ENCAPSULATION AND RELEASE OF NON-FLUORESCENT BIO-ACTIVE DRUG CONFINED IN BILE-SALT AGGREGATES

A dissertation report submitted

in partial fulfilment of the requirement for

the award of degree of

Master of Science

in
Chemistry

By

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Dedicated to my parents
for their
enduring love &
unconditional support

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Certificate

This is to certify that the dissertation entitled "**Encapsulation and release of non-fluorescent bio-active drug confined in bile-salt aggregates**" is submitted by **Prachi Sharma** 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.

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ABSTRACT

In this thesis, the entrapment of anti-fungal, anti-bacterial non-fluorescent dye Crystal Violet (CV) in presence of bio-mimetic confined bile-salt aggregates has been studied. The photophysical characteristic behaviour of CV has been carried out by changing different kinds of hydrophilic head groups and hydrophobic skeletons of bile-salt aggregates (e.g. NaC, NaDC, NaTC and NaTGC). The main aim of the thesis is to modulate the solubility behaviour, fluorescence property and elucidation of different kinds of non-covalent interaction of CV confined in bile-salt aggregates. To interpret the result, steady state absorption and fluorescence emission techniques have been employed. In aqueous buffer, CV molecule exhibits non-fluorescent in nature. The value of fluorescence quantum yield (Φ) is $\sim 10^{-4}$. It has been observed that CV molecule confined in bile-salt aggregates becomes highly fluorescent in nature. The enhancement of 'Φ' value of CV in bile-salt aggregates is ~1000 folds compared to that of aqueous buffer medium. It has also been observed that in presence of different bile-salt aggregates, CV molecule exhibits remarkable enhancement of absorption and fluorescence emission spectral behaviour. The ground state and the excited state binding constant values of CV molecule in presence of different bile-salt aggregates have been determined by using non-linear regression analysis method. In presence of different bile-salt aggregates; the partition coefficient values of CV are very high. This clearly depicts the dye molecule resides in bile-salt aggregates.

Moreover, another aim of the thesis is to release of the dye molecule from the confined bile-salt aggregates to the aqueous medium. Since, the dye molecule exhibits strong fluorescent in presence of bile-salt aggregates, therefore the target was by addition of foreign substance (non-toxic and green), the dye molecule return backs (from confined bile-salt environments) to its original position (aqueous buffer medium). It will be possible only when CV molecule will exhibit again non-fluorescence property. It has been found that addition of very minute concentration of KCl salt (100 nM) to the bile-salt aggregates leads to extreme modification of the photophysical property of CV molecule. The absorption, fluorescence intensity, fluorescence quantum yield, ground state and excited state

binding constant values, partition coefficient and aggregation number of CV molecule entrapped in bile-salt aggregates significantly reduces by addition of KCl. This result clearly confirms that the studied molecule releases from the confined system to the aqueous medium. The work involved in this thesis might be valuable for potential targeted drug-delivery implications, detection analysis, sensors, and also in physiological systems.

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CHAPTER-1

INTRODUCTION

1.1 Crystal Violet (CV)

Triphenylmethane dyes are generally known as aniline dyes. They are one of the most important classes of commercial dyes, which have a wide variety of potential applications in photographic industry, dyeing, and textile industry, sensitizers for photoconductivity in environmental science, medicinal and biological chemistry.[1-7] Crystal violet (also known as Gentian violet or methyl violet 10B) is chemically assigned as Tris(4-(dimethylamino)phenyl)methylium chloride It is an inexpensive cationic dye with molecular formula ($C_{25}H_{30}N_3Cl$) (**Figure 1.1**). X-Ray studies[5] indicate that the structure of CV molecule is propeller-shaped with its aromatic rings rotated by 32˚ from the central horizontal plane. In aqueous solution, CV molecule is surrounded by water molecules and chloride counter ions, which interact with the positive charge present on central C and N of CV molecule and results in changes in symmetry [8,9]. This dark purple coloured dye (due to its conjugative structure) shows striking optical behaviour as it exhibits intense visible absorption bands of molar extinction coefficient (ε_{max}) is 10^5 cm^2 M^{-1} upon excitation. In aqueous medium, CV molecule exhibits weak fluorescence due to very fast rotation of its aromatic rings. The absorption spectrum of CV displays one shoulder band along with the absorption maxima. This characteristic feature of the absorption spectra of CV can be explained on the basis of the existence of two ground state isomers. The two type of ground state isomers **(Figure 1.1)** are planar form and the distorted or the pyramidal form.[6]

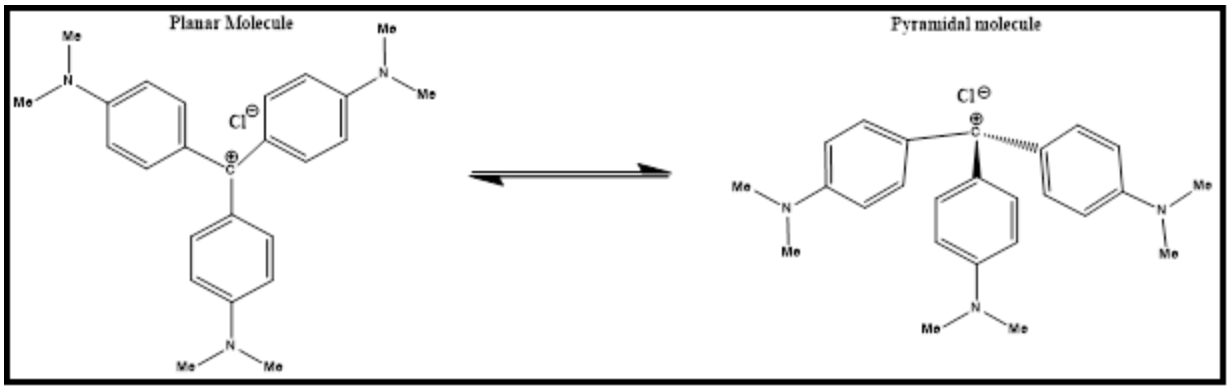

Figure 1.1: Schematic representation of two isomeric form of Crystal Violet.

1.2 Pharmaceutical importance of CV

Crystal violet exhibits potent clinically useful properties and exhibits various pharmacological activities. [10-15] CV is one of the most important dye molecule which is used for the histological staining, classifying bacteria (Gram's Method), topical treatment of skin infections and wounds like burns, ringworm and eye infections like pink eye, due to its antiseptic, anti-fungal, anti-bacterial properties. From almost 100 years, it has been used for medicinal treatment for anti-angiogenic, anti-trypanosomal, anti-helminthic and anti-tumour activities. Recently it has also been used as a blood additive during the transfusion to inactivate *Trypanosoma cruzi* (causative agent), for the prevention of the transmission of Chagas' disease.[11]CV can also be used for the treatment of *Ichthyophthiriusmultifiliis*, which leads to 'white spot disease' in fish.

1.3 Bio-surfactants

Bio-surfactants are several groups of amphiphilic compounds, synthesized by living organisms that contains both hydrophobic and hydrophilic moieties. Most of the bio-surfactants are either anionic or neutral having a hydrophilic moiety that can be a phosphate group, hydroxyl group, carbohydrate moiety or an amino acid. The hydrophobic moiety consists of a long carbon chain fatty acid or bulky steroidal rings. These are grouped as glycolipids, phospholipids, lipopeptides, neutral lipids and fatty acids. These surfactants are completely dissolved in aqueous medium. They also have ability to organize spontaneously by themselves in a definite fashion to form bi-layers, micelles, reverse micelles, lipids, vesicles, etc. The aggregation property of bio-surfactants depends upon their concentrations, molecular structure and experimental conditions of the medium like solvent, pH, temperature etc. The biological membranes consists of complex multi-component structures, hence bio surfactant having much less complexity are used as model systems for bio-membranes for investigation of the interactions of various drug molecules with biological membranes.

1.4 Bile-salts

Bile salts are naturally occurring non-conventional bio-surfactants, synthesized in the liver in form of greenish yellow secretion called bile, with the help of a cytochrome named P450 through well-moderated oxidation of cholesterol. Bile is made up by different

constituents. Bile acid is one of the constituents of bile, which is composed of deoxycholic, cholic, chenodeoxycholic, and lithocholic. These acids interact with taurine or glycine, forming various complex bile derivatives. The biological importance of bile-salts is their capability to solubilize fats, monoglycerides, and emulsification of cholesterol, as well as fat-soluble vitamins in a human digestive system. [18-21] Bile salts play an important role in assisting the digestion of lipids and fats inside human system. Apart from these physiological applications, bile salts play a pivotal role in pharmaceutical and biochemical applications as vehicles for carrying drugs. Several biopharmaceutical factors like drug stability, solubility in the gastrointestinal fluids, metabolic stability and sufficient intestinal permeability for absorption of drug molecule. More than 50% of drug molecules have low aqueous solubility.[22] The enterohepatic organotropism of bile-salts convert them into intriguing drug delivery vehicles for targeting selective drugs or enhancing bioavailability of the drug molecule by improvising their intestinal absorption and metabolic stability. Such compounds are well solubilized in bile salts and thus transported to different parts of body, hence increasing its bio-availability.[23] Due to their biocompatible and biodegradable nature. [16,17] and ability to undergo supramolecular host-guest interactions, bile-salts have gained a lot of attention for various drugs, cosmetic materials, vitamins *etc.* making them very cheap, non-toxic and efficacious drug carrier vehicle for medical applications.

Bile-salts are amphiphilic compounds like surfactants but instead of having a small polar head and a flexible non-polar tail, bile-salts are rigid in structure. Bile-salts have convex side of the steroid moiety, which is hydrophobic in nature and a concave side, which is made up of hydrophilic polar groups (generally hydroxyl). This facial amphiphilicity imparts a unique property in bile-salts which leads to selective binding with different molecules. This property of bile-salts can be exploited in medicinal field. [24]

Bile-salts (**Figure 1.2**) are easily recognized by its four-membered hydrocarbon core known as "cyclopentanoperhydrophenanthrene". All the natural as well as synthetic steroids are derivatives of this core. Ring 'A' is the cyclohexane ring which is fused to another six-membered ring called ring 'B', followed by ring 'C' and a cyclopentane system called ring 'D'. On the basis of different number, position and orientation of hydroxyl group at C-3, C-7 and C-12 position of the steroid ring human bile-salts are categorized as various names (tabulated in **Table 1.1**).

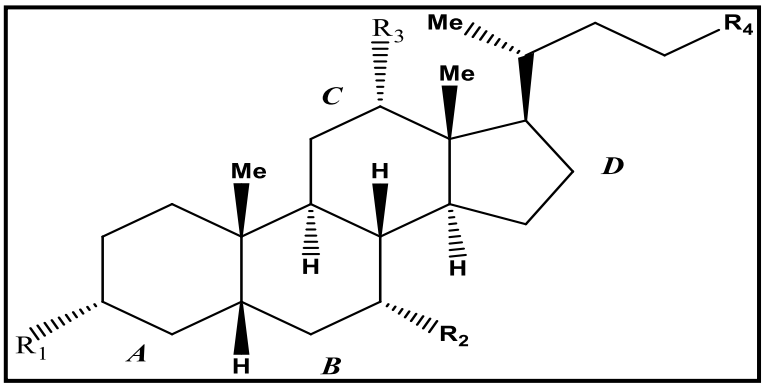

Figure 1.2: Schematic representation of carbon backbone of bile-salts.

Table 1.1: Nomenclature of different bile-salts

Bile-salts	**R_1**	**R_2**	**R_3**	**R_4**
Sodium Cholate (NaC)	α-OH	α-OH	α-OH	-COOH
Sodium Taurocholate (NaTC)	α-OH	α-OH	α-OH	$-CONHCH_2CH_2SO_3H$
Sodium Deoxycholate (NaDC)	α-OH	-H	α-OH	-COOH
Sodium Tauroglycocholate (NaTGC)	α-OH	α-OH	α-OH	$-CONHCH_2CONHCH_2CH_2SO_3H$

1.5. Difference between micellization between conventional surfactants and bile-salts

Conventional surfactants show well-defined polarity gradient between the long alkyl hydrophobic tail and small polar hydrophilic heads which at a particular concentration called critical micellar concentration (CMC) pack into thermodynamically stable molecular clusters called micelle.[18]

The formation of the micelles are favoured by the decrease in free energy of the system due to breaking of hydrogen bonds around the hydrophobic tail and reconstruction of hydrogen bond network in a bulk water. This rearrangement simultaneously leads to the formation of van der Waals bond between hydrophobic patches in the core of the spherical shell. The intricate interplay between various electrostatic, hydrophobic, van der Waals, and steric interactions and their delicate balance plays an important role in aggregate formation. The formation of the micelles by aggregation of amphiphilic surfactants above CMC at a particular temperature is shown in **Figure 1.3**.

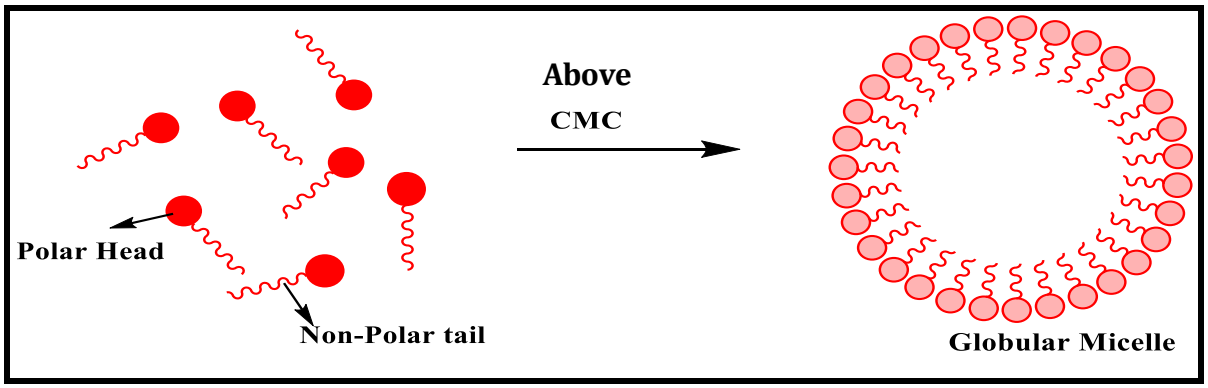

Figure1.3: Schematic representation of the formation of micelles.

Although bile-salts are anionic surfactants but the steroidal core of bile-salts makes them different from conventional surfactants by providing facial polarity gradient. Unlike conventional surfactants, bile-salts do not have well separated hydrophilic and hydrophobic domains.

The *CMC* value of bile-salt is lower than ordinary surfactants, which is usually characterized by a range instead of a particular value. Bile-salts show more micro-viscosity, rigidity, higher polydispersity and higher charge density [25, 26] as compared to conventional surfactants. Bile-salt have characteristic micellization or aggregation behaviour which is highly controversial. Several techniques such as freezing point depression, conductivity, absorption, dynamic light scattering, [30.31] fluorescence, calorimetry, and capillary, electrophoresis, surface tension were employed to study the aggregation behaviour of bile-salts. [27-29]

Numerous models of bile-salt micellization have been proposed since last few decades and have lot of controversy. The two most important models in this context are (a) Small's model and (b) Oakenfull-Fisher's Model. Out of the two models, "Small's model" is considered as the most accepted one. The formation of bile-salts micellization is shown in **Figure 1.4**.

1.5.1 Small's Model of bile salt aggregation

D. M. Small's.[32] worked on high-resolution ^{1}H nuclear magnetic resonance (NMR) technique and predicted that micellization of bile-salts are governed by formation of primary aggregates, followed by formation of secondary aggregates (**Figure 1.4**). At low salt concentrations, about 2-10 monomers of bile-salt combine to form globular primary aggregates. This primary aggregates is due to hydrophobic-hydrophobic interactions of

convex side of steroidal core, resulting in exposed concave hydrophilic side of the bile molecule towards water and shielding the hydrophobic parts from water on gradually increasing the bile-salt concentration. The intermolecular hydrogen bonding between hydroxyl groups dominate and agglomeration of primary aggregates takes place, which results in the formation of prolate or ellipsoidal micelle morphology, called secondary aggregates. For example, NaDC exhibits two critical micelle concentrations, at 10mM (primary aggregates, CMC_1), and 60mM (secondary aggregates, CMC_2). The formation of the secondary aggregates of bile-salts looks like an elongated rod with a central hydrophilic core filled with water. [33. 42] In NaDC, the radius of the rod is about 8 $\AA$ and length of the rod is about 40 $\AA$.

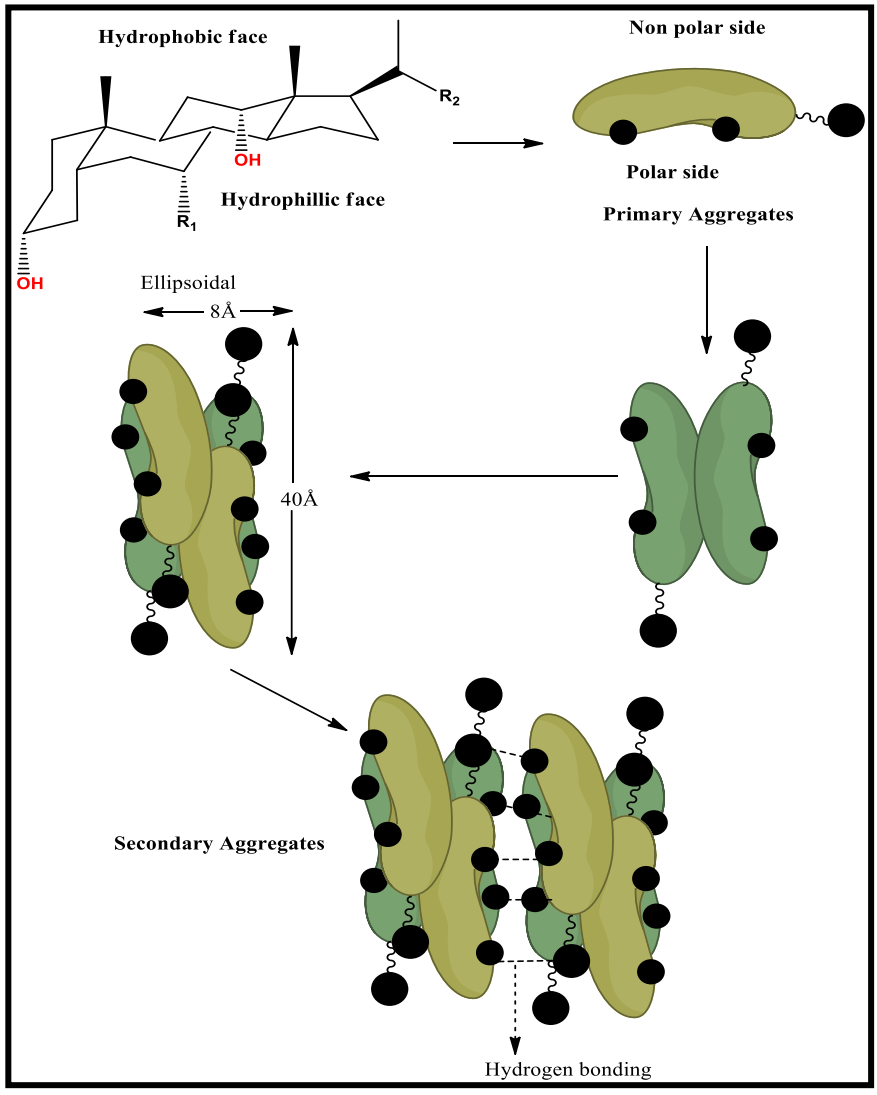

Figure 1.4: Schematic representation of the bile-salt micellization.

1.5.2. Oakenfull and Fisher model

They suggested that the first stage aggregation of bile-salts is mainly because of dimer formation, which involves the maximum number of hydrophilic groups (hydroxyl and charged carboxylic groups) forming hydrogen bonding, followed by back-to-back hydrophobic interactions from the convex side of bile salt dimmers,[34.35] which leads to the formation of layer aggregates of bile-salts. In synchronization with Small's model these stacked micelles give rise to a lengthened rod-like structure to the secondary aggregates which are governed by both hydrophobic interactions and hydrogen bonding between the hydroxyl groups.

1.6. What is confined medium?

A reaction cavity is said to be a confined space or medium when it contains well-marked boundary which separates the enclosed guest reactant molecule(s) from the bulk .This cavity may or may not contain some amount of free volume after entrapment of guest molecule. Selectivity of host-guest interaction within confined space depends on various factors like , shape and size of the cavity, free volume, rigidity (more or less) of the boundary, and the supramolecular interactions that hold the guest molecule(s) within the confinement. Hence, the guest species remain confined in a small cavity of few nanometres. [36] Such confinement of chemical species in small volume imposes severe restrictions on free motion of solvent and solute molecules. Therefore, molecules confined within geometrical restraints generally exhibit behaviour that is quite different from bulk solutions, where the motion of molecules are random and can take place in all degree of freedom. The micropolarity of bulk water molecule gradually drops from the outer boundary surface to the inner core region of micelles.[37] The most intriguing feature of such organised assembly is that they have tendency to bind and stabilize various guest molecule(s) that are insoluble or partially soluble in bulk solvents. Micelles, reverse micelles, cyclodextrins, lipids, vesicles, bile-salt aggregates [38-40] are regarded as model system for organised bio-mimic confined environment. The importance of confined media is very much useful because maximum physicochemical and biological phenomenon take place in the confined media rather than the bulk media. Bile-salt aggregates can act as potential supramolecular host systems and confined environments

due to its amphiphilic nature that can encapsulate both hydrophilic and hydrophobic guest molecules and severe interaction also takes place inside its restricted cavity.

1.7 Elementary idea of fluorescence phenomena

The processes that occur between the absorption of light by fluorophore and emission of radiation are usually illustrated by the Jablonski diagram (**Figure 1.5**). In ground state, the singlet electronic state is designates as S_0, first excited state as S_1, and second excited electronic state as S_2 and so on. The electronic levels are further composed of various vibrational energy levels, and are designated as 0,1,2,3 etc. According to Franck-Condon principle, [41] the fluorophore is photchemically excited by the energy of photons (absorption of light) from ground state (S_0) to higher excited electronic state. The molecule reaches in the excited state and becomes unstable due to its high energy. It has tendency return to the lowest excited singlet state and the emission of photon releases from further return to the ground state. This phenomenon is called fluorescence. The fluorophore is excited to some higher vibrational level like S_1 or S_2. When fluorophore suddenly relaxes back from higher singlet excited states to lower vibrational level of S_1 state, this process is called internal conversion (IC). The fluorescence emission occurs from thermally equilibrated lowest energy vibrational excited state of S_1.[4]

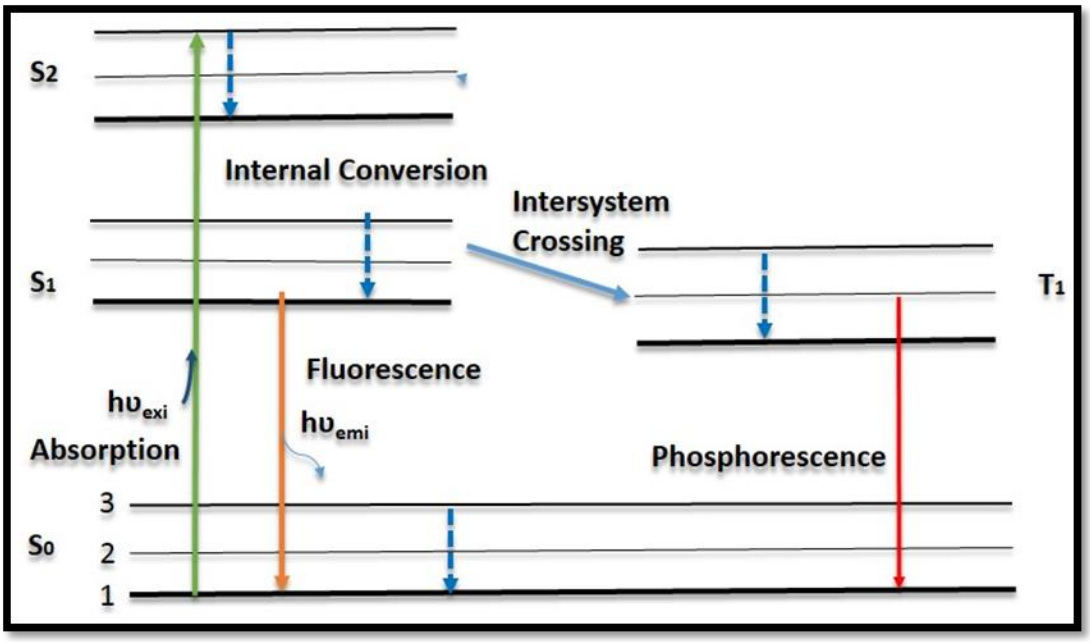

Figure 1.5: Schematic representation of Jablonski diagram.

Sometimes in viscous medium or at low temperatures or many other factors molecules in S_1 state can undergo a spin conversion to the first triplet excited state T_1. This phenomenon is termed as intersystem crossing (ISC), which is spin forbidden transition. The emission from T_1 state to S_0 state is termed as phosphorescence, which results in generally longer wavelengths (lower energy) shifts as compared to fluorescence.

CHAPTER-2

LITERATURE REVIEW

2.1 Basic literature review on bile-salts and CV

Reis *et al.* (2004)[43] determined the critical micellar concentrations (*cmc*) of different bile salts, sodium cholate, sodium deoxycholate, sodium glycocholate and sodium glycodeoxycholate. They demonstrated three independent non-invasive methodologies (such as derivative spectrophotometry, potentiometry and light scattering) for determination of *CMC*. A critical comparison of the *CMC* values of bile-salt with or without the influence of salt (NaCl) was carried out in literature that were determined with various complex techniques like high-performance liquid chromatography, electron paramagnetic resonance, light scattering, refractometry , microcalorimetric titration techniques and reversed-phase.

Faustino *et al.* (2016)[44] demonstrated that bile-salts are not solely functional excipients used to improve drug bio-availability but also effective drug carriers as well as therapeutic agents, which can be optimized for drug targeting and controlled as well as sustained drug delivery. They also reported that bile-salts are used in prodrug design and augmented the oral absorption and metabolic stability of feebly aqueous-soluble drugs.[35] The cytotoxic properties of bile-salts are also used in the development of drugs for cancer treatment , anti-proliferative activities.[35]

Enache *et al.* (2017)[45] investigated the interaction of therapeutic drug (mitoxantrone) with two different bile-salts namely sodium taurodeoxycholate (NaTDC) and sodium taurocholate (NaTC) with the help of UV-Vis absorption and electron paramagnetic resonance (EPR) spectroscopic technique and the reported the location of the drug molecule in bile-salt aggregates. They also demonstrated at pre-micellar concentration, the drug molecule have tendency to aggregate itself, whereas in the micellar concentration only the monomer of the drug entrapped inside the bile-salts aggregates. They also analysed different parameters (binding coefficients, partition coefficients and various other thermodynamic parameters) and EPR measurements, to probe the location of the drug molecule in confinement of the bile-salt aggregates.[45]

Malik (2016) [46] reported bile-salt-drug and bile-salt-surfactant interactions. He discussed different physicochemical parameters like, enthalpy, Gibbs free energy, *CMC*, entropy ,binding constants in order to get a better insight to the interaction of bile salts with biologically important drugs like curcumin, iodomethacin, amphotericin B, phenylbutazone, imipramine hydrochloride, promethazine hydrochloride, proazamine hydrochloride, which have very low solubility in aqueous medium but upon interaction with various bile-salts they showed increased solubilisation as well as modulated the phase forming behaviour, micro-emulsion and clouding phenomenon, absorption intensity. They also reported that in biotechnology and pharmaceutical industry this system have manifold impact without side effects of human systems.

Moore *et al.* (2007)[47] reported that the effects of the addition of varying concentrations of two different non-ionic polyoxyethylene (POE) surfactants on the photophysical properties of CV. In order to get a better insight and comprehends the interaction of CV with miceller environment, they determined various parameters like binding constant and aggregation number. They also showed that the absorption and fluorescence intensity gradually enhances and undergoes bathochromic shift on gradual increase of the concentration of the non-ionic surfactants. They also explained that due to dye-micelles interaction and increase of the confinement the photophysical properties are significantly modulated.

Ghosh *et al.* (2012)[48] informed the interaction of CV molecule with different types of ionic and non-ionic surfactants (for cationic surfactants such as DTAB, TTAB, CTAB, anionic surfactants such as SDS, SDBS and neutral surfactants such as Tween 20, Tween 40, Tween 60). They studied the photophysics of CV in different kinds of surfactants in their premicellar and post micellar concentration regions with the help of UV-Vis and fluorescence techniques. They demonstrated that in cationic and non-ionic surfactants, the interaction of CV is less compared to that of anionic surfactants. They also explained that CV is cationic dye and it has tendency to interact with the anionic surfactants through electrostatic interactions and increases the solubilisation of CV molecule inside the hydrophobic core of the micelles. Since, the dye molecule possesses strong hydrophobicity of the aromatic

group. Therefore, hydrophobic-hydrophobic interaction also leads to enhancement of the solubility of drug molecule. As a result, the fluorescence spectra of CV showed bathochromic shift as well as enhancement of the fluorescence intensity with increase of the micellar concentration. They also reported that the studied dye molecule have no effect with cationic surfactant CTAB.

Maity *et al.* (2015)[49] demonstrated the binding interaction and photophysics of non-steroidal anti-inflammatory drug (indomethacin) in presence of different micelles as potential drug delivery vehicles. With the help of several spectroscopic techniques (steady-state absorption and fluorescence emission, isothermal titration calorimetry (ITC), time-resolved fluorescence emission measurements) they demonstrated the spectral properties of the drug molecule are significantly modulated in presence of both cationic and anionic surfactants. Moreover, they claimed that poorly soluble and non-fluorescent indomethacin in aqueous medium turns into highly soluble and highly fluorescent in presence of the confined micellar environments. Most interesting part of their research was effect of addition of salt in the respective micelles which leads to the release of drug confined micellar environments to the aqueous medium. They also demonstrated that loosely bound drug molecule is more easily released from the confined micellar media to the aqueous environment. They also claimed that the rate of release of IMC molecule decreases gradually with increase of the non-polar alkyl chain length of the surfactants.

2.2 Research gap

Based on the literature review the following research gaps have been identified:

1. CV is used as histological stain, antiseptic, anti-fungal, anti-bacterial properties. So the target is to enhance its solubility property in the confined environments.
2. Since, CV molecule exhibits non-fluorescent in nature in aqueous solution. Therefore, the target is to improve its fluorescence property in presence of confined environments. So that CV molecule can act as detection analysis and sensing agent in physiological system.

3. There are very limited reports on the photophysics of CV molecule. Only interaction between CV and different types of surfactants was reported till date.[47.48]
4. Since, bile-salts have potential impact on drug-delivery vehicles and drug-carrier. But there is no literature report on the interaction of CV in bile-salt aggregates.
5. There is no literature report that how the photophysics of CV will change by addition of small concentration of electrolyte (nM range KCl salt), to the encapsulated drug-bile-salt aggregates.
6. Since, KCl plays vital role in human body, used to treat low blood potassium and intravenously injectable. Bile-salt aggregates also have manifold applications in drug-delivery approaches. Therefore, systematic study should be required to comprehend the role of KCl in encapsulated CV-bile-salts aggregates. But, till date there is no systematic study reported elsewhere.

2.2 Aim of this project

Very limited studies on interaction of CV in confined environments have reported in literature.[47.48] Therefore, the main aim and motivation of the thesis is to endeavour the interaction of CV confined in different kinds of bile-salt aggregates and compare the result with aqueous buffer medium. CV is non-fluorescent in aqueous medium; therefore the main aim of this thesis is to improve the fluorescence property and solubility in bile-salt aggregates. Since, bile-salts are regarded as potential supramolecular receptor (host) and have ability to interact with definite size and shaped substrate (guest) through different kinds of non-covalent supramolecular interactions. Therefore, to get more insight and comprehend the interactions of encapsulated complex, the photophyics of CV molecule have been carried out by modulating several kinds of hydrophilic head groups and hydrophobic skeletons of bile-salt aggregates (e.g. NaC, NaDC, NaTC and NaGDC) and to rationalize the location of CV molecule in confined environment. Another aim of this thesis is to release the CV molecule from encapsulated bile-salt aggregates to the aqueous medium by addition of foreign substance (non-toxic and green method). This will be possible if the studied CV

molecule will exhibits strong fluorescence to non-fluorescence property or in other words, fluorescence turn-on-off property. The detection analysis of the bio-mimetic confined bile-salt aggregates on the studied biologically active CV molecule and its release phenomenon is very much important in biological model systems. Addition of KCl salt perturbs the micellization process of bile-salt aggregates. As a result, CV molecule releases from the confined environments to aqueous medium. Moreover, different kinds of physical parameters such as ground state and excited state binding constants, partition coefficients, aggregation numbers, fluorescence quantum yield have been calculated for explaining the result of the work in thesis. The graphical representation of the work involved in the thesis is shown in **Figure 2.1**.

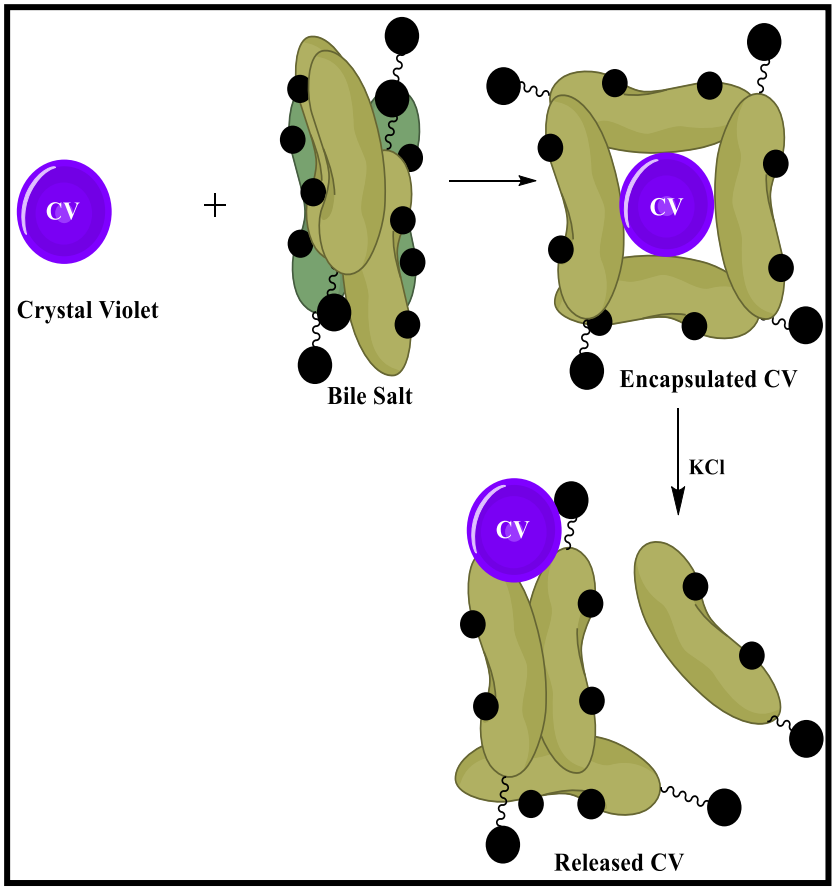

Figure 2.1: Graphical representation of the thesis.

CHAPTER-3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Pharmaceutical compound

Crystal violet (CV) was purchased from Loba Chemie and used as received. The purity of the compounds was more than 97%. Fresh solution of CV was prepared every time to avoid any problem of degradation and aggregation. The concentration of CV solution was maintained at 1×10^{-5} (M).

3.1.2 Different bile-salts and chemical used

Bile-salts such as sodium cholate (NaC), sodium deoxycholate (NaDC), sodium taurocholate (NaTC) and sodium tauroglycocholate (NATGC) were obtained from Loba Chemie. Potassium Chloride (KCl) was obtained from Sigma-Aldrich, India. All the bile-salts used of very high purity grade (≥97%).

3.2 Experimental methods

3.2.1 Preparation of sample solution

The stock solution of CV was prepared in aqueous medium. From the stock solution, required amount of aliquots (200 µl) was aliquot was pipette out by using micro-liter syringe in a cuvette, followed by addition of 1800 µl 0.1 (M) phosphate buffer solution to achieve the concentration of the experimental solution as 1×10^{-5} (M), which is regarded as CV in aqueous buffer medium. Such small concentration of the dye molecule would not affect the spectral and aggregation behaviour of bile-salts.

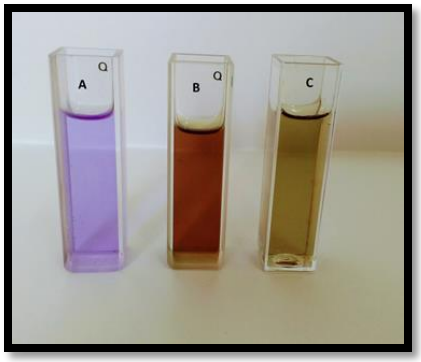

Figure 3.1: Photograph of (a) CV in buffer (b)CV in NaTGC bile-salt(100mM)(c) CV in NaTGC bile-salt(100mM) KCl (100nM).

3.2.2 CV-Bile-KCl system

To the aqueous buffer medium of CV, required amounts of respective bile-salts were successively incorporated to the solution of the cuvette and mixed thoroughly with the help of micro-pipette and sufficient time was given to the bile-salt allowed to dissolve completely. The concentrations of different bile-salts were varied from the lower concentration to higher concentration range (starting from below *CMC* value that is pre-micellar concentration, followed by at *CMC,* that is at critical micellar concentration and above *CMC* that is higher micellar concentration) to achieve final concentration as 100 mM. In all the respective bile salts, the maximum concentration was fixed such that the studied dye molecule gets completely saturation in the respective concentration region. After that to the respective bile-salts, very low concentration of KCl solution was progressively added and the concentration of KCl was varied from 1nM to 100 nM region.

3.2.3 CV-KCl-Bile system

In order to study the effect of the role of salt (KCl) on interaction of CV with bile-salt, another set of solution was prepared. 100 nM KCl was transferred to the cuvette containing 1×10^{-5} (M) CV in aqueous buffer medium. After that, respective concentrations of different bile-salts were varied from the lower concentration to higher concentration range (100 mM) to achieve the saturation of the studied dye molecule in bile-salts.

3.2.4 Conductometric measurements

Conductometric measurements were performed on Equiptronics (Model number: EQ 664A) conductometer. The conductometer was calibrated with KCl solution of the appropriate concentration range prior to use. Each of the experiments was repeated thrice in order to minimize any scope of error. Specific conductance was recorded for varying concentrations of NaTGC.

3.2.5 Ultraviolet–Visible absorption measurements

Absorbance measurements were performed by Sepcord 205 Analyttik Jena spectrophotometer using 1cm path length quartz cuvette. The spectra were recorded for 400-800 nm wavelength range. The absorbance intensity was measured for both the systems (CV-Bile-KCl and CV-KCl-Bile) respectively.

3.2.6 Fluorescence measurements

The fluorescence emission spectra of the experimental solution were measured by Perkin Elmer LS 55 fluorescence spectrometer using quartz cuvette of a 1 cm path length. Fluorescence spectra were recorded at two different excitation wavelengths (λ_{exi} = 550 nm and 590 nm) Two different excitation wavelengths were selected since the studied dye molecule displayed shoulder band (550 nm) followed by absorption maxima (590 nm). The emission slit widths were fixed at 15 nm and 15 nm respectively. The scan time was fixed at 250 nm per minute.

3.3. Analysis methods

3.3.1 Determination of *CMC* of NaTGC

In literature, *CMC* values of NaC, NaTC, NaDC bile-salts are reported, which are found as 18 mM, 8 mM and 6 mM respectively.[50.51] But the *CMC* value of NaTGC was not reported in literature. Hence with the help of Conductivity measurements *CMC* of NaTGC was determined. Concentration of CV in beaker was kept constant at 10^{-5}M and concentration of NaTGC was varied from 0-20 mM .The specific conductivity (κ) was recorded after each addition followed by thorough mixing. Two intercepts were found at varying the concentration of NaTGC. From the intersection of both the intercepts, *CMC* value was determined.

3.3.2 Determination of ground state and excited state binding constant

The ground state and the excited state binding constant values were obtained from UV-Vis and fluorescence emission techniques. The ground state binding constant (K_1) values of CV were determined from the variation of absorbance against the micellar concentration of different bile-salts by using the following 1: 1 nonlinear least-squares regression analysis method.[52]

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

where, 'A_{buffer}' and '$A_{micelle}$' are the absorption intensities of CV in buffer and respective highest micellar concentration of bile-salts. 'K_1' is ground state 1:1 binding constant value of CV-bile aggregates.

Similarly, excited state binding constant values are also determined from the variation of fluorescence intensity against the micellar concentration of different bile-salts by using the following 1: 1 nonlinear least-squares regression analysis method.[53]

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

where, 'F_{buffer}' and '$F_{micelle}$' are the fluorescence intensities of CV in buffer and respective highest micellar concentration of respective bile-salts. 'K_1'' is the excited state 1:1 binding constant value of CV-bile aggregates.

3.3.3 Determination of fluorescence quantum yield (Φ)

Fluorescence quantum yield is individual property of any fluorophores. It gives the nature of fluorescence property of the fluorophore. Fluorescence quantum yield values are determined from the fluorescence emission intensity (integrated area) and the absorbance value at the particular wavelength of excitation. The fluorescence quantum yield can be mathematically expressed as: [37.41]

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

Where, 'Φ_S' and 'Φ_R' represents the fluorescence quantum yield of sample (CV) and reference (Rhodamine B), '*Abs*' denotes absorbance, '*A*' represents the area under the fluorescence emission, '*n*' is the refractive index of the solvent used. The subscripts '*S*' and '*R*' denotes the corresponding parameters for the CV (sample) and Rodamine B (reference) respectively. The fluorescence quantum yields of CV in different bile-salt systems were determined by using 'Rhodamine B' as reference solution in aqueous medium (Φ_R = 0.31). [56]

3.3.4 Determination of ground state and excited state partition coefficient

The remarkable spectral changes were observed in the absorption and fluorescence studies of CV in aqueous medium with gradual increment in the concentration of bile-salts due to entrapment of CV in bile-salts aggregates. As a result, CV molecule causes significant partitioning inside the hydrophobic nano-cavities of different bile-salt aggregates. In order to prove that bile-salts are good drug transportation vehicles, it is very necessary to have an estimative idea of the amount of penetration. The extent of

penetration of the studied dye molecule inside the bile-salt aggregates can be quantified by the value of partition coefficient. The partition coefficient (K_P) of the molecule between two different phases (aqueous and confined) is mathematically expressed as following.[54.55]

$$K_P = \frac{\frac{\left(\frac{C_m}{C_w}\right)}{[bile\ salt]}}{\frac{\left(\frac{C_w}{C_t}\right)}{[water]}} \qquad Eq.3.4$$

where, 'C_t', 'Cm' and 'C_W' represents total concentration of dye molecule, concentration of dye bile-salt aggregates and buffer medium respectively. Experimentally, the partition coefficient [43] can be determined from absorbance (ground state partition coefficient) as well as fluorescence intensity (excited state partition coefficient) data of CV in buffer with varying concentration of bile-salts using the following equation.[55]

$$\frac{I_\infty - I_0}{I_t - I_0} = 1 + \frac{[water]}{Kp}\frac{1}{[bile\ salt]} \qquad Eq.3.5$$

Where, 'I_0', 'I_t' and 'I_∞' represents the absorption and/or emission intensities of the dye molecule in aqueous buffer medium, at different concentrations (above their CMC values) of respective bile-salts and at highest micellar concentrations. 'K_P' is the partition coefficient value.

3.3.5 Determination of aggregation number (Nagg)

The aggregation number (N_{agg}) can be determined as following [53]

$$N_{agg} = \frac{(B - CMC)}{[Micelle]} \qquad Eq.3.6$$

Where, 'B' represents the highest micellar concentration of respective bile-salt at saturation, CMC is the critical micellar concentration. Due to the presence of different hydrophobicity and hydrophilicity, bile-salts aggregates have different aggregation number. The aggregation number gives quantitative idea that how strongly the amphiphilic surfactant molecules agglomerate together. Greater the number of aggregation number, greater will be the stability of the system. In other words, much more interaction operates in case of greater aggregation number of the surfactants and vice-versa.

CHAPTER-4

RESULTS AND DISCUSSION

4.1. *CMC* of NaTGC

Unionized water is a good insulator of electricity, but even if small number of ions present in the medium it will show certain amount of current. The amount of current that flows through aqueous solution is directly proportion to the number of charge /ions present, but at a certain concentration of ions current becomes constant. In this experiment micellization tendency of NaTGC in phosphate buffer solution (pH-7.4) was determined with the help of conductometric measurements [57.58] .Since, phosphate buffer already consists of various ions initial current was recorded and then concentration of NaTGC was varied until current reached saturation. It is difficult to determine accurately a particular value of *CMC* for bile- salts, as *CMC* lies in a broader range because on increasing the bile salt concentration the aggregates grow in size, which is not observed in conventional micelles. Hence, the range of *CMC* value of NaTGC was found to lie in between 8mM-10mM (**Figure 4.1**), which was determined through inflection point obtained from plotting specific conductivity (κ) against different concentration of NaTGC at room temp (298K).

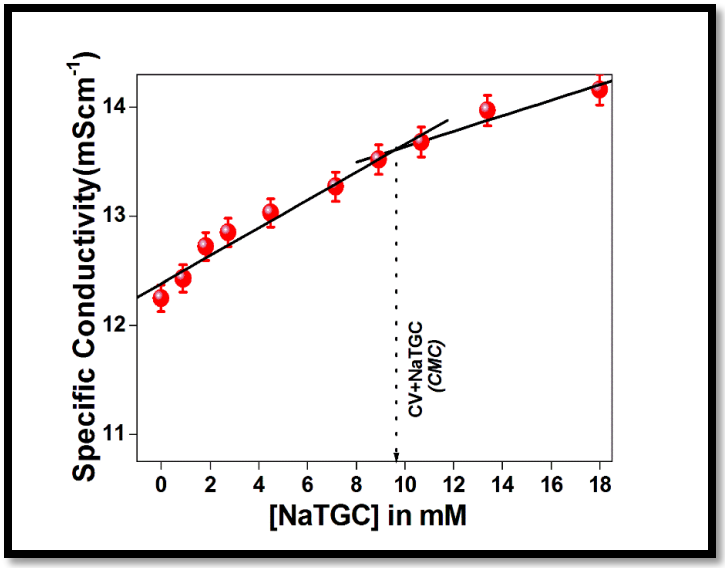

Figure 4.1: Plot of specific conductivity against [NaTGC].

4.2. UV-Vis absorption studies

In phosphate buffer, CV showed shoulder band ~550nm along with the absorption maxima at 590nm. The origin of the shoulder band of CV has remained a topic of argument from past many years. Lueck *et al.*[8] proposed shoulder band occurred due to formation of dimeric structure in which the dimethylamino groups overlap in a head-to-tail fashion resulting in increased hydrophobic interactions which are the driving forces for dimer formation. Garcia-Rio *et al.*[59] explained the presence of CV shoulder band is due to the existence of two ground state isomers in the aqueous medium, one is the pyramidal form (C_3 symmetry) or distorted form, which is caused by rotation of phenyl rings and another is propeller structure (D_3 symmetry). On gradual addition of the respective bile-salts, CV molecule undergoes significant bathochromic shift (**Figure 4.2**) as well as enhancement of the absorbance values (**Figure 4.3**).

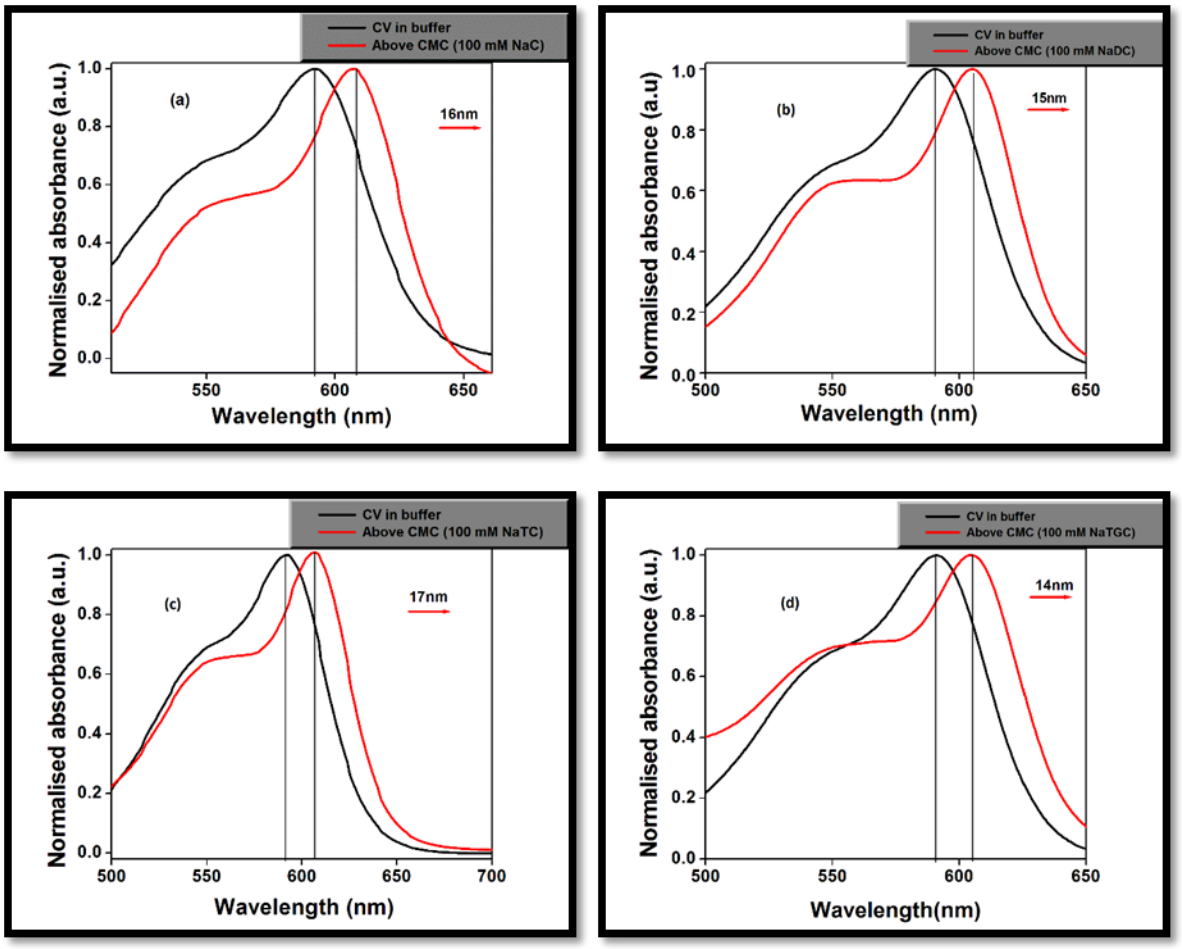

Figure 4.2: Normalised absorption of spectra of CV in (a) NaC (b) NaDC (c) NaTC (d) NaTGC.

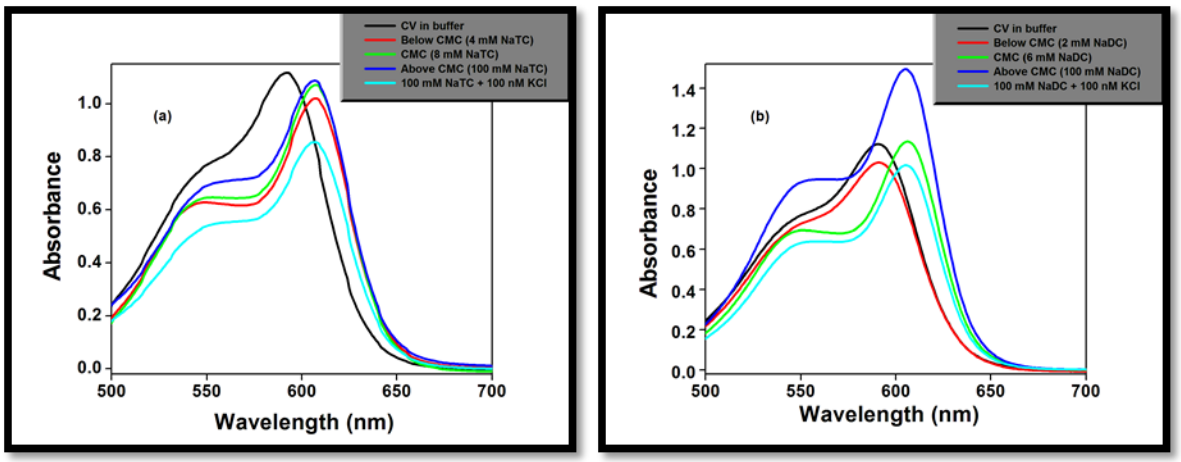

Figure 4.3: Absorption studies of CV on gradual addition of (a) NaTC (b) NaDC in phosphate buffer medium.

The enhancement of the absorbance values of CV in the presence of bile-salt aggregates is a clear manifestation that the micro-environment around CV molecule has been modified due to formation of bile-aggregates. [60.61] The specific interactions or changes in the micro-environment around the dye molecule might occur, which can cause redistribution of electron densities in the chromophore leading to the spectral changes. This result clearly suggested that electrostatic interactions takes places between the cationic dye molecule with the hydrophilic anionic parts of the respective bile-salts. The enhancement of the absorbance value signified that the extent of solubility of CV molecule confined in bile-salt aggregates significantly enhances due to hydrophobic-hydrophobic interaction. Due to the presence of strong hydrophobic aromatic group of CV, it has tendency to dissolve in hydrophobic core of the bile-salts. Therefore, both the electrostatic and hydrophobic interactions occur between CV and bile-salt aggregates. This analysis may also give a preliminary idea that the dye molecule undergoes to the confined bile-salt aggregates from the aqueous solution.

The absorbance value of the CV-bile aggregates astonishingly decreases on progressive addition of lower concentration (100 nM) KCl salt (**Figure 4.3**). The decrease of the absorbance value may be due to the reason that the solubility of the dye molecule becomes comparatively less than the solubility of the dye molecule entrapped in bile-salt aggregates. Since, the dye molecule is hydrophobic in nature. Therefore, in bile-salt aggregates hydrophobic-hydrophobic interaction occurs which leads to encapsulate CV molecule. In presence of KCl, the dye molecule may perturbs CV-bile complex and release from the confined hydrophobic core of the bile-salt aggregates to the

hydrophilic regions and/or to the aqueous medium. As a result, comparatively less interaction of the dye molecule occurs upon addition of KCl salt. It is noteworthy to mention that at gradual addition of KCl salt to the encapsulated CV-bile aggregates, beyond 100 nm (higher concentration KCl); there is no change on the absorption spectra of CV. Therefore, from this study it may be concluded that lower concentration of salt (nM) senses the release of the drug molecule from the confined environments.

4.3. Fluorescence emission studies at different excitation wavelengths

In phosphate buffer, the studied drug molecule (CV) displayed unstructured fluorescence emission maxima and the fluorescence quantum yield (Φ) was very low ($\sim 10^{-4}$) at both the excitation wavelengths (λ_{exi} = 550 nm and 590 nm). Therefore, the dye molecule present in bulk aqueous buffer was non-fluorescent in nature. Since, the studied molecule showed shoulder band (550 nm) along with the absorption maxima (590 nm) in phosphate buffer as well as in aqueous medium. Therefore, molecule was excited at both the wavelengths to understand the excited state dynamics and nature of interaction of the fluorophore entrapped in bile-salt aggregates. On progressive incorporation of the respective bile-salts to the buffer, the fluorescence intensity of the drug molecule (CV) at both the excitation wavelengths significantly enhanced. This characteristic modification of the emission spectra clearly demonstrated that the microenvironment of the studied molecule inside the bile-salt medium gets modulated compared to that buffer medium. **Figure 4.4** depicts the fluorescence intensity of CV molecule with varied concentration of NaTC bile-salts (below *CMC*, *CMC*, highest *CMC* values).

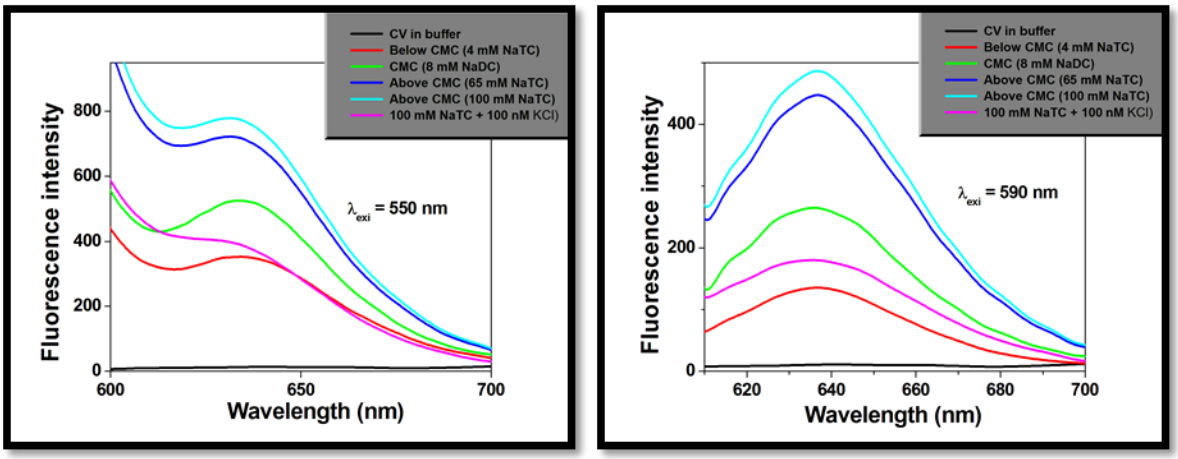

Figure 4.4: Fluorescence emission spectra studies of CV with increasing concentration of NaTC at different excitation wavelengths (λ_{exi} = 550 nm and 590 nm).

The fluorescence quantum yield values (Φ) of CV in different bile-salt aggregates significantly enhanced (~1000 folds) (**Table 4.1**). This clearly suggests that CV molecule becomes strong fluorescence in nature confined in encapsulated bile-salt aggregates. From the results, it may be demonstrated that gradual addition of the respective bile-salts have tendency to agglomerate the dye molecule through hydrophobic interaction.

Table 4.1: Fluorescence quantum yield values (Φ) of CV in different systems

System	Φ_{550nm}	Φ_{590nm}
CV (10^{-5} M) in buffer	6.79×10^{-4}	6.54×10^{-4}
CV (10^{-5} M)+KCl (100 nM)	1.98×10^{-3}	1.98×10^{-3}
CV (10^{-5} M)+NaC (100 mM)	0.12	1.6×10^{-2}
CV+NaC (100 mM)+KCl (100 nM)	0.12	2.5×10^{-2}
CV+KCl (100 nM)+NaC (100 mM)	9×10^{-2}	2.6×10^{-2}
CV (10^{-5} M)+NaDC (100 mM)	0.27	0.18
CV+NaDC (100 mM)+KCl (100 nM)	9.8×10^{-3}	1.8×10^{-3}
CV+KCl (100 nM)+NaDC (100 mM)	1.96×10^{-3}	1.52×10^{-3}
CV (10^{-5} M)+NaTC (100 mM)	0.52	0.19
CV+NaTC (100 mM)+KCl (100 nM)	4.54×10^{-3}	1.72×10^{-3}
CV+KCl (100 nM)+NaTC (100 mM)	9.6×10^{-3}	2.4×10^{-3}
CV (10^{-5} M)+NaTGC (100 mM)	0.10	1.54×10^{-2}
CV+NaTGC (100 mM)+KCl (100 nM)	2.4×10^{-3}	1.3×10^{-3}
CV+KCl (100 nM)+NaTGC (100 mM)	3.84×10^{-3}	1.71×10^{-3}

The addition of lower concentration of KCl salt (100 nM) to the encapsulated bile-salts causes remarkable decrease of fluorescence intensity (**Figure 4.4)** and fluorescence quantum yields (**Table 4.1**). From literature, [62] it has been found that incorporation of salts to the bile-aggregates results more aggregation of the bile-salts, leading to enhancement of the fluorescence intensity and fluorescence quantum yield values. They also explained that

addition of salts also responsible for the conformational and structural change of the bile-aggregates.[62] But in our case, opposite result was found. Increasing the concentration of KCl salt beyond 100 nM, there is not found any change of the fluorescence intensity and fluorescence quantum yield values. This exciting result may be due to the reason that the studied drug molecule may disrupts CV-bile complex and release from the confined hydrophobic core of the bile-salt aggregates to the hydrophilic regions and/or to the aqueous medium. Similar kind of phenomenon was also obtained from the absorption study. Here, it is important to note that if the drug molecule (CV) releases from the confined bile-aggregates after the addition of small concentration of KCl salt, then the binding constant of the drug-bile aggregates should be significantly lowered.

4.4 Determination of binding constant values of CV in bile-salt aggregates and effect of salt to the confined system

In order to get more insight the stability of the studied drug molecule (CV) in bile-salt aggregates, the binding constant values of CV molecule was evaluated by non-linear 1:1 regression analysis method (**using Equation 3.1**). The ground state binding constant values were calculated from the absorbance data of CV with different concentration of the respective bile-salts and are tabulated in **Table 4.2**.

Table 4.2: Binding constant values of CV in different bile-salt aggregates from absorption study.

Bile-salt [100mM]	Binding Constant (M^{-1}) of CV-bile-salt (Absence of KCl)	Binding Constant (M^{-1}) of CV-KCl-bile-salt (Presence of KCl)
NaC	24 ±(6)	19±(4)
NaDC	50±(10)	32±(7)
NaTC	80±(21)	42±(10)
NaTGC	26±(7)	14±(3)

Similarly, in presence of KCl (100 nM), the binding constant values of CV with varying concentration of CV were also evaluated and tabulated in **Table 4.2**. From the table, it has been found that presence of KCl salt results decrease of the binding interaction between

CV-bile aggregates. **Figure 4.5** represents the binding constant plot of one representative model of CV-bile-salt aggregates

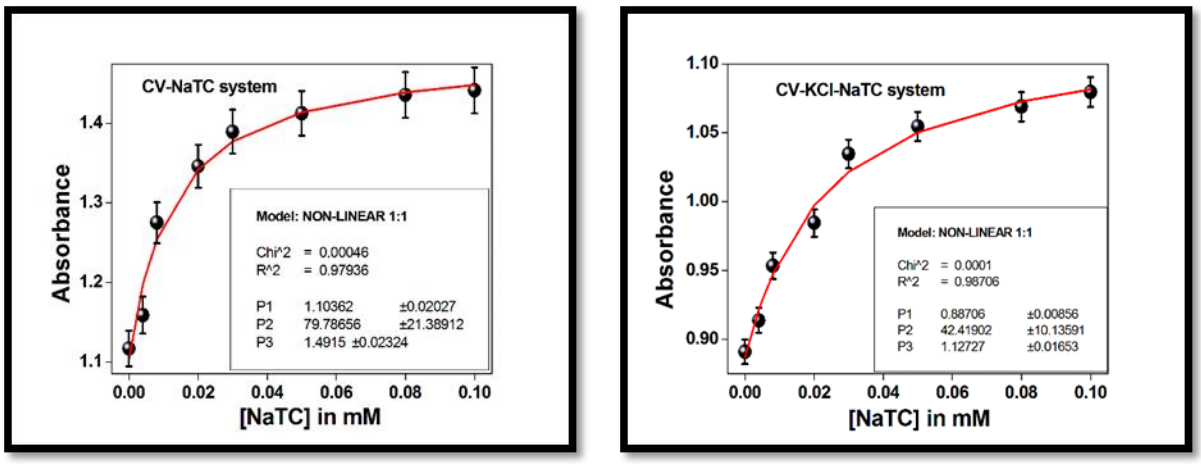

Figure 4.5: Ground state binding constant plot of CV-NaTC and CV-KCl-NaTC.

The excited state binding constant values of CV-bile aggregates in absence of KCl and in presence of KCl were also obtained from the fluorescence intensity data with varying the concentration of bile-salts using **Equation 3.2**. The excited state binding constant data were also tabulated in **Table 4.3**.

Table 4.3: Binding constant values of CV in different bile-salt aggregates from fluorescence study at two different excitation wavelengths (λ_{exi} = 550 nm and 590 nm).

Bile-salt [100mM]	Binding Constant (M^{-1}) of CV-bile (Absence of KCl)	Binding Constant (M^{-1}) of CV-KCl-bile (Presence of KCl)	Binding Constant (M^{-1}) of CV-bile (Absence of KCl)	Binding Constant (M^{-1}) of CV-KCl-bile (Presence of KCl)
	λ_{exi} = 550 nm		λ_{exi} = 590 nm	
NaC	110±(16)	75±(10)	60±(11)	35±(7)
NaDC	189±(25)	114±(17)	93±(14)	53±(11)
NaTC	206±(31)	69±(7)	103±(15)	54±(2)
NaTGC	92±(6)	44±(7)	78±(5)	47±(5)

From the table, it was also clear that at two different excitation wavelengths (λ_{exi} = 550 nm and 590 nm), the presence of KCl salt suppress the binding interaction between CV-bile aggregates in the excited state. From the analysis of both the ground and the excited state binding studies, it can be clearly demonstrated that addition of salt drives out the drug molecule from the confined hydrophobic region of bile-aggregates to outside. As a result, binding constant values significantly dropped both in ground state and the excited state. It was also noticed that the extent of binding interaction at the excitation of shoulder band (λ_{exi} = 550 nm) is greater compared to excitation of absorption maxima band (λ_{exi} = 590 nm). **Figure 4.6-4.7** depicts the binding constant plot of one representative CV-bile-salt aggregates in absence (CV-NaTC) and in presence of salt (CV-KCl-NaTC) respectively.

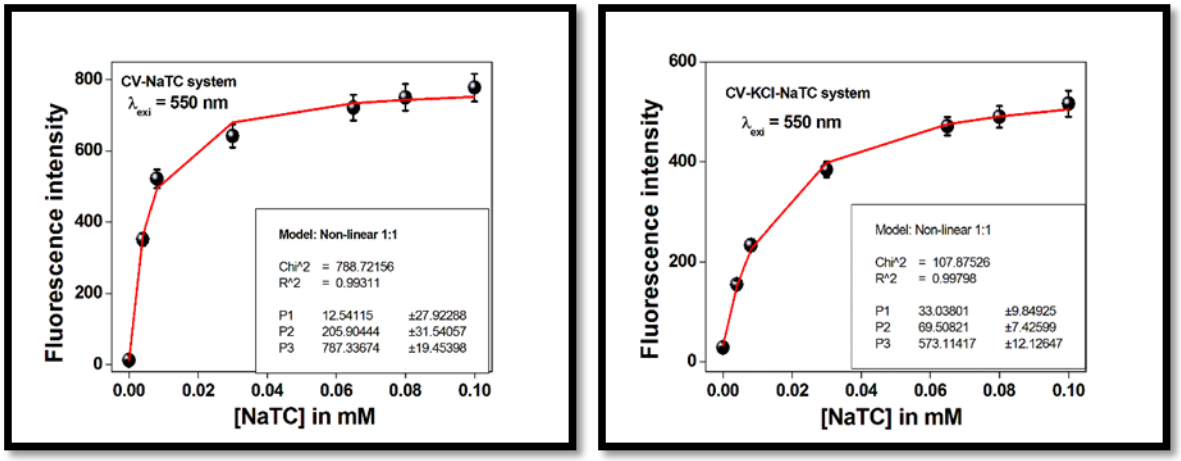

Figure 4.6: Excited state binding constant plot of CV-NaTC and CV-KCl-NaTGC at λ_{exi} = 550 nm.

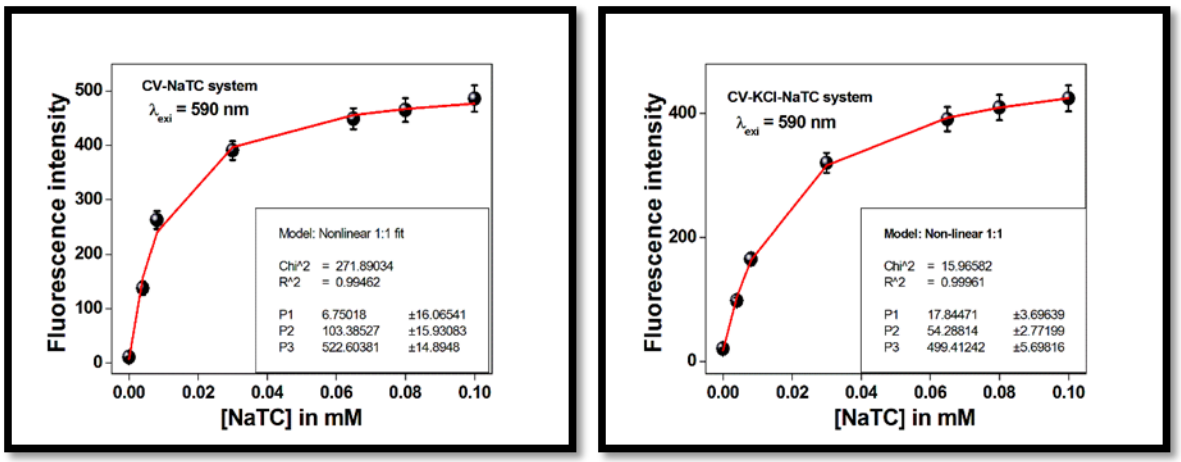

Figure 4.7: Excited state binding constant plot of CV-NaTC and CV-KCl-NaTGC at λexi = 590 nm .

4.5 Determination of partition coefficient values of CV in bile-salt aggregates and effect of salt to the confined system

To elucidate the location of the studied drug molecule (CV) at highest micellar concentration of the respective bile-salt aggregates (100 mM), the ground state and excited state partition-coefficient values were evaluated (**using Equation 3.5**) by absorption and fluorescence data. The partition coefficient values were tabulated in **Table 4.4**.

Table 4.4: Partition coefficient values of CV in different bile-salt aggregates.

Bile-salt [100mM]	**Partition coefficient (K_P) of CV-bile in M^{-1} (Absence of KCl)**	**Partition coefficient (K_P) CV-KCl-bile in M^{-1} (Presence of KCl)**	**Partition coefficient (K_P) of CV-bile in M^{-1} (Absence of KCl)**	**Partition coefficient (K_P) CV-KCl-bile in M^{-1} (Presence of KCl)**
	Ground state		**Excited state (λ_{exi} = 550 nm)**	
NaC	1,748	76	8,546	4,751
NaDC	2,112	4,89	14,317	5,668
NaTC	1,903	1,791	10,540	3,703
NaTGC	1,804	1,385	5,903	2,708

It was observed that magnitude of partition coefficient is very high (in order of 10^3).This significantly higher values of partition coefficient clearly suggest that the drug molecule resides at the confined environment rather than the aqueous medium.

The partition coefficients values are in the order of NaDC>NaTC>NaTGC>NaC. Thus NaTC and NaDC have high binding as well as partition coefficient, which is surprising as NaDC has high hydrophobicity index as compared to that of NaTC. The hydrophobicity index of NATC, NaDC and NaC are 0, 0.72 and 0.13 respectively. [63] Since CV exists in two isomeric form, it might be possible that the two forms binds in different fashion with amphiphilic bile-salts, where electrostatic interaction due to cationic form of CV is responsible for higher binding and partitioning for NaTC, while the hydrophobic interactions due to the presence of aromatic hydrophobic moieties of CV molecule are responsible for higher binding efficiency as well as partition coefficient for NaDC.

From the **Table 4.4**, it has been noticed that addition of KCl results significant decrease of the respective partition coefficient values both in ground as well as excited state. This clearly demonstrated that addition of KCl salt to the CV-bile aggregates the studied drug molecule comes out from the confined hydrophobic environments to the aqueous medium.

4.5 Determination of percentage release of CV in bile-salt aggregates after incorporation of KCl

Addition of KCl (100nM) to the respective bile salts drives out the studied drug molecule (CV) from confined environment to the surface. Therefore, the release of drug molecule from the confined environment of bile-salts have been carried out using the fluorescence intensity data. The release of CV molecule in different bile salts are tabulated in **Table 4.5**

Table 4.5: Percentage (%) of release of drug molecule from bile-salt confinement

Bile-salt [100mM]	**Percentage(%) of Release of drug molecule**
NaC	48%
NaDC	62%
NaTC	63%
NaTGC	54%

From the above **Table4.4**, it has been found that the release order is NaTC > NaDC > NaTGC > NaC. From the binding constant data, the course of binding value also follows the same order **Table 4.3.**Therfore after analysing it has been found that more strongly bound bile-salt have propensity to release the drug molecule.

4.6. Determination of aggregation number (Nagg) of CV in bile-salt aggregates and effect of salt to the confined system.

Aggregation number were calculated using **Equation 3.6** for different bile salt system in order to get an insight of effect of salt on micellization process of bile salt. According to present literature bile salts aggregation numbers ranges between 2-15.

Table 4.6: Aggregation number (Nagg) of CV in bile-salt system.

Bile-salt [100mM]	**Aggregation number (N_{agg}) in CV-bile - KCl system**	**Aggregation number (N_{agg}) in CV-KCl - bile system**
NaC	4	2
NaDC	4	3
NaTC	3	2
NaTGC	3	2

It has been reported that for conventional surfactants increase in ionic strength, temperature and decrease in pH leads to growth of the micelles. In contrast, bile-salt aggregates do not follow a general growth behaviour and their growth depends upon various factors, such as concentration which varies from different bile species. [64-67] Zana *et.al*[62] have reported that with the increase in concentration of NaCl salt, there is a prominent increases in the aggregation number due to salting out effect. As a result, the hydrophobic interaction enhances, which results decrease in *CMC* values and increase the aggregation number.

But in our case a completely opposite trend **TABLE 4.6** was observed. Addition of KCl to the respective CV-bile system leads to decrease in aggregation number. This result clearly confirms that KCl disrupts the bile-aggregates. Our result also match with the literature data in which Maity *et al*. showed addition of salt causes the drug molecule drive out from micellar confinement. [49]

CHAPTER- 6

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