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A Versatile Method for Determining the Molar Ligand-Membrane Partition Coefficient

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Abstract A novel method for the quantitative assessment of the membrane partitioning of a ligand from the aqueous phase is described, demonstrated here with the thoroughly studied antipsychotic chlorpromazine (CPZ). More specifically, collisional quenching of the fluorescence of a pyrene labeled fluorescent lipid analog 1-palmitoyl-2[10-(pyren-1-yl)]decanoyl-*sn*-glycero-3-phosphocholine (PPDPC) by CPZ was utilized, using 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholine and -serine (POPC and POPS) liposomes as model membranes. The molar partition coefficient is obtained from two series of titrations, one with constant [phospholipid] and increasing [drug] and the other with constant [drug] and varying total [phospholipid], the latter further comprising of large unilamellar vesicles (LUVs) of POPC/POPS/PPDPC at a constant concentration of 10 μM and indicated concentrations of POPC/POPS LUVs. Notably, the approach described is generic and can be employed in screening for the membrane partitioning of compounds, providing that a suitable fluorescence parameter can be incorporated into one population of liposomes utilized as model membranes.

Keywords Chlorpromazine · Phosphatidylserine · Drug-lipid interaction · Partition coefficient · Quenching

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Introduction

Poor absorption, distribution, metabolism, and excretion properties and toxicity (ADME/tox) are by far the most common cause for the failure of new chemical entities in pharmaceutical R&D. Accordingly, the possibility for the use of predictive screens for ADME/tox properties already in the early phases of drug development would be highly desirable. One of the key parameters influencing ADME/tox is the partitioning of a compound between the lipid bilayer membrane and the aqueous phase. Various techniques such as centrifugation, radioactivity assays (i.e. radioimmunoassays), monolayers, parallel artificial membrane permeability assays (PAMPA) [1], and fluorescence spectroscopy have been used for this utility [2, 3]. However, these methods are tedious and require quantities of the compounds studied. Accordingly, it would be of interest to establish simple and robust methods for the determination of the partition coefficients for compound libraries, for instance.

In this study we used chlorpromazine (CPZ), a widely used classical antipsychotic drug as a model. As an amphiphile with an appropriate surface activity profile it readily crosses the blood-brain barrier [4], partitions into cellular membranes and elicits its effects on the central nervous system by influencing various transmitter systems in the human brain [5, 6]. Conventionally specific receptor-mediated interactions have been thought to account for most of the observed actions of CPZ in humans and that binding to dopaminergic receptors is mostly responsible for the beneficial effects of CPZ seen in patients suffering from schizophrenia. It remains uncertain, however, if binding to different receptors and the effects mediated through them are alone enough to describe all the diverse actions of this compound. Along this line of thinking, there are

several examples where no receptor protein has been found. Many examples stand in which compounds such as antimicrobial peptides for example temporins, indolicidin and magainins [7] interact directly with phospholipid membranes modulating their structure. Drugs may also displace peripheral membrane proteins as shown for cytochrome c [8]. Altering of the physical properties of membranes has been demonstrated for cyclosporin A [9] and for general anesthetics [10].

CPZ binds avidly to phospholipid membranes [11, 12] with direct effects on their structure, organization, and dynamics [13]. CPZ has a high affinity to acidic phospholipids, such as phosphatidylserines (PS) and phosphatidylinositols (PI), which are commonly found in the inner leaflet of the plasma membrane [14, 15]. Interestingly, in mouse synaptosomal membranes up to 20% of the total PS is located in the outer leaflet [16]. Considering the affinity of CPZ to negatively charged lipids the availability of PS on the cellular surface could thus provide an additional receptor for this drug, for instance when considering potential interference with lipid-protein interactions of membrane associating proteins by CPZ [8]. Nuclear magnetic resonance and electron spin resonance studies on model membranes have shown that CPZ binds to the head group region as well as intercalates into the membrane between the acyl chains [17, 18]. This is supported also by monolayer studies showing that CPZ increases the surface area/molecule for certain phospholipid species [19].

The molar partition coefficient of CPZ into dipalmitoyphoshpatidylcholine (DPPC) bilayers has been determined as $(8.0 \pm 0.7) \times 10^4$ [20]. The presence of negatively charged lipids enhances the binding of CPZ to membranes. The partition coefficient of CPZ to acidic phospholipids containing membranes has been calculated in a second-derivative spectrophotometry study and reported as $(6.03 \pm 0.17) \times 10^5$ for vesicles containing egg yolk phoshpatidylcholine (PC) and bovine brain PS, 70% and 30% respectively in the presence of 50 mM NaCl [21].

In this study we use CPZ as a model compound to demonstrate a novel method for a fast assessment of the partition coefficient of this drug. The intrinsic fluorescence of CPZ can be used to monitor its membrane binding [20, 22, 23]. In this study we introduce an additional approach, using CPZ as a collisional quencher for the fluorescent phosphatidylcholine analog 1-palmitoyl-2[10-(pyren-1-yl)] decanoyl-*sn*-glycero-3-phosphocholine (PPDPC), yielding direct information of the binding of this drug to membranes consisting of 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphoserine (POPS). Importantly, collisional quenching is not affected by the formation of fluorescent CPZ aggregates such as micelles in the bulk aqueous phase or in the vicinity of the membrane. By comparing the extent of quenching of pyrene fluorescence obtained by different [drug] and [phospholipid] the molar partition coefficient K_p can be calculated. The values for K_p for vesicles with $X_{POPS} = 1.0$ and vesicles with $X_{POPS} = 0.2$ and $X_{POPC} = 0.8$ without added salt were determined to be $(2.2 \pm 0.2) \times 10^7$ and $(5.55 \pm 1.1) \times 10^5$, respectively. For comparison K_p for vesicles with $X_{POPS} = 0.3$ and $X_{POPC} = 0.7$ with 50 mM NaCl was determined to be $(6.6 \pm 0.5) \times 10^5$.

Materials and methods

Chemicals

POPS and POPC were from Avanti Polar lipids (Alabaster, AL) and PPDPC was from K&V Bioware (Espoo, Finland). CPZ, HEPES, EDTA and NaCl were from Sigma. Deionized Milli-Q (Millipore, Bedford, MA) filtered water was used in all experiments. The different lipids were dissolved in chloroform (Merck) and their concentrations were determined gravimetrically using a high precision balance (Cahn Instruments Inc., Cerritos, CA). The concentrations of PPDPC and CPZ were determined by absorption spectroscopy employing 42,000 cm^{-1} at 342 nm and 24,000 cm^{-1} at 254 nm as the molar extinction coefficients for PPDPC and CPZ in ethanol (Altia Oy, Rajamäki, Finland), respectively. The lipids were also analyzed by thin layer chromatography on a silica plate, using chloroform/methanol/water/ammonia (65/20/2/2 by volume) as the solvent. No impurities were detected upon examination after iodine staining.

Preparation of liposomes

Solutions of the lipids used were made in chloroform and were subsequently mixed so as to obtain the desired final molar stoichiometries. The solvent was removed using a gentle stream of nitrogen where after the dry lipid residues were maintained under reduced pressure for at least 2 h in order to remove trace amounts of chloroform. The lipids were hydrated into 20 mM HEPES, 0.1 mM EDTA, pH 7.0, except for determining the K_p in the presence of 50 mM NaCl where the buffer contained 10 mM HEPES, 0.1 mM EDTA and pH was 7.4 to meet the conditions used by Takegami et al. [21]. During hydration the dispersing was aided by a shaking water bath at room temperature so as to yield multilamellar vesicles. Large unilamellar vesicles (LUV) were prepared by extrusion using a LiposoFast (Avestin, Ottawa, Canada) small-volume homogenizer. The lipid dispersions were passed 19 times through Millipore polycarbonate filters with an average pore diameter of 100 nm (Millipore, Bedford, MA), in order to yield LUVs with an average diameter of 80 ± 25 nm [24].

Steady-state fluorescence spectroscopy

The steady-state fluorescence spectroscopy measurements were conducted in quartz cuvettes, using a Varian Cary Eclipse (Varian Inc., Victoria, Australia) spectrofluorometer equipped with a four-position Peltier element thermostated cuvette holder. Excitation through a monochromator was set to 344 nm, corresponding to the absorption maximum for pyrene. Emission was collected in the range of 370 to 500 nm allowing the detection of the monomer peak at 398 nm which was utilized for the calculations. The emission and excitation bandpasses were set at 5 nm. CPZ is itself fluorescent, with an absorption peak at 254 nm and an emission band centered at 450 nm. For the pyrene monomer emission peak at 398 nm the intrinsic emission of CPZ does not interfere.

All measurements were conducted at 25°C, i.e. above the main phase transition temperatures for the lipids used. The molar fraction of the pyrene labeled lipid (X_{PPDPC}) was 0.01. All data were corrected for dilution by increase in volume due to the addition of CPZ or liposomes. The binding kinetics was found to be very rapid and repeated measurements of the samples produced stable fluorescent signals within a few seconds. The data points depicted in the graphs were taken after a 5 min.

The fluorescence data were analyzed using the Cary Eclipse Bio software (provided by the instrument manufacturer) and Origin professional 5.0 (Microcal, Northampton, MA, USA). For calculating the partition coefficient for the drug between the lipid bilayer and the aqueous solution basic functions in Matlab (The MathWorks, Natick, MA) and Microsoft Excel (Microsoft Corporation, Redmond, WA) were used.

Results

Reversible binding of CPZ to liposomes

Our preliminary experiments demonstrated that CPZ quenches pyrene fluorescence. Lack of spectral overlap of the absorption spectrum of CPZ and the emission spectrum of pyrene indicates that this quenching is not due to resonance energy transfer, but collisional, most likely involving the double bonds in the ring structure of CPZ (Fig. 1). Accordingly, binding of CPZ to liposomes can be monitored by observing changes in the fluorescence from the pyrene-labeled phospholipid analog PPDPC (Fig. 2).

For PPDPC liposomes with $X_{PC} = 1.0$ (20 μM total lipid), 40 μM CPZ reduced the fluorescence intensity to 15% of the initial value, with no further decrease at higher [CPZ] (data not shown). In keeping with the high affinity of CPZ to the anionic phosphatidylserine (POPS) [23], significantly enhanced quenching was observed in the presence of this

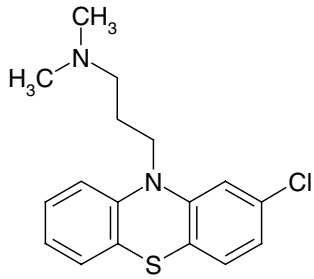

Fig. 1 Molecular structure of the amphiphilic antipsychotic drug chlorpromazine (CPZ)

lipid in the bilayers (Fig. 2). Accordingly, only 4 μM CPZ was needed for similar quenching with liposomes containing $X_{PS} = 0.50$, compared to neat POPC liposomes requiring ten times higher [CPZ]. Saturation was observed already at [CPZ] = 6μM. Upon further increase of X_{PS} up to 0.99 no further enhancement was observed. Cholesterol (X = 0.5) did not alter the characteristics of the quenching with the anionic lipid (X = 0.5) present (data not shown).

For studying the reversibility of the binding and in order to determine the partition coefficient of the drug the dissociation of bound CPZ from different liposome species was studied. First [CPZ] = 4 μM was added to quench pyrene monomer intensity (I_m) from vesicles with $X_{PPDPC} = 0.01$ and $X_{PS} = 0.2$, and 1.0. Subsequently, [phospholipid] was increased by adding unlabeled liposomes to reverse the

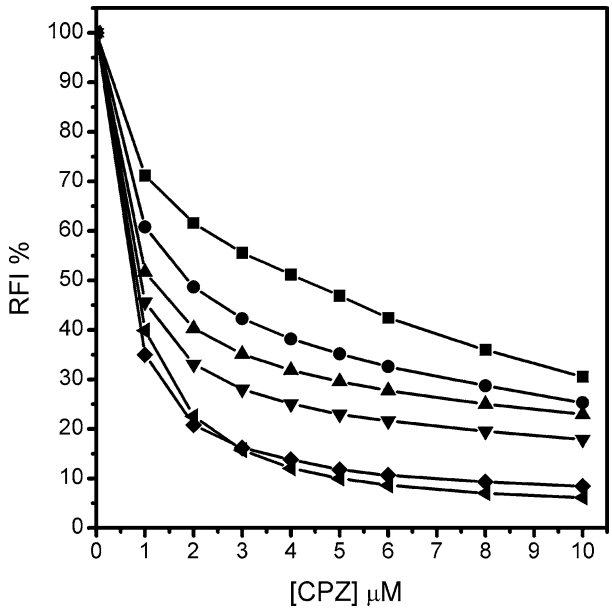

Fig. 2 Quenching by CPZ of I_m from the pyrene-labeled phospholipid analog PPDPC (X = 0.01) in POPC vesicles (20 μM total phospholipid concentration in 20 mM HEPES, 0.1 mM EDTA, pH 7.0) with increasing mole fractions of negatively charged POPS (X_{POPS}): 0.0 (-■-), 0.05 (-●-), 0.10 (-▲-), 0.20 (-▼-), 0.50 (-◆-), and 0.99 (-◀-). Temperature was 25°C

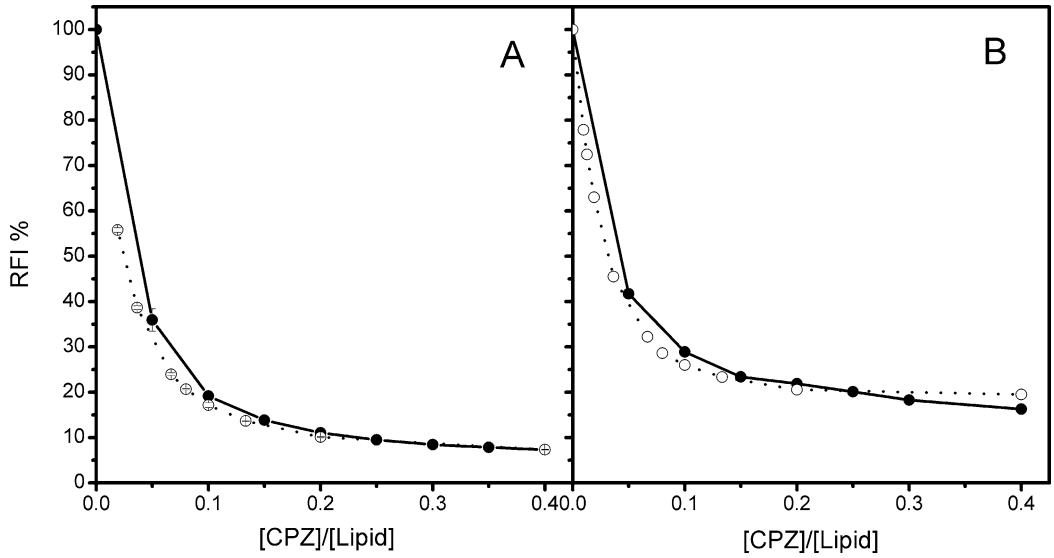

Fig. 3 Panels A and B Comparison of the quenching of PPDPC labeled vesicles by CPZ and subsequent reversal by the addition of liposomes lacking the fluorophore. In the experiment illuminated in panel A CPZ is added to LUVs with POPS/PPDPC (0.99/0.01) at [phospholipid] = 20 μM series A (-●-) and to LUVs with POPC/POPS/PPDPC (0.79/0.20/0.01) in panel B. In series B (-○-) vesicles with the above compositions, [phospholipid] = 10 μM are initially quenched by 4 μM CPZ. The quenching is reversed by adding unlabeled vesicles containing either POPS (panel A) or POPC/POPS (0.8/0.2), (panel B). The data are plotted against the assumed molar proportion of CPZ (X_D) residing in the vesicles ([CPZ]/[total lipid]). Conditions as described in the legend for Fig. 2

quenching. For 10 μM vesicles with $X_{PS} = 0.99$ quenched by 4 μM CPZ yielding $I_m = 10\%$, the addition of 300 μM of liposomes with $X_{PS} = 1.0$ increased the RFI to 80% of the initial value. Assuming that CPZ is evenly distributed between the liposomes, we can depict the values for RFI against the assumed mole fraction of CPZ in the labeled liposomes. The exact matching of the data (Fig. 3A) reveals that the drug is evenly distributed between the labeled and unlabeled vesicles, reflecting their respective concentrations. Similar experiments conducted for vesicles containing $X_{PS} = 0.2$ (Fig. 3B) and $X_{PS} = 0.3$ with 50 mM NaCl (data not shown) again showed that the binding of the drug to PPDPC labeled liposomes could be readily reversed by adding unlabeled liposomes.

Partition coefficient for CPZ between buffer and POPS vesicles

In order to determine the molar partition coefficient for CPZ-liposome interaction quenching data from two series of measurements were collected (Fig. 3). More specifically, in the first series the [phospholipid] was maintained constant and [CPZ] was progressively increased. In the second series [CPZ] was constant and [phospholipid] was increased by adding increasing amounts of unlabeled liposomes. [PPDPC] was held constant throughout the titrations in order to have an indicator of drug distribution as described below in detail. These data are compiled in Table 1.

The molar partition coefficient K_p is defined by

$$K_p = \frac{[D_L]}{[L]} \cdot \frac{[H_2O]}{[D_W]}, \quad (1)$$

where $[D_L]$, $[L]$, $[H_2O]$, and $[D_W]$ stand for the concentrations of drug in the lipid phase, lipid, water, and drug in water

Table 1 Values for the relative fluorescence intensities from the two series of experiments as follows

[CPZ] at [phospholipid] = 10 μM	RFI-1 (series 1)	[POPS] at [CPZ] = 4 μM	RFI-2 (series 2)
0	1		
0.5	0.359	210	0.558
1	0.191	110	0.387
1.5	0.138	60	0.240
2	0.111	50	0.207
2.5	0.0953	40	0.171
3	0.0845	30	0.137
3.5	0.0785	20	0.101
4	0.0733	10	0.0733

Note. In the first series the total phospholipid concentration was maintained constant and [CPZ] was progressively increased, while measuring the emission intensity (RFI-1) after each addition of the drug. In the second series the concentration of CPZ was constant and the phospholipid concentration was increased while recording fluorescence intensity (RFI-2). See results for further details. The RFI values were normalized with respect to the initial fluorescence intensity recorded in the absence of the drug

phase, respectively. Of course, this partition coefficient can only be applied for relatively low drug concentrations when the drug does not significantly change the properties of the bulk phase (usually the lipid phase). Thus, for relatively low membrane drug concentration we have

$$X_D \approx \frac{[D_L]}{[L]}, \text{ and further} \tag{2}$$

$$K_p = X_D \cdot \frac{[H_2O]}{[D_W]}, \tag{3}$$

in which X_D is the mole fraction of drug in the membrane. Using the relationships between the total drug concentration and the drug concentrations in the different phaseswe can easily get

$$X_D = \frac{K_p\,[D_T]}{[H_2O] + K_p\,[L]}, \tag{4}$$

where D_T stands for total drug concentration. Evaluation of the partition coefficient was based on the assumption that in both of the above two experimental conditions at identical mole fractions of drug in the bilayer (X_D) the quenching efficiency should always be identical. Accordingly, each RFI value corresponds to a unique X_D, although the absolute numerical value of X_D is not known. The cubic interpolation function of Matlab (The MathWorks, Natick, MA) was used to retrieve the [CPZ] in the second series that would produce identical RFI values to those of the first series. This yielded a series of paired values (Table 2), in which each unknown X_D corresponds to two different combinations of [CPZ] and [phospholipid].

Next we used the assumption that the data could be described in terms of a single, concentration independent lipid/water partition coefficient. An initial guess for the partition coefficient was made and subsequently the mole fractions for constant [CPZ] ($X_{C,i}$) and constant [phospholipid] ($X_{L,i}$) corresponding to each RFI_i were calculated. Ideally, with the correct K_p, for each value of i the equality $X_{C,i} = X_{L,i}$ should apply. Subsequently, by varying K_p we minimized the following sum:

$$\sum_{i=1}^{5} \left[\frac{(X_{L,i} - X_{C,i})^2}{\frac{1}{2}(X_{L,i} + X_{C,i})} \right] \tag{5}$$

in which $1/2(X_{L,i} - X_{C,i})$ is the average of the two fitted values. The term $(X_{L,i} - X_{C,i})^2$ requires that the values in each pair should be equal, however, without the divider it would give identical weight to differences from pairs like $X_{C,i} = 0.02$, $X_{L,i} = 0.04$ and $X_{C,i} = 0.28$, $X_{L,i} = 0.30$.

Table 2 Paired values obtained from the data sets in Table 1 using the cubic interpolation function of Matlab

RFI-2	[CPZ] (interpolated) at [phospholipid] = 10 μM that produces RFI-2	[phospholipid] at [CPZ] = 4 μM corresponding to RFI-2	Calculated X_{CPZ} in the membrane
0.558	0.254	210	0.020 ± 0.002
0.387	0.462	110	0.036 ± 0.001
0.240	0.79	60	0.063 ± 0.001
0.207	0.926	50	0.075 ± 0.002
0.171	1.14	40	0.092 ± 0.003
0.137	1.52	30	0.122 ± 0.002
0.101	2.29	20	0.180 ± 0.005

Note. Assuming that at identical mole fractions of drug in the bilayer (X_D) the quenching efficiency should also be identical in both series, each RFI value corresponds to a specific, yet unknown numerical value for X_D. The cubic interpolation function of Matlab was utilized to retrieve those drug concentrations in series 2 that would produce identical RFI values to those of series 1. This procedure yields the series of paired values shown, in which each unknown X_D is obtained by two different combinations of [CPZ] and [phospholipid]

On the other hand, using $(X_{L,i} - X_{C,i})^2/[1/2(X_{L,i} + X_{C,i})]^2$ would give identical weights to differences from pairs like $X_{C,i} = 0.02, X_{L,i} = 0.03$ and $X_{C,i} = 0.20, X_{L,i} = 0.30$. The weighing scheme used by us is thus an intermediate between the two extremes, though care should be taken to select the local minimum corresponding to real K_p, and not the global minimum with K_p close to zero. This can be accomplished by first retrieving the approximate value of K_p by the last weighing scheme.

For vesicles with $X_{PS} = 1.0$ the above sum could be minimized by setting the value of the molar partition coefficient to $(2.2 \pm 0.2) \times 10^7$, which corresponds to $(5.1 \pm 0.6) \times 10^5$ on volume/volume basis. Decreasing the PS content to $X_{PS} = 0.2$ while $X_{PC} = 0.8$ the partition coefficient was determined to be $(5.55 \pm 1.1) \times 10^6$. To further evaluate the precision of the method we determined the partition coefficient to vesicles with $X_{PS} = 0.3$ and $X_{PC} = 0.7$ in the presence of 50 mM NaCl. The obtained value $K_p = (6.6 \pm 0.5) \times 10^5$, is in accordance with the value $K_p = (6.03 \pm 0.17) \times 10^5$ presented by Takegami et al. [21].

Discussion

In this study we introduce a facile method for determining the molar partition coefficient of a drug to model membranes. The values determined by second derivative absorption spectroscopy ($6.03 \pm 0.17 \times 10^5$) [21] and by our method ($6.6 \pm 0.5 \times 10^5$) agree well, corroborating the validity of the presented approach. Our approach is based on equilibrium partitioning of the drug between liposomes causing a change in a fluorescent property (in this case emission intensity) of

a dye embedded in liposomes. Fluorescence-based assays of substrates binding to liposomes typically yield relatively good signal to noise ratio. In practice, this can be achieved if the substance is (i) an efficient quencher by whatever means (collisional quenching, resonance energy transfer) or (ii) in ratiometric measurements of some other bilayer property, which is affected by the substance. Our method can be applied in both cases. However, the latter case is ambiguous as such a change in bilayer properties likely affects the partitioning of the drug in question. Our preliminary experiments (data not shown) revealed that many drugs (i.e. amiodarone, clozapine, doxorubicin, haloperidol and promazine) highly efficiently quench pyrene fluorescence and are thus suitable for further studies using the presented method.

Our approach requires two titrations and not only allows calculation of the partition coefficient for simple equilibrium partitioning but also reveals deviations from such simple behavior. In the first series [phospholipid] is held constant and [drug] is varied, whereas in the second series [drug] is maintained constant and the total [phospholipid] is increased. More specifically, the content of POPC/POPS/PPDPC LUVs is maintained constant, while POPC/POPS LUVs are added. Accordingly, after each addition of the latter a new equilibrium is reached, due to dissociation of the bound drug from the vesicles labeled with fluorescent lipid analog. The method is based on the assumption that an equal molar fraction of the drug in the fluorescently labeled bilayers always causes equal signal to be measured, irrespective of the total lipid concentration. Importantly, this requires no assumptions with respect to the bilayer behavior. Likewise, the mode of quenching, possible lateral phase separation in the bilayer, changes in phase behavior due to drug, and unusual quenching kinetics should not influence on the results, as long as their impact is equal for equal X_D in the bilayer. Our method can also be easily automated and can be conducted as dilution series measured by a fluorescence plate reader.

We have used a fluorescence-based approach to determine the amount of CPZ bound to lipid bilayers. Yet, as we made no assumptions about the mode of quenching, we can without prejudice evaluate the quenching efficiency by the drug in the membrane. We first approximate the anisotropic bilayer by an isotropic three-dimensional system. We use the values 0.77 l/mol and 0.26 l/mol for the molar volumes of phospholipid and drug, respectively. The former was evaluated by the assumption that lipid density is approximately equal for POPS and DMPC [25] and the latter from structure optimized by Gaussian 98 (Gaussian, Inc., Carnegie, PA) at the level HF/6-31G. With the known amounts of lipid and lipid-bound CPZ we can now easily calculate the concentration of CPZ in the lipid phase, amount of CPZ / volume of lipid phase. A Stern-Volmer plot of these results (Fig. 4) gives a quenching constant of 40.5 M^{-1}, and with the estimated PPDPC lifetime of 100 ns [26] a quenching rate of 4×10^8 M^{-1}/s is obtained. For diffusion-controlled reactions in water and in three dimensions a value of 10^{10} M^{-1}/s is typically obtained, and the value is lower, if the fluorophore is poorly available to quencher [27]. The smaller value obtained by us could be related to many things, among the most likely is difference in the vertical position of the quenching moiety of CPZ and pyrene in the bilayer.

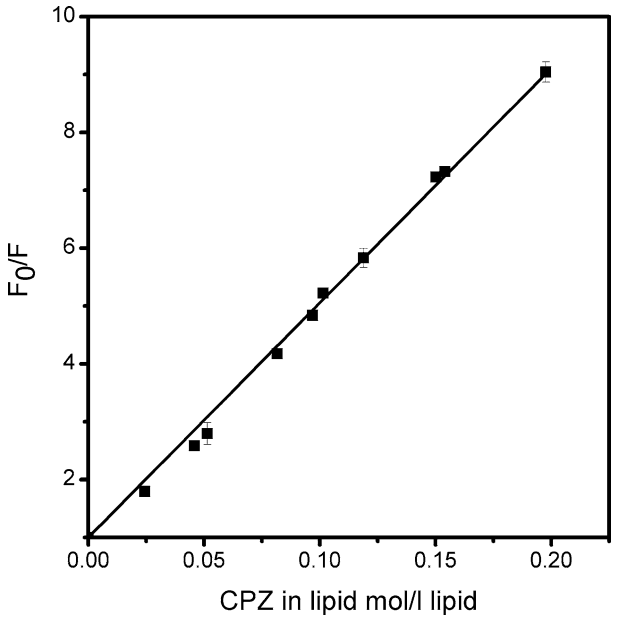

Fig. 4 Stern-Volmer plot of initial intensity F_0/quenched intensity F vs. calculated molar concentration of CPZ in the lipid phase (moles of CPZ/liters of lipid). For details of calculations see text. Notice that the plot includes both the dataset with CPZ titrated at constant lipid concentration and the dataset with lipid titrated at constant CPZ concentration

Obviously, as we use simple partition efficient approach to describe binding, the method described will fail if this is not true. Nevertheless, we may expect that the partition coefficient approach provides good approximation for low concentrations of the drug. Additionally, the method can be extended to more complex binding models, although this requires more data points as there are more parameters to be fitted. A more fundamental issue is that the measured property, e.g. fluorescence intensity, should be strictly increasing or decreasing with increasing X_D, i.e. there should be no local minima or maxima as a function of X_D as this would yield at least two different values for X_D with the same RFI. Finally, if the label affects the partition coefficient giving different coefficients for populations of labeled and unlabeled vesicles, the fits will be poor and the method will yield inaccurate values. Yet, one may argue that this is an advantage, as for typical, traditional fluorescence-based approach we would get a partition coefficient, but would remain unaware of the fact that it applies only for the vesicles labeled containing the label in question.

Using PPDPC as a probe for monitoring drug binding to model membranes offers certain advantages compared to using the fluorescence of the drug for exploring the binding of CPZ to membranes. First, knowing that the fluorescent dye is located in the liposomes gives direct information on the membrane association of the drug, as the fluorescence from drug micelles or aggregates in the aqueous solution does not interfere. Second, liposomes with different compositions can be employed, some of which are labeled and others not, in order to compare the binding affinities to liposomes with different lipid compositions [28]. Finally, this approach should be easily extended to other types of macromolecular interactions [29, 30].

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