INTERACTION OF AMANTADINE WITH EGG ALBUMIN IN SLS : SPECTROSCOPIC STUDIES

*,[1]S. Bakkialakshmi, and [2]V.Barani

Department of Physics, Annamalai University, Annamalai nagar,
Tamilnadu, India-608 002.

Abstract: The binding of Amantadine (AMT) to egg albumin (EA) in sodium lauryl Sulfate (SLS) solution was investigated by molecular spectroscopy at pH 7.4. The steady-state fluorescence, time-resolved fluorescence and UV – absorption spectroscopies were employed to study the mode and the mechanism for this interaction. Amantadine binding is characterized by one by high affinity binding site with the binding constant of the order of 10^5. The micelle parameters have been calculated and tabulated. FTIR spectra and SEM photographs have also been used to know the formation of complex of Egg albumin and Amantadine in SLS solution

Key words: Amantadine, Egg albumin, fluorescence spectra, UV absorption spectra, Sodium lauryl sulphate.

1. Introduction

The study of quenching of fluorescence has been an active area of research for the last few decades. Quenching can take place through different mechanisms and finds extensive application in the study of biophysical systems. Hence, this much explored area continues to attract considerable interest even today. A wide variety of substances can act as quenchers. Some examples are molecular oxygen (Masami Okamoto et al, 2002, Masami Okamoto and Oh Wada, 2001), chlorinated hydrocarbons (Joseph R. Lakowicz and Delman Hogen, 1980), aliphatic and aromatic amines (Kuzmin et al, 1969, Siegfried Schneider et al, 1994 and Uwe Pischel , 2000), aromatic nitro compounds (Germanenko et al, 2001), metal ions (Li et al, 2003, Luigi Fabbrizzi and Antonio Poggi, 1995) and halides (Huber et al, 2001, Calafut et al, 1995, Biwersi et al, 1994, Marek Mac et al, 1991, Mehata et al, 2002, Debi Pant et al, 1992 , Debi Pant et al, 1990 and Mehata et al, 2001). Fluorescence quenching is widely used in the investigation of phenomena like partitioning of fluorophores into membranes (Fastenberg et al, 2003, Menger et al, 2002) and dynamics in proteins (Ghosh et al, 2003, Cioni et al, 1998). Very recently, the technique has been used elegantly to develop a scale for flexibility of amino acid in peptides (Fang Huang et, al 2003). It is often observed that some classes of fluorephores are quenchers. A fluorophore with such selectivity is often used in qualitative and quantitative analysis of small amounts of its specific quenchers (Luigi Fabbrizzi and Antonio Poggi, 1995, Huber et al, 2001, Calafut et al, 1995, Biwersi et al, 1994, Marek Mac et al, 1991, Jeff T. Suri et al, 2003, Patricia Choppinet et al, 1999, Guo et al, 1998 and Zhu et al, 1990).

Amantadine is an antivirotic drug that has been used to treat influenza and Parkinson disease (Prud'homme et al, 1997, Sweet et al, 1997, Woo-Young choi et al, 2009, S.Kano, 2009, Brenner et al, 1989 and Nishikawa et al, 2009).The molecular structure of Amantadine is shown in Fig.1.However in recent years, influenza is often caused by viruses and germs at the same time (Yazawa et al, 2004 and Hirano 1999). Because it has only antivirotic activity. Amantadine cannot be used to treat current influenza. Therefore, it is necessary to develop the difunctional or multifunctional anti-influenza drug, which not only has antiviral activity but also does antibacterial action (Gomez-Gallego et al, 2000).

2. EXPERIMENTAL DETAILS

2.1. Materials

Egg albumin, amantadine and sodium lauryl sulphate (SLS) were purchased from **Sigma Aldrich Company, Bangalore** and were used without further purification. Triply distilled water was used throughout the study.

2.2. Sample preparation

The concentration of Egg albumin was maintained at 1.0×10^{-4} M and the concentration of the quencher (AM) was varied in the range 0.2-0.14mM. The concentration of SLS was varied in the range 0.02 – 0.10 M which is above the critical micellar concentration (CMC) of SLS (8.8mM) (J.H.Fendler et al,1975).

2.3 Methods

2.3.1. UV/Vis absorption experiments

The absorption spectra of Egg albumin in water and in different micellar concentrations of SLS both in presence and absence of the quencher, Amantadine have been recorded using **Shimadzu 1650PC UV-Visible Spectrophotometer**.
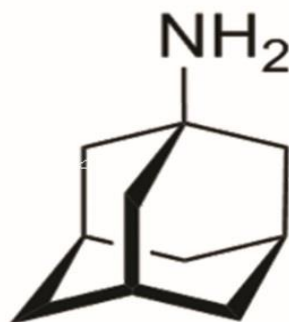
Fig.1: Structure of Amantadine

2.3.2. Fluorescence steady – state measurements

The steady – state fluorescence quenching measurements were carried out in a **Shimadzu RF5301PC Spectrofluorophotometer**. The excitation wavelength was 280 nm. The emission wavelength was monited at 332nm. The excitation and emission slit widths (5nm) and scan rate (200 nm/s) were constantly maintained for all the experiments.

2.3.3 Fluorescence quenching experiments

For the quenching experiments, various concentrations of quencher were chosen. Fluorescence intensities were obtained for different quencher concentration and plotted according to the equation,

$$\frac{I_o}{I} = 1 + K_q \tau_0 [Q] \tag{1}$$

The slopes afforded the K_{sv} values. The lifetime τ_o of EA without any added quencher and with different concentrations of the quencher were recorded. To extract K_q values, the experimental lifetimes were plotted against quencher concentration according to the Stern – Volmer equation,

$$\tau_0 / \tau = 1 + K_q \tau_0 [Q] \tag{2}$$

Where τ_0 and τ are lifetime of EA in the absence and present of quencher.

2.3.4. Fluorescence lifetime measurement

Fluorescence lifetime measurements were carried out in a **Hariba – Jobin Yvon [spex-sf 13-11] Spectrofluorimeter.** The interchangeable nano LED (280 nm) was used as excitation source. The fluorescence decay of EA was measured with a monochromator – Photo multiplier setup. The data points were fitted by mono exponential decay functions. The data analysis was carried out by the software.

2.3.5 FTIR measurements

FTIR spectra of Egg albumin without and with Amantadine in different concentrations of SLS were recorded using **Thermonicolet iS₅ FTIR Spectrophotometer.**

2.3.6 SEM analysis

Joel Sem Model, Jsm – 5610 Lv Scanning Electron Microscope was used to record the SEM photographs of Egg albumin with different concentrations of SLS in the presence and absence of Amantadine.
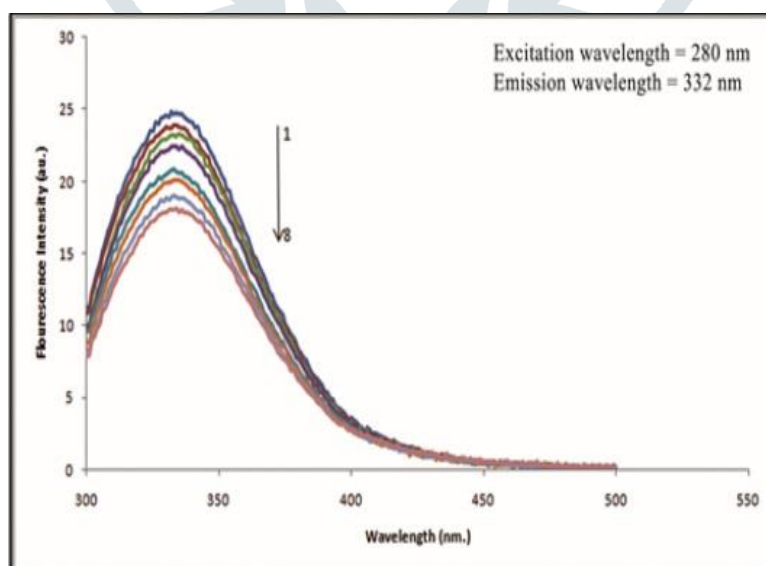

Fig.2: Fluorescence quenching spectra of EA without and with different concentrations of Amantadine (Mol L^{-1}) (1) 0, (2) 0.2 (3) 0.4 (4) 0.6 (5) 0.8 (6) 1.0 (7) 1.2 and (8) 1.4 in 0.02 M concentration of SLS.
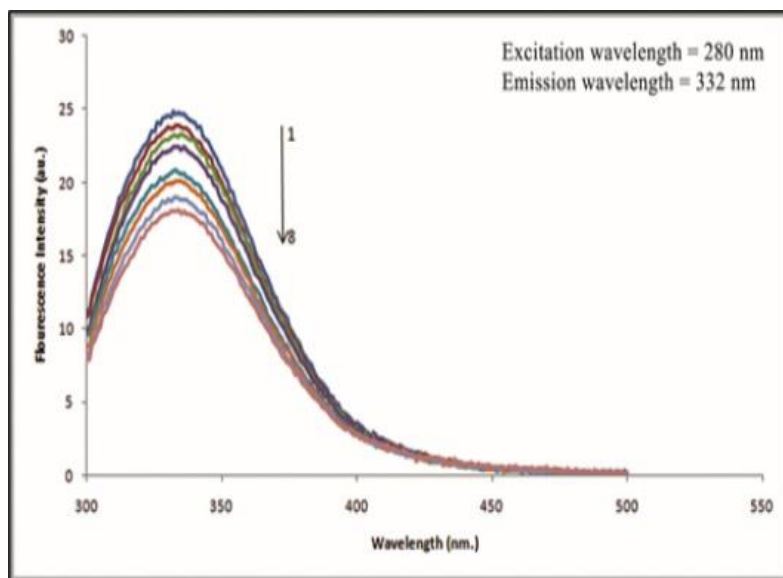
Fig.3: Fluorescence quenching spectra of EA without and with different concentrations of Amantadine (Mol L^{-1}) (1) 0, (2) 0.2 (3) 0.4 (4) 0.6 (5) 0.8 (6) 1.0 (7) 1.2 and (8) 1.4 in 0.02 M concentration of SLS

3. Results and Discussion

3.1 UV/Vis absorption studies

The ground state complex formation if any between Egg albumin and Amantadine was checked by recording the absorption spectra of a mixture of EA and Amantadine in different concentrations of SLS using concentration similar to those used in quenching studies. The absence of any new peak and the fact that absorption spectrum of EA was unaltered in the presence of the quencher eliminate the possibility of ground state charge transfer complex formation. For example, as a typical case, the absorption spectrum of Egg albumin in the absence and presence of Amantadine in 0.02 M concentration of SLS is shown in Fig. 2. It may be noted that other concentrations, (0.04, 0.06, 0.08 and 0.10M) of SLS also exhibited a similar behavior (Supplementary figures).

3.2 Steady – state fluorescence study

The fluorescence spectra of Egg albumin in different micellar concentrations of SLS both in presence and absence of the quencher [Fig. 3 (0.02 M concentration of SLS)], show no observable change in spectral shape and maxima. Although there is appreciable quenching even at low concentration of Amantadine (0.2 x 10^{-5}M).
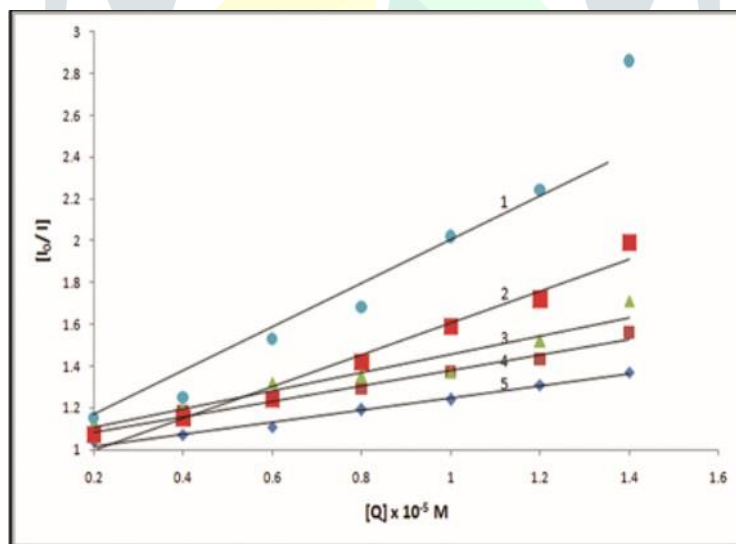

Fig.4: Stern – Volmer Plots of Egg albumin with amantadine in different concentration of SLS ((1) 0.02 M, (2) 0.04 M, (3) 0.06 M, (4) 0.08 M (5) 0.1 M)
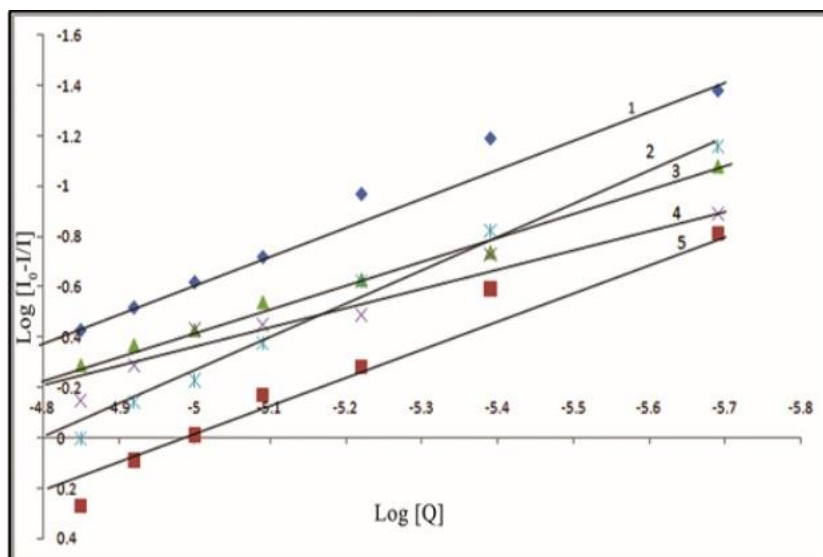
Fig.5: Double long plot of amantadine quenching effect on Egg – albumin fluorescence in different concentrations of SLS ((1) 0.02 M, (2) 0.04 M, (3) 0.06 M, (4) 0.08 M (5) 0.1 M

The shape of the fluorescence spectra remains the same with no change in the position of the maxima. Furthermore, observation of similar absorption spectra of a solution containing any concentration of the quencher after carrying out the fluorescence indicates that no detectable photoproduct is formed under the experimental condition. No new fluorescence peak is also observed at longer wavelength. The excitation spectra monitored at different emission wavelengths also remain the same in all the media. These observations indicate that there is no ground state complexation of Egg albumin and Amantadine. Decrease in the fluorescence intensity of Egg albumin in all concentrations of SLS (0.02, 0.04, 0.06, 0.08, 0.10M) without the appearance of any new band in the presence of Amantadine indicates that no emissive exciplex is formed between the Egg albumin and Amantadine(Supplementary figures).

Fig. 3 shows the effect of increasing concentration of Amantadine on the fluorescence emission of Egg albumin in 0.02 M concentration of SLS. Addition of Amantadine to the solution of Egg albumin resulted in the quenching of its fluorescence emission. According to eqn (1) we got linear plot [shown in Fig. 4] of the I_0/I against Amantadine concentration in the SLS solution. Stern-Volmer quenching constants (K_{sv}) have been calculated from the slope of the plots. The bimolecular quenching rate constant (K_q) was obtained and the corresponding electrochemical data were compiled in Table 1.

The obtained K_q values differ among the different concentrations of SLS studied. The observed minimum K_q value may be due to a weak quenching.
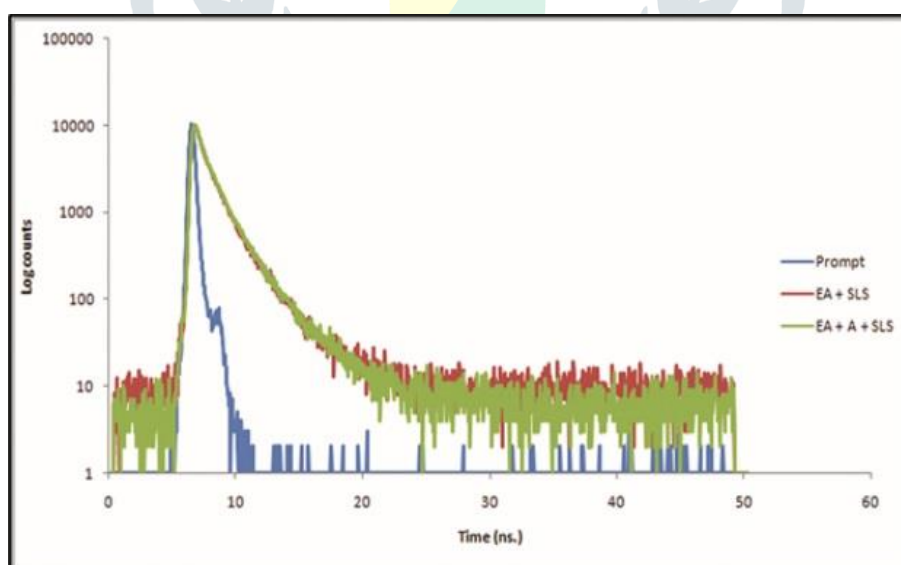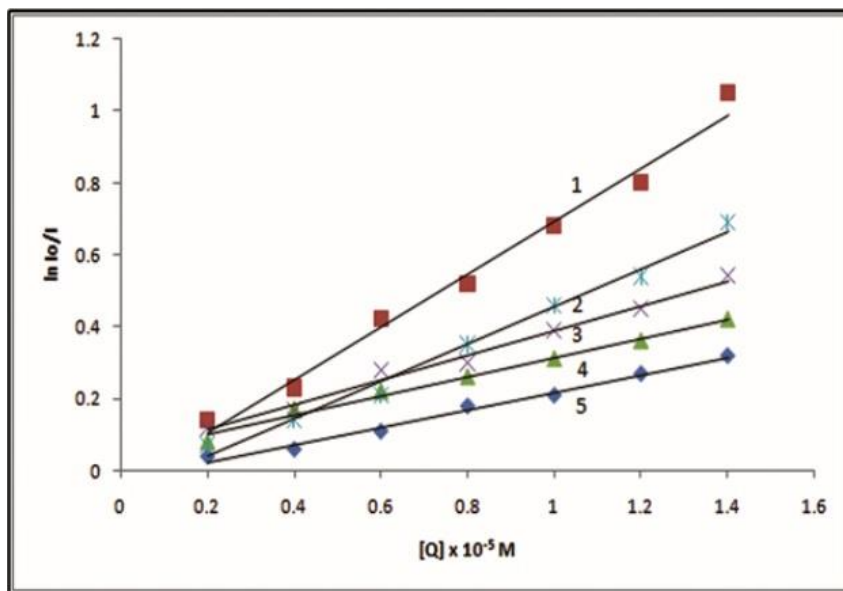

Fig.6: Decay curves of Egg albumin

Fig.7 : Plot of In (I_0 / I) V$_S$ $[Q] \times 10^{-5}$ M of egg albumin with amantadine in different concentration of SLS
((1) 0.02 M, (2) 0.04 M, (3) 0.06 M, (4) 0.08 M (5) 0.1 M)

3.3 Binding constant and number of binding sites

Large K_q beyond the diffusion – controlled limit indicates that some type of binding interaction exists between fluorophore and quencher (H.R. Park et al, 2006). For static quenching, the relationship between the intensity and the concentration of the quencher can be described by the binding constant formula (Y.Xu et al, 1997).

The relationship between the fluorescence intensity and the quencher medium can be deduced from the following equation.

$$nQ + B \rightarrow Qn \ldots B \tag{3}$$

where B is the fluorophore, Q is the quencher and Qn….B is the postulated complex between a fluorophore and n molecules of the quencher. The constant K is given by,

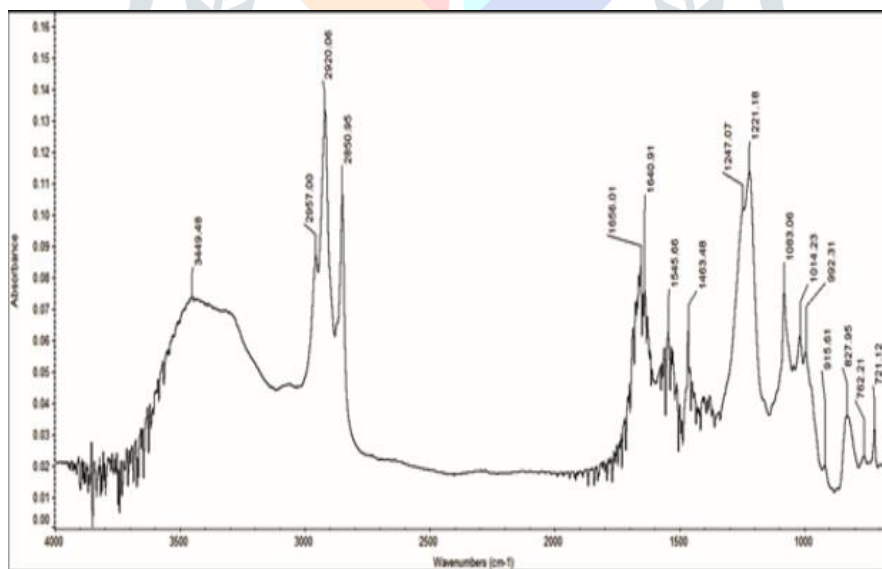$$K = [Q_n \ldots B] / [Q] [B] \tag{4}$$

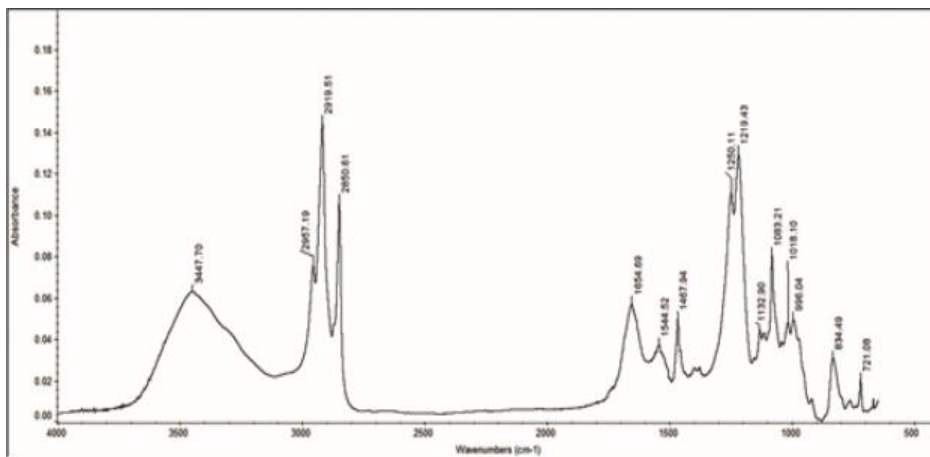Fig .8: FTIR spectra of Egg albumin in SLS solution (0.02 M)

Fig 9: FTIR Spectra of Egg albumin with amantadine in SLS solution (0.02 M)

If the overall amount of biomolecules (bound or unbound with the quencher) is B_0, then $[B_0] = [Q_n....B] + [B]$, here $[B]$ is the concentration of unbound biomolecules, then the relationship between fluorescence intensity and the unbound biomolecular as $[B] / [B_0] = I/I_o$ that is,

$$\log\left[\frac{F_0 - F}{F}\right] = \log k + n \log[Q] \tag{5}$$

Where K is the binding constant and n is the number of binding sites.
The value of K was determined from the intercept of $\log[(I_o - I)/I]$ versus $\log[Q]$ as shown in Fig. 5. The value of binding constant [K] and number of binding sites (n) for Amantadine in all SLS concentrations have been calculated and shown in Table. 2. The correlation coefficient for all the curves were larger than 0.97 indicating that the interaction between Egg albumin and Amantadine in SLS solution agrees well with the site binding model underlying eqn. (5).
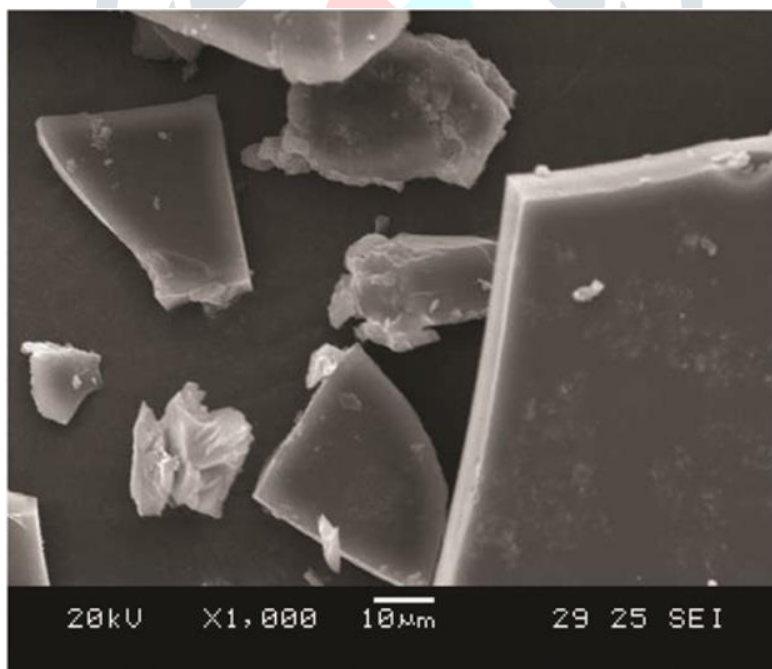

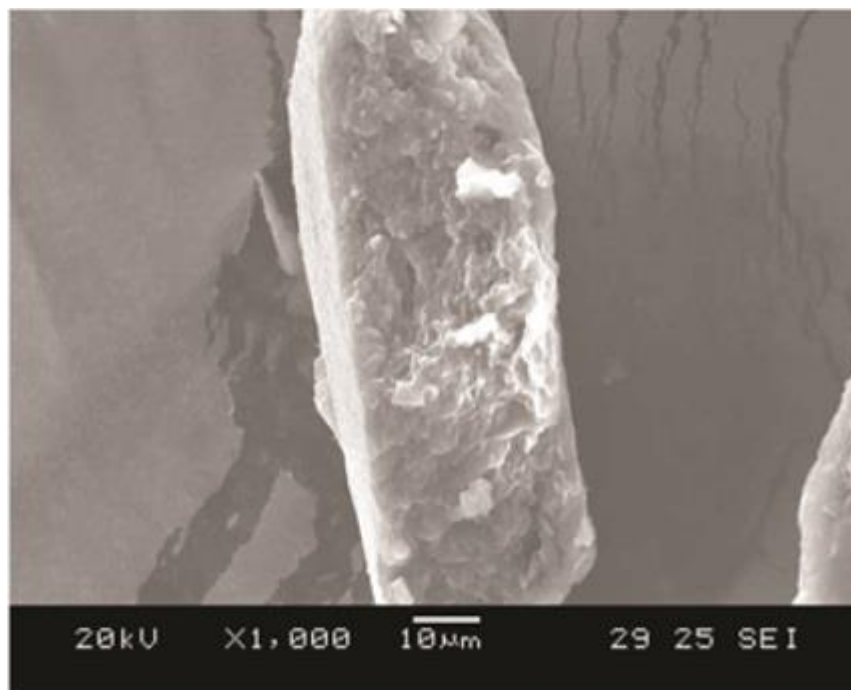Fig.10: SEM images of Egg albumin with Amantadine

Fig.11: SEM image of Egg albumin with Amantadine in SLS solution (0.02 M)

3.4. Mechanism of quenching

The quenching of Egg albumin can be explained by a number of possible mechanisms such as electron transfer, energy transfer, and proton transfer or hydrogen atom transfer. Fig. 3 shows the fluorescence emission spectra of EA with various quantities of Amantadine in 0.02M concentration of SLS. It can be seen from a scrutiny of the all the concentrations of SLS (0.02, 0.04, 0.06, 08, 0.1M).Fluorescence intensity of EA decreases steadily and with the addition of quencher there is almost no shift in the emission wavelength (λ_{emi} = 332nm). The quenching rate constant K_q are much higher than the maximum scatter collision quenching constant of the various quenchers [2.0×10^{10} L mol^{-1} s^1] which indicates that the quenching mechanism of Amantadine – EA interaction is not initiated by dynamic collision but by compound formation (Yao-hai Zhang et al, 2009). That is, drug is bound to EA and a drug – EA complex is formed, which resulted in the quenching of the fluorescence of the fluorophore.

Essentially, there exist four types of non-covalent interactions in the binding of the ligands to proteins. These are hydrogen bonds, Van der waals forces, hydrophobic and electrostatic interactions (Jun Wang, et al, 2009). Thermodynamic parameters, free energy (ΔG), standard enthalpy (ΔH) and standard entropy (ΔS) will provide an insight into the binding mode. Among these parameters, ΔG reflects the possibility of reaction; ΔH and ΔS are principal evidence for determining the active forces.
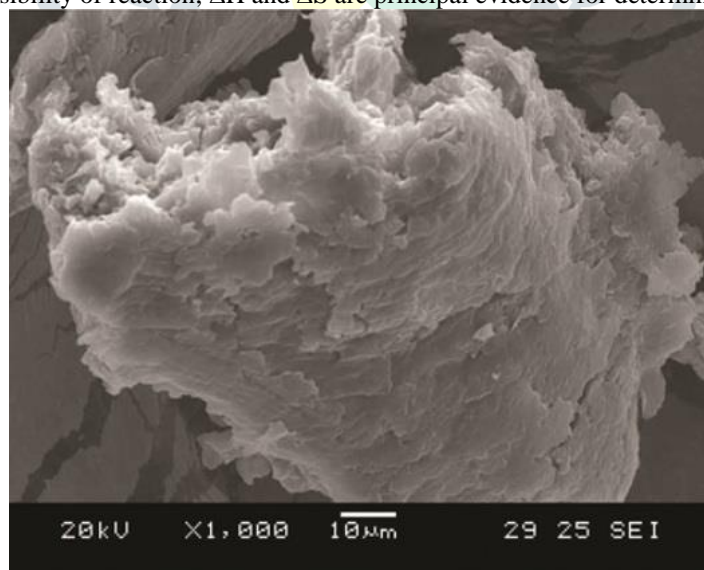

Fig.12: SEM image of Egg albumin with Amantadine in SLS solution (0.04 M)
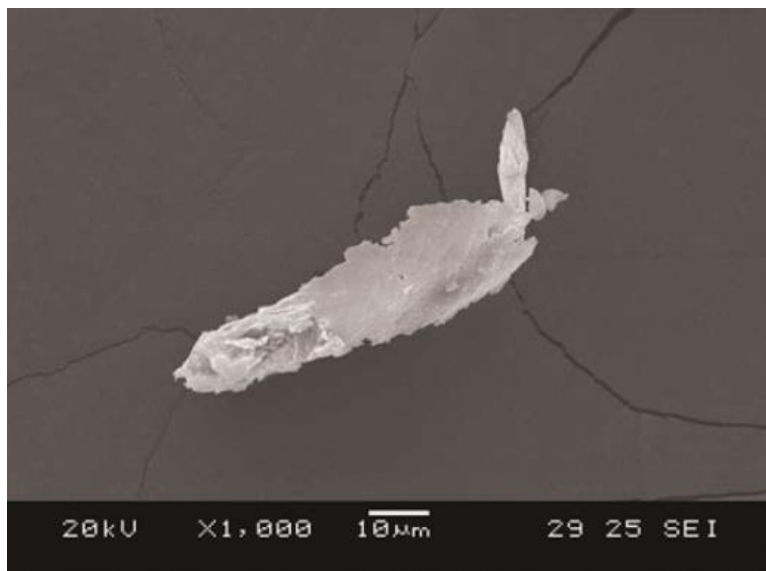
Fig.13: SEM image of Egg albumin with Amantadine in SLS solution (0.06 M)

Through, the binding constant K_a, thermodynamic parameter is evaluated using the following equation,

$$\Delta G = - RT \ln K_a \tag{6}$$

R is the gas constant; ΔG value is given in Table 2.

The negative sign for ΔG means that interaction is spontaneous and also indicates that the electron transfer processes studied are thermodynamically favourable. The hydrophobic force may play a major role in the reaction (Leckband et al, 2000).

3.5 Fluorescence lifetime measurements of Egg albumin with Amantadine in different SLS concentrations

Fluorescence lifetime measurement is a very useful technique for understanding the type of interaction between the donor and the acceptor systems. In general, the measurement of fluorescence lifetime is the most definite method to distinguish static quenching and dynamic quenching (Ross et al, 1981).

Fig.14: SEM image of Egg albumin with Amantadine in SLS solution (0.08 M)

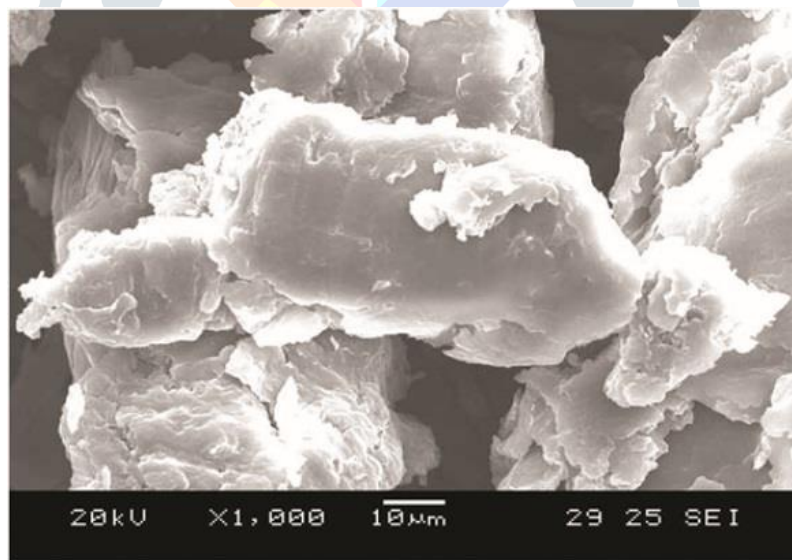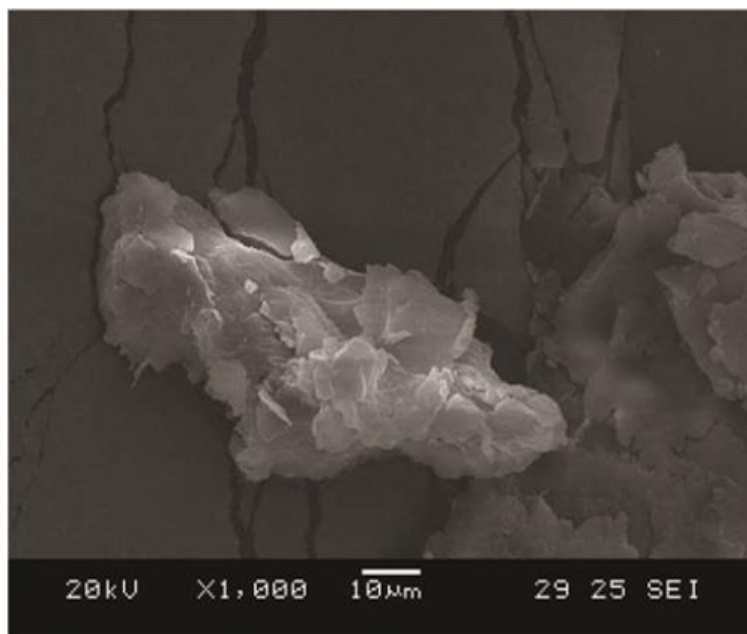Fig.15: SEM image of Egg albumin with Amantadine in SLS solution (0.10 M)

The decay curves of Egg albumin in the absence and presence of Amantadine in different SLS concentration were shown in Fig. 6. The lifetime of EA remains not same in both conditions; hence the merging of the kinetic traces was not observed (The plots not look like a single decay curve). This shows that the quenching of Egg albumin was dynamic in nature. The same case was observed for all concentrations of SLS (Table.3).

3.6. Micellar size

From a structural point of view, the most relevant parameter of a micellar system is the mean micellar aggregation number. To analyse the effect of Egg albumin addition on the mean aggregation number of SLS micelles, the well-established quenching method firstly proposed by Turro and Yekta (Turro et al, 1978) on the basis of previous analysis performed by Tachiya (Tachiya , 1975). This procedure is based upon the quenching of a luminescent probe by a known concentration of quenchers. The quenching experiments were analysed by using the following equation,

$$\ln \frac{I_0}{I} = \frac{N_{agg}}{[S]-CMC}[Q] \tag{7}$$

Where I_o and I are the fluorescence intensities in the absence and presence of the quenchers respectively. N_{agg} is the mean aggregation number, [S] is the total surfactant concentration and [Q] is the quencher concentration.

The results obtained in this quenching studies show how the Egg albumin fluorescence emission is quenched as the quencher concentration in the micellar system increase. Fig. 7 shows the obtained quenching results according to equation (7). The mean aggregation numbers of SLS micelles are listed in Table 4.

Table 1 : Stern – Volmer (K_{SV}) and biomolecular quenching rate constant (K_q) of Egg Albumin with Amantadine in different concentration of SLS

Concentration of SLS (M)	K_{sv} x 10^5 (L mol^{-1})	K_q (L mol^{-1}s^{-1})	R^2	S.D
0.02	0.25	9.26 x 10^{12}	0.99	0.12
0.04	0.50	2.15 x 10^{13}	0.94	0.60
0.06	0.65	2.85 x 10^{13}	0.99	0.16
0.08	0.75	3.45 x 10^{13}	0.93	0.19
0.10	0.85	3.66 x 10^{13}	0.97	0.33

Table 2 : Binding constant (K_a), binding numbers (n), correlation coefficient (R), change in free energy ΔG_g (for ground state) and ΔG_e (for excited state).

Concentration of SLS (M)	K_a (L mol^{-1})	n	R	ΔG_g KJ mol^{-1}	ΔG_e KJ mol^{-1}
0.02	4.27x 10^5	1.25	0.97	-65.56	31.18
0.04	2.87x 10^5	1.20	0.97	-44.29	66.99
0.06	1.73x 10^5	1.13	0.99	-60.49	53.41
0.08	6.22x 10^4	1.03	0.95	-47.80	88.33
0.10	2.33x 10^4	0.99	0.99	-47.42	35.95

It has been proposed (Israelachvili et al, 1985) that the surface area per head group, a_o, is the most important controlling factor for micelle size. According to Tanford (Tanford , 1980), the hydrophobic chain volume of the micelle, v, and the critical chain length, l_c, can be obtained from,

$$v, = (27.4 + 26.9\ n_c)\ (\text{Å}^3) \tag{8}$$
and
$$l_c = (1.5 + 1.265\ n_c)\ (\text{Å}) \tag{9}$$

where n_c is the number of carbon atoms in the hydrophobic chain of the surfactant. In this way, assuming a spherical geometry, the micellar radius, R_o, and the surface area per head group were obtained. The corresponding values are listed in Table 5.

It is also included the critical packing parameter, $v/a_o l_c$, which is a parameter controlling the micelle shape (Israelachvili et al, 1985].

Table 3 : Fluorescence life time and amplitudes of Egg albumin without and with Amantadine in different concentration of SLS

Concentration of SLS (M)	Concentration of Amantadine (ml)	Lifetime (ns)			Average life time x 10^{-9} sec	Relative amplitude			χ^2	S.D x 10^{-11} sec		
		τ_1	τ_2	τ_3		B_1	B_2	B_3		τ_1	τ_2	τ_3
0.02	0	1.69	4.56	4.19	2.69	38.24	14.27	47.49	1.18	1.17	3.53	4.59
	1.4	1.78	4.32	4.26	2.64	40.53	16.31	43.16	1.28	1.19	2.93	5.46
0.04	0	1.32	2.39	3.67	2.32	37.31	13.65	49.03	1.56	9.02	3.94	4.06
	1.4	1.51	2.62	3.98	2.25	42.71	14.78	42.51	1.59	8.30	3.22	5.29
0.06	0	1.50	2.13	4.04	2.28	42.43	18.03	39.54	1.28	6.64	2.57	4.79
	1.4	1.52	1.79	4.18	2.24	43.27	19.41	37.32	1.13	5.19	2.21	4.72
0.08	0	1.24	1.57	3.74	2.17	39.67	16.05	44.28	1.22	5.88	2.65	3.79
	1.4	1.32	1.90	3.77	2.10	39.51	16.64	43.85	1.07	5.63	2.38	3.88
0.10	0	1.60	3.02	4.23	2.33	43.86	19.08	37.06	1.19	8.43	2.51	5.51
	1.4	1.49	1.82	4.11	2.17	41.37	21.87	36.76	1.26	6.23	2.28	5.06

Table 4: Aggregation number, (N_{agg}), radius (R_o), surface area per head group (a_0), and packing parameter ($v/a_0 l_c$) of SLS micelle

Concentration	Aggregation number	Radius of the micelle (R_0)Å	Area of the micelle (a_0) Å^2	Critical aggregation parameters
0.02	354	29.12	30.09	0.697
0.04	1295	44.67	19.35	1.084
0.06	1987.5	51.45	16.73	1.253
0.08	2156.16	52.86	16.28	1.288
0.10	5049	69.99	12.19	1.720

3.7. Fourier Transform Infrared Spectra

The changes of the FTIR spectra shown in Figs. 8 & 9 can reveal the formation of the Egg albumin and Amantadine complexes in 0.02 M concentration of SLS. The same can be observed for all other concentrations of SLS (0.04, 0.06, 0.08 and 0.1 M) and the values are shown in Table 5.

The absorption intensity of the complexes are significantly weaker then that of Egg albumin. The complex infra-red peaks are observed in the range 1000 cm^{-1}– 3000 cm^{-1} and are 0.01 to 0.16 % weaker than that of the Egg albumin molecule. As there is no change in the wavenumber other than the change in the absorption intensities, it can be concluded that 0.01 to 0.16% weaker complexes were formed of Egg albumin and Amantadine in different SLS concentrations.

3.8. Scanning electron microscope (SEM) observation of Egg albumin with Amantadine in SLS

Egg albumin with amantadine (without SLS) was powdered separately and the structure of their particles in this powder was observed first, in the scanning electron microscope (Fig.10). Then the particles of the powdered form of the complexes (EA+AMT+SLS) were also studied. These are shown in Figs. 11& 15. The SEM images of (EA+AMT) with 0.02, 0.04, 0.06, 0.08, and 0.10M concentration of SLS are shown in figs 11, 12, 13, 14&15 respectively .The structure of the particles of the complexes EA without SLS appears different from that of Egg albumin with SLS and it can be assumed as proof of the formation of new complex.

Table 5: Difference in FTIR absorption peak Intensities of Egg Albumin before and after complex formation in different concentrations of SLS

Intensities (cm^{-1})				Difference in intensities prior to and after (%)		Tentative Assignment
EA SLS		EA A SLS				
0.02	0.10	0.02	0.10	0.02	0.10	
2957	2956	2956	2957	0.02	0.07	C – H stretching
2919	2918	2919	2918	0.02	0.16	C – H stretching
2850	2850	2850	2850	0.02	0.12	C – H stretching
1654	1652	1654	1655	0	0	C = O Stretching
1467	1468	1467	1467	0.02	0.03	C – O Stretching
1083	1082	1083	1083	0.01	0.06	C – O – H stretching
1018	1018	1019	1019	0.01	0.02	C – O – H stretching

Conclusion

In this paper, the interaction of Amantadine with Egg albumin has been investigated in vitro under simulated physiological conditions using different optical and computational techniques. These studies show that Amantadine binds to Egg albumin with high affinity and quenchers the intrinsic fluorescence of Egg albumin efficiently. Binding constants are evaluated. The results of fluorescence, time – resolved fluorescence, UV absorption spectra, FTIR spectra and SEM analysis are suggest that the conformation of Egg albumin is changed obviously on the binding of Amantadine in SLS solution. The binding study of drugs of Egg albumin is of great importance in understanding chemio-biological interactions for drug design, pharmacy, pharmacology and biochemistry. Furthermore, these studies are expected to provide important insight into the interaction of the physiologically important protein Egg albumin with an important drug used in various therapeutic regimes.

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