrum of compound 5

^{13}C NMR spectrum of compound **5** showed δ 171.17, and 169.80 ppm due to carboxyl of amide group & carboxyl group of two coumarins, signals at δ 158.56, 153.86, 150.13,

140.97, 132.76, 128.87, 128.43, 128.14, 126.11, 124.83, 121.91, 117.65, 116.59, 101.52 ppm due to aromatic carbons of two coumarins, and signal at δ 82.34 corresponding to C of ethylene CH, signal at δ 30.89 and 30.77 corresponding to aliphatic C adjacent to carboxyl group and quaternary carbons, and signal at δ 18.66 ppm due to CH_3 which confirmed the formation of compound **5** (**Figure 2**).

3.2 Photophysical Studies

3.2.1 Fluorescence studies with serum albumins and other bioanalytes

Competitive fluorescence measurement experiment was performed with different bioanalytes like fructose, cysteine, methionine, tryptophan, arginine, proline, aspartic acid, glutathione, histidine, asparagine, glycine, fructose, dextrose, glutamic acid, glutamine, galactose, leucine, lysine, phenyl alanine, serine, threonine, tyrosine, uric acid and urea to identify the selectivity of compound **5 (Figure 3)**. When excited at 280 nm, a solution of **5** (10 μM) showed emission bands at 400 nm and 560 nm in phosphate buffer solution (pH = 7.4). On adding serum albumins and different bio-analytes (10 μM) to **5**, the emission spectra in phosphate buffer solution were noted at the same excitation wavelength. Interestingly, a new band was seen at 350 nm and 354 nm were observed, which is 6 times stronger for HSA and 5 times more intense in case of BSA. On the other hand, the emission pattern of compound **5** did not change for any of the other bio-analytes. Therefore, the developed compound **5** can be utilized to differentiate serum albumins in the presence of different bio-analytes.

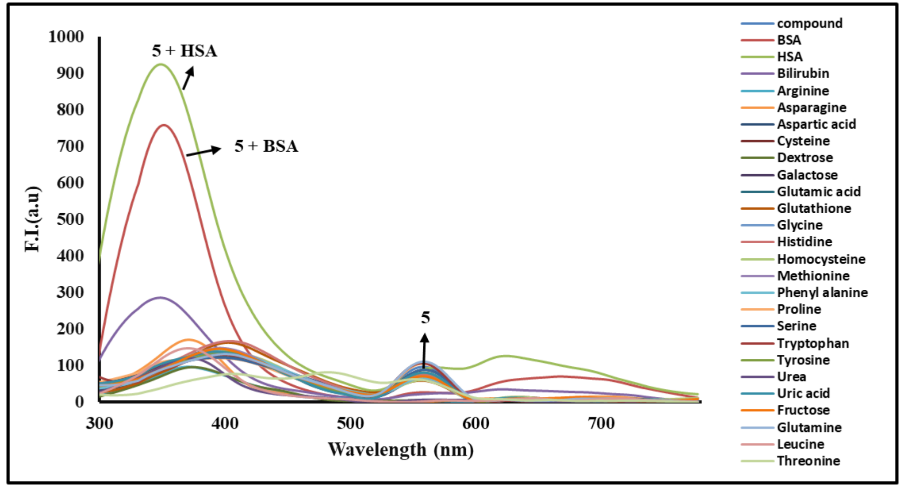

Figure 3. Change in emission spectrum of compound 5 (10 μM) with different bioanalytes in phosphate buffer solution (pH = 7.4) at 298K.

3.2.2 Evaluation of photophysical properties of compound 5 towards serum albumin

The photophysical properties of the synthesized compound was first analysed by UV–vis and fluorescence spectroscopy techniques. Human serum albumin (HSA) showed a distinct strong absorption band at 280 nm which was due to π–π* electronic transitions of aromatic acids like tryptophan, tyrosine, and phenylalanine. Tryptophan was the most significant contributor among these three. When a ligand interacts with HSA, it cause conformational changes in the protein structure and microenvironmental changes around the aromatic residues. These can lead to change in intensity or position (λ max shift) of absorption band. Continuous addition of compound **5** (21 μM) to HSA solution (10 μM) (phosphate buffer, pH = 7.4) resulted in a hyperchromic shift at 280 nm with emergence of a new band at 360 nm in case of HSA and at 320 nm in case of BSA (**Figure 4(A)** and **4(B)).** These changes in the absorption band are likely attributed to conformational alterations in the protein structure, induced by environmental variations resulting from chemical binding interactions with the protein. Benesi-Hildebrand equation (eq 1) was used to obtain the binding constants (K_b) for compound **5** and found to be 2.5×10^4 M^{-1} and 1.9×10^4 M^{-1} for HSA and BSA (**Figure 5(A)** and **5(B))**, respectively. These data confirmed the strong binding of compound with protein.

$$\frac{A_0}{A-A_0} = \frac{\varepsilon_f}{\varepsilon_b-\varepsilon_f} + \frac{\varepsilon_f}{(\varepsilon_b-\varepsilon_f)\, K_b[\text{compound}]} \quad (1)$$

where A_o is the initial absorbance of the free analyte (HSA or BSA), A gives the absorbance of the analyte in the presence of the compound, and ε_f and ε_b are the molar extinction coefficients of the analyte in its free and fully bound forms, respectively. Using the titration data and linear fitting, the plot of $A_o/(A-A_o)$ versus 1/[compound] was made and the values of K_b were found by calculating the ratio of intercept to slope.

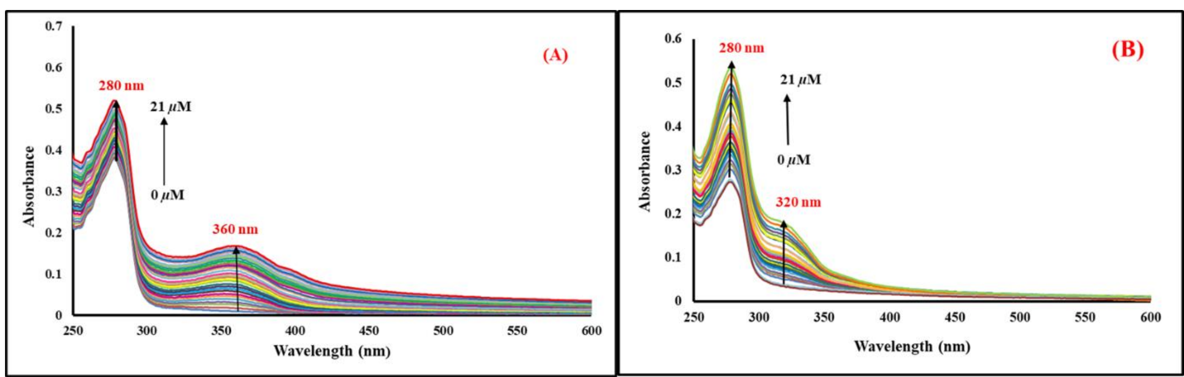

Figure 4. Change in absorption spectra of (A) HSA and (B) BSA with increasing concentration of compound 5 in phosphate buffer solution (pH = 7.4) at 298 K.

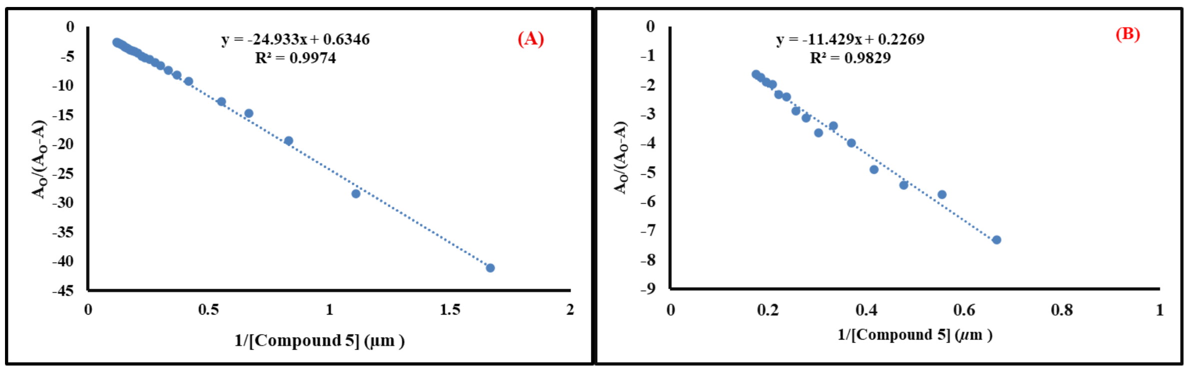

Figure 5. Benesi-Hildebrand plots of (A) HSA and (B) BSA for absorption spectra on incremental addition of compound 5.

Further fluorescence studies were conducted to investigate changes in the microenvironment surrounding specific amino acid residues, primarily tryptophan, in HSA and BSA. Shifts in fluorescence wavelength were observed, indicating alterations in the polarity around tryptophan, phenylalanine and tyrosine residues. On excitation at 280 nm in phosphate buffer (pH = 7.4), HSA exhibited an intense fluorescence band at 340 nm, while BSA at 347 nm, due to transitions in the aromatic rings of amino acid residues (**Figure 6(A)** and **6(B)**). On incremental addition of compound **5** (0-62 μM), a significant decrease in fluorescence intensity in the emission bands of HSA and BSA has been observed with slight red shift (10 nm) in both cases. A substantial quenching effect was observed upon titration with compound **5**, indicating strong interaction between the compound and the protein. This quenching suggests changes in the microenvironment around Trp-214, the primary fluorophore in serum albumins, likely due to conformational alterations induced by binding. At a concentration of 62 μM of compound **5**, fluorescence intensity was quenched by approximately 80%, suggesting compound **5** and serum albumin formed a strong complex. Further, the modified Stern−Volmer equation (eq 2) has been used to analyze the binding constants, and were found to be 7.4×10^5 M^{-1} and 6.7×10^5 M^{-1} in case of HSA and BSA, respectively where HSA showed better binding interactions than BSA (**Figure 7(A)** and **7(B)**).

$$\log \frac{Fo-F}{F} = \log K_b + n\log[\text{analyte}] \quad (2)$$

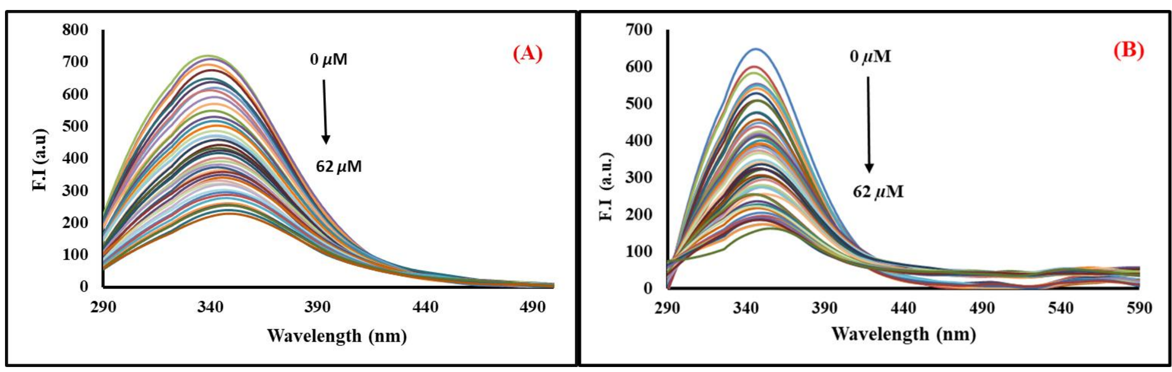

Figure 6. Changes in emission spectra of (A) HSA and (B) BSA with increasing concentration of compound 5 in phosphate buffer solution (pH = 7.4) at 298 K.

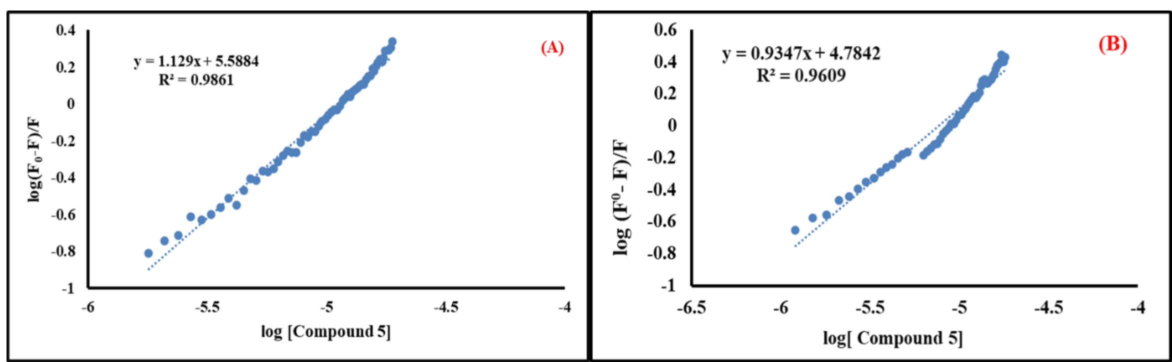

Figure 7. Modified Stern–Volmer plots for interaction of (A) HSA and (B) BSA with compound 5 at 298K.

3.2.3 Drug Displacement study

Drug displacement studies were performed using ibuprofen, and warfarin as site markers to identify the specific binding site of compound **5** on human serum albumin (HSA). HSA mainly have two main drug-binding regions: Sudlow's site I, binds to warfarin, and Sudlow's site II, corresponds to ibuprofen. Site I is in domain IIA and has two hydrophilic centres in a hydrophobic cavity, whereas domain IIIA contains site II and is primarily hydrophilic. To determine the potential binding sites of compound **5**, fluorescence titration experiments were performed at 280 nm using equimolar concentrations (10 μM) of HSA and each site marker with successive additions of compound to determine the possible binding site of compound. When compound **5** was added gradually, the emission band at 380 nm was quenched in the HSA-warfarin and at 346 nm in case of HSA-ibuprofen (**Figure 8(A)** and **8(B)**). The modified Stern−Volmer equation was used to find the binding constants (K_b) for compound−HSA when ibuprofen and warfarin were present and found to be 6.9×10^5 M^{-1} in case of warfarin and 7.8

× 10^5 M^{-1} in case of ibuprofen (**Figure 8(C)** and **8(D)**). Notably, the binding constant of compound **5** to HSA was decreased significantly when warfarin was present, indicating that warfarin could prevent the binding of compound **5**. These findings suggest that compound **5** primarily binds to HSA at Sudlow's site I.

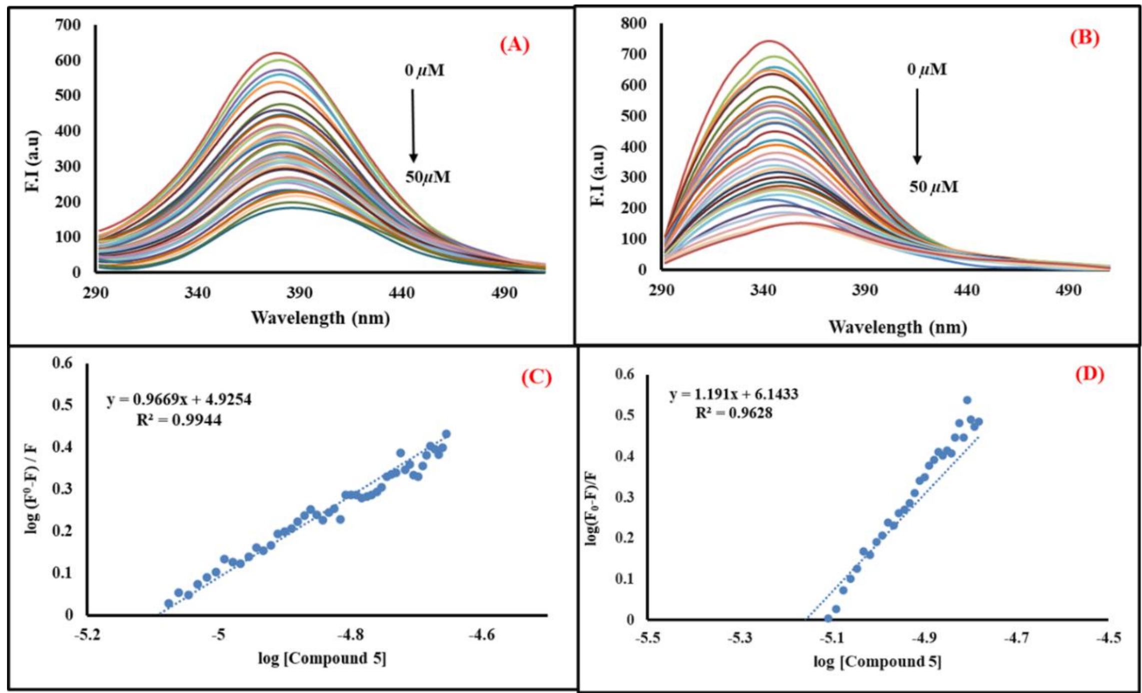

Figure 8. Effect of compound 5 on emission spectra of (A) HSA-warfarin and (B) HSA-ibuprofen complexes. Modified Stern-Volmer plots for (C) HSA-warfarin and (D) HSA-ibuprofen site markers.

Chapter 4

Conclusion and Future Prospective

4.1 Conclusion

We successfully developed and characterized 3,3'-(4-methyl-6-oxo-1,4,5,6-tetrahydropyridine-2,4-diyl)bis(2*H*-chromen-2-one) (compound **5)** to investigate its interaction with various biologically active analytes where compound **5** showed a distinct and selective detection towards serum albumins, such as human serum albumin (HSA) and bovine serum albumin (BSA). HSA showed 80% quenching at a concentration of 62 μM as compared to BSA which showed 75% quenching under the same conditions. To evaluate the strength of these interactions, binding constants (K_b) were calculated using fluorescence titration data. The binding constant for HSA–probe complex was found to be 7.4 × 10^5 M^{-1}, which is higher than

the value obtained for the BSA–probe complex (6.7×10^5 M^{-1}), proves that compound **5** has a stronger affinity for HSA than BSA.

The results indicated that compound **5** primarily binds to Sudlow's site I, which is the binding site for warfarin on HSA. These results showed that the bis-coumarin based probe has the potential to be a useful tool for selectively and effectively detection of serum albumins, which may have applications in bioanalytical or diagnostic applications.

4.2 Future Prospective

1. Structural analogues of probe **5** could be synthesized to explore interaction with other biologically active compounds like enzymes, or nucleic acids, thereby increasing the scope of bis-coumarin-based probes in chemical biology.

2. By modifying the core structure, it may be possible to extend the probe's detection capabilities to other biologically active proteins, such as enzymes, transport proteins, or disease-specific markers. This would significantly expand the applicability of the probe series in clinical diagnostics and chemical biology.

3. In addition to sensing capabilities, the biological activity (e.g., cytotoxicity, antioxidant activity, or antimicrobial properties) of the new derivatives can be studied.

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