"Synthesis and interaction studies of Naphthalimide-Azide based probes with Serum Albumins"

A dissertation

Submitted in partial fulfilment of the requirement for the degree of

Masters of Science

In

Biochemistry

Submitted By:

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July 2023

CANDIDATE DECLARATION

I hereby declare that the work being presented in thesis entitled **"Synthesis and interaction studies of Naphthalimide-Azide based probes with Serum Albumins"** submitted in partial fulfilment of requirements for the award of the degree of **Masters of Science in Biochemistry,** submitted in the **School of Chemistry and Biochemistry, Thapar Institute of Engineering and Technology, Patiala** under the guidance and supervision of **Dr. Kamaldeep Paul,** Professor and **Dr. Diptiman Choudhury** Associate Professor, School of Chemistry and Biochemistry, Thapar Institute of Engineering and Technology, Patiala. All the laboratory work is done on my own under the supervision of my guides during the period of January 2023 to June 2023. I have not submitted the matter embodied in the thesis for the award of any other degree.

Komal Verma
(302107002)

Place: Patiala, Punjab
Date: 29/07/23

CERTIFICATE

This is to certify that the thesis entitled **"Synthesis and interaction studies of Naphthalimide-Azide based probes with Serum Albumins"** submitted by **Ms. Komal Verma (Roll no. 302107002)** in the partial fulfillment of the requirement for the degree of **Masters of Science in Biochemistry**, from Thapar Institute of Engineering and Technology, Patiala is a bona fide piece of work carried out under the guidance and supervision of **Dr. Kamaldeep Paul**, Professor and **Dr. Diptiman Choudhury,** Associate Professor, School of Chemistry and Biochemistry and no part has been submitted for the award for any other degree in any other university.

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

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Komal Verma

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ABBREVIATIONS

BSA	Bovine Serum Albumin
HSA	Human Serum Albumin
PBS	Phosphate Buffered Saline
NMR	Nuclear Magnetic Resonance
$CDCl_3$	Deuterochloroform
ICT	Intramolecular Charge Transfer
µM	Micromolar
mg	Milligram
ns	Nanosecond
TICT	Twisted Internal Charge Transfer
DMSO	Dimethyl sulphoxide
DMF	Dimethylformamide
FBS	Fetal Bovine Serum
Eq	Equation
DMEM	Dulbecco's Modified Eagle Medium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazoliumbromide

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ABSTRACT

Naphthalimide based chemosensors (Probe **1** and Probe **2**) were developed for sensing Bovine Serum Albumin and Human Serum Albumin. Preliminary selectivity analysis confirmed that both the probes were highly selective towards BSA and HSA as compared to other bio analytes. Fluorescence spectroscopy analysis showed that addition of both probes **1** and **2** lead to hypochromic shift in the spectra of both BSA and HSA, at 330nm. The binding affinity of BSA-probe complex was found to be 1.7 x 10^5 M^{-1} and 3.1 x 10^5 $M^{-1}$for probes **1** and **2**, respectively and for HSA-probe were 0.5 x 10^5 $M^{-1}$and 3.1 x 10^5 M^{-1} for probes **1** and **2**, respectively. This indicated that probe **2** has better sensing ability towards both BSA and HSA. The probes and albumin proteins were found to bind in 1:1 stoichiometry, as the number of binding sites were 1.0 for both BSA-probe and HSA-probe complexes. Furthermore, the protein-ligand binding site studies indicated that both probes bind at Suldow's site III, (binding site for Bilirubin) of the BSA. Cell viability assay confirmed the newly synthesised probe **2** is non-toxic towards Human Embryonic Kidney (Hek293) cell line, therefore, it can be explored for its biological applications.

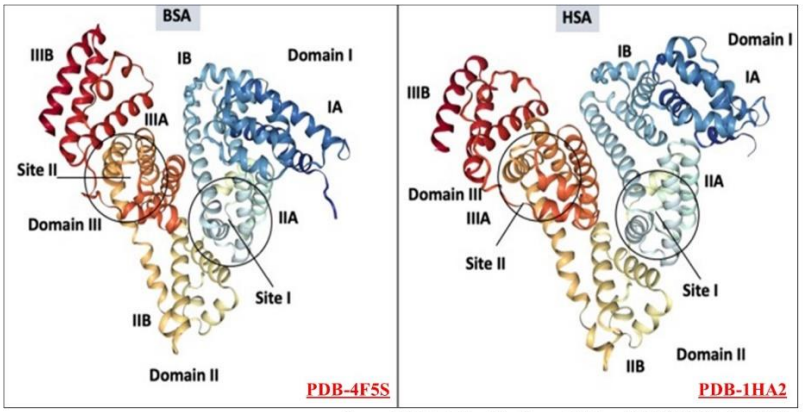
Chapter 1

Introduction and Literature Review

1.1 Introduction

The most prevalent members of the albumin family are the Serum Albumins. These are monomeric, globular, extracellular proteins weighing ~ 66KDa. Human Serum Albumin (HSA) accounts for half of the human plasma proteins (3.5g/dL- 5g/dL) [1]. Its homolog (with 75.6% structural identity), found in cows, is called Bovine Serum Albumin (BSA).

Albumin is synthesized by hepatocytes, i.e., liver cells (approximately 10-15 g/day), and released into the bloodstream [2]. When albumin enters the tissues, it is spontaneously recycled by the lymphatic system and returned to the circulatory space, maintaining high quantities everywhere it circulates. The primary function of Albumin protein is the maintenance of plasma oncotic pressure [3]. It also involves transporting fat-soluble hormones (thyroid and steroid hormones), fatty acids, bilirubin, and various drugs [4]. Hypoalbuminemia can be a marker of liver diseases (cirrhosis), nephrotic syndrome, neurometabolic disorders, coronary artery disease, Ménétrier disease, and malnutrition [5–9]. Hyperalbuminemia can indicate a state of excessive dehydration [10]. Since an imbalance in albumin levels is an indicator of various diseased state, thus quantification of albumin level is essential.



Source- Medicinal Chemistry Research (doi.org/10.1007/s00044-021-02748-z)

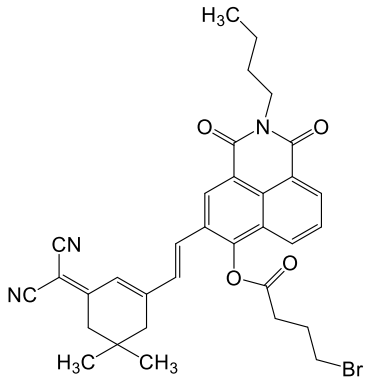
Figure 1: Biochemical conformations and binding sites of BSA and HSA

Nowadays, chemosensors are vividly being used for the detection of various biologically significant moieties. The chemosensors involving fluorophores are favoured as they conveniently and specifically detect the targeted moiety using conventional methods like UV-

visible and fluorescence spectroscopy. These fluorescent probes are non-invasive and have been used for various biomedical applications [11,12]. Since albumin has three binding sites: Suldow's sites I, II & III (**Figure 1)**, which regulate interactions between proteins and ligands [13,14], many chemosensors which can bind to these sites have been developed. Among the various HSA/ BSA sensing approaches, many naphthalimide-based chemosensors are being used. Here, we have used naphthalimide-azide and naphthalimide-azide-tryptamine based chemosensors for efficient and specific detection of BSA and HSA.

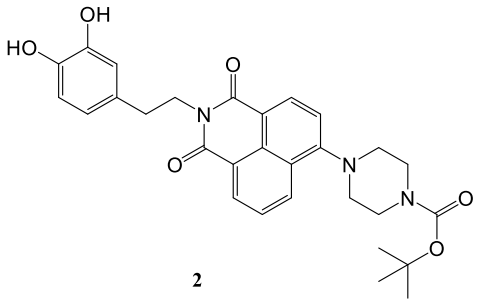
1.2 Literature Review

Yue Ke *et al.* synthesized (sensor **1**), a "turn-on" near-infrared fluorescence probe to find HSA in lysosomes. It shows very little fluorescence because of an intramolecular twisted internal charge transfer (TICT) action between the unsaturated bonds. The TICT action is blocked by the presence of HSA in the lysosome, which increased the fluorescence of sensor **1**. As a result, it could sense HSA in the lysosome with precision. Since fluorescence response of sensor **1** to HSA varies significantly than BSA, it can effectively distinguish between them [15].



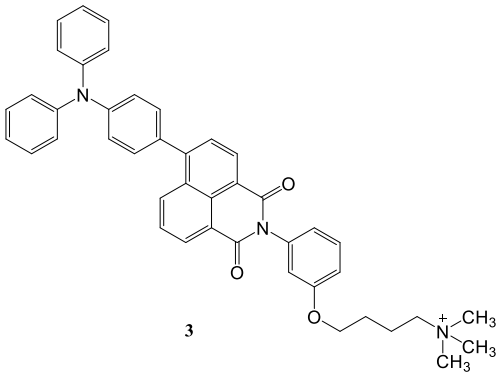
1

Liuting Mo *et al.* devised and created a fluorescent probe (sensor **2**) for HSA. They involved adding a piperazine group to the 1,8-naphthalimide's 4-position. The N-C single bond initially had a low degree of conjugation with the probe due to its highly spinning condition (TICT) in the initial state. A feeble fluorescence signal was produced as a result of the probe's energy being released through a nonradiative transition. The rotation action (TICT) was bound when the probe encountered HSA, enhancing conjugation and producing a potent fluorescence signal [16].



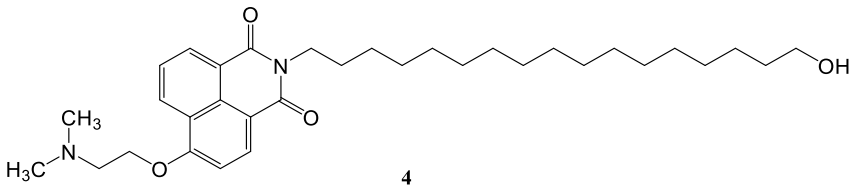
2

Junying *et al*. designed a new water-soluble biological naphthalimide probe (sensor **3**). It is a near-infrared fluorescent dye. The findings demonstrated that the probe's "turn-on" reaction to BSA is extremely sensitive in near- infrared range. In an aqueous solution, the free sensor **3** emits almost no light, but a dramatic increase in near-infrared emission is seen as a result of the electrostatic interaction that forms an aggregation state and self-assembly between the positively charged sensor **3** ammonium group and the negatively charged protein [17].



3

Yang Sun *et al*. synthesized a novel spectrofluorimetric probe (sensor **4**). Fluorescence findings showed that sensor **4**-HSA/BSA complexes were produced, which showed quenching. It was used to determine the total proteins in humans that matched with the hospital reports [18].



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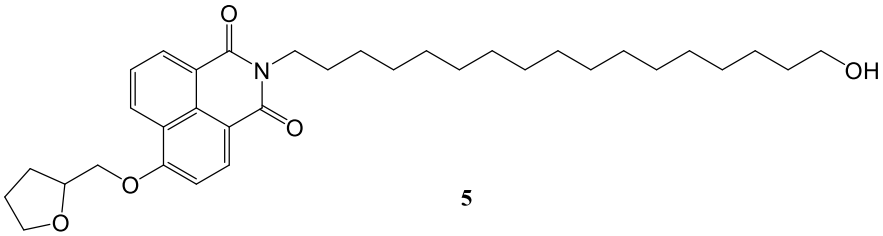
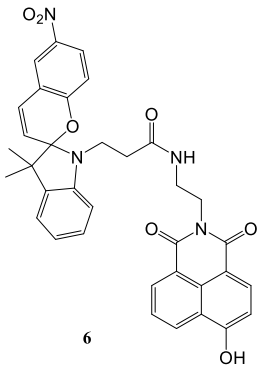
Yang Sun *et al.* created a novel long alkyl chain naphthalimide based probe for the identification of serum albumins (sensor **5**). The findings demonstrated that the influence of internal charge transfer caused sensor **5** to exhibit dependent solvent polarity features. The

interactions between sensor **5** and HSA were investigated using fluorescence and absorption spectroscopy. According to fluorescence measurements, the formation of the sensor **5** -HSA complex caused sensor **5** to quench the fluorescence of HSA [19].



5

Using naphthalimide and spiropyran as inspiration, Jun Tang *et al.* synthesized two novel fluorescent probes (sensor **6** and sensor **7**). After the addition of human serum albumin (HSA), sensor **6** displayed emission at 540 nm. The sensor **6**/HSA hybrid was then found to emit red fluorescence at 610 nm as a result of the spiropyran group of sensor **6** being isomerized into the merocyanine by UV light irradiation, demonstrating the probe's unique "double-check" process for accurately detecting the serum albumin. The fact that probe MSP's recognition effect on HSA is less pronounced than probe sensor **6** indicated that the hydrophilic group hydroxyl significantly influences probe sensor **6**'s response to HSA [20].



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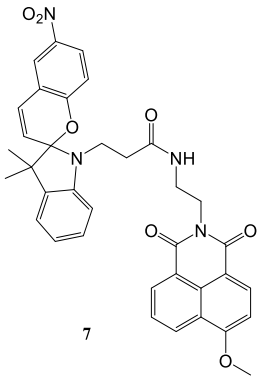

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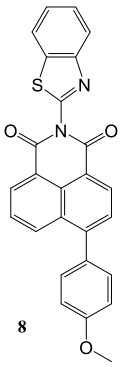
Rani *et al.* synthesized fluorescent probes for the selective detection of serum albumins among various bio analytes. This probe worked on the principle on internal charge transfer (ICT). Interactions of naphthalimide derivatives containing an electron-donating group (sensor **8**) and containing an electron-withdrawing group (sensor **9**) were investigated. In comparison to sensor **8**, sensor **9** had a higher binding affinity for both HSA and BSA. The binding of the compounds with albumin proteins were explained by the effects of both the substituents and

locations. These fluorescent probes are useful for clinical diagnosis when quantifying HSA [21].



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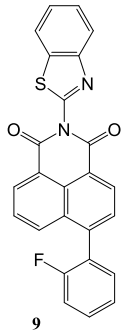

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1.3 Research gap in studies

After an extensive literature review, it was found that various naphthalimide-based chemosensors exist for serum albumin sensing. These sensors have different alkyl or aryl substitutions at 4^{th} position of naphthalimide. However, azide substituted sensors with a biological moiety are rarely reported. Considering this, probe **1** and probe **2** were developed for sensing serum albumin proteins.

Objectives

In this research work, we aimed at developing chemosensors using an already synthesized naphthalimide-azide based organic compound, which can easily detect serum albumin by producing significant changes in its fluorescence spectra in the visible range. In order to achieve this, following objectives are designed:

- To synthesize naphthalimide-azide based chemosensors
- To explore the effect of the synthesised probes on the fluorescence spectra of serum albumin proteins
- To determine the binding site of the probes on serum albumin proteins

Chapter 2

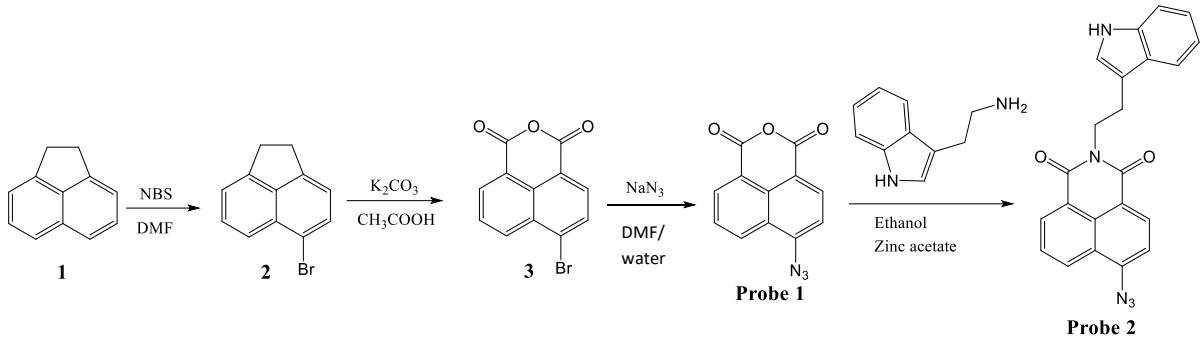
Materials and Methodology

2.1 Materials

The chemicals and solvents used in the synthesis of probes were sourced from Loba Chemie and Himedia. All the solvents used were of spectroscopic grade. Thin Layer Chromatography was used to monitor the reactions involved in synthesis of probes. NMR studies were performed using a Jeol ECS-400 MHz spectrometer. The stock solutions of concentration 1mM of probes **1** and **2** were prepared in DMSO. All the bio analytes were produced from Sigma-Aldrich, and their solutions of 1 mM concentration were prepared using distilled water. In order to check the pH of the buffer (pH=7.4), the Eutech pH meter was used. Quartz cuvettes were used for spectrophotometric studies. For UV-visible spectroscopic analysis, Shimadzu UV-2600 spectrophotometer was used. Fluorescence spectrophotometric studies were carried out using RF-6000-Shimadzu spectrophotometer and Cary Eclipse fluorescence spectrophotometer.

2.2 Methodology

2.2.1 Synthesis of probes 1 and 2



Scheme 1: Synthesis of probes **1** and **2**

2.2.1.1 Synthesis of compounds 2 and 3

Synthesis of compound **2** and **3** were done as described in literature reports [22]

2.2.1.2 Synthesis of Probe 1

Compound **1** was taken in DMF: water (9:1) and sodium azide was added to it. The reaction

was refluxed for 3 h. On completion, the precipitate was obtained by addition of water in the reaction mixture. The precipitate formed was filtered, and the final product was obtained.

2.2.1.3 Synthesis of probe 2

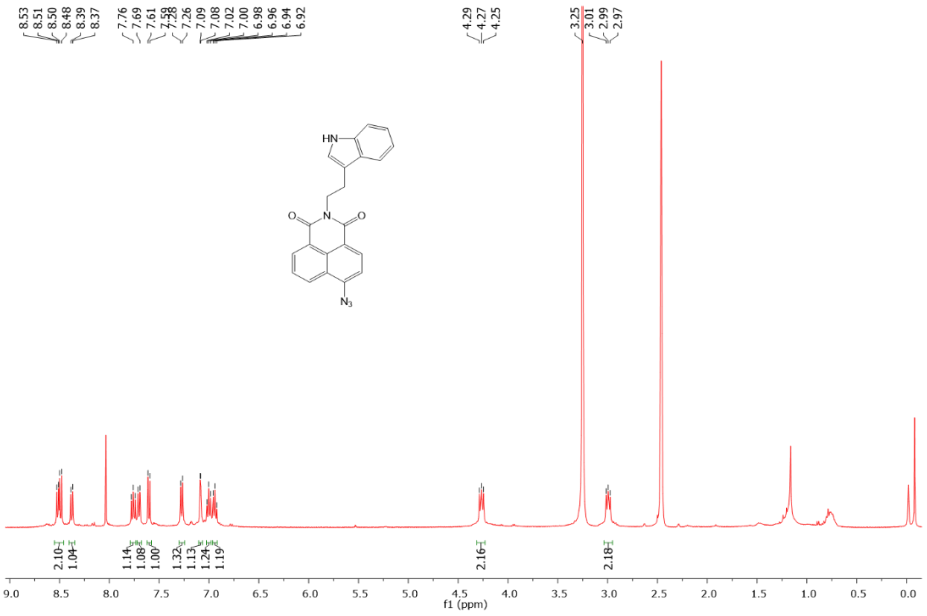


Figure 2: ^{1}H NMR spectrum of probe **2**

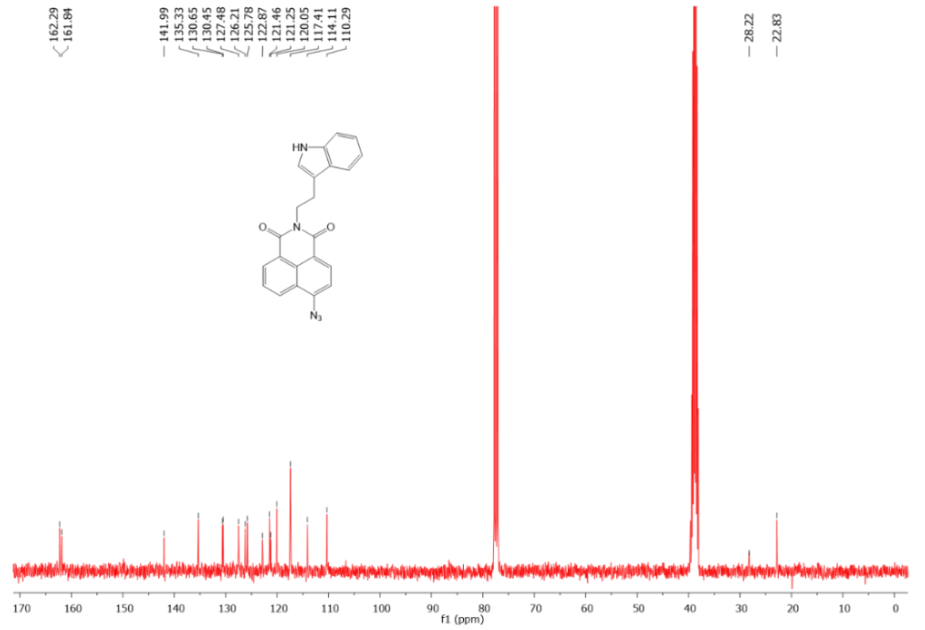


Figure 3: ^{13}C NMR of probe **2**

Tryptamine (278 mg, 1.7 mol) and a catalytic amount of zinc acetate were added to a stirred solution of probe **1** (200 mg, 0.83 mol) in ethanol. The reaction was refluxed for 6 h while continuously being monitored by Thin Layer Chromatography (TLC). Ethanol was evaporated on completion of the reaction. Water was added to the reaction mixture and the precipitate obtained was filtered. The final brown coloured solid product was obtained; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.50 (dd, ^{1}J = 11.9, ^{3}J = 7.5 Hz, 2H, ArH), 8.38 (d, J = 7.7 Hz, 1H, ArH), 7.79 – 7.73 (m, 1H, ArH), 7.70 (d, J = 7.7 Hz, 1H, ArH), 7.60 (d, J = 8.0 Hz, 1H, ArH), 7.27 (d, J = 7.9 Hz, 1H, ArH), 7.09 (d, J = 2.0 Hz, 1H, ArH), 7.00 (t, J = 7.1 Hz, 1H, ArH), 6.94 (t, J = 7.2 Hz, 1H, ArH), 4.31 – 4.23 (m, 2H, CH_2), 3.04 – 2.95 (m, 2H, CH_2) (**Figure 2)**; ^{13}C NMR (DMSO-d_6, 100 MHz): δ (ppm) 162.2, 161.8, 141.9, 135.3, 130.6, 130.4, 127.4, 126.2, 125.7, 122.8, 121.2, 120.0, 117.4, 114.1, 110.2, 28.2, 22.8 (**Figure 3).**

2.2.2 Preliminary analysis for selective sensing ability

The selectivity assay was done to evaluate the selective sensing ability of probes **1** and **2** towards BSA and HSA over other bio analytes. 1 mM stock solutions of bio analytes (glycine, proline, threonine, glutamic acid, tryptophan, glutamine, phenylalanine, methionine, histidine, aspartic acid, leucine, isoleucine, lysine, arginine, asparagine, tyrosine, L-cystine, BSA and HSA) were prepared in deionized water. 20 µM of each of these bio analytes were added to 20 µM of probes **1** and **2**, individually. The emission spectra were recorded at 280 nm as the excitation wavelength and 290-450 nm as the emission range.

2.2.3 UV-visible Spectrophotometric analysis

UV-visible spectrophotometric analysis was done to examine the interaction of probes **1** and **2** with HSA as well as BSA. UV-visible spectra of serum albumins (10 µM) were recorded in PBS (10 mM, pH=7.4), followed by incremental addition of each, probe **1** (0-33 µM) and probe **2** (0-33 µM), separately. The spectra were recorded in the range of 250 nm – 500 nm.

2.2.3.1 Determination of binding affinity

The binding constants of UV-visible spectra were determined by using the Benesi-Hildebrand equation:

$$\frac{Ao}{A-Ao} = \frac{\varepsilon_f}{\varepsilon_b-\varepsilon_f} + \frac{\varepsilon_f}{\varepsilon_b-\varepsilon_f}\frac{1}{K_b[Q]} \qquad \ldots\ldots. (1)$$

where A_o is the initial absorbance of the free serum albumin; A is the absorbance of the serum albumin in the presence of probes **1** and **2**; [Q] is the concentration of analyte; K_b is the binding constant; ε_f and ε_b are molar extinction coefficients of the serum albumin in its free and bound forms, respectively [23].

2.2.4 Fluorescence spectrophotometric analysis

Emission studies were performed to check the interaction of probe **1** and probe **2** with HSA and BSA. For this, HSA and BSA emission spectra (10 µM) at its excitation wavelength, i.e., 280 nm, were recorded in PBS. Probe **1** (0-33 µM) and Probe **2** (0-33 µM) were added incrementally to it.

2.2.4.1 Quenching Mechanism

The quenching constants of fluorescence spectra were determined by using the Stern-Volmer equation:

$$\frac{Fo}{F} = 1 + K_{sv}[Q] = 1 + K_q\tau_o[Q] \qquad \ldots\ldots\ldots (2)$$

where F_o and F are the intensities of albumin protein in the absence and presence of the Probes; τ_0 is the experimentally determined value of the lifetime of the albumin proteins (4.07 ns), [Q] is the concentration of analyte; K_{sv} is the quenching constant and K_q is the bimolecular quenching constant [24].

2.2.4.2 Binding affinity and number of binding sites

The binding constants and number of binding sites can be determined by the double logarithmic of the Stern-Volmer equation from the linear regression plot:

$$\log\left(\frac{Fo-F}{F}\right) = logK_b + nlog[Q] \qquad \ldots\ldots\ldots (3)$$

where n is the number of binding sites; F_o and F are the intensities of albumin protein in the absence and presence of the probes; [Q] is the concentration of analyte and K_b is the binding constant.

2.2.5 Protein-ligand binding site study

Albumin protein has three biomarkers: Warfarin, Ibuprofen and Bilirubin. These biomarkers bind at different ligand binding sites on serum albumins, called Suldow's sites. Warfarin binds at site I, Ibuprofen binds at site II, and Bilirubin binds at III. Hence, in order to find the specific binding site for probes **1** and **2** on BSA, the protein-ligand binding site studies were performed with the site markers. A solution of HSA and BSA with different site markers was titrated with incremental addition of probes **1** and **2**. To record the fluorescence emission spectra, BSA was excited at 280nm and the emission range was 290 nm- 450 nm.

2.2.6 Competitive assay of selectivity

To further confirm the sensing ability of the probes towards BSA and HSA, the fluorescence response of the probes was observed for various bio analytes in the presence of BSA and HSA. 1 mM stock solutions of various bio analytes were prepared (glycine, proline, threonine, glutamic acid, tryptophan, glutamine, phenylalanine, methionine, histidine, aspartic acid, leucine, isoleucine, lysine, arginine, asparagine, tyrosine, L-cystine, HSA, and BSA). 20 µM of HSA was added to each 20 µM of probe **1** and probe **2**. Further, 20 µM of each bio analyte was added to the probe-protein complex. A similar study was done with BSA, 20 µM of BSA was added to 20 µM of probe **1** and probe **2**. Further, 20 µM of each bio analyte was added to the probe-protein complex.

2.2.7 Cell viability assay

To check the toxic effect of probe **2,** MTT assay or cell viability assay was performed on Human Embryonic Kidney cell line (Hek293). The cells were cultured at 37 °C and 5 % CO_2 in Dulbeco's Modified Eagle's Medium (DMEM), containing FBS (10 %) and antibiotics. Once the cells were confluent, these were treated with different concentrations of probe **2** (1 µM- 50 µM) for 48 h.

Chapter 3

Results and Discussion

3.1 Preliminary analysis for selective sensing ability

Preliminary analysis was done to check the sensing ability of probes **1** and **2** towards BSA, HSA, and various bio analytes, including glycine, proline, threonine, glutamic acid, tryptophan, glutamine, phenylalanine, methionine, histidine, aspartic acid, leucine, isoleucine, lysine, arginine, asparagine, tyrosine, L-cystine, by fluorescence spectroscopy. The probes showed significantly higher selectivity towards BSA and HSA than other bio analytes (**Figure 4**).

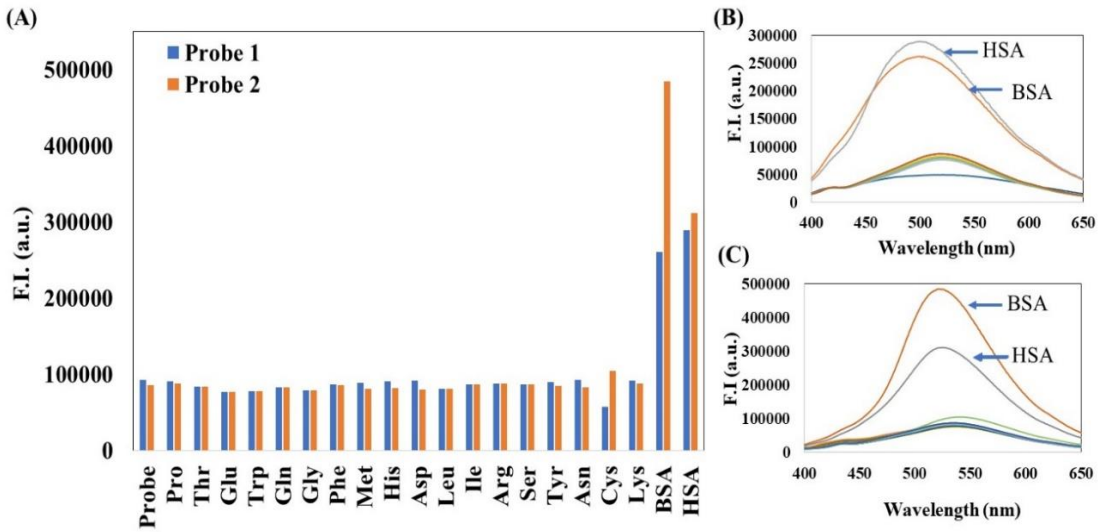


Figure 4: (A) Data of selective sensing ability of probes **1** and **2** showing significant selectivity towards BSA and HSA; Fluorescence spectra of **(B)** probe **1** and (**C)** probe **2** for various bio analytes.

3.2 Sensing ability towards Bovine Serum Albumin (BSA)

3.2.1 UV-visible spectral response

The absorbance spectra of BSA (10 µM) in PBS (pH = 7.4) exhibited an intense absorption peak at 280 nm due to amino acid residues in its structure. A hyperchromic shift was observed during the incremental addition of probes **1** (0 - 9 µM) and **2** (0 - 9 µM) (**Figure 5**). The

progressive addition of probe **2** to a solution of BSA resulted in the development of new band at 374 nm along with the enhancement.

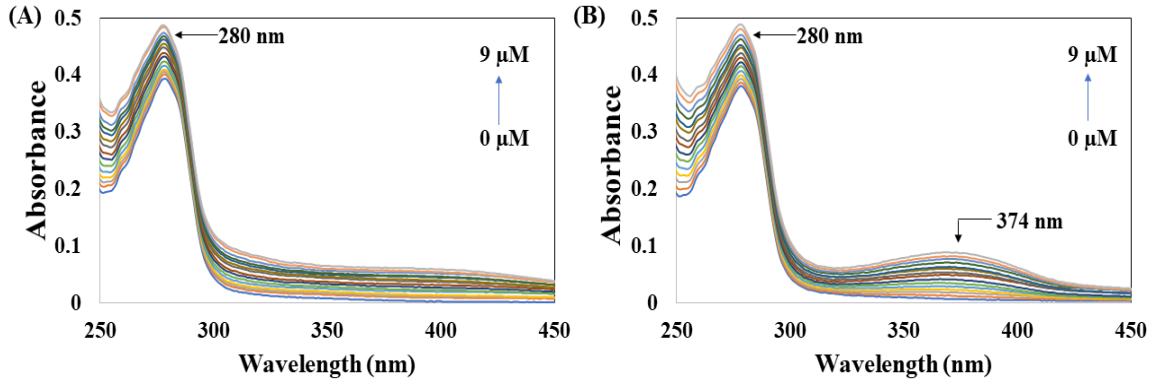


Figure 5: Effect of probes **(A) 1** and **(B) 2** on absorption spectra of BSA in PBS (pH = 7.4)

The binding constants for BSA with probes **1** and **2** were determined using Benesi- Hildebrand equation (eq 1) and were found to be 1.4 x 10^4 M^{-1} and 9.2 x 10^4 M^{-1}, respectively (**Figure 6)**. The high value of the binding constant suggested the significant interaction between BSA and probes, where probe **2** binds more firmly with BSA than probe **1**.

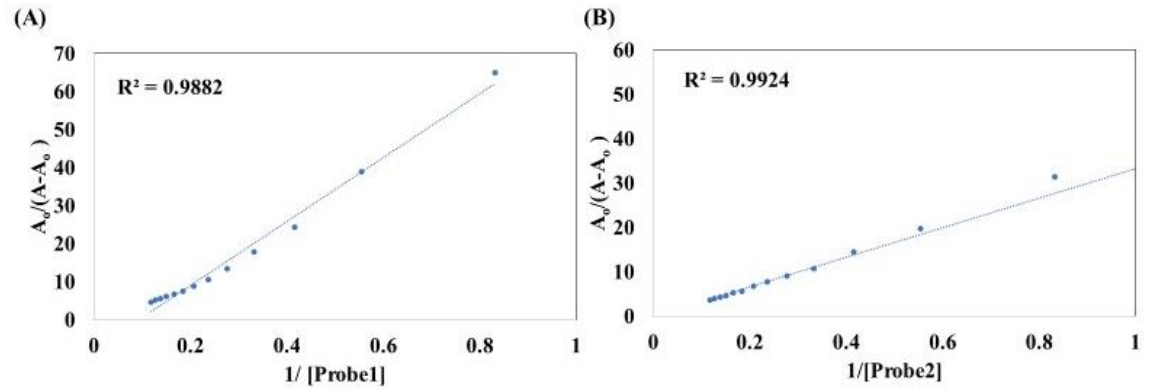


Figure 6: Calculation of binding constant of probes **(A) 1** and **(B) 2** with BSA using Benesi-Hildebrand equation.

3.2.2 Fluorescence spectral response

Upon excitation at 280 nm, BSA showed emission maxima at 330 nm (λ_{em}). The concentration-dependent study showed a hypochromic shift i.e., a decline in the fluorescence intensity of BSA spectra after the incremental addition of probes **1** (0 - 33 µM) and **2** (0 - 33 µM)) (**Figure**

7). Determination of the quenching mechanism in BSA was done using Stern-Volmer equation (eq 2). This equation helps to understand whether the quenching is static or dynamic.

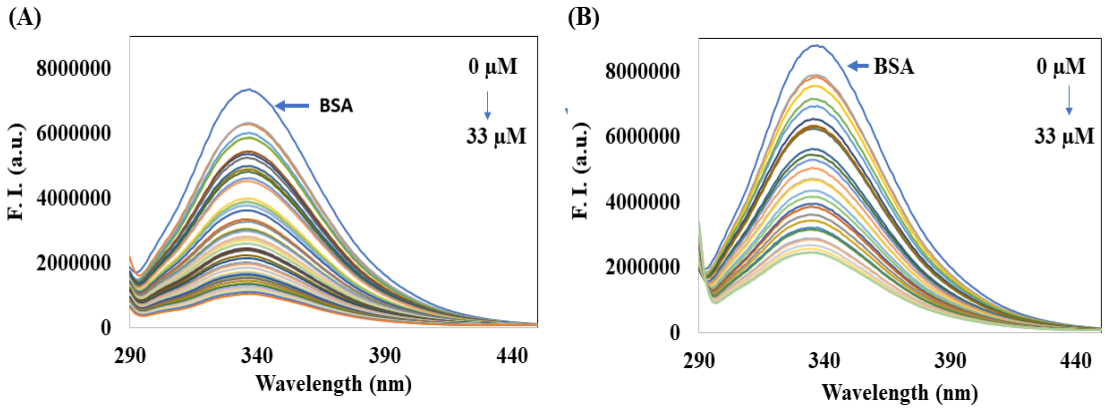


Figure 7: Effect of probes **(A) 1** and **(B) 2** on the fluorescence intensity of BSA in PBS buffer (pH = 7.4)

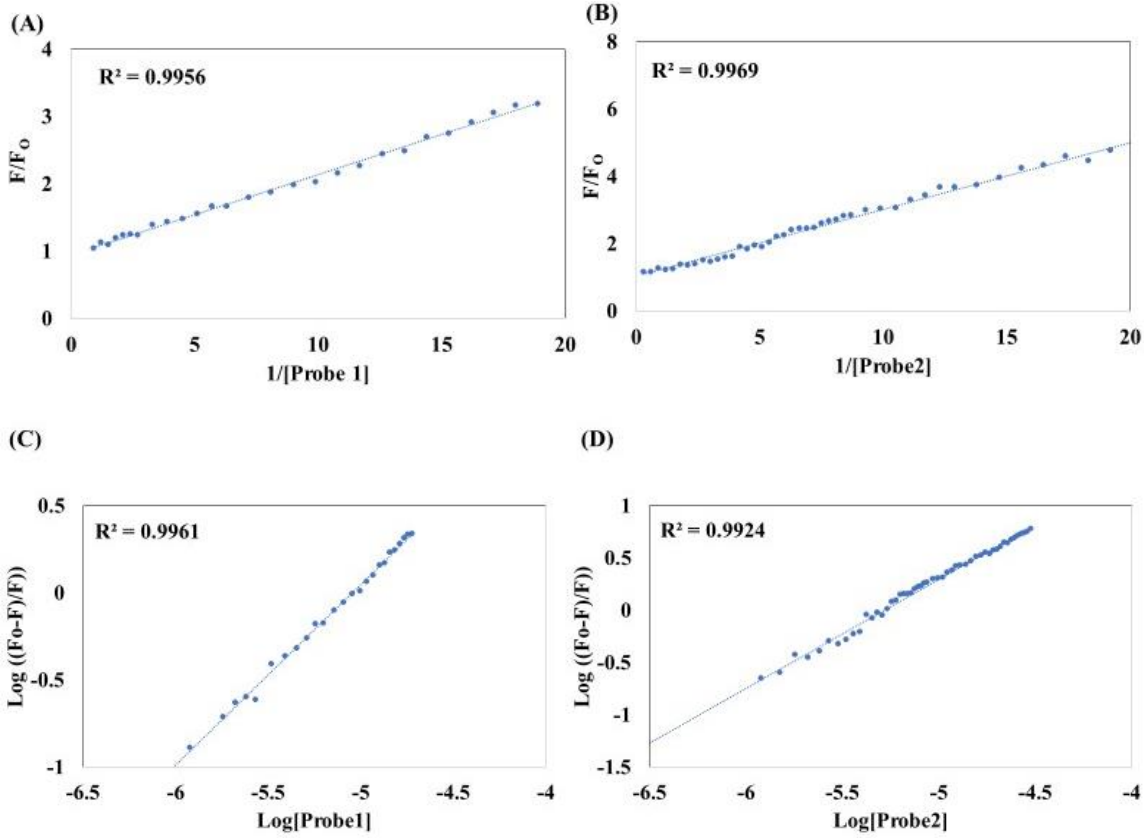


Figure 8: Calculation of quenching constant of probes **(A) 1** and **(B) 2** with BSA using Stern-Volmer equation; Calculation of binding constant of probes **(C) 1** and **(D) 2** with BSA using Stern-Volmer equation

The quenching constant (K_{sv}) of BSA for probe **1** was found to be 1.2 x10^5 M^{-1} and for **2** was found to be 1.9 x 10^5 M^{-1}. The bimolecular quenching constant (K_q) for probes **1** and **2** were calculated to be 1.2 x10^{13} M^{-1} s^{-1} and 1.9 x 10^{13} M^{-1} s^{-1}, respectively. The binding constants for both the probes were determined by using the Stern - Volmer equation (eq 3) were found to be 1.7 x $10^5 M^{-1}$ (probe **1**) and 3.1 x 10^5 M^{-1} (probe **2**). These results suggested a strong interaction between the probes and BSA. The number of binding sites of probes **1** and **2** with BSA were 1.0 and 1.0, respectively (**Figure 8, Table 1)**. This indicated that the probes bind in 1:1 stoichiometry with BSA. The diffusion-limited quenching in water for BSA is ~10^{10} $M^{-1}s^{-1}$ [25]. The calculated values of bimolecular quenching constant (K_q**)** for probe-BSA complex were found to be higher than the reported value of diffusion-limited quenching in water. This indicated static quenching due to complex formation between probes and BSA in the ground state (**Figure 8, Table 1**). Between the two probes studied, probe **2** showed better quenching and binding ability towards BSA.

Table 1: Quenching and binding parameters for BSA interaction with probes **1** and **2**

Probe	**K_{sv} (×10^5) (M^{-1})**	**K_q (×10^{13}) ($M^{-1}s^{-1}$)**	**R^2**	**K_b (×10^5) M^{-1}**	**n**	**R^2**
1	1.2	1.2	0.9956	1.7	1	0.9961
2	1.9	1.9	0.9969	3.1	1	0.9924

3.2.3 Protein-ligand binding site analysis

Serum albumins have three binding sites called the Suldow's sites, where ligands (probes in this case) bind and interact for efficient complex formation. The protein-ligand binding site analysis was done based on drug displacement method to find the binding site of probes on BSA using three biomarkers: Warfarin (for site I), Ibuprofen (for site II), and Bilirubin (for site III). A solution of BSA (10 μM) with different site markers was titrated with incremental addition of probe **1** as well as probe **2**.

The binding constants and number of binding sites for each biomarker were calculated using Stern-Volmer equation (eq 3) and a comparison was made to find whether the probes bind to Suldow's site I or II or III (eq 3).

3.2.3.1 Binding site analysis with Warfarin

Probe **1** and probe **2** were added incrementally to the Warfarin-BSA complex. A hypochromic shift was observed. The binding constants for probes **1** and **2** were observed to be respectively 1.9 x 10^4 M^{-1} and 3.9 x 10^4 M^{-1}, with warfarin. The binding constant for the probes **1** and **2** were found to decrease significantly, manifesting that warfarin prevented the binding of both the probes to Suldow's site I. Also, the number of binding sites were less than 1 for both the probes in presence of site marker (**Figure 9, Table 2**).

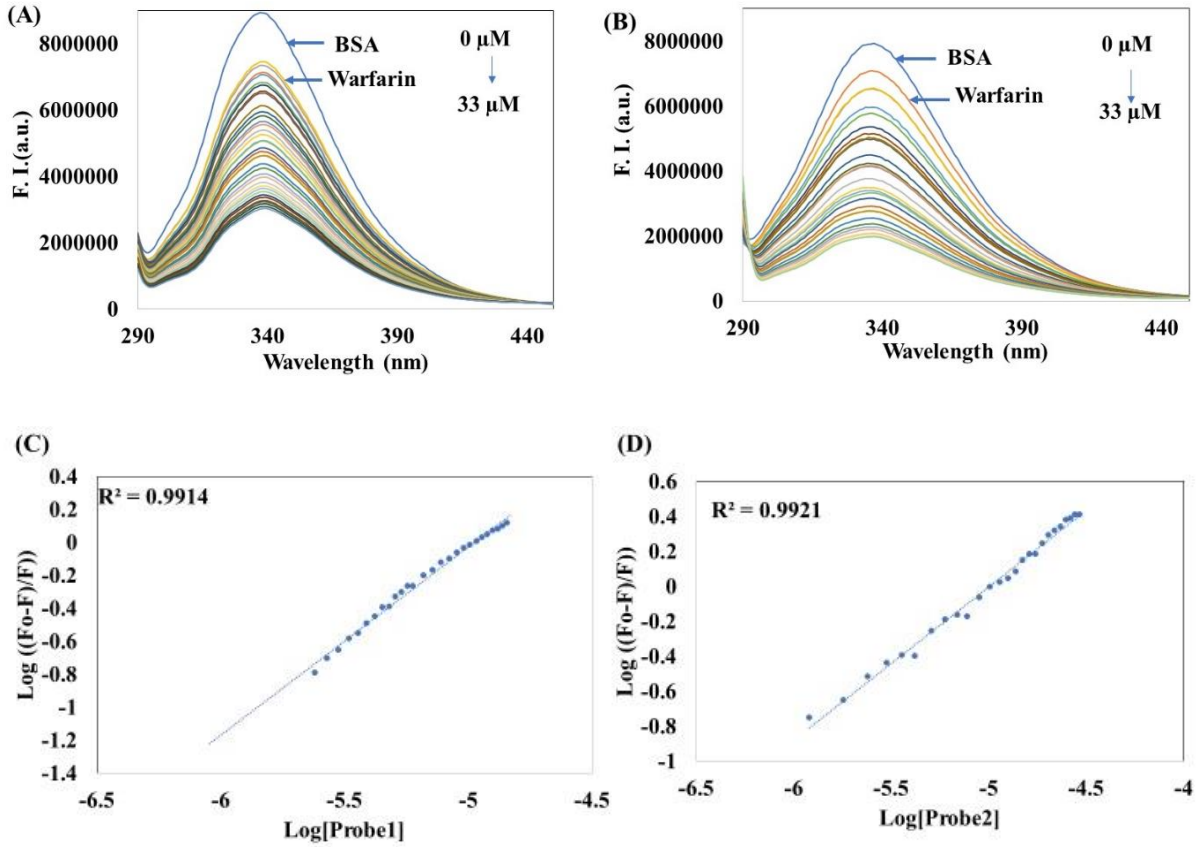


Figure 9: Effect of probes **(A) 1** and **(B) 2** on BSA - Warfarin complex in PBS (pH = 7.4); Calculation of binding constants of probes **1 (C)** and **(D) 2** with BSA - Warfarin complex using Stern-Volmer equation.

3.2.3.2 Binding site analysis with Ibuprofen

Similarly, probes **1** and **2** were added incrementally to the Ibuprofen-BSA complex,where hypochromic shift was observed. The binding constants for probes **1** and **2** were observed to

be 5.1 x 10^3 M^{-1} and 8.9 x 10^3 M^{-1}, respectively. There was decrease in binding constants of both the probes in the presence of Ibuprofen, suggesting that Ibuprofen inhibited the binding of probes with BSA at Suldow's site II (**Figure 10, Table 2**). Moreover, the number of binding sites for both probes were decreased to 0.8 from 1 in the presence of Ibuprofen.

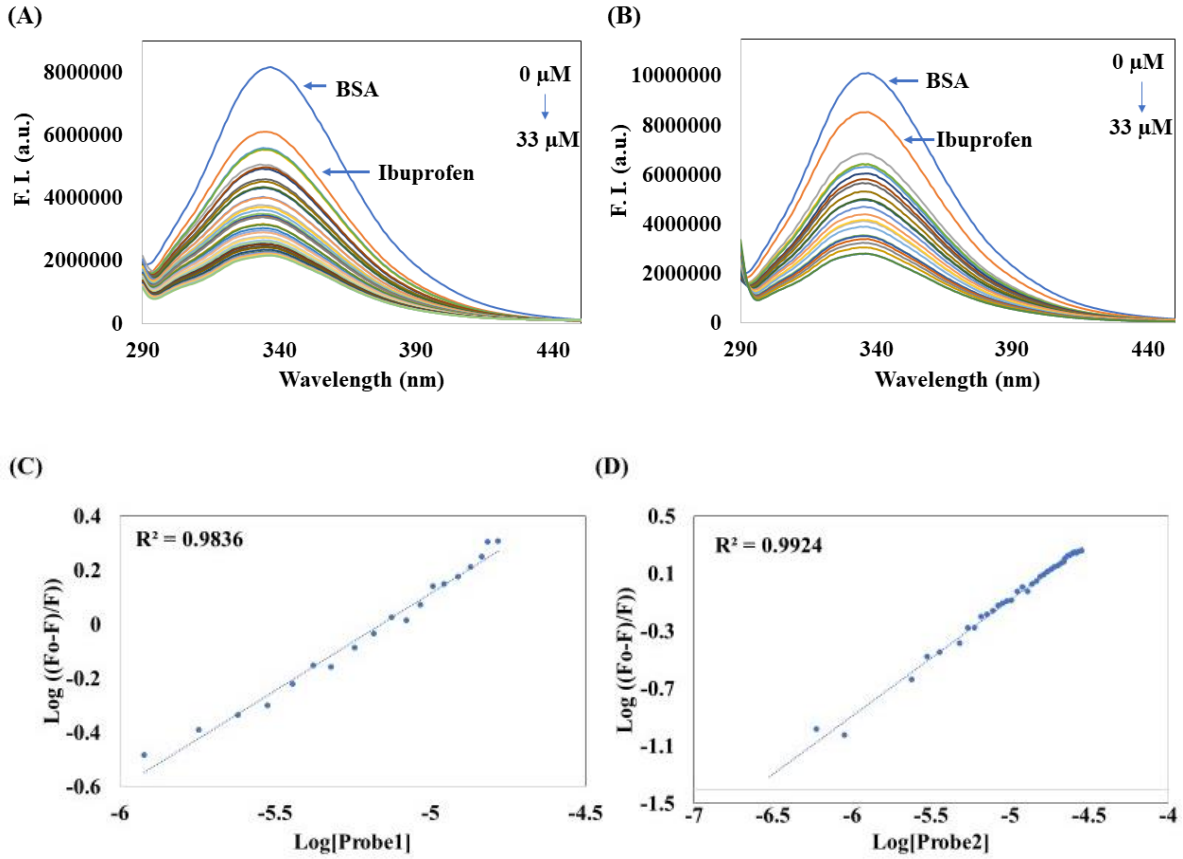


Figure 10: Effect of probes **(A) 1** and **(B) 2** on BSA- Ibuprofen complex in PBS (pH=7.4); Calculation of binding constant of probes **(C) 1** and **(D) 2** with BSA-Ibuprofen complex using Stern-Volmer equation.

3.2.3.3. Binding site analysis with Bilirubin

Likewise, probe **1** and probe **2** were added incrementally to the BSA-Bilirubin complex. A hypochromic shift was observed. The binding constants for probe **1** and probe **2** were observed to be 2.5 x 10^5 M^{-1} and 6.3 x 10^5 M^{-1}. The binding constants for both the probes were found to be same in the presence of bilirubin as in absence of it. Also, the number of binding sites were 1.1 for both the cases indicating that both the probes bind to site III of BSA significantly (**Figure 11, Table 2**).

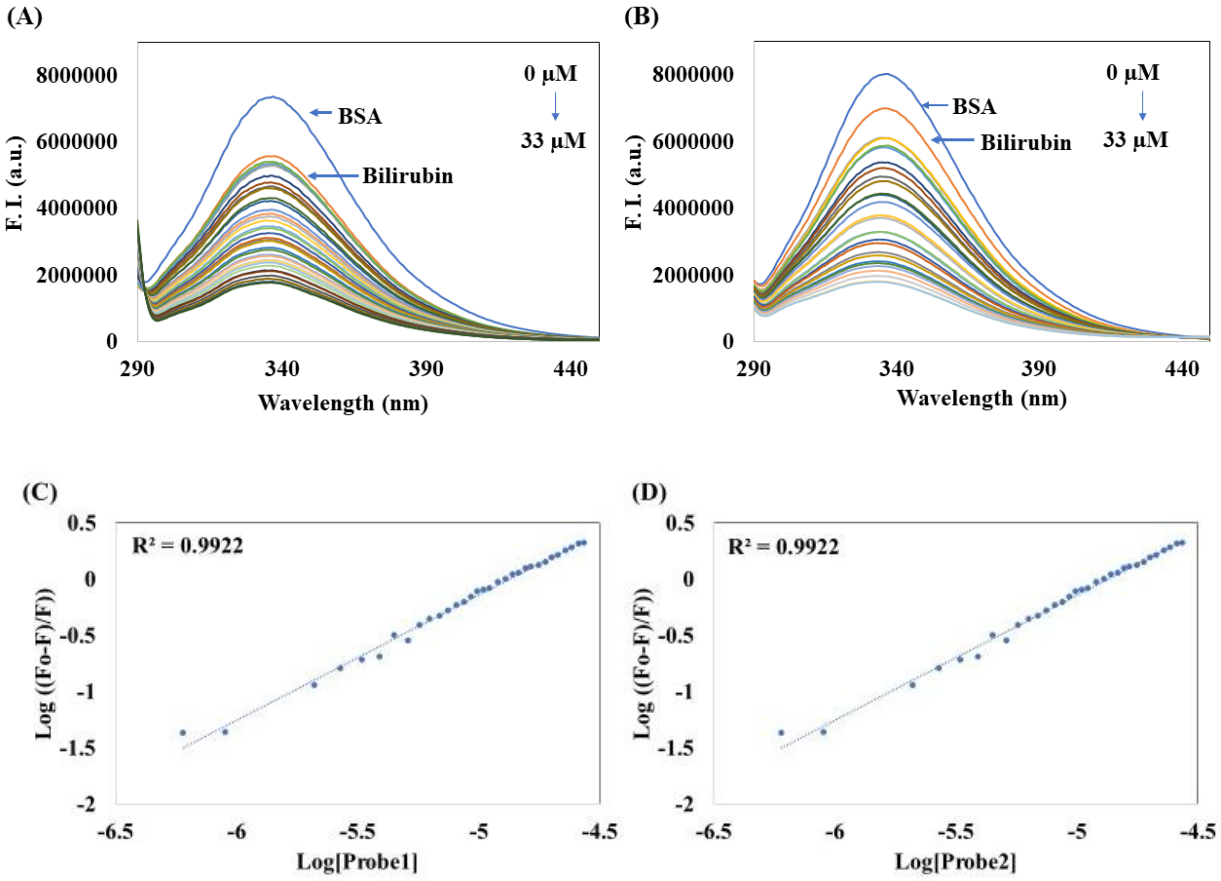


Figure 11: Effect of probes **(A) 1** and **(B) 2** on BSA - Bilirubin complex in PBS (pH = 7.4); Calculation of binding constants of probes **(C) 1** and **(D) 2** with BSA-Bilirubin complex using Stern-Volmer equation.

Table 2: Binding constants K_b of probes **1** and **2** with BSA and site markers

Probe	**Probe 1**		**Probe 2**	
	K_b (10^4 M^{-1})	**n**	**K_b (10^4 M^{-1})**	**n**
Blank	17	1.0	31	1.0
Ibuprofen	0.51	0.8	0.89	0.76
Warfarin	1.9	0.85	3.9	0.87
Bilirubin	25	1.1	63	1.1

Protein-ligand binding site analysis showed that the binding constant of probe-albumin complex was the highest in the presence of Bilirubin, indicating that both probe **1** and probe **2** bind at the binding site of Bilirubin i.e., at Suldow's site III.

3.2.4 Competitive assay for selectivity

Competitive assay for selectivity further confirmed the probes' selectivity towards BSA. No significant change was observed in the fluorescence spectra of the probe-BSA complex in the presence of various bio analytes. Both the probes were found to be highly selective towards BSA (**Figure 12**).

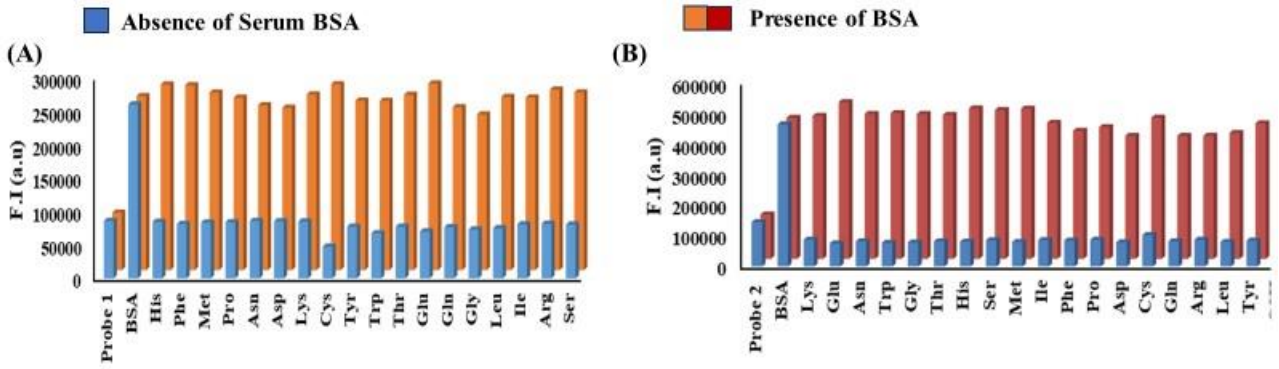


Figure 12: Interference studies of **(A)** probe **1**-BSA; **(B)** probe **2**- BSA.

3.3 Sensing Ability towards Human Serum Albumin (HSA)

3.3.1 UV-visible spectral response

Further, binding of both the probes with HSA was also explored. At 280 nm, HSA (10 µM) exhibited an intense band due to transition in aromatic rings of amino acid residues. Hyperchromic shift in the HSA spectra was found upon incremental addition of probe **1** (0 µM – 9 µM) as well as probe **2** (0 µM – 9 µM) (**Figure 13**).

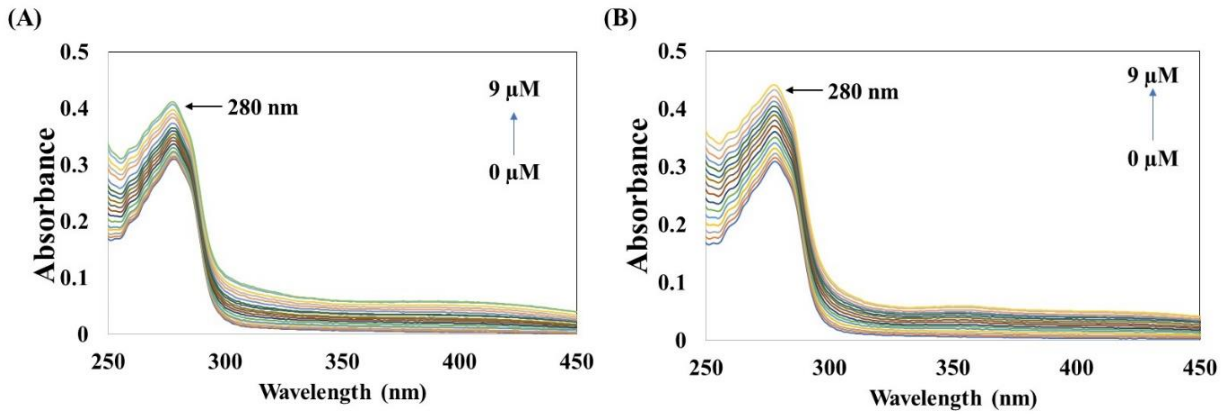


Figure 13: Effect of probes **(A) 1** and **(B) 2** on absorbance spectra of HSA (10 µM) in PBS (pH = 7.4).

The binding constants calculated (using eq 1) for HSA with probe **1** and probe **2** were found to be 1.3 x 10^4 M^{-1} and 3.7 x 10^4 M^{-1}, respectively (**Figure 14**), suggesting effective binding of the probes with HSA. This suggested a significant interaction between the probes and HSA, making them suitable for sensors. Furthermore, the higher binding constant of probe **2** as compared to probe **1**, showed that probe **2** has better sensing ability towards HSA.

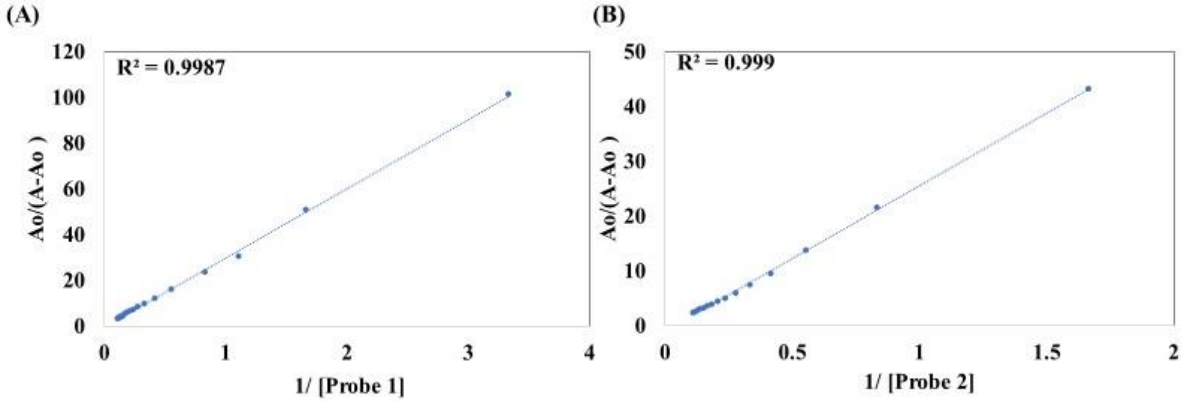


Figure 14: Calculation of binding constant of probes **(A) 1** and **2** with HSA using Benesi-Hildebrand equation.

3.3.2 Fluorescence spectral response

On excitation at 280 nm, HSA exhibited an emission maximum at 330 nm (λ_{em}). The study showed a decrease in the fluorescence intensity i.e., quenching was observed on addition of probe **1** and probe **2** (**Figure 15**). Thus, both the probes showed sensing abilities towards HSA.

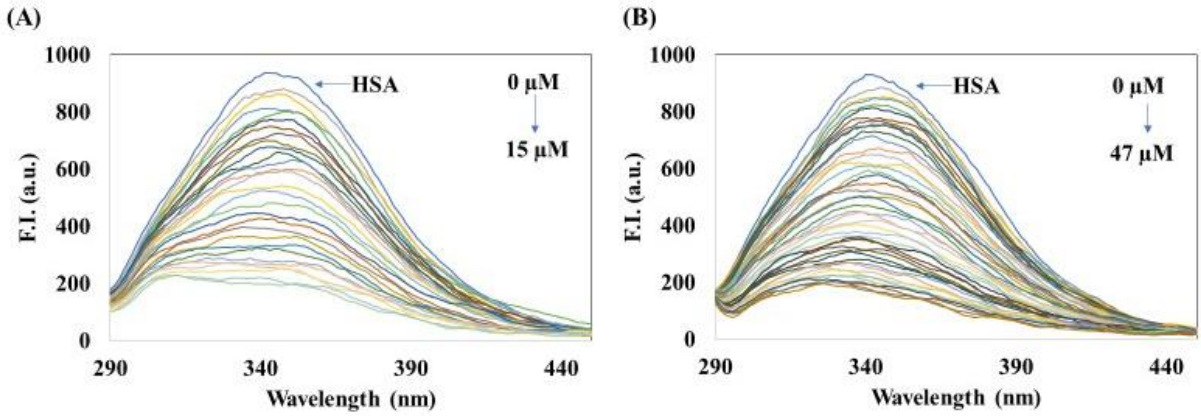


Figure 15: Effect of probes **(A) 1** and **(B) 2** on the emission spectra of HSA in PBS (pH = 7.4)

Determination of the quenching mechanism in HSA was done using the Stern-Volmer equation (eq 2). This equation helps to understand whether the quenching is static or dynamic. The quenching constants (K_{sv}) for HSA for probe **1** and probe **2** were found to be 1.9 x10^5 M^{-1} and 1.2 x 10^5 M^{-1}, respectively. The bimolecular quenching constants (K_q) for probes **1** and **2** were 1.9 x10^{13} M^{-1} s^{-1} and 1.2 x 10^{13} M^{-1} s^{-1}, respectively. The binding constants of BSA with probe **1** was calculated as 5 x 10^4 M^{-1} and for **2** was calculated as 3.1 x 10^5 M^{-1}. The binding sites of probe **1** and probe **2** with BSA were found to be 1.0 and 1.0, respectively. The diffusion-limited quenching in water is ~10^{10} $M^{-1}s^{-1}$ for HSA [26]. The calculated values of bimolecular quenching constants for HSA-probes complexes were found to be higher than the reported value of diffusion-limited quenching in water. This suggested static quenching in the fluorescence upon HSA-probes complex formation in the ground state (**Figure 16** and **Table 3**). Comparing these two probes, probe **2** showed better binding ability towards HSA.

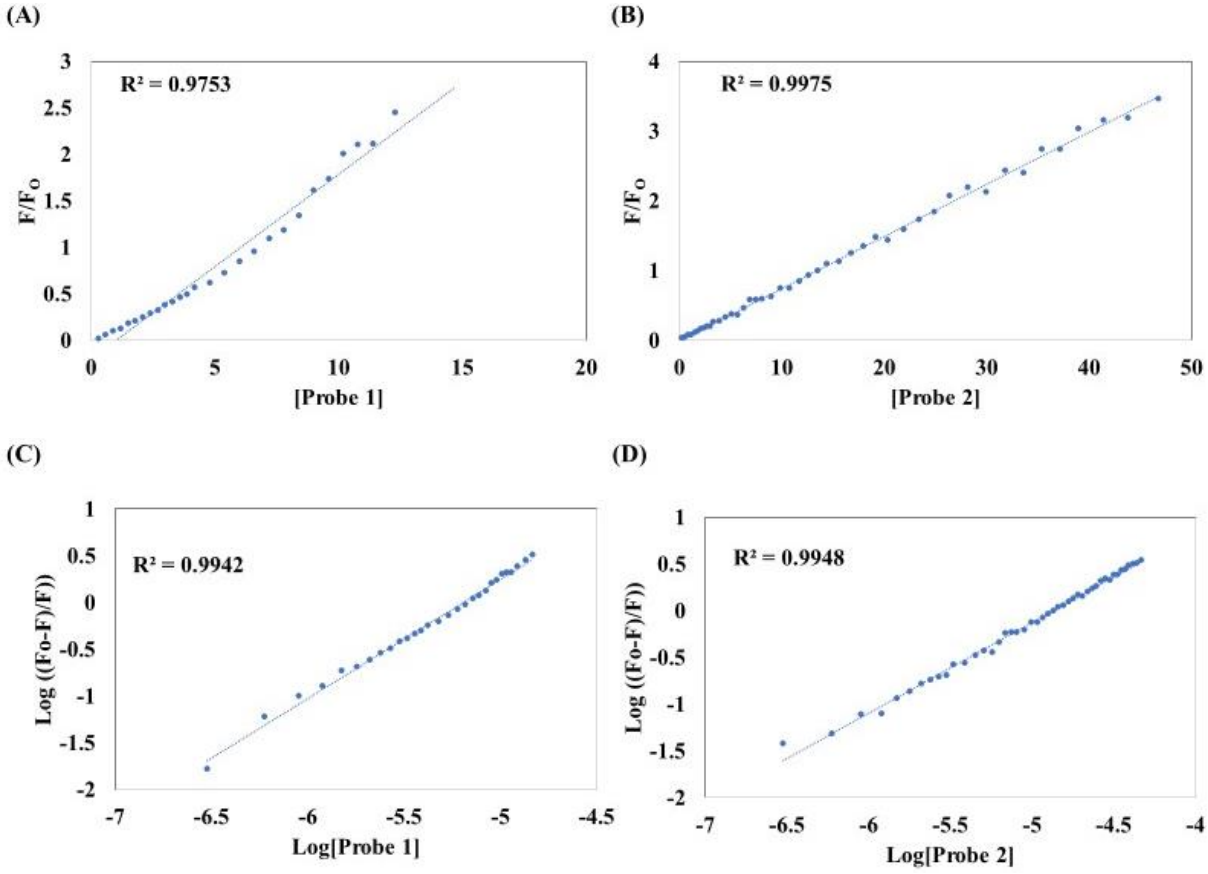


Figure 16: Calculation of quenching constant of probes **(A) 1** and **(B) 2** with HSA using Stern-Volmer equation; Calculation of binding constant of probes **(C) 1** and **(D) 2** with HSA using Stern-Volmer equation

Table 3: Quenching and binding parameters for HSA interaction with probes **1** and **2**

Probe	K_s ($\times 10^5$) (M^{-1})	K_q ($\times 10^{13}$) ($M^{-1}s^{-1}$)	R^2	K_b ($\times 10^5$) M^{-1}	n	R^2
1	1.9	1.9	0.9753	0.5	1.0	0.9942
2	1.2	1.2	0.9975	3.1	1.0	0.9948

3.3.3 Competitive assay for selectivity

Competitive assay for selectivity further confirmed the probes' selectivity towards HSA. No significant change was observed in the fluorescence spectra of the probe-HSA complex in the presence of various bio analytes. Both the probes were found to be highly selective towards HSA (**Figure 17**).

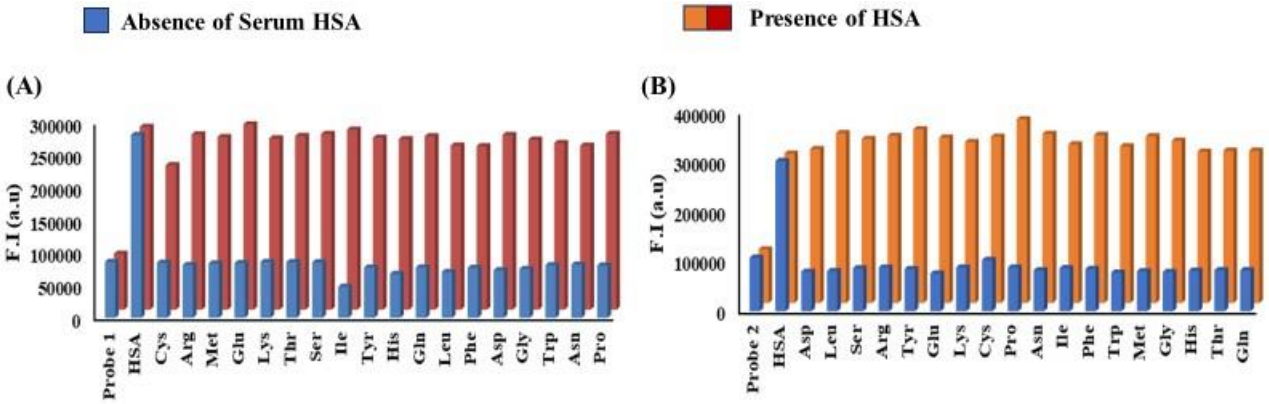


Figure 17: Interference studies of **(A)** probe **1**- HSA; **(B)** probe **2**- HSA.

3.5 Cell viability assay

To check the toxic effect of probe **2**, an MTT assay or cell viability was performed on the normal kidney cell line Hek293. The Probe was incubated at various concentrations for 48 h. The cell viability was more than 80% at all the concentrations (1-50 µM). This suggests that probe **2** is non-toxic toward human cell lines (**Figure 18**).

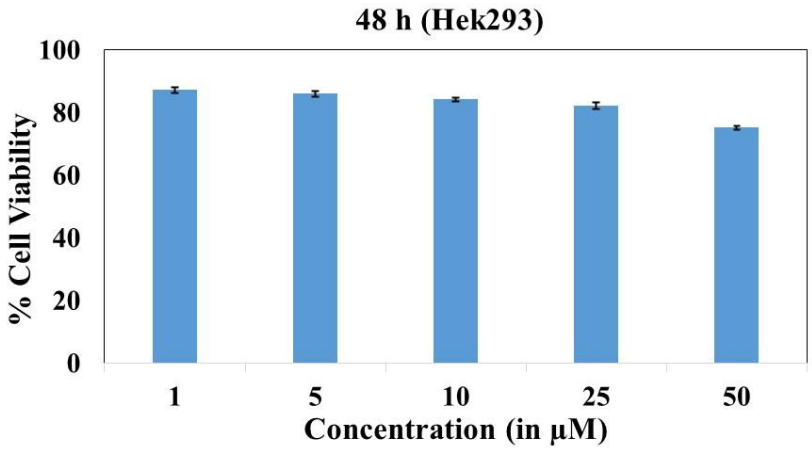


Figure 18: Cell viability of HeK293 cells in the presence of probe **2**.

Chapter 4

Conclusion

Here in this research work, two probes: probe **1** and probe **2** were developed for sensing serum albumin proteins. Preliminary analysis showed high selective sensing ability of probes **1** and **2** towards BSA as well as HSA, as compared to other bio analytes. Fluorescence study showed that the probes were interacting with the albumin proteins through static quenching, indicating that the albumin-probe complexes were forming in the ground state. Further, the binding constant of BSA-probe complex was found to be 1.7×10^5 M^{-1} and 3.1×10^5 M^{-1} for probe **1** and **2**, respectively and the binding constants of the HSA-probe were 0.5×10^5 M^{-1} and 3.1×10^5 M^{-1} for probe **1** and probe **2**, respectively. This inferred that probe **2** has better sensing ability towards serum albumin proteins than probe **1**. The number of binding sites were 1.0 for both the probes, indicating that the probes and albumin proteins bind in 1:1 stoichiometry. To further explore the binding site of the probes on BSA, protein-ligand binding study was done. It was found that the both the probes were binding at Suldow's site III. Furthermore, the MTT assay was performed to check the cell viability of the human embryonic kidney cell line (Hek293) in the presence of probe **2**. The non-toxic nature of the probe showed that this probe can further be used for biological applications.

Future Scope

Moving forward, the binding site of the probes on HSA can be determined by protein- ligand binding site studies. Furthermore, the interaction mechanism between the probes and Albumin proteins can be explored. The alteration in the size of serum albumin upon interaction with the probes can be found out with DLS. The changes in the morphology of proteins can be determined by SEM. The probes can be used for estimation of albumin concentration in urine.

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Komal Verma

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