MOLECULAR MODELING AND MULTISPECTROSCOPIC STUDIES
OF THE INTERACTION OF AMANTADINE WITH EGG ALBUMIN

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ABSTRACT
The binding of the drug amantadine (A) with Egg albumin (EA) was investigated by fluorescence quenching, absorption spectroscopy, FRET and molecular docking. UV/Vis absorption spectroscopy results showed that the binding of the drug to EA induces some conformational changes in EA. The values of Ro (the distance when 50% energy is transformed) and r (binding distance) are less than 7nm, it indicates the presence of interaction between amantadine and egg albumin along with the opportunity of energy transfer from the protein to drug. In SPAN 40 solution.

KEYWORDS: Amantadine, Egg Albumin, Molecular Docking, Fluorescence, FRET, SPAN 40.

1. INTRODUCTION
Plasma proteins play an important role in the transportation and deposition of substances such as fatty acids, hormones and medicinal drugs in the circulatory systems. Therefore, it is important to reveal the interaction between drugs and protein in the blood stream, at it may affect the bioavailability, distribution and elimination of pharmaceutically or netraceutical active compounds albumin is the main plasma protein, and its main function is to regulate colloidal osmotic pressure and transport substances in the blood stream.¹

Docking is frequently used to predict the binding orientation of small drug candidates to their protein targets in order to predict the affinity and activity of the small molecule.
In the present work, we have investigated the binding of amantadine with egg albumin in different concentration of SPAN 40 solution using UV/Vis, fluorescence and molecular docking studies. The binding affinities, number of binding sites and binding distances are determined for EA-polyphenol complexes. Depending on various biological activities, the dietary flavonoids is extensively studied by researches in the field of biological and pharmaceutical sciences. These compounds exhibited their affinity towards different biomacro molecules and also established their interactions on various biological process.

2. MATERIALS AND METHODS
Egg albumin, querceten and SDAN 40 were obtained from sigma Aldrich Company Bangalore. They were used for taking reading without farther purification. UV – Vis studies were conducted on a SHIMADZU 1650 PC UV-VISIBLE SPECTROPHOTOMETER, in the region of 250 – 350 nm, at room temperature. The fluorescence measurements were carried out at room temperature on SHIMADZU RF 5301 PC SPECTROFLUOROPHOTOMETER using a 1 cm quartz cell.

The 3D crystal structure of Egg albumin (PDB ID 4XEN) is obtained from the protein Data Bank.

3. RESULTS AND DISCUSSION
3.1. Absorption characteristics of Egg albumin with Amantadine
UV/Vis absorption measurement is a simple but effective method of detecting complex formation.\textsuperscript{[2,3]} Complex formation between Amantadine and EA is evident from the data UV/Vis absorption spectra.

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 SPAN 40 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. UV/Vis absorption spectra of EA and those of EA with the addition of different concentrations of Amantadine were recorded. It is obvious that the intensity of UV absorption of EA decrease with the addition of Amantadine in SPAN 40 solution.
Fig. 1: UV/Vis absorption spectra of EA with different concentrations of Amantadine (mol L$^{-1}$) (1) 0, (2) 0.02, (3) 0.04, (4) 0.06, (5) 0.08, (6) 0.10, (7) 0.12, (8) 0.14 in 0.02 M concentration of SPAN 40.

Fig. 1 shows the absorption spectra of EA from 250 nm to 350 nm in the presence of different concentrations of Amantadine in 0.02 M concentration of SPAN 40. It is evident that absorbance of EA decreased regularly with the variation of Amantadine concentrations and the maximum peak position of Amantadine-EA shifted from 280 nm 278 to nm. Moreover, a slight blue shift of the maximum peak of albumin at 278 nm, probably due to complex formation between quencher and albumin was also noticed.\cite{4}

Fig. 2: UV/Vis absorption spectra of EA with different concentrations of Amantadine (mol L$^{-1}$) (1) 0, (2) 0.02, (3) 0.04, (4) 0.06, (5) 0.08, (6) 0.10, (7) 0.12, (8) 0.14 in 0.04 M concentration of SPAN 40.
Fig. 3: UV/Vis absorption spectra of EA with different concentrations of Amantadine (mol L\(^{-1}\)) (1) 0, (2) 0.02, (3) 0.04, (4) 0.06, (5) 0.08, (6) 0.10, (7) 0.12, (8) 0.14 in 0.06 M concentration of SPAN 40.

Fig. 4: UV/Vis absorption spectra of EA with different concentrations of Amantadine (mol L\(^{-1}\)) (1) 0, (2) 0.02, (3) 0.04, (4) 0.06, (5) 0.08, (6) 0.10, (7) 0.12, (8) 0.14 in 0.08 M concentration of SPAN 40.

The absorption of spectrum of Egg albumin in the absence and presence of quecertin in 0.04, 0.06 and concentrations of SPAN 40 are shown in Figs.2, 3 and 4 and respectively. It may be noted that all the other concentrations of APAN 40 (0.04, 0.06, and 0.08M and) also exhibited a similar behavior as like as 0.02 M concentration of SPAN 40.

**Fluorescence Quenching of Egg albumin by Amantadine in SPAN 40**

The fluorescence spectra of Egg albumin in different micellar concentrations of SPAN 40 both in presence and absence of the quencher, show no observable change in spectral shape and maxima. Fig.6 shows the fluorescence quenching spectrum of Egg albumin without and with different concentrations of Amantadine in the solution of 0.02M SPAN 40.
concentration. There is appreciable quenching even at low concentration of Amantadine (0.2 x 10^{-5} M), the shape of the fluorescence spectra remains the same with no change in the position of the maxima. Further 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 is all the media. These observations indicate that there is no ground state complexation of Egg albumin and Amantadine.

Fig. 5: Steady – state fluorescence spectra of EA with different concentrations of Amantadine (mol L^{-1}) (1) 0, (2) 0.02, (3) 0.04, (4) 0.06, (5) 0.08, (6) 0.10, (7) 0.12, (8) 0.14 in 0.02 M concentration of SPAN 40.

Fig. 6: Steady – state fluorescence spectra of EA with different concentrations of Amantadine (mol L^{-1}) (1) 0, (2) 0.02, (3) 0.04, (4) 0.06, (5) 0.08, (6) 0.10, (7) 0.12, (8) 0.14 in 0.04 M concentration of SPAN 40.
Fig. 7: Steady – state fluorescence spectra of EA with different concentrations of Amantadine (mol L$^{-1}$) (1) 0, (2) 0.02, (3) 0.04, (4) 0.06, (5) 0.08, (6) 0.10, (7) 0.12, (8) 0.14 in 0.06 M concentration of SPAN 40.

Fig. 8: Steady – state fluorescence spectra of EA with different concentrations of Amantadine (mol L$^{-1}$) (1) 0, (2) 0.02, (3) 0.04, (4) 0.06, (5) 0.08, (6) 0.10, (7) 0.12, (8) 0.14 in 0.08 M concentration of SPAN 40.

Figs. 5, 6, 7 and 8 show the fluorescence quenching spectra of egg albumin without and with different concentrations Amantadine in the solutions of 0.02 M, 0.04M, 0.06M and 0.08M concentrations of SPAN 40. Decrease in the fluorescence intensity of egg albumin in all concentrations of SPAN 40 (0.02M, 0.04M, 0.06M and 0.08M) 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.

Addition of Amantadine to the solution of Egg albumin resulted in the quenching of its fluorescence emission. Fluorescence quenching of EA results from a decrease of quantum yield of fluorescence. The Stren-Volmer equation is often applied to describe fluorescence quenching and analyze the quenching mechanism (lakowicz, 2006).
\[ \frac{I_o}{I} = 1 + K_q [Q] = 1 + K_q \tau_o [Q] \]

Where \( I_o \) and \( I \) are the fluorescence intensities before and after addition of the quencher, \( K_q \) is the quenching rate constant, \( K_{sv} \) is the Stern-Volmer quenching constant, \([Q]\) is the quencher concentration, and \( \tau_o \) is the average lifetime without quencher.

According to eqn (1.1) a graph was drawn for \( (I_o / I) \) against quencetin concentration \([Q]\) in SPAN 40 solution. A linear plot was observed. Fig. show the linear plot of \( I_o / I \) against \([Q]\) in all the concentrations (0.02M, 0.04M, 0.06M, and 0.08M) of SPAN 40. From the slope the Stern-Volmer quenching constants \((K_{sv})\) were calculated. The bimolecular quenching rate constants \((K_q)\) were obtained for the different concentrations of SPAN 40 and the corresponding electrochemical data were compiled in Table 1.1.

Table 1 Stern – Volmer \((K_{sv})\) and bimolecular quenching rate constant \((k_q)\) of Egg Albumin with Amantadine in different concentrations of SPAN 40.

<table>
<thead>
<tr>
<th>Concentration of SPAN 40 (M)</th>
<th>( K_{sv} \times 10^5 ) (L Mol(^{-1}))</th>
<th>( K_q \times 10^{13} ) L mol(^{-1})S(^{-1})</th>
<th>( R^2 )</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.02</td>
<td>0.15</td>
<td>3.88 \times 10^{12}</td>
<td>0.98</td>
<td>0.196</td>
</tr>
<tr>
<td>0.04</td>
<td>0.30</td>
<td>1.32 \times 10^{13}</td>
<td>0.98</td>
<td>0.217</td>
</tr>
<tr>
<td>0.06</td>
<td>0.40</td>
<td>3.51 \times 10^{13}</td>
<td>0.99</td>
<td>0.134</td>
</tr>
<tr>
<td>0.08</td>
<td>0.50</td>
<td>4.97 \times 10^{13}</td>
<td>0.97</td>
<td>0.137</td>
</tr>
<tr>
<td>0.10</td>
<td>0.55</td>
<td>1.14 \times 10^{16}</td>
<td>0.99</td>
<td>0.176</td>
</tr>
</tbody>
</table>

Fig. 9: Stern-Volmer plots of Egg albumin with Amantadine in different concentration of SPAN 40.

The obtained \( K_q \) values differ among the different concentrations of SPAN 40 studied. The observed minimum \( K_q \) value may be to a weak quenching.
Binding constants and number of binding sites

Large $K_q$ beyond the diffusion controlled limit indicates that some type of bindings interaction exists between fluorophore and quencher\(^\text{[6]}\). For static quenching, the relationship between the intensity and the concentrations of quencher can be described by the binding constant formula.

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

$$nQ + B \rightarrow Q_n \ldots B$$

where $B$ is the fluorophore, $Q$ is the quencher and $Q_n \ldots B$ is the postulated complex between a fluorophore and $n$ molecules of the quencher. The constant $K$ is given by,

$$K = \frac{[Q_n \ldots B]}{[Q_n]} \frac{1}{[B]}$$

If the overall amount of biomolecular (bound or unbound with the quencher) is $B_o$ then,

$$[B_o] = [Q_n \ldots B] + [B],$$

Here, $[B]$ is the concentration of unbound biomolecules, then the relationship between fluorescence intensity and the unbound biomolecules as $[B] / [B_o] = I / I_o$

That is

$$\log \left( \frac{I_o - I}{I} \right) = \log K + n \log [Q] \quad (7.5)$$

Where $K$ is the binding constant, and $n$ is the number of binding sites. Thus, a plot of $\log (I_o - I / I)$ versus $\log [Q]$ can be used to determine $K$ as well as $n$.

The value of $K$ was determined from the intercept of $\log (I_o - I / I)$ versus $\log [Q]$ as shown in Fig. 10. The value of $K$ are $1.76 \times 10^6$, $5.75 \times 10^5$, $3.14 \times 10^5$ and $5.96 \times 10^4$ L mol$^{-1}$ for EA in 0.02, 0.04, 0.06 and 0.08M concentrations of SPAN 40 respectively and the values of $n$ were found to be nearly 1.3 (above 0.90) for all concentration of SPAN 40. The linear correlation interaction between Amantadine and EA agreed well with the site-binding model according to equation (7.5).
Fig. 10: Double log plot of Amantadine quenching effect on EA fluorescence at different concentrations of SPAN 40.

Table 2: Binding constant ($K_a$), binding numbers (n), correlation coefficient (R), change in free energy $\Delta G_g$ (for ground state) and $\Delta G_e$ (for excited state).

<table>
<thead>
<tr>
<th>Concentration of SPAN 40 (M)</th>
<th>$K_a$ Lmol$^{-1}$</th>
<th>n</th>
<th>R</th>
<th>$\Delta G_g$ KJmol$^{-1}$</th>
<th>$\Delta G_e$ KJ mol$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.02</td>
<td>4.75X10$^{-5}$</td>
<td>1.22</td>
<td>0.98</td>
<td>-26.99</td>
<td>62.69</td>
</tr>
<tr>
<td>0.04</td>
<td>3.63 X10$^{-7}$</td>
<td>1.17</td>
<td>0.97</td>
<td>-19.98</td>
<td>92.93</td>
</tr>
<tr>
<td>0.06</td>
<td>1.12 X10$^{-8}$</td>
<td>1.11</td>
<td>0.98</td>
<td>-29.30</td>
<td>81.09</td>
</tr>
<tr>
<td>0.08</td>
<td>3.88 X10$^{-4}$</td>
<td>1.01</td>
<td>0.96</td>
<td>-31.56</td>
<td>70.87</td>
</tr>
<tr>
<td>0.10</td>
<td>1.59 X10$^{-9}$</td>
<td>0.92</td>
<td>0.99</td>
<td>-29.14</td>
<td>80.47</td>
</tr>
</tbody>
</table>

The result illustrates that there is a strong binding force between Amantadine and EA, and that the binding site formed would be one. Table 2 presents the calculated binding constant and binding site values.

**Mechanism of quenching**

The quenching of Egg albumin can be explained by a number of possible mechanisms such as electron transfer, energy transfer, proton transfer, or hydrogen atom transfer. It can be seen from a scrutiny of the above said figures, fluorescence intensity of EA decreases steadily and with the addition of quencher there is almost no shift in the emission wavelength ($\lambda_{emi} = 336$ nm). The quenching constant $K_q$ are much higher than the maximum scatter collision quenching constant of the various quenchers [2.0 x 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.$^{[7]}$ 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 exists four types of non-covalent interactions in the binding of the ligands to proteins. These are hydrogen bonds, van der walls forces, hydrophobic and electrostatic interactions.\[8\] Thermodynamic parameters, free energy ($\Delta G$), standard enthalpy $\Delta H$ and standard entropy ($\Delta S$) will provide an insight into the binding mode. Among these parameters, $\Delta G$ reflects the possibility of reaction, $\Delta H$ and $\Delta S$ are principal evidence for determining the active forces. Through the binding constant $K$, thermodynamic parameter is evaluated using the following equation,

$$\Delta G = -RT \ln K \quad (7.6)$$

$R$ is the gas constant and this value is given in Table 2.

The negative sign for $\Delta G$ 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.\[9\]

**Fluorescence resonance energy transfer between Amantadine and egg albumin in SPAN 40.**

A lot of information about molecular details of the donor – acceptor pair can be obtained from non-radiation energy transfer\[10\], fluorescence resonance energy transfer occurs when the emission spectrum of the donor overlaps the absorption spectrum of the acceptor. The dependence of the energy transfer rate on the interaction distance has been widely used to measure the distance between the donor and the acceptor.

Generally, the maximum distance is in the range of 7-10 nm.\[10\] According to the Forster non-radiation energy transfer theory\[11,10\], energy transfer is related not only to the distance between the acceptor and donor, but also to the critical energy transfer distance ($R_0$),

$$E = \frac{R_0^6}{R_0^6 + r^6} \quad (7.7)$$

Where $R_0$ is the critical transfer distance when the transfer efficiency is 50% and $r$ the mean distance between the centers of the donor and acceptor dipoles. The donor and acceptor here are EA and Amantadine respectively. $E$ is the energy transfer efficiency calculated with equation (7.8),

$$E = 1 - \frac{I}{I_0} \quad (7.8)$$
Where I and $I_0$ are the fluorescence intensity of EA with and without Amantadine respectively. And $R_0$ can be given by,

$$R_0^6 = 8.8 \times 10^{-25} K^2 N^4 \phi J \quad (7.9)$$

where $K^2$ is the spatial orientation factor of the dipole, $N$, the refractive index of the medium, $\phi$ the fluorescence quantum yield of the donor in the absence of the acceptor, $J$ expresses the overlap integral of the fluorescence emission spectrum of the donor and the absorption spectrum of the acceptor, $J$ is given by,

$$J = \frac{\sum F(\lambda) \varepsilon(\lambda) \lambda^4 \Delta \lambda}{\sum F(\lambda) \Delta \lambda} \quad (7.10)$$

where $F(\lambda)$ is the fluorescence intensity of donor at wavelength range $\lambda$ and $\varepsilon(\lambda)$, the molar absorption coefficient of the acceptor at wavelength $\lambda$ with unit of Cm$^{-1}$mol$^{-1}$ integrating the overlap of the UV absorption spectrum of Amantadine and the fluorescence emission spectrum of EA.

The calculated $E = 0.4566$; $R_0 = 38.94$ Å and $r = 40.09$ Å. The overlap spectrum is shown in Fig.11. The compiled data are present in Table. 3.

![Fig. 11: The overlap of UV absorption of Amantadine (solid line) with the fluorescence Emission Spectrum of EA (Dotted line) with SPAN 40.](image)

Table 3. Efficiency transfer energy ($E$), Critical energy transfer distance ($R_0$) of EA with Amantadine

<table>
<thead>
<tr>
<th>Quencher</th>
<th>Energy (E)</th>
<th>$R_0$ Å</th>
<th>$J$ (cm$^3$M$^{-1}$)</th>
<th>$r$Å</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amantadine</td>
<td>0.3173</td>
<td>22.03</td>
<td>2.73X10$^{-15}$</td>
<td>25.04</td>
</tr>
</tbody>
</table>
Micellar size

From a structure point of view, the most relevant parameter of a micellar system is the mean micellar aggregation number. To analyze the effect of Egg albumin addition on the mean aggregation number of SPAN 40 micelles, the well established quenching method firstly proposed by\textsuperscript{[12]} on the basis of previous analysis performed by\textsuperscript{[13]} The procedure is based upon the quenching of a luminescent probe by a known concentration of a quencher. The quenching experiments were analysed by using the following equation,

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

(7.11)

where $I_0$ and $I$ are the fluorescence intensities in the absence and presence of the quenchers respectively, $N_{agg}$ is the mean aggregation number, $[S]$ the total surfactant concentration and $[Q]$ is the quencher concentration.

Fig. 12: Plot of $\ln (I0-I)$ Vs. $[Q] \times 10^{-5}$ M of Egg albumin with Amantadine in different concentration of SPAN 40.

Table 4. Aggregation number, $(N_{agg})$, radius $(R_0)$, surface area per head group $(R_0)$, and packing parameter $(\nu/a_{00})$ of SPAN 40 micelle

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Aggregation number</th>
<th>Radius of the micelle $(R_0)$</th>
<th>Area of the micelle $(a_0)$</th>
<th>Critical aggregation parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.02</td>
<td>597.00</td>
<td>42.87</td>
<td>38.67</td>
<td>0.5459</td>
</tr>
<tr>
<td>0.04</td>
<td>1399.44</td>
<td>56.79</td>
<td>28.95</td>
<td>0.7292</td>
</tr>
<tr>
<td>0.06</td>
<td>1859.50</td>
<td>62.38</td>
<td>26.28</td>
<td>0.8033</td>
</tr>
<tr>
<td>0.08</td>
<td>3359.33</td>
<td>75.82</td>
<td>21.49</td>
<td>0.9824</td>
</tr>
<tr>
<td>0.10</td>
<td>3799.39</td>
<td>78.96</td>
<td>20.61</td>
<td>1.024</td>
</tr>
</tbody>
</table>
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. 12 shows the obtained quenching results according to equation (7.11). The mean aggregation numbers of SPAN 40 micelles, are listed in Table 4.

**Molecular docking results**

Recently molecular docking becomes an important tool to investigate the protein – ligand binding study. The docking poses of Egg albumin and Amantadine in Egg albumin are represented in figs 13 (a) and (b) respectively. For discussion we have chosen the first docked conformation in each cash because it posses the minimum energy.

It has been found that Amantadine bind within H-bonding distance (Fig. 13) to the tryptophan residues. (Trp 62 and Trp 63) of Egg albumin. The expected interaction of the ligand with two tryplophan residues, Trp 62 and Trp 63 observed quenching of fluorescence intensity of egg albumin by Amantadine. The distance of Amantadine to Trp 108 to the FRET results it has also been found that Amantadine is present in the close vicinity to egg albumin. To recognize the residues involved in the binding, we have estimated me accessible surface area (ASA) of residency of nature and complexed protein Table (5). High binding energy or total score represents a greater binding affinity of quereetis towards Egg albumin. The negative values of bindings energy or total score indicate the spontaneous binding of the polyphenol towards the protein.

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**Fig 13(a): 2-dimensional view of molecular docking of EA with Amantadine**
Fig 13(b): 3-dimensional view of molecular docking of EA with Amantadine.

Table 5: Docking results

<table>
<thead>
<tr>
<th>FLAVONOID</th>
<th>docking score</th>
<th>glide ligand efficiency</th>
<th>XP GScore</th>
<th>glide evdw</th>
<th>glide ecul</th>
<th>glide energy</th>
<th>XP HBond</th>
<th>XP LipophilicEvdW</th>
<th>Potential Energy-OPLS-2005</th>
<th>Ionization Penalty</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amantadine</td>
<td>-1.614</td>
<td>-0.147</td>
<td>-1.614</td>
<td>-5.558</td>
<td>-4.96</td>
<td>-10.518</td>
<td>-1.401</td>
<td>-0.222</td>
<td>124.196</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

4. CONCLUSION

The interaction of amantadine with Egg albumin in SPAN 40 solution using UV/vis, fluorescence and also by molecular modeling studies has been studied. The absorption spectrum of the EA shows that the amantadine led to the increase in absorbance of EA at 280 nm, which indicates formation of a complex between EA and A and change in protein conformation. The docking results shows that the Amantadine mainly interacts with EA through hydrogen bonding. The experimental results of fluorescence showed that the quenching of EA by amantadine is the results of the formation of (EA-A) complex.
REFERENCES