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Partitioning of fluoxetine into mixed lipid bilayer containing 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (DPPG) and 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC)

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Abstract:

In this study, the partitioning of fluoxetine, an antidepressant of selective serotonin reuptake inhibitor class into a mixture containing anionic and zwitterionic lipid vesicles was evaluated using second derivative spectrophotometry. The partition coefficients (K_p) of fluoxetine into the large unilamellar vesicles (LUVs) composed of zwitterionic 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) containing 0 mol%, 10 mol%, 20 mol%, and 30 mol% of anionic 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (DPPG) were measured in HEPES buffer at pH 7.4. The result revealed that when more negatively charged lipids incorporated into the LUVs, the condensing effect on the binary phospholipid membrane impeded the partitioning of positively charged fluoxetine, resulting in the decrease in the K_p values. This study adds a deeper understanding of how antidepressant fluoxetine exerts its effect on anionic-containing biological membranes, shedding light onto drug delivery systems in the pharmaceutical field.

Keywords: **binary phospholipid membrane, electrostatic interaction, fluoxetine, partition coefficient, second derivative spectrophotometry.**

Classification number: **2.2**

Introduction

Depression is one of the most widespread mental disorders among humanity and up to 15% of the population might experience a series of symptoms ranging from the persistent state of low mood to suicidal behaviors during their lifetime [1]. The discovery of SSRIs (selective serotonin reuptake inhibitors) in the late 1980s marked a milestone in the therapeutic orientation towards depressive disorder [2]. In recent times, SSRIs have emerged as the most prescribed antidepressants [3] since they have better efficacy, tolerability, lower cost and fewer side effects compared to the old generation depression-resistant drugs [4, 5]. Fluoxetine is a well-known antidepressant, which belongs to the SSRI group that serves as a highly active serotonin reuptake blocker in vitro and in vivo by impeding the action of serotonin transporter [2, 6, 7] (Fig. 1). The therapeutic mechanism of fluoxetine is closely associated with TREK-1 ion channel protein, which is highly distributed in the central nervous system and the cell membrane [8-11]. Fluoxetine blocks the activity of the TREK-1 channel by truncating the C-terminal domain, which causes the loss of channel function, resulting in the depression-resistant phenotype [12, 13]. Being a lipophilic compound, fluoxetine must enter the interior of the lipid membrane to perform the inhibition [14]; hence, the study of fluoxetine partitioning into lipid bilayer could provide a better understanding of how such a common antidepressant exerts its therapeutic effect.

Liposomes are artificially prepared vesicles consisting of natural and synthetic phospholipids and are widely used as cell membrane mimicking platforms to study the drug delivery systems [15-17]. Drug partitioning, a powerful indicator to evaluate the physical activity of drugs towards lipid membranes is obtained by liposome/water partition coefficient (Kp) of drugs. In previous studies, the partition

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coefficient of drugs was determined by different methods such as phase separation, hygroscopic desorption and the octanol/water system [18-21]. As the lipid vesicles cause high apparent background signals derived from the light scattering, these techniques aimed to separate drugs and vesicle suspensions into aqueous and lipid phases [22]. However, they were either time-consuming, disturb the equilibrium state of sample solutions, and more importantly were too simplified to study the drug and membrane interactions or might introduce a huge discrepancy between Kp values of different drugs [23, 24]. Later, the second derivative spectrophotometry was employed as a newly developed method to eliminate the background signals from the absorption spectra without the old methods' drawbacks [25-27].

The lipid bilayer, a core component of the cell membrane, is made of two layers of lipid molecules and each molecule has a hydrophilic headgroup and two hydrophobic tails. The properties of highly dominant lipids in the cell membrane have been in the spotlight for a certain time. Despite the fact that charged lipids are seemingly minor but incident to many crucial biologically relevant processes, the understanding of how they function solitarily and collectively with other cell components is still at the tip of the iceberg [28]. Consequently, examining the role of charged lipids especially the negative ones in form of liposomes mimicking the cell membranes has risen as a great biological interest in recent times [28]. Heterogeneities in lipid membranes comprising of negatively charged lipids have recently attracted considerable attention [29] including lipid-protein interactions e.g. the interplay of peripheral proteins with phosphatidylinositol (PI) [30], the interactions of phosphatidylserine (PS) with the Tim4 protein characterized by all-atom molecular dynamics data combined with interfacial X-ray scattering and membrane binding essays [31], and lipid-cholesterol interactions e.g. the behaviors of cholesterol towards the PC/PS asymmetric model bilayers [32]. However, the interplay between mixed protein-free lipid bilayers comprising of a negatively charged lipid and nanosized molecules, like drugs, are considerably few [28]. For the above reasons, this study aimed to examine the partitioning of fluoxetine, a positively charged drug molecule, into a mixture of anionic-zwitterionic lipid bilayers via derivative spectrophotometry under the viewpoint of electrostatic interactions. By incorporating charged lipids into the membrane components, the lipid-water interface region might unveil some interesting features. The partition coefficients of fluoxetine into LUVs composed of pure zwitterionic 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and DSPC containing 10 mol%, 20 mol%, 30 mol% of anionic 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (DPPG) (Fig. 1) were determined using second derivative spectrophotometry. Phosphatidylcholine (PC) is the most abundant constituent of cell membranes which has a zwitterionic headgroup [33, 34]. Though the anionic phosphatidylglycerol (PG) is reported to account for a minority in cells, it is commonly representative of the charged lipids [29]. PG is fairly distributed in the pulmonary surfactant [35] and the thylakoid membrane of the chloroplast [36], it also has a part in ATP production via the cooperative function with the pulmonary surfactant proteins and cardiolipin [37, 38]. This study focused on the partitioning of fluoxetine in the mixtures of DSPC:DPPG bilayers at a molar ratio of 7:3, which is the ideal molar fraction between the zwitterionic and anionic lipid species in the lung surfactant [38]. DSPC and DPPG transition temperatures are 55^0C and 41^0C, respectively; thus at the experimental temperature of 37^0C, they both remain in the solid-gel state.

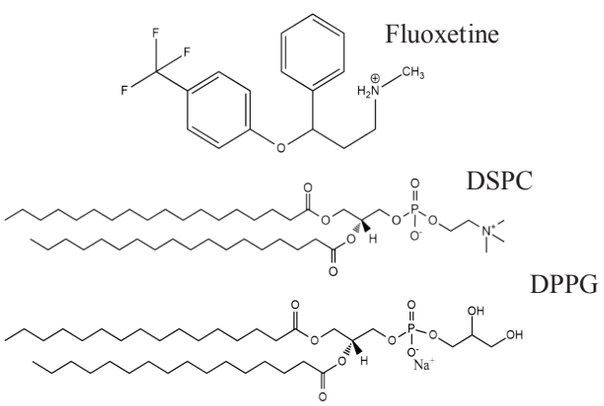

Fig. 1. Molecular structures of fluoxetine, DSPC and DPPG.

With pK_a 10.1, fluoxetine carries a net positive charge at pH 7.4 [27]. Thus, at this physiological pH the presence of the anionic DPPG in the DSPC bilayer would induce the electrotratic interaction between the drugs and the lipid bilayers, affecting the partitioning of fluoxetine into the lipid bilayers. There were few works paid attention to the interaction of fluoxetine with LUVs by differential scanning calorimetry and spin labeling EPR techniques [39-42] but still, the insight of fluoxetine partitioning into LUVs under the electrostatic perspective has not been profusely investigated. Therefore, this study was carried out to add further understanding of how fluoxetine interacts towards heterogeneous anionic membranes.

Experimental

Materials

Fluoxetine hydrochloride was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and used without further purification. The buffer was composed of 50 mM NaCl and 10 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES buffer) and adjusted to pH 7.4. 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol C16:0 (DPPG) and 1,2-distearoyl-*sn*-glycero-3-phosphocholine C18:0 (DSPC) of 99% purity were purchased from Avanti Polar-Lipids Inc. (Alabaster, AL, USA) and used without further purification. Stock DSPC (20 mg/ml) was supplied as a 2% (w/v) chloroform solution and stock DPPG (20 mg/ml) was supplied as a 10% (w/v) chloroform solution containing 5% methanol. Both solutions were stored at -20^{0}C before usage. Nanopure water distilled from the Nanopure system with an impedance of 18.2 MΩ (Ultrapure, USA) was used to prepare all solutes.

Methods

Stock solution of drug preparation: a stock solution of the drug was prepared at 1 mg/ml concentration in 50 mM NaCl - 10 mM HEPES buffer.

Liposome preparation: appropriate amounts of DSPC and DPPG stock solutions were mixed and evaporated with a gentle stream of nitrogen. Further removal of the solvent residue was performed by applying a high vacuum at room temperature for more than 4 hours. Thereafter, the unused dried lipid cakes were stored at -20^{0}C for further use.

The resulting dried lipid cake was dispersed with 50 mM NaCl - 10 mM HEPES buffer to produce large homogeneous multilamellar vesicles (LMVs). The suspension was subsequently vortexed. Five consecutive cycles of 5 min freeze at -20^{0}C and 5 min thaw at 60^{0}C were repeated. Suspensions of LMVs were extruded 30 times through 100 nm pore size polycarbonate filters (Avanti Polar-Lipids Inc., AL, USA) at a temperature which is higher than both lipids' transition temperatures to produce LUVs.

Drug-Liposome environment preparation: the extruded suspensions were diluted to different concentrations for further analysis. Sample solutions were prepared by mixing a known volume of the drug solution (67.5 µM) and a suitable aliquot of the vesicle suspensions in HEPES buffer. The correspondent reference solutions were prepared identically but without the drug. Sample solutions and reference solutions were all prepared in 1-ml Eppendorf and the final volume was 800 µl. All samples were vortexed 5 min and incubated at 37^{0}C for 30 min before being measured to collect the UV-Vis absorption spectra.

UV-Vis absorption spectra collection and second derivative spectrophotometry: each absorption spectrum of the sample solution was measured against the correspondent reference solution by using a microcell cuvette with the chamber volume of 700 µl on the Agilent Cary 60 UV-Vis spectrophotometer (Agilent, USA), with a temperature regulated cell holder set at 37^{0}C. The spectral window was from 190 nm to 300 nm. Thereafter, the second derivatives of absorption spectra were obtained from Origin 9.1.0 software (Origin Lab, WA, USA) based on the Savitzky-Golay method [42], in which the second-order polynomial convolution of 20 points was employed. A wavelength interval (Δλ) of 1 nm was incorporated in the calculation.

Partition coefficient determination: the partition coefficient of drugs between lipid bilayer vesicle suspensions and aqueous solutions is defined as [23].

$$K_p = \frac{fraction\ of\ drug\ in\ lipid\ /[lipid]}{fraction\ of\ drug\ in\ aqueous\ phase/[aqueous\ phase]} \quad (1)$$

where:

[lipid]: lipid molar concentration [43]

[aqueous phase]: water molar concentration (55.3 mol/dm^3 at 37^{0}C)

The fraction of the bound fluoxetine is defined as ΔD/ΔDmax, where ΔD=D-Do is directly proportional to the fluoxetine concentration in the membrane [26] and ΔD is the second derivative intensity difference between absorption in the presence and absence of liposomes.

From equation (1), K_p value could be presented as

$$K_p = \frac{\left(\frac{\Delta D}{\Delta Dmax}\right)[aqueous\ phase]}{\left(\frac{1-\Delta D}{\Delta Dmax}\right)[lipid]} \quad (2)$$

After simple transformations of the equation (2), equation (3) was obtained as follows

$$\Delta D = \frac{Kp\ \Delta Dmax\ [lipid]}{[aqueous\ phase]+Kp\ [lipid]} \quad (3)$$

The value of K_p and ΔD_{max} can be calculated from the experimental values of [lipid] and ΔD by employing a non-linear least-squares fitting to equation (3).

The second derivatives of the absorption spectra were obtained from Origin 9.1.0 software (Origin Lab, WA, USA) and the K_p values were calculated by Sigmaplot 12.0 software (Systat Software Inc., CA, USA).

Results

Absorption spectra of fluoxetine in LUVs

Absorption spectra of fluoxetine at a concentration of 67.5 μM recorded in the presence of different liposome concentrations of pure DSPC and mixed DSPC:DPPG = 9:1, 8:2, and 7:3 are depicted in Fig. 2, respectively. It is requisite to point out that the concentration of fluoxetine used in this study conforms to the Beer-Lambert Law for absorbance. The curves (2-8) in Fig. 2 were obtained by subtraction of the absorption spectrum of liposomes without fluoxetine from the absorption spectrum of liposomes with the drug recorded at the same lipid concentration.

In respect to four different compositions i.e. pure DSPC, DSPC:DPPG = 9:1, 8:2, 7:3 with increased lipid concentrations, absorption maxima (λ_{max}) of fluoxetine decreased and shifted to the longer wavelength (bathochromic shift) as compared to the maximum in the buffer solution (spectrum 1). The bathochromic shift was caused by the decrease of polarity in fluoxetine molecules' surrounding, indicating the incorporation of fluoxetine into the hydrophobic cores of the lipid bilayers. This behavior are observed on other drugs namely phenothiazine (Poła et al. 2004), chlorpromazine and methochlorpromazine [43], trifluoperazine [44], and promazine [26] when they partitioned into lipid membranes.

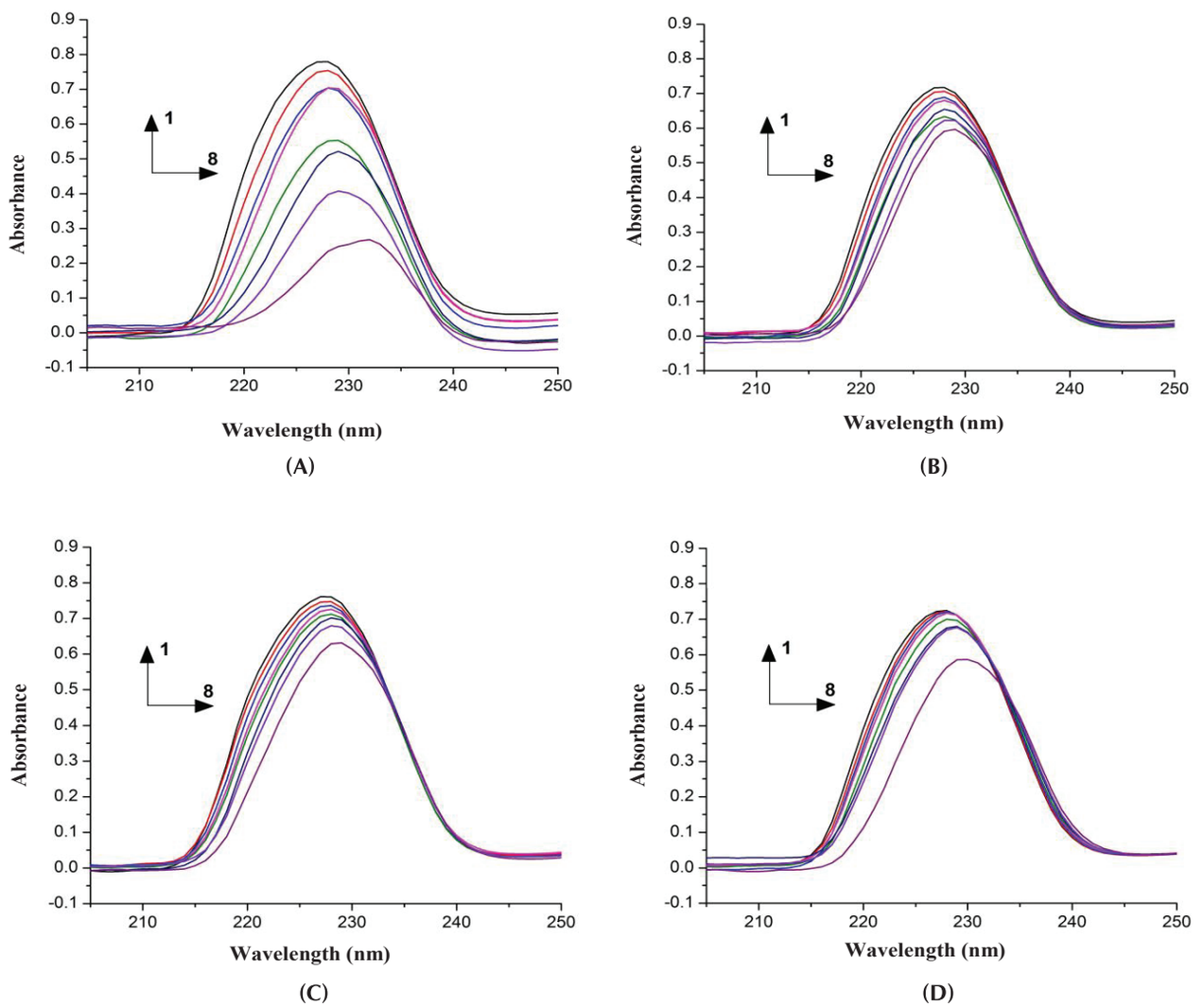

Fig. 2. Absorption spectra of fluoxetine in HEPES buffer (pH 7.4, 37°C) containing various amounts of pure DSPC (A), and mixtures of DSPC: DPPG = 9:1 (B), 8:2 (C), 7:3 (D). LUVs concentrations (mM) (1) 0; (2) 0.025; (3) 0.050; (4) 0.075; (5) 0.10; (6) 0.15; (7) 0.20; (8) 0.30.

Second derivative spectra of absorption

The apparent background signals caused by light scattering in the liposome suspensions could be eliminated by applying the second derivative spectrophotometric method [45, 46]. The second derivatives of the absorption spectra of fluoxetine in the HEPES buffer containing the pure DSPC and the mixtures of DSPC and DPPG LUVs are depicted in Fig. 3.

Despite the fact that the same amount of LUVs was purposely prepared in the sample and reference solutions, no isosbestic points are observed in the absorption spectra figures. It was obvious that strong background signals impeded the complete baseline correction. Therefore, the second derivative spectrophotometric method was then applied to eliminate those background noises, allowing isosbestic points to be obtained, and enabling the partition coefficients to be determined. In previous studies, the partition coefficient of drugs into the DMPG liposomes [23] and phenothiazine into the phosphatidylcholine bilayer vesicles and water [47] were also determined by using second derivative spectrophotometry. With the increasing of lipid concentrations, the second derivative minima increase in intensity and shift toward the longer wavelength in all four conditions (Fig. 3). Two isosbestic points at 218 nm and 229 nm were obtained, proving that the apparent background signals were entirely eliminated [24-26, 48].

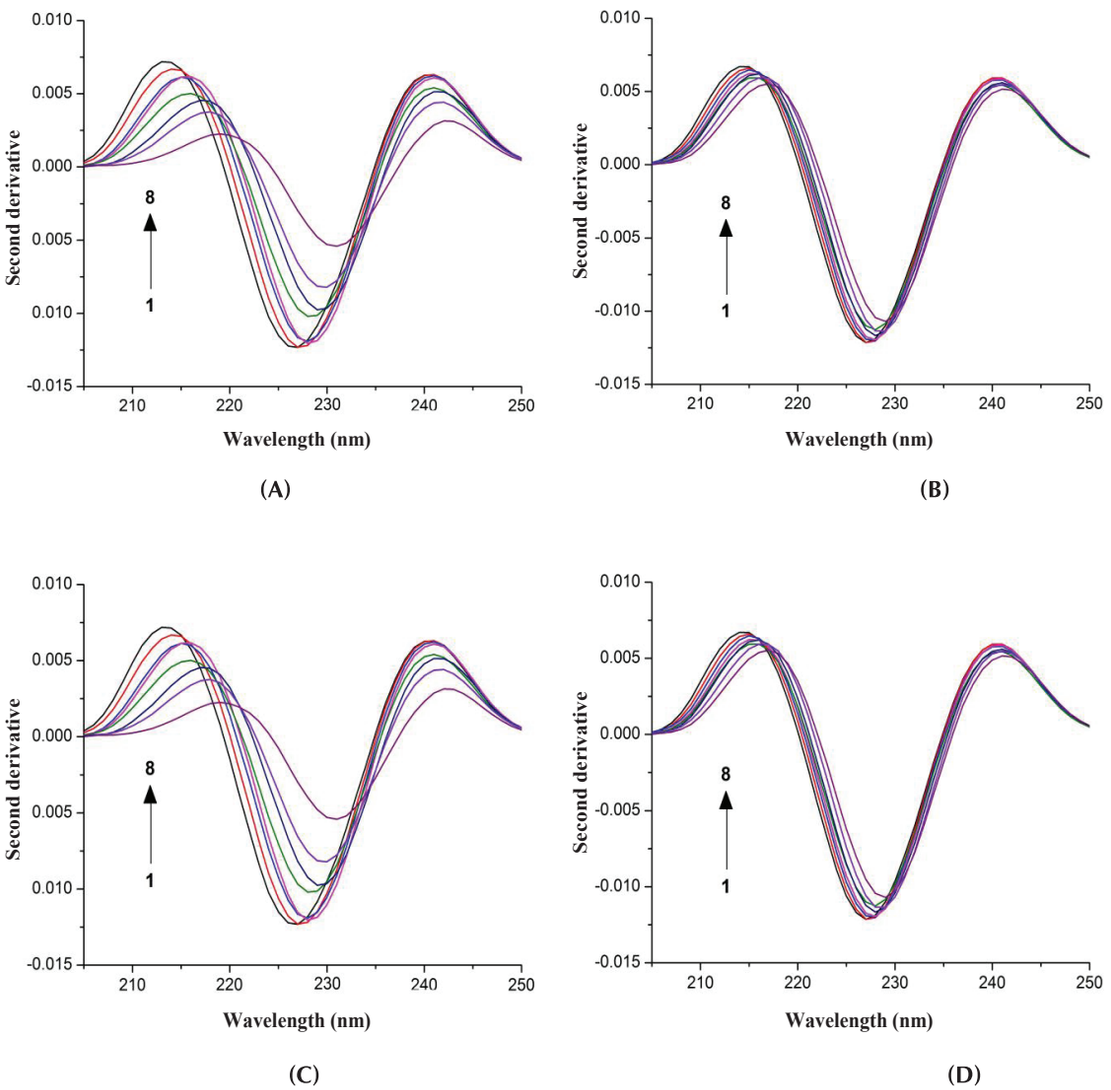

Fig. 3. Second derivative spectra of fluoxetine in HEPES buffer (pH 7.4, 37^{0}C) calculated from the absorption spectra in Fig. 2: pure DSPC (A), and mixtures of DSPC:DPPG = 9:1 (B), 8:2 (C), 7:3 (D).

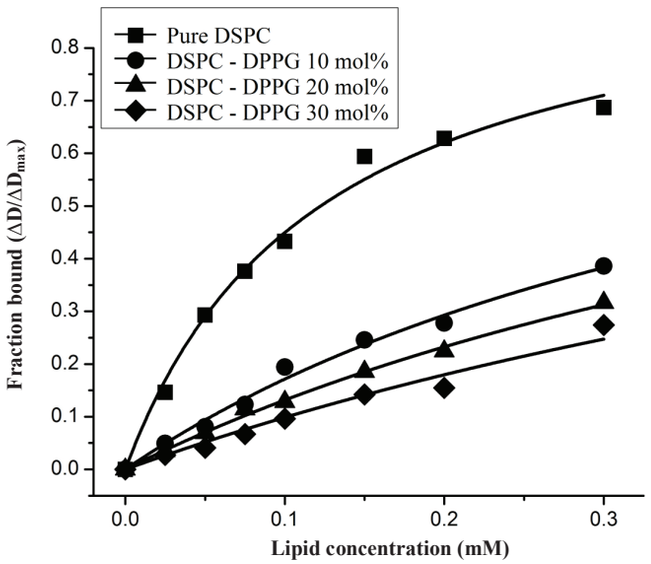

Fig. 4. The fraction bound of fluoxetine in LUVs ($\Delta D/\Delta D_{max}$) as a function of lipid concentration (mM).

The fraction bound of partitioned fluoxetine in the lipid vesicles ($\Delta D/\Delta D_{max}$)

The values of $\Delta D/\Delta D_{max}$, i.e. the fraction of fluoxetine partitioned into the LUVs, were plotted against the concentrations of the LUVs and shown in Fig. 4. The parition coeeficients were obtained by non-linear fitting the ΔD values and the LUVs' concentration to equation (3) and listed in Table 1.

Table 1. The partition coefficient of fluoxetine at a concentration of 67.5 µM into mixed lipid vesicles composed of DSPC and DPPG at pH 7.4, 37°C.

Liposome component(s)	Partition coefficient ($K_p \times 10^{-5}$)*
Pure DSPC	2.04±0.17
DSPC:DPPG = 9:1	1.32±0.26
DSPC:DPPG = 8:2	0.69±0.17
DSPC:DPPG = 7:3	0.53±0.02

*The value shown in the results section was the mean of at least two independent determinations. Kp value was presented as mean ± s.d.

Discussion

As seen in Table 1, the K_p values tendentiously decrease with the increase of the molar fraction of the negatively charged DPPG in the binary membrane of DSPC - DPPG LUVs. This event indicates that fluoxetine had lower affinity to the binary DSPC - DPPG bilayer membrane than the pure DSPC membrane. The figures of K_p themselves do not provide insights into how each lipid species was arranged on the liposome membrane. For this reason, three potential regions which were believed to have great impact on the final K_p values should be taken into account: DSPC - rich regions, DPPG - rich regions, and DSPC - DPPG rich regions. On each lipid region, different driving forces were responsible for the partitioning of fluoxetine into the lipid hydrophobic core.

In the DSPC - rich regions, the zwitterionic PC headgroup of DSPC lipids composed of a positive choline group and a negative phosphate group is being ionized at physiological pH [33]. The electrolytes were found not to have any interactions with the functional groups of the lipids [38]. The sodium and the chloride ions were shown to remain homogenously in the buffer and no headgroup modification was recorded in the presence of salt in the binary lipid system [38]. DSPC itself has strong steric headgroup repulsions between the same charges of two adjacent lipids [49]. It also has two attractive intermolecular forces that help to stabilize the membrane. The first intermolecular force is the hydrogen bonds that are formed between the water molecules and two phosphocholine molecules, and the second one is the weak electrostatic interactions between the positive choline and the negative phosphate groups of the neighboring lipids [50]. The electrolytes that are distributed homogeneously in the aqueous media indeed has no influences on physical properties in general and the packing density of DSPC lipids in specific. Besides that, the electrostatic interaction was further demonstrated not to be the driving force for the partitioning of charged molecular particles. Phan, et al. and Pola, et al. reported that the disordering in the lipid acyl chains, in which the hydrophobic part of fluoxetine interacts with the hydrophobic tails of DSPC lipids, gives rise to the partitioning of fluoxetine into the lipid hydrophobic core of DSPC [39, 44]. The DSPC - rich regions were believed to be the main Kp contributor since the deduction of DSPC molar fractions in the binary lipid system led to the significant decrease in the K_p values.

Regarding the DPPG - rich regions, these regions are anionic since DPPG itself possesses a net negative charged at physiological pH [29]. Despite the fact steric repulsions exist between the neighboring lipids that push them apart from each other, there is a source of attraction in the lateral directions which helps to stabilize the membrane [38]. Dicko, et al. proposed that glycerol hydroxyl is hydrogen-bonding to phosphate or carbonyl groups of the phospholipids [51]. Later research confirmed the experimental suggestion and stated that this specific hydroxyl-phosphate molecular interaction accounts for the event [38]. Do, et al. suggested that at 50 mM NaCl, fluoxetine was found to be located at

the interfacial part of the DPPG - rich regions due to the strong electrostatic attraction between the positive NH_3^+ moiety of fluoxetine and the negative phosphate moiety of the DPPG lipid [41]. This attractive force could lead to the drug accumulation on the interface of the lipid membrane, which interfered with fluoxetine penetrating more deeply into the lipid bilayers' hydrophobic core. This event led to the fact that the natural negative charges of DPPG - rich regions were neutralized by the screening effect upon the addition of positive fluoxetine molecules. Indeed, the steric repulsions between two adjacent lipid headgroups were significantly reduced by the addition of the positive-charge drug agents into the media, thus more densely packed regions on the bilayer surface were expected. Thanks to the neutralizing effect, the phase separation caused by the DPPG lipids in an anionic-zwitterionic mixture was prevented, which further helped to stabilize the membrane structure [29]. In short, the electrostatic interactions were responsible for the partitioning of fluoxetine into DPPG lipid bilayers. As similar to the case of DSPC lipids, the electrolytes were found not to have any great impact on the lipid membrane surface [41]. The DPPG - rich regions by some means or other have a part in the final partition coefficients. However, no matter how much DPPG lipids were added to the binary mixtures, the partitioning of fluoxetine into the LUVs decreased, indicating that the contribution of such regions to the final K_p values was negligible.

In regards to the DSPC - DPPG rich regions, the zwitterionic PC headgroup of DSPC lipids consists of a single dipole including an immobile negative phosphate moiety and a mobile positive amine moiety [49]. The PC headgroup orientation was found to remain essentially unaffected in the anionic-zwitterionic membrane [49, 52]. This indicates that there was no attraction between the positive end of DSPC lipid and the negative end of the adjacent DPPG lipid. Thus, hardly any cluster was formed in terms of a strong electrostatic interaction between these two lipid species. Indeed, as in the case of the DMPC/DMPG mixture, only few and short-lived hydrogen bonds between them were recorded [52]. However, there must have a structure that keeps the lipid system in shape and prevents the phase separation. In this case, the electrolytes are believed to play an essential role in stabilizing and increasing the packing density of the DSPC - DPPG rich regions. Before the drug is added to the vesicle suspensions, in the area where DSPC and DPPG lipids are held next to each other, the sodium cations neutralize the negative moieties of each lipid (Fig. 5). This screening effect could prevent the repulsion between two adjacent negative phosphate groups; therefore, it helps to tighten the membrane structure. Later on, upon the addition of fluoxetine, the positively charged fluoxetine could be repelled by the positive choline moieties of DSPC lipids, which inhibits the partitioning of fluoxetine. Besides that, the sodium cations which occupied considerable spaces on the membrane created the steric hindrances that further impeded the partitioning of fluoxetine into the lipid bilayers. With the decline in K_p values, the data in Table 1 clearly shows that the DSPC - DPPG rich regions were not the main K_p contributors.

On the whole, not only is electrostatic interaction indispensable in a homogenous lipid bilayers but its role is also recognizable in a heterogenous lipid system which has at least a charged lipid agent in the combination. Once the positive fluoxetine makes its appearance, it initially targets the DPPG - rich regions on the liposomes due to the strong attractive forces between the opposite charged species. When all DPPG - rich regions were thoroughly absent, the two remaining areas were DSPC - rich regions and DSPC - DPPG rich regions, which would compete for contacting with the drug molecules. Nevertheless, both the impeditive coordination of the sodium ions association on the vesicles and the repulsions between same charged species evidently demanded more energy for fluoxetine to partition into this area. Therefore, the DSPC - rich regions should interact with the drugs prior to the DSPC - DPPG rich regions.

To sum up, the decline in molar fractions of the main K_p contributor, DSPC, causes the reduction of the final K_p values. The DPPG - rich region shows not to contribute much to the outcome since its increased molar fractions still associates with a lessoned partition of fluoxetine. The DSPC - DPPG rich regions are propably formed more than the DPPG - rich regions. This could occur because the hindering effects are much greater than the contribution of DPPG lipids themselves, which results in a significant reduction of the final K_p values. Electrostatic interaction between the sodium cations and the natural negative moieties of each lipid plays an important role in the binary anionic-zwitterionic lipid system. It helps to prevent phase separation caused by the repulsion between two adjacent negative moieties of each lipid. It also increases the packing density of the lipid bilayers, which hinders the drug partitioning.

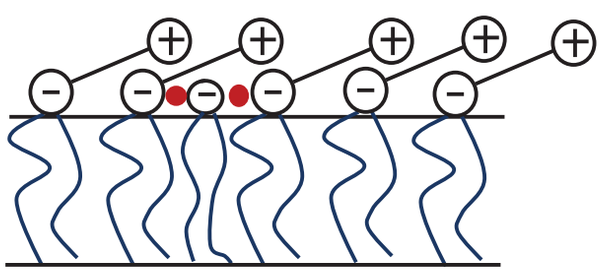

Fig. 5. Schematic illustration of a DSPC - DPPG rich membrane leaflet. The headgroup of DPPG lipid species is presented as a single negative charge, whereas the headgroup of DSPC lipid species consists of a mobile positive choline group and a fixed negative phosphate group. Sodium cations are depicted as red dots.

Conclusions

In this study, the partitioning of fluoxetine into DPPG - DSPC binary lipid bilayers was investigated under the viewpoint of electrostatic interaction by varying the molar fractions of DPPG in the lipid system. It was found that the increase of negative charges on the membrane surface impeded the partitioning of fluoxetine into the anionic DPPG - zwitterionic DSPC LUVs. As the molar fraction of DPPG increased, the partition coefficient decreased. The condensing effect on the membrane under the impact of electrolytes strongly demonstrated that the electrostatic interaction between the oppositely charged ions in the aqueous solution played such an important role in the partitioning of the positive charged drug into binary membranes composed of anionic and zwitterionic lipids. This study also highlighted how seemingly small variations in the lipid system could affect biophysical membrane properties and proved how fundamental membrane measurements were crucial in the interpretation of lipid-drug delivery mechanisms.

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