PHOTOPHYSICAL ENCAPSULATION AND RELEASE OF ACRIDINE ORANGE CONFINED IN GRAPHENE-OXIDE MEDIATED BILE-SALT AGGREGATES

A dissertation Report Submitted

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By
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Dedicated to my family
for their
constant love &
unconditional support

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Certificate

This is to certify that the dissertation entitled **“Photophysical Encapsulation And Release Of Acridine Orange Confined In Graphene-Oxide Mediated Bile-Salt Aggregates”** is submitted by **Jaideep Gujral** to the School of Chemistry and Biochemistry (SCBC), Thapar Institute of Engineering and Technology (T.I.E.T), Patiala, Punjab for the award of the degree of Master of Science is a record of bonafide research carried out by her under my supervision and guidance. To the best of my knowledge, the matter embodied in the dissertation has not been submitted to any other University / Institute for the award of any Degree or Diploma.

15/07/2019

Date: 15-07-2019

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Declaration

I certify that

a. The work contained in this thesis is original and has been done by myself under the general supervision of my supervisor.
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ABSTRACT

In the present work, Acridine Orange, a cationic fluorescent dye has been made to interact with three different bile-salt aggregates (NaC, NaDC and NaTC). The photophysical studies of AO have been carried out by varying the concentrations of different bile salts in the presence/absence of Graphene Oxide. An important aim of the present thesis is to extensively study the UV and the fluorescence properties of AO-BileSalt-GO and AO-GO-BileSalt systems. In PBS medium, AO molecule exhibits fluorescence at λ_{exi}= 465 nm and λ_{exi}= 490 nm. It has been observed from the UV and fluorescence studies that AO shows ambiguous behavior with different bile salts. GO acts as a quencher generally, and thus leads to the decrease in the fluorescence and UV absorbance values.

In AO-BileSalt-GO system, the fluorescence and UV absorbance values of AO increase on addition of the different bile salts. On further addition of GO in the system resulted in the decrease in the fluorescence and absorbance values. This has also been confirmed by calculating the fluorescence quantum yield by using Quinine Sulphate as the standard. Moreover, the binding constant results confirm the phenomenon.

For the reverse system, i.e. AO-GO-BileSalt, the fluorescence and UV absorbance values of AO decreases on addition of GO. On addition of BileSalt to this system leads to the increase in fluorescence and absorbance values. The results have been verified by calculating the fluorescence quantam yield and binding constant.

The results suggest that AO molecules get entrapped in the bile salt aggregates. While the addition of GO in all the three bile-salt systems results quenching of absorption, fluorescence emission and quantum yield values. Moreover, in presence of GO, the studied fluorophore (AO) releases from the encapsulated bile-salt complexes to the aqueous medium of GO.

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CHAPTER-1

INTRODUCTION

1.1 Acridine Orange (AO)

AO is an important aza-aromatic compound. [1] It belongs to the class of aminoacridines. [Figure 1.1].

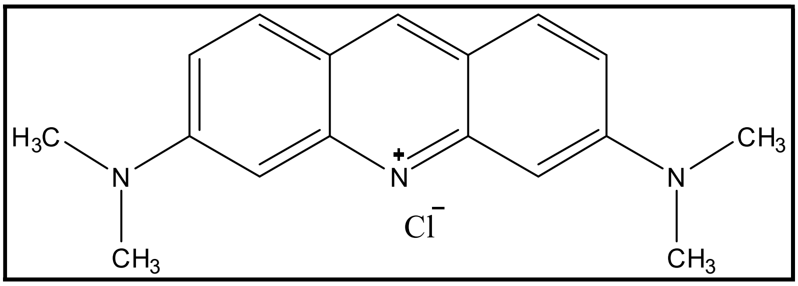

Figure 1.1: Schematic representation of Acridine Orange.

AO is a cationic dye that has a strong inclination to get adsorbed on surfaces, emitting orange color at low pH values. Due to the metachromatic property of AO, it is widely used in fluorescence microscopy to study the properties of microorganisms, microheterogenous and biological systems like micelles, vesicles, microemulsions, DNA and nucleosides.[2]AO's metachromism can be explained on the basis of interaction between the π electrons of the aromatic ring when aggregates of the dye get formed both in water and in hydrophobic medium. At micromolar (10^{-6} M) concentration, aqueous AO shows absorbance maxima at 491 nm due to the formation of monomer and at 469 nm due to the formation of dimer. Its emission maxima are visible at 527 nm.[2] The chemiluminiscence of AO has high quantam yield. The photophysical aspects are greatly influenced by temperature, protonation and environment of AO. Two major bands have been observed in the absorption spectra of AO. The first is a low lying and a structured band in the visible region starting at 3.2eV and peaking at 3.5 eV, while the second band observed is an intense one peaking near 5eV. In acidic conditions, a broad shoulder at around 3.10eV is observed. The emission spectra of AO consist of low-lying singlet and triplet states[1]. A wide variety of clinical microbiological techniques incorporate AO to stain DNA(yellow) and RNA(orange) at low pH(acidic pH).Hence, diseases like malaria, gonorrhoeae and Trichomonas Vaginalis in vaginal secretions can be easily detected.[3] Use of regular dyes like Gram stain and methylene blue for staining biological samples like blood cultures is time consuming and mostly insensitive. AO can affectively be used to stain biological samples at low pH (~4) and at low concentrations can be easily be detected using AO as it is more sensitive than Gram stain and methylene blue. Thus, AO is a sensitive, rapid and reliable stain for the use in biological field. Modifications in wood biopolymers on exposure to fungus can be observed using fluorescence spectroscopy with AO as stain. This method has a high potential to enhance the studies of the mechanisms that are active during fungal biodegradation of wood.[4] AO also has remarkable chelating properties and so are also used as ligands for heavy metals.[1]

1.2 Bio-surfactants

Surfactants, also known as surface active agents, are an interesting class of substances that have unique structural features. These are mostly amphiphilic in nature possessing both hydrophilic (head) and hydrophobic (tail) groups [Figure 1.2]

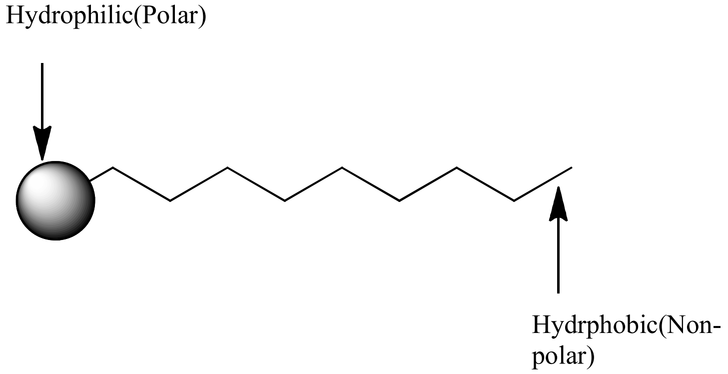

Figure 1.2: General representation of a surfactant molecule.

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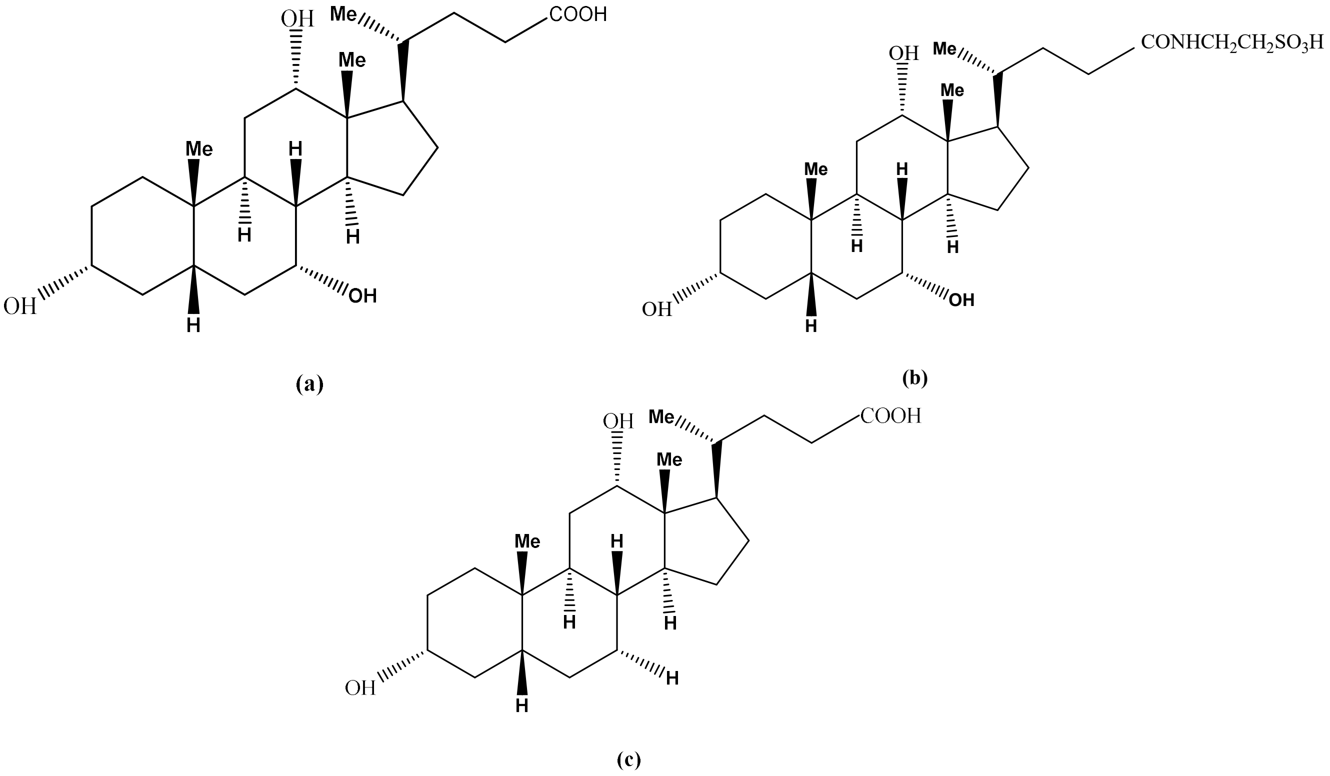
Bio-surfactants are biological surfactants that are produced on microorganisms like lactobacilli and yeasts containing hydrophobic and hydrophilic groups.[5] The hydrophobic part of the bio-surfactant molecule is made up of long-chain fatty acids. The hydrophilic portion, on the other hand, consist of polar moieties like carbohydrate, amino acid, carboxylic acid or alcohol. These two groups are responsible to reduce both the interfacial and surface tensions between two bio-surfactant molecules. Because of their low toxicity and simple preparation, bio-surfactants are used in several industries including petroleum, mining, food and beverages, cosmetics, fertilizers, pharmaceuticals etc. On dissolution in water, they form micelles, microemulsions etc. The shape and size of the bio-surfactant are vital as they determine its properties. [6] Due to the unique properties associated with them, they can also be used as wetting agents, foaming agents, demulsifiers, detergents etc. [5]

1.3 Bile-salts

Bile salts are the salts of bile acids, which are an important bio-surfactant found in mammals and other vertebrates. The liver produces bile and it gets stored in gall bladder for use in biological functions like digestion, gall stone formation. [7]Thus, they can be differentiated from other amphiphiles present in our body on the basis of their special role in our system. Bile salts are synthesized from cholic acids and hence the basic structure of bile salts includes rigid and curved tetracyclic steroid ring system. The system consists of hydrophobic ring joined with the hydrophilic group [Figure 1.3]. Some of the bile salts extensively studied are:



(a)

(b)

(c)

Figure 1.3: Schematic representation of structures of bile salts: (a) NaC (b) NaDC (c) NaTC.

Each bile salt can be differentiated on the basis of the number, position and stereochemistry of hydroxyl groups. The molecule of bile salt contains a large and rigid hydrophobic steroid group along with hydrophilic groups that provide the molecule planar polarity. It is because of this molecular structure aspect of bile salt that they do not behave analogous to conventional surfactants with clear cut polarity.[8] Bile salts can solubilise and emulsify non-polar moieties present in our biological system like dietary lipids, fatty acids, cholesterol and fat soluble vitamins in the gastronomical tract. Bile salts act as drug carrier, thereby influencing the biopharmaceutical factors like drug stability, metabolic stability, solubility in gastrointestinal fluids and intestinal permeability for absorption of the drug. They target a particular drug molecule, enhancing the bioavailability of the drug. [8]

1.3.1 Critical micellar concentration (CMC) of Bile Salts

Micelles are small colloidal particles that get formed when the concentration of surfactant is very high. This high concentration of surfactant is called the Critical Micelle Concentration (*CMC*). The aqueous solution of surfactants starts behaving like a micro-heterogenous system when the concentration of surfactant reaches CMC value. [9] Bile salts form micelle because of the presence of amphiphilicity in their molecule. Due to the micellization process of bile salts, the critical micelle concentration (*CMC*) becomes a major criterion to determine their biological activity.[8]The physiochemical properties of the surfactant solution are important aspects to study the value of *CMC* for any surfactant. The experimental techniques that are employed to determine the *CMC* value are: (i) Fluorescence spectroscopy (ii) UV-Vis spectroscopy (iii) Electrical conductance of ionic surfactant.[9]Many methods have been suggested to define the self assembly of bile salts. These are:

Small's Model (Primary and secondary micelles): [7-10]

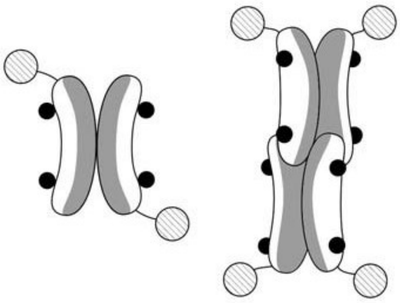


Figure 1.4: Schematic representation of primary and secondary micelles.

This is one of the earliest models which is associated with the formation of primary and secondary micelles. Firstly small aggregates are formed that are called primary micelles.In primary micelles the bile salts are associated hydrophobically with their hydophobic faces towards each other present in the interior of the bile salt aggregate. The hydrophilic group,on the other hand, is in direct contact with water and hence faces outside [Figure 1.4]. A maximum of ten bile salts can be associated in such a fashion due to space factors. Secondly, the hydrophilic groups of the primary micelles form H-bond that results in the formation of elongated secondary micelles.

Disclike model

This model is associated with the thought that the hydrophobic core is present at the interior and the hydrophilic gropus are present outside such that the long axes of the molecules are parallel in orientation [Figure 1.5]. This model helps to explain that continuous increase in the micelle size. This model can be applied to both trihydroxy and dihydroxy bile salts.

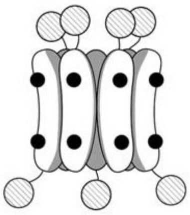


Figure 1.5: Schematic representation of disclike micelles.

Helical model [7]

This model considers the molecule to be in crystalline state rather than in liquid state. X-ray diffraction studies suggest helical arrangement of molecules in aggregate. Cations surrounded by water are present in the interior of the aggregate while the hydrophobic surfaces point outside [Figure 1.8].

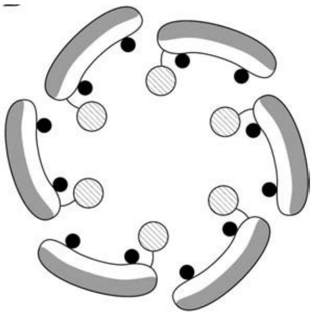


Figure 1.6: Schematic representation of helical micelles.

CHAPTER-2

LITERATURE REVIEW

2.1 Basic literature review on Acridine Orange and Bile Salts

Feng *et al.* (1998) [15] demonstrated the fluorescence and absorption spectra of AO at different concentrations. They reported that the two spectra greatly depend on the concentration of AO in the solution. Absorption spectrum at 492nm is observed that corresponds to the low concentration of AO in monomeric form. The spectra changes to 465 nm when the concentration of AO is changed. They suggested that this could be due to the aggregation of AO. As the concentration of AO is increased, red shift is observed and fluorescence intensity decreases drastically. From both the spectral results, they inferred that the aggregation of AO depends on its concentration in the solution.

Jahanmehr *et al.* (1987) [11] experimentally demonstrated that staining of clinical samples by AO is not only simple but also a reliable and reproducible method. They showed that the stock solution prepared using AO as a dye is more stable than stock solution prepared from other fluorophores provided it is excluded from light. It was also suggested by them that AO can also be used to study mature cells without adjusting the pH of its stock solution again and again. It was found by them that AO could successfully assess the morphological features of red blood cells. This technique is can also be used to detect malarial parasites. It was expressed that this methodology is superior to the standard methods.

Chiessi *et al.* (1991) [12]reported on the basis of UV-Vis and NMR studies that AO forms aggregates in aqueous solutions and on addition of bile salt (NaDC), these aggregates break. It was shown that AO interacts with the methyl group of NaDC present at C-18 & 19 positions. Thus, it was outlined that this interaction was due to the helical micellar aggregate formation of NaDC similar to that of NaTDC.

Coello *et al.*(1993) [13],while determining the depression in freezing point of aqueous NaC obtained that aggregation number is low (~5). Due to this low aggregation number, the aggregated structure can be modified using a guest molecule like NaCl. They analysed that this aggregation number can be increased by addition of guest moieties and thus bring about a change in the absorptive and fluorescent properties of NaC in aqueous media. They also confirmed this result by viscosity measurements.

Matsuoka *et al.* (2001) [14] determined the CMC and aggregation number of NaDC and NaUDC taking NaC as the reference. They reported that aggregation number increases in the order NaUDC<NaC<NaDC. This was attributed to the position of OH at C-7 in the bile salt.The second effective position of OH is at C-12 rather than C-3.

Subuddhi *et al.* (2007) [8] studied the micellisation of bile salts using DPH as the fluorescent probe in aqueous medium. They reported that the CMC of NaC and NaDC are 16mM and 6mM respectively at 25°C.They also obtained the effect of temperature and ionic strength on the micellization of bile salts. They concluded that on increasing the temperature fluffier micelles are formed with less rigid interior. They also reported that that the micellar core of NaC is less disturbed in the presence of NaCl whereas, the aggregates of NaDC five the fluorescent probe a rigid and a non-polar environment when NaCl is added.

2.2 Research gap

On the basis of the literature review, multiple numbers of research gaps have been identified. These are:

1. Firstly, AO is a cationic dye that has been previously used in a number of fields like biology, textile industry etc. Taking advantage of its excellent fluorescent properties, activities and properties of a wide number non-fluorescent compounds like bile salts can be studied.
2. There are very few reports on the interaction of AO with bile salts. Thus, a good amount of work needs to be done in this area as well to utilise the properties of bile salts both in vitro and in vivo.
3. No work has been reported till date regarding the interaction of AO and graphene oxide in the presence of bile salts. The effect of GO on the fluorescence of AO can be studied.
4. There is no literature report regarding the photophysics of AO-GO-Bile Salt system and thus the effect of GO on the limited photophysical features of AO-Bile Salts is yet to be known.

Bile salts are produced in our body and have a vital role to play. As the number of reports on the interaction of AO-Bile salt system is very less, so a lot is still unknown regarding this system. Moreover the effect of GO on the system is yet to be explored. A systematic study on this entire system can comprehend the role of GO encapsulated in AO-Bile Salt.

2.3 Aim of this project

Not much literature work has been reported on the interaction of AO in confined environments. Thus, the main aim of the thesis is to attempt and report the interaction of AO with three kinds of bile-salt aggregates and compare the result. AO is a fluorescent cationic dye, so the main focus of this project is to utilise this fluorescent property and incorporate it into the bile salt system. Bile salts are bio-surfactants that have both hydrophilic and hydrophobic group in a molecule. These groups interact with guest molecules (AO) via non covalent supramolecular interactions. These interactions are studied thoroughly through fluorescence and absorption measurements. Another aim of this thesis is to study the photophysical effect of addition of GO in the AO-Bile salt system. The study has been made on the basis of physical parameters like binding constant, fluorescence quantam yield.

CHAPTER-3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Pharmaceutical compound

Acridine Orange (AO) was purchased from Loba Chemie. The purity of the compound was more than 97% and recrystallised with HPLC graded methanol prior to use. Each time freshly prepared stock solution of the dye molecule was prepared to avoid any degradation and photochemical decomposition. The concentration of AO solution was maintained as 2 μM throughout the experiment.

3.1.2 Different bile-salts and chemicals used

Bile salts sodium cholate (NaC), sodium deoxycholate (NaDC) and sodium taurocholate (NaTC) were obtained from Loba Chemie. All the bile-salts used were of extremely high purity grade. HPLC grade methanol was obtained from SDFCL and was used as received. The concentration of phosphate buffer saline (PBS) was 0.1 M and the pH value was maintained ~7.4 throughout the experiment. Graphene oxide was synthesized from fine graphite powder, which was also procured from Loba Chemie.

3.2 Experimental methods

3.2.1 Synthesis of Graphene Oxide (GO)

Graphene oxide (GO) was prepared using Hummer's method.[24] Fine graphite powder (0.5 g), $NaNO_3$ (0.5g), concentrated H_2SO_4 (23mL) were added to an Erlenmeyer flask (50 mL) in an ice bath such that the temperature of the system was below 20ºC. The mixture was stirred continuously for 4 hours using a magnetic stirrer. Then $KMnO_4$ (3g) was slowly added to the reaction mixture while the flask was still in the ice bath. The mixture was again stirred for an hour. After stirring, ice bath was removed and the reaction mixture was heated at 35ºC using water bath followed by an hour stirring. At this stage, distilled water (46 mL) was added and the entire mixture was transferred to another Erlenmeyer flask (100 mL).The mixture was stirred well and again heated at 95ºC for 2 hours. The mixture was then allowed to cool at room temperature followed by addition of distilled water (10 mL) with stirring. Finally 30% H_2O_2 (10 mL) was added dropwise. After that, the mixture was washed with 5% HCl and distilled water to obtain graphene oxide. Then, it was dried overnight in an oven to obtain dry graphene oxide. This was stored in microcentrifuge tubes at room temperature for later use. Aqueous solution of GO was Graphene oxide solution was prepared by addition of distilled water (10 mL) in GO (1 mg).

3.2.2 Preparation of Phosphate Buffered Saline

The PBS solution was prepared in vitro by mixing NaCl (8 g), KCl (0.2 g),Na_2PO_4 (1.44 g) and KH_2PO_4 (0.24 g) with distilled water(500 mL) in a beaker. After the addition, the pH of the solution was adjusted to 7.4 using HCl solution. Finally, distilled water (500 mL) was added to make the volume of the solution 1L.The buffer solution prepared was transferred to a volumetric flask with a tight lid and stored in the refrigerator for later use.

3.2.3 Preparation of sample solution

The stock solution by dissolving AO (2 mg) in HPLC methanol (3 mL) . The cuvette was thoroughly washed with dilute HNO_3, rinsed with methanol and then finally dried using a dryer. From the stock solution, required amount of aliquots (10 µl) was pipette out by using micro-liter pipette in a cuvette. This was dried again till the faint smell of methanol departs. After this, PBS (2 mL) was added and mixed well, which is considered as AO in buffer medium. The dye at this low concentration would neither affect the spectra nor get aggregated.

3.2.4 AO-Bile Salt-GO

To the buffer medium of AO, required amounts of respective bile-salts were successively added in the cuvette and mixed thoroughly with the help of micro-pipette such that the bile salt dissolved completely. The concentrations of different bile-salts were varied from the lower concentration to higher concentration range, starting from below *CMC* value (pre-micellar concentration), followed by at *CMC* (critical micellar concentration) and above *CMC* (higher micellar concentration). After that, GO solution was added gradually to the saturated AO-bile salt solution.

3.2.5 AO-GO-Bile Salt

To AO buffer medium, added high amount of GO solution in the cuvette and mixed thoroughly with the help of micro-pipette such that GO and AO buffer medium get mixed well. The concentrations of different bile-salts were varied from the lower concentration to higher concentration range , starting from below *CMC* value ,followed by at *CMC(*critical and above *CMC* (higher micellar concentration) were gradually added along with constant mixing .

3.2.6 Ultraviolet-Visible absorption measurements

UV-Vis absorbance measurements were performed by using Perkin Elmer UV (Lambda 35) spectrophotometer using 1cm path length quartz cuvette. The wavelength range was set at 400-800nm.The UV-Vis spectra were recorded for both AO-GO-Bile Salt and AO-Bile Salt-GO systems.

3.2.7 Fluorescence measurements

The fluorescence emission spectra were performed by using Perkin Elmer LS 55 fluorescence spectrometer using quartz cuvette of a 10mm path length. The excitation wavelengths were fixed at 465 nm and 490 nm. The slit widths for recording the spectra were fixed at 15 nm and 4 nm respectively with the scan speed of the instrument being fixed to 250 nm per minute. The fluorescence spectra were also recorded for both AO-GO-Bile Salt and AO-Bile Salt-GO systems.

3.3. Analysis methods

3.3.1 Determination of ground state and excited state binding constant

The data obtained from the fluorescence spectra and the UV-Vis spectra was used to calculate the ground state and the excited state binding constant values .K_1 is the 1:1 gound state binding constant for AO was determined using the non- linear least-squares regression analysis method. The mathematical equation associated with the ground state binding method is:

$$A = \frac{\mathbf{A_{buffer}} + \mathbf{A_{micelle}} \times \mathbf{K_1[Micelle]}}{\mathbf{1 + K_1[Micelle]}} \qquad (Eq. 3.1)$$

Where, A_{buffer} = absorption intensity of AO in buffer

$A_{micelle}$ = absorption intensities respective micellar concentration of bile-salts.

K_1 = 1:1 ground state binding constant

The mathematical equation associated with the excited state binding method is:

$$\boldsymbol{F} = \frac{\mathbf{F_{buffer}} + \mathbf{F_{micelle}} \times \mathbf{K'_1}[\mathbf{Micelle}]}{\mathbf{1} + \mathbf{K'_1}[\mathbf{Micelle}]} \qquad (\ Eq. 3.2)$$

Where, F_{buffer} = Fluorescence intensity of AO in buffer

$F_{micelle}$ = Fluorescence intensity of respective micellar concentration of bile-salts.

K_1' = excited state 1:1 binding constant value of CV-bile aggregates.

3.3.2 Determination of fluorescence quantum yield (Φ)

Flurophores exhibit fluorescence and thus their fluorescence quantam yields are calculated from the emission intensities and absorbance at a particular wavelength. The mathematical formula to calculate the fluorescence quantum yield is:

$$\boldsymbol{\Phi_S} = \boldsymbol{\Phi_R} \times \left(\frac{A_S}{A_R} \times \frac{(Abs)_R}{(Abs)_S} \times \frac{n_S{}^2}{n_R{}^2}\right) \qquad (Eq. 3.3)$$

Where, Φ_S = Fluorescence quantum yield of sample

Φ_R = Fluorescence quantum yield of reference

Abs_R= Absorbance of reference

Abs_S= Absorbance of sample

A_R = Area under the fluorescence emission of reference

A_S = Area under the fluorescence emission of sample

n_S = Refractive index of sample

n_R = Refractive index of reference

CHAPTER-4

RESULTS AND DISCUSSION

4.1 UV-Vis absorption studies

In phosphate buffer saline (PBS), AO showed absorption maxima at 490 nm followed by a shoulder band ~465 nm. Feng *et al* [15-16] proposed that with increase the concentration of AO, the shoulder band becomes prominent due to dimer formation whereas at low concentration of the fluorophore, an absorption maximum becomes prominent. Figure 4.1 represents the absorption spectra of AO in PBS medium.

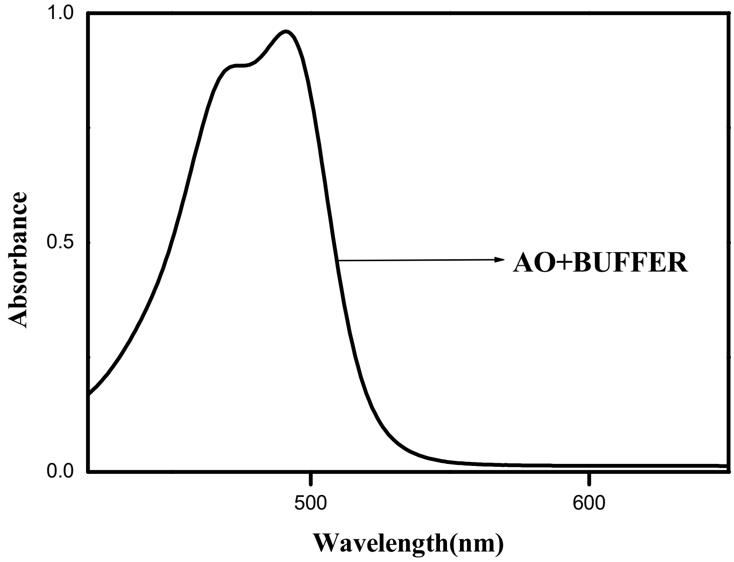


Figure 4.1: Absorption spectra of AO in PBS.

Increasing the concentration of the bile-salts, the absorbance of the dye molecule gradually enhances along with the bathochromic shift of the absorption maxima.[17] The increase in the absorption of AO on gradual addition of different bile salts is attributed to the fact that bile salt gets aggregates. The addition of aqueous suspension of GO into AO-Bile salt system showed a drastic decrease in the absorbance value. Figure 4.2 represents the addition of graphene oxide in AO-Bile salt systems.

This decrease can be attributed to the fact that AO molecule is encapsulated in the bile salt micelles. As GO is added into the AO-Bile salt system, the aggregates formed get disturbed resulting in the breakage of hydrophobic interaction between the dye and bile salt molecules. Thus, it can be inferred that addition of GO caused release of AO molecules from the encapsulated bile salt aggregates.

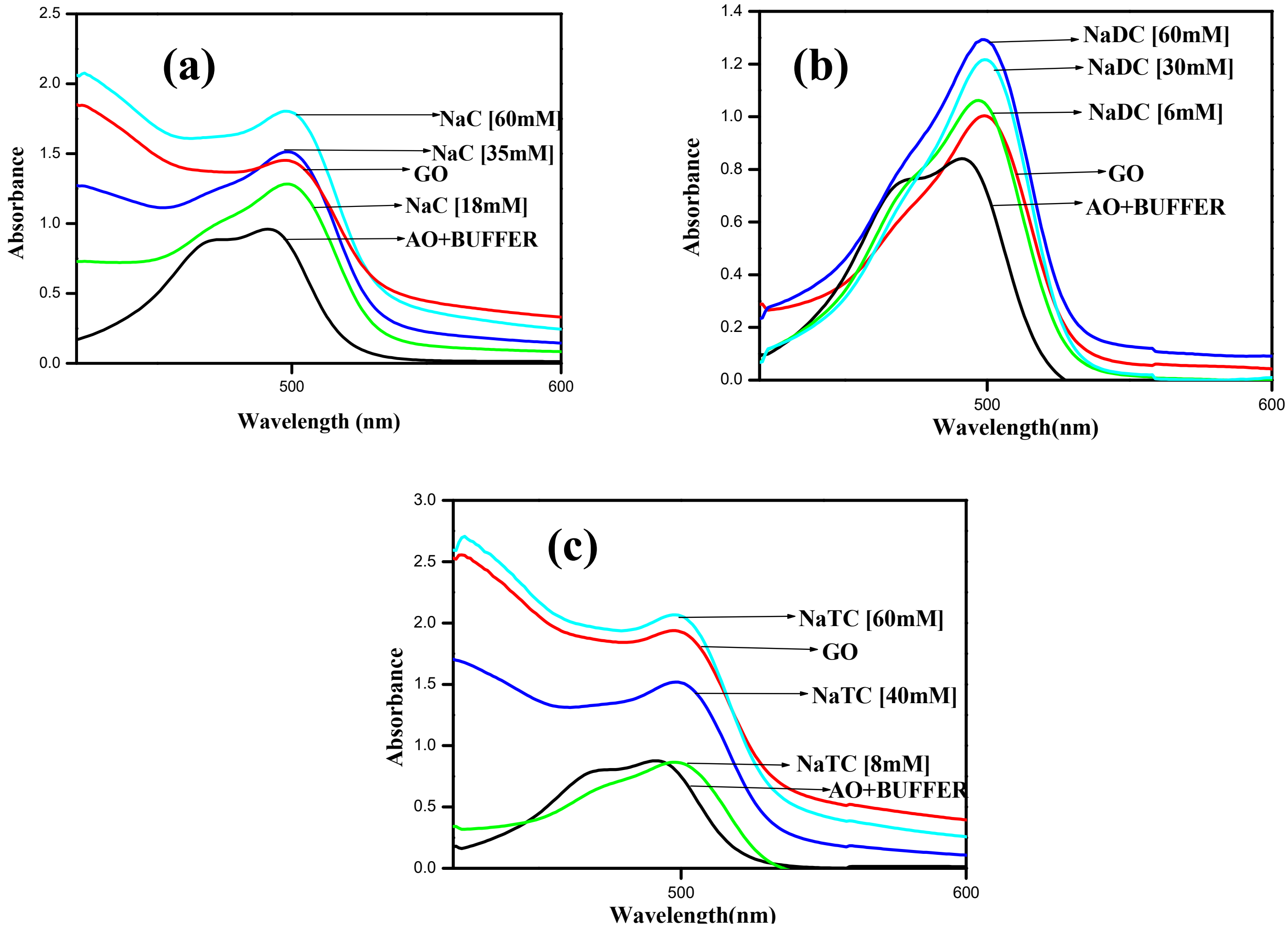


Figure 4.2: Absorption spectra of (a) AO-NaC-GO (b) AO-NaDC-GO (c) AO-NaTC-GO.

On studying the reverse system i.e. addition of bile salt into AO-GO system leads to the increase in the absorbance values. The absorbance values are extremely high when bile salt concentrations reach above *CMC* values. This can be due to the fact that when the concentration of bile salt in AO-GO system is below *CMC*, comparatively less hydrophobic interactions are possible between AO and the bile salts. As the bile salt concentration reaches the *CMC* value, the AO-Bile salt interactions increases that results in enhancement of the absorption value. At above *CMC* concentration of the bile salt, the absorbance value reaches the maximum value (Figure 4.3). Beyond this concentration, the AO-GO-Bile salt system reaches the saturation point in absorbance and thus no effect is seen on addition of bile salt beyond this point.

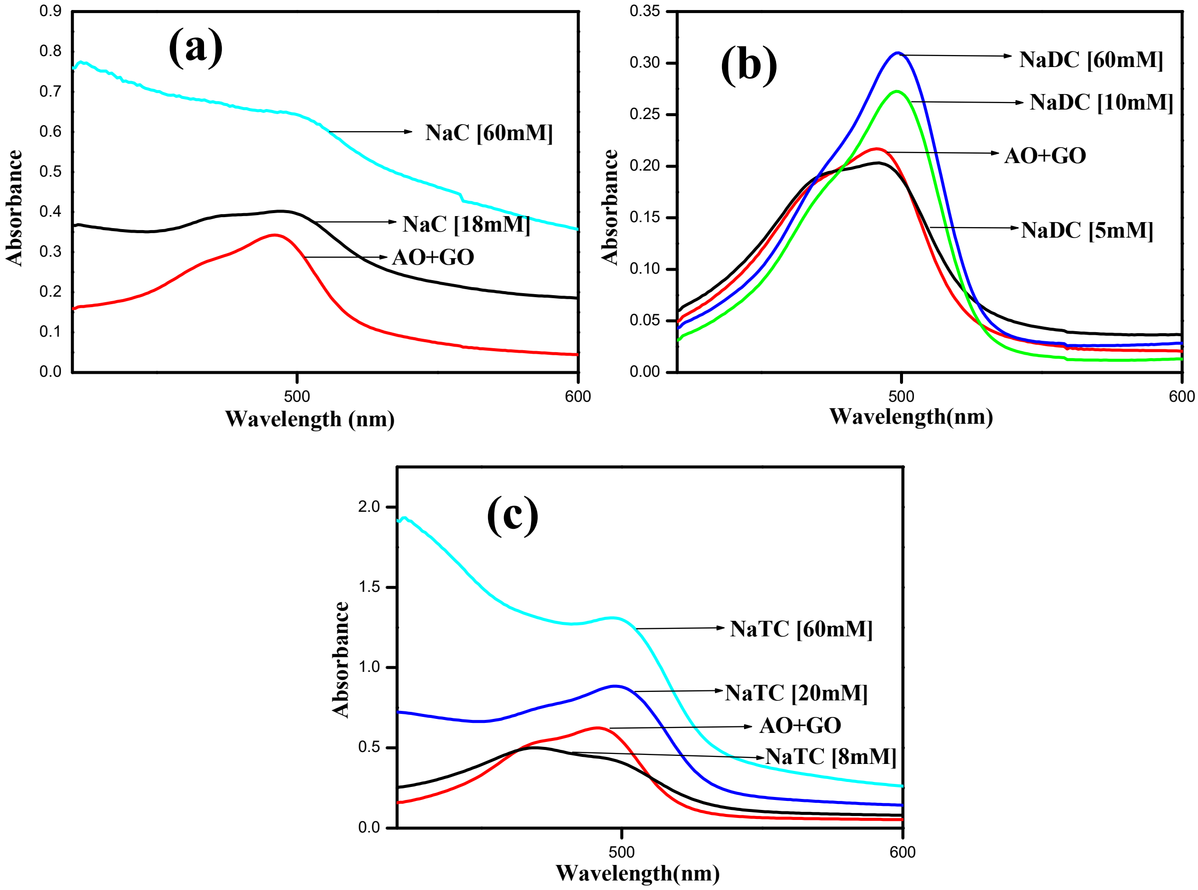


Figure 4.3: Absorption spectra of (a) AO-GO-NaC (b) AO-GO-NaDC (c) AO-GO-NaTC.

4.2 Fluorescence emission studies

The fluorescent emission spectra of AO were recorded at two different excitation wavelengths (λ_{exi}= 465 nm and 490 nm). (Figure 4.4)

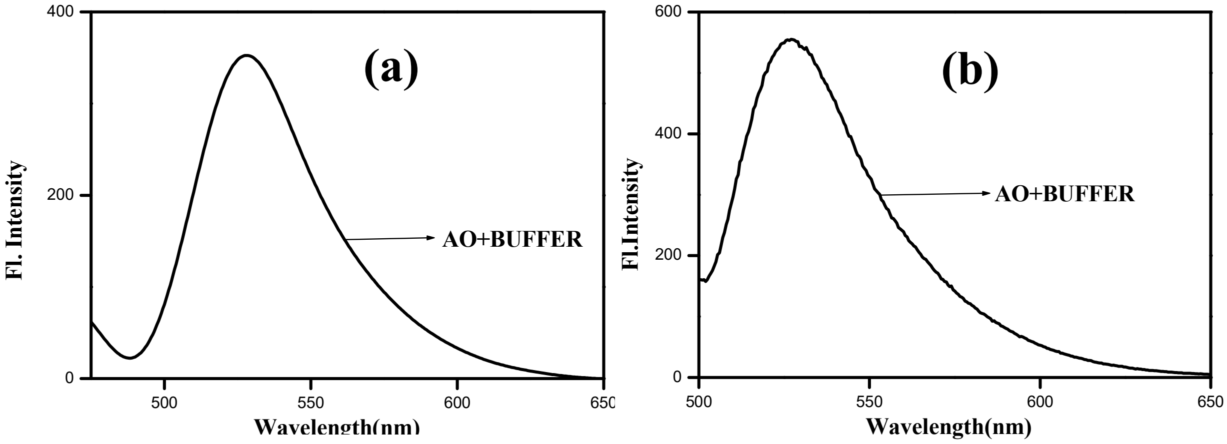


Figure 4.4: Fluorescence emission spectra of AO in PBS medium at (a) λ_{exi}= 465 nm (b) λ_{exi}= 490 nm.

In PBS, AO showed the fluorescence emission maxima at ~530 nm. Incorporation of the bile-salts to the buffer solution caused significant increase of the fluorescence emission intensity of AO, which clearly shows the formation of the encapsulated host-guest complex (Figure 4.5). The fluorescence spectra arise when AO molecules get encapsulated in the bile salt aggregates. Thus fluorescence exhibited depends on the extent to which the AO molecules get entrapped in the bile salt aggregates. A very obvious difference among the three bile salts is that of head groups. From the Figure 1.3, it can be clearly inferred that the structures of NaC and NaDC are very similar, except that NaC consists of an extra hydroxyl group on its polar part which makes it less hydrophobic as compared to NaDC. Also, NaDC has an non-conjugated head group due to which it is considered as a weak acid. NaTC has a conjugated head group that provides it more hydrophilicity. Thus it can be easily concluded that NaDC is the most hydrophobic among the three bile salts. [18]

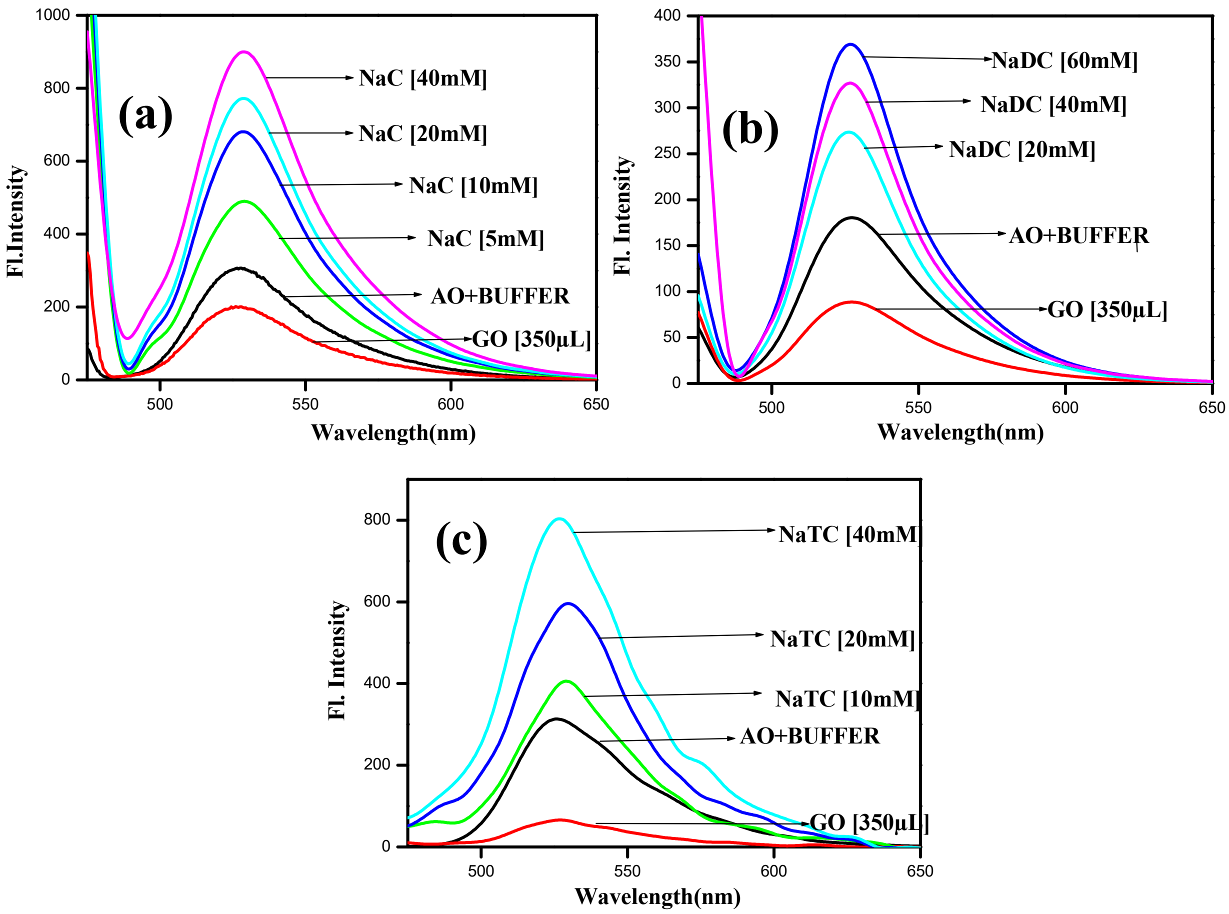


Figure 4.5: Fluorescence emission spectra of (a) AO-NaC-GO, (b) AO-NaDC-GO and (c) AO-NaTC-GO.

GO acts as a quencher[19-20] in the AO-Bile salt systems through a process called Forster Resonance Energy Transfer (FRET). The quenching takes place by the means of the non-covalent interaction between AO-Bile salt and GO. The addition of GO reduces the fluorescence from a maximum value to the lowest fluorescence value. [21] Figure 4.5 shows the fluorescence effect on addition of GO in AO-Bile salt systems

The efficiency of the fluorescence emission can be analyzed by evaluating the fluorescence quantum yield.[22]The fluorescence quantum yield values (Φ) of AO in different bile-salt aggregates was calculated using Quinine Sulphate (Φ=0.546) as the reference and the results have been tabulated in Table 4.1

Table 4.1: Fluorescence quantum yield values (Φ) of AO in different Bile salt systems.

System	Φ_{465nm}	Φ_{490nm}
AO (2µM) in buffer	0.15	0.14
AO (2µM)+NaC (100 mM)	0.38	0.36
AO (2µM)+NaC (100 mM)+GO (350µL)	0.08	0.07
AO (2µM)+NaDC (100 mM)	0.68	0.64
AO (2µM)+NaDC(100mM)+GO(350µL)	0.10	0.09
AO (2µM)+NaTC(100 mM)	0.46	0.42
AO (2µM)+NaTC(100mM)+GO(350µL)	0.10	0.08

Error range: ±10%

It can be easily concluded from the above results that the fluorescence quantum yield values for NaC and NaTC are quite different from that of NaDC at both the wavelengths (λ_{exi}= 465 nm and 490 nm). Due to the large hydrophobicity of NaDC, it encapsulates the AO molecules more than NaC and NaTC and thus exhibit higher fluorescence emission. Hence the fluorescence quantum yield of NaDC is also high. Moreover, it has been also found that addition of GO results fluorescence quenching in all the three bile salt systems. As a result, fluorescence quantum yield drastically reduced.

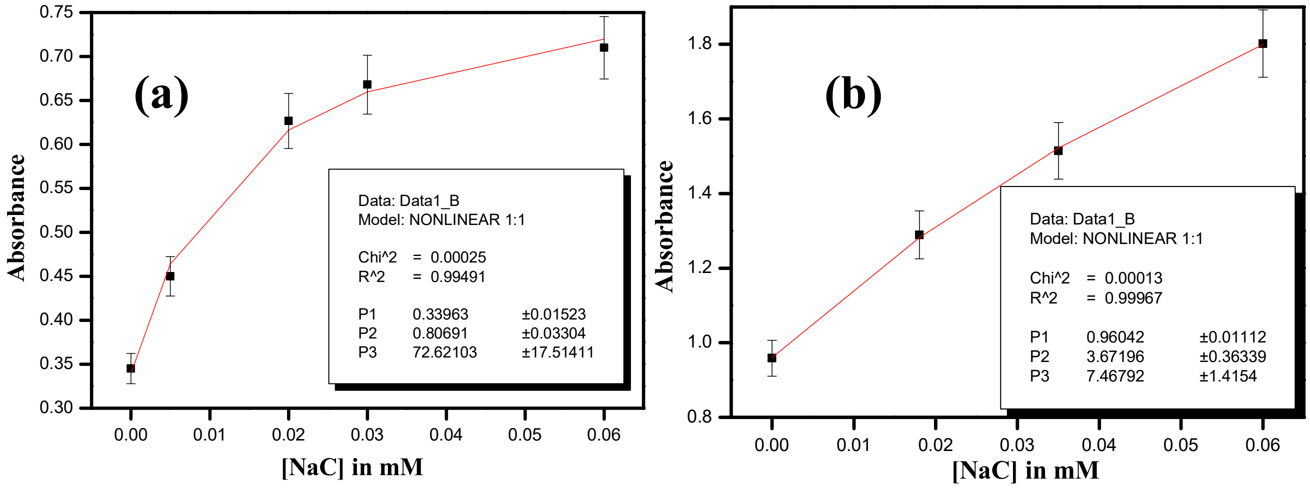
4.3 Determination of binding constant values of AO-Bile Salt systems in ground state and excited state

It has been previously discussed that AO-Bile salt interacts with each other through non-covalent interactions. The AO molecules get encapsulated in the bile salt aggregates. In other words bile salt acts as hosts for AO (guest) molecules. [23] In order to quantitatively study the extent of binding between the host and guest moieties, binding constant has to be determined. The binding constant was assessed using the non-linear analysis method for AO-Bile salt system and also for AO-GO-Bile salt system by taking help from UV absorbance and fluorescence data, which showed 1:1 stoichiometric complex is formed.

Table 4.2: Binding constant values of AO from UV-Vis study

BINDING CONSTANTS	
AO+NaDC	**AO+GO+NaTC**
123.0 ± 6	48.0 ± 15.0
AO+NaC	**AO+GO+NaC**
72.6 ± 17.5	7.5 ± 1.4
AO+NaTC	**AO+GO+NaTC**
90 ± 11	19 ± 8

From Table 4.2, it was found that the binding constant value of AO molecule significantly increases which indicating that the interaction of AO with bile salts takes place in ground state. Therefore, AO molecule forms 1:1 encapsulated host- guest complex in presence of bile-salt aggregates. The ground state binding constant order of AO molecule follows the order: NaDC>NaTc>NaC. Addition of GO to the encapsulated bile-salt complexes results significant decline of the binding constant values. This result clearly states that AO molecule releases from the bile-salts to the GO system. Figure 4.6 represents binding constant plot of AO in NaC bile salt and GO-mediated NaC bile salt aggregates.



```
Data: Data1_B
Model: NONLINEAR 1:1

Chi^2   = 0.00025
R^2     = 0.99491

P1      0.33963      ±0.01523
P2      0.80691      ±0.03304
P3      72.62103     ±17.51411
```

```
Data: Data1_B
Model: NONLINEAR 1:1

Chi^2   = 0.00013
R^2     = 0.99967

P1      0.96042      ±0.01112
P2      3.67196      ±0.36339
P3      7.46792      ±1.4154
```

Figure 4.6: Binding constant value for (a) AO-NaC and (b) AO-GO-NaC.

On similar grounds, the binding constants were calculated for AO-bile salt system using the fluorescence emission spectra.

Table 4.3: Binding constant values of AO from fluorescence study

BINDING CONSTANTS (λ_{exi} = 490 nm)	
AO+NaC	**AO+GO+NaC**
122 (±17)	30 (±4)
AO+NaDC	**AO+GO+NaDC**
752 (±70)	21 (±3)
AO+NaTC	**AO+GO+NaTC**
144 (±22)	27 (±4)

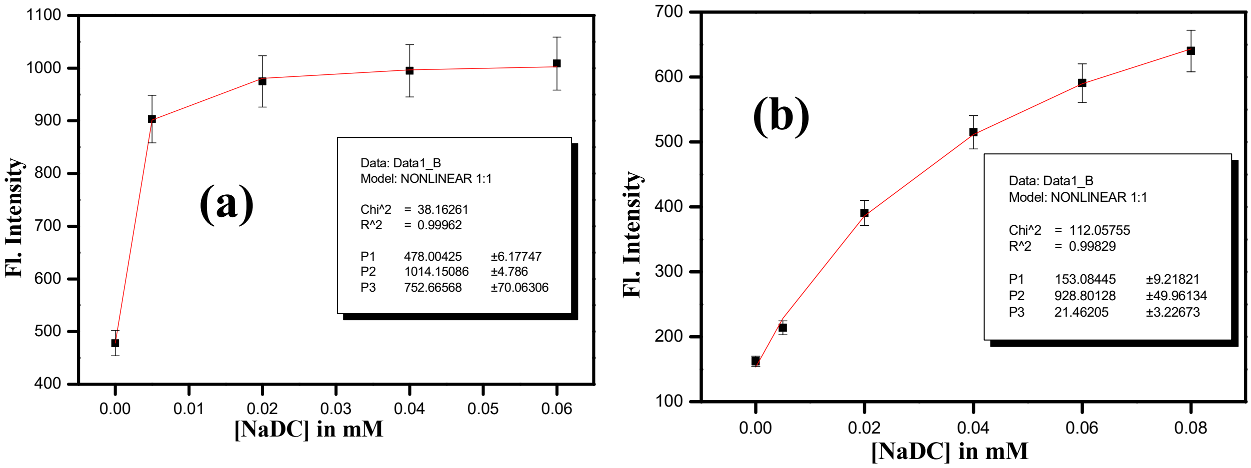


Figure 4.7: Binding constant value for (a) AO-NaDC (b) AO-GO-NaDC.

From the excited state fluorescence emission studies, it has been found that in the excited state, the binding constant values of AO in bile-salts are more compared to that GO-mediated bile-salt. The excited state binding constant values of AO in presence of bile-salt aggregates follows the same order of ground state binding constant, i.e., NaDC>NaTC>NaC. Moreover, it was also observed from Table 4.3 that in presence of GO, the binding constant value significantly quenches, which indicates that AO molecule releases from the encapsulated bile-salt aggregates to the aqueous GO medium. Figure 4.7 represents binding constant plot of AO in NaDC bile salt and GO-mediated NaDC bile salt aggregates.

CHAPTER-5

CONCLUSION

The present thesis evaluates the photophysical interaction between AO molecules and different kinds of bile salt aggregates (NaC, NaDC, NaTC) in the presence of GO (quencher). Two techniques were employed to study the properties of the bile salt namely- UV-Vis and fluorescence emission. It is quite evident from the spectral results of both the techniques that the dye molecule studied (AO) shows ambiguous behaviour with different bile salts. To confirm our results fluorescence quantum yield and binding constants were also calculated. The results clearly showed that AO molecules get entrapped in the bile salt aggregates. While the addition of GO in all the three bile-salt systems results quenching of absorption, fluorescence emission and quantum yield values. Moreover, the ground state and excited state binding efficacy were also determined, which shows that in presence of GO, the studied fluorophore (AO) releases from the encapsulated bile-salt complexes to the aqueous medium of GO. Therefore, the present study may be applied as drug delivery vehicles and fluorescence sensors as fluorescence turn-on/turn off phenomenon in physiological system.

CHAPTER-6

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