Characterisation of chlorpromazine binding to lipid bilayer membranes

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***Abstract*—With the aid of Atomic Force Microscopy (AFM) and Surface Plasmon Resonance (SPR), the interaction of chlorpromazine (CPZ) with phosphatidylcholine bilayer membranes was investigated. The interaction of CPZ had inherent effects on the transition temperature (T_M), and hence, fluidity of DMPC phospholipid membranes. It was discovered that CPZ intercalates within the membrane bilayers to form a drug/phospholipid complex.**

Keywords-phospholipids; microscopy; surface plasmon resonance

I. INTRODUCTION

Previous approaches in drug design have typically focused on interactions of ligand molecules with proteins, such that the lipid environment has been considered to play a more passive role. However, it is now apparent that drug substrates may interact with membrane constituents, particularly via charged head groups [1]. It is recognised that such interactions can influence drug partitioning, orientation and conformation within the membrane. Although there are intrinsic effects on drug substrates, complimentary effects on membrane fluidity, curvature or phase separation may also potentially occur. Ultimately, these alterations can induce changes in the performance of cells, with the ability to affect the function of transmembrane receptor proteins and/or proteins responsible for signal transduction [1]. In view of the current need for further understanding of drug-membrane interactions, the aim of this research is to investigate membrane interactions using SPR and AFM.

In order to promote an understanding of membrane related processes, supported phospholipid bilayers (SPBs) can be used as simple model systems for biological membranes [2, 3]. In this paper we will present the formation and characterisation of SPBs on mica, in addition to lipid membrane vesicles anchored to lipophilic groups covalently attached to Au substrates. The integrity of the bilayer structure is maintained whereby the interaction of CPZ has also been demonstrated.

II. EXPERIMENTAL

Multilamellar vesicles (MLVs) were prepared by first dissolving aliquots of lipid (either 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) or 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC)), in chloroform/methanol (3:1 v:v), followed by evaporation of the solvent under nitrogen. Lipid samples were further dried under vacuum for 3hr prior to being suspended in 10mM Hepes buffer containing 150mM NaCl (pH 5.5). The final concentration of lipids was 1mM. Samples were left to hydrate overnight, followed by sonication for 1hr. During sonication, periodic vortex mixing was carried out prior to their extrusion (Avanti Mini-Extruder, Avanti Polar Lipids, Birmingham, AL) 30 times through a polycarbonate membrane filter of defined pore diameter, typically 100 nm. Extrusion was performed at temperatures higher than the transition temperature (T_M) of the component phospholipids, as gel-state lipids are difficult to extrude at lower temperatures. Resultant small unilamellar vesicles (SUVs) yielded a homogenous size distribution and were used for all further experiments.

The visualization of SPBs were performed using a commercial AFM (Nanoscope IV, Digital Instruments, Santa Barbara, CA). All images were obtained by means of *in situ* tapping mode using triangular Si_3N_4 cantilevers (Digital Instruments) which had a spring constant of 0.15 N/m. Formation of SPBs was achieved by depositing 100µL of 100nm SUV solution (10mM Hepes 150mM NaCl 2mM $CaCl_2$, pH 5.5 or 7.4) to a freshly cleaved mica surface. Prepared surfaces were then incubated at temperatures greater than their gel-fluid phase T_M for 3hrs to promote SPB formation. Prior to imaging, bilayer surfaces were washed 5 times with an appropriate buffer.

SPR studies were performed at 25°C using a Biacore 2000 (Biacore AB, Uppsala Sweden) biosensor equipped with an L1 sensor chip (Biacore AB, Uppsala Sweden). Fig 1. depicts a typical binding cycle of SUVs on an L1 sensor chip. Prior to each experiment sensor chips were preconditioned with 100µL of non-ionic detergent (40mM Octyl β-D-glucopyranoside). Membrane vesicles were subsequently attached to lipophilic groups of the L1 sensor chip at a flow rate of 2µL/min in a running buffer of 10mM Hepes, 150mM NaCl, 1% DMSO, pH 5.5. Resulting phospholipid bilayers were washed with 20mM NaOH prior to drug analysis. Drug injections were performed at a flow rate of 100µL/min and were preformed in triplicate. Regeneration of the L1 sensor chip was accomplished by performing a second injection of non-ionic detergent. To avoid carryover between experiments, a new lipid membrane surface

was regenerated for each drug-binding cycle. All data were double-referenced to account for bulk refractive index changes and systematic effects throughout the course of an experiment.

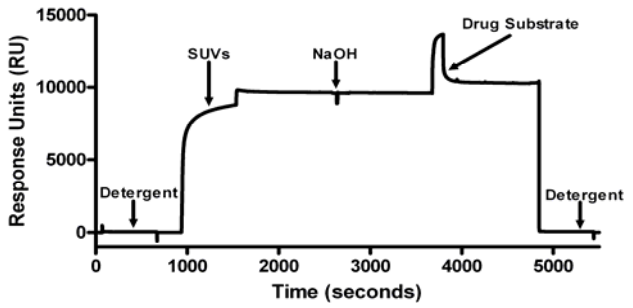

Figure 1. Sensorgram illustrating preconditioning, vesicle capture, NaOH wash, drug binding and regeneration. DOPC vesicles and CPZ (500µM) were utilised for the current sensorgram, representative of vesicle capture and drug binding respectively.

III. Results & Discussion

Upon exposing a SUV suspension to a 'muscovite' mica surface, vesicles adhere to the surface, rupture, and spread to form a planar phospholipid bilayer [2]. As the zwitterionic lipid phosphatidylcholine is the most abundant lipid found in biomembranes, investigations utilised phospholipid vesicles that contained DMPC. As DMPC has a T_M of 23°C, the gel and fluid phases were distinguished by topography AFM images (Fig. 2a) at ambient room temperature. The immiscibility between the gel and fluid phase exhibited a height difference of ~ 0.57nm which is in close agreement with previous studies [4, 5]. Fig. 2b illustrates a typical topographical image of DMPC where no phase separation is observed. Numerous elongated defects were present, due to 'cracks' in the planar bilayer. Cross-section analysis (Fig. 2c, d) of defects located at different locations demonstrated a height difference of ~4.1nm confirming the observed bilayer was in its gel phase [4, 5].

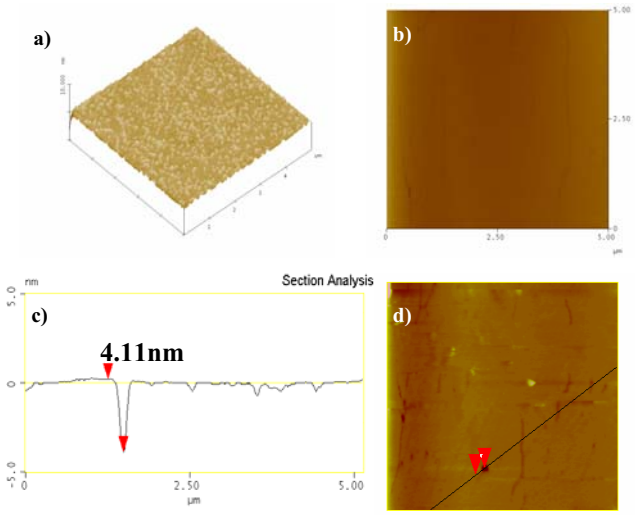

Figure 2. AFM images of DMPC on mica. a) Surface plot image exhibiting phase separation between fluid and gel domains. b) Height image of gel phase bilayer. c) Section analysis of d) membrane defects.

Membrane surfaces of DOPC, DMPC and DSPC were prepared. To each membrane surface the interaction of CPZ was analysed. A concentration range between 15.625 to 1500µM was investigated (Fig. 3b). In a plot of equilibrium drug-binding response (R_{eq}) versus concentration (C), two processes were evident. Based on data for concentrations betweeen 15.625 and 500µM, fitted single-site equilibrium-binding isotherms (1) were extrapolated to 1500µM. When the experimentally determined single-site equilibrium-binding isotherm between 750 and 1500µM was overlaid (Fig. 3b), it was evident a second process was taking place for each phospholipid bilayer.

$$R_{eq} = R_{max}/((K_D/C) + 1) \quad (1)$$

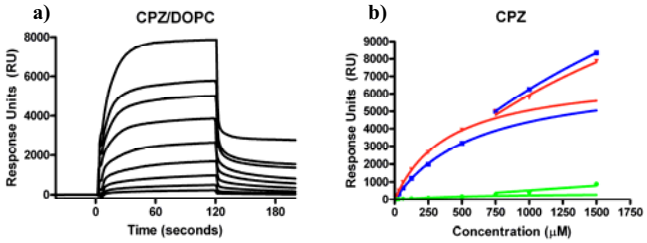

Figure 3. Concentration dependent (a) sensorgrams of CPZ interacting with DOPC membrane surfaces and (b) response plots for CPZ binding to DOPC (▼), DMPC (■) and DSPC (●) membrane surfaces for CAD concentrations between 15.625 and 1500µM. Points are experimentally determined values while the continuous curves from 0-1500µM are from model fitting.

The first process, which was observed for concentrations between 15.625 and 500µM, exhibits saturable binding. This process, also reported by others, presumably arises from electrostatic and hydrophobic interactions where the negatively charged phosphate headgroup interacts with the positively charged amine group of the drug while the lipophilic groups align with the hydrophobic carbon chains of the bilayer [6-8]. Recent studies have also yielded results consistent with the electrostatic interactions being of paramount importance [9, 10]. The second process may be considered a high capacity, low affinity process. It has only been referred to previously in relation to SPR sensorgrams where a heterogeneous interaction was established [11, 12]. Previous studies utilising 1, 2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) phospholipid vesicles and lamellar bodies has also reported two binding affinities for CPZ over the concentration range employed in the current study [13, 14].

A comparison between the alkyl chain lengths and molecular structure for three phospholipid molecules (DOPC, DSPC and DSPC) on drug binding responses was investigated. It was demonstrated that each drug exhibited a much larger interaction to bilayers comprising DOPC. DOPC phospholipid molecules possess a T_M of -20°C and therefore exhibit greater membrane fluidity than bilayer surfaces comprised of either DMPC (T_M of 23°C) or DSPC (T_M of 55°C). As experiments were performed at 25°C, the trend observed suggests binding is a function of membrane fluidity. CPZ exhibited greater binding to DOPC phospholipid vesicles which were in their liquid crystalline state, followed by DMPC surfaces that displayed a

gel-liquid crystalline phase [15] and, lastly, DSPC vesicles that were in their gel phase. Membranes that exhibit greater fluidity provide a more flexible structure for drug binding, and hence the ability of each drug molecule to partition into the bilayer is a more readily achievable process.

With respect to Fig. 3b, the uptake of CPZ to DMPC phospholipid bilayers increased markedly at concentrations greater than 750µM. In particular, the increase in response was greater than those observed for experiments utilising DOPC within the same concentration range. This is related to a lowering in T_M of the phospholipid which is an effect that has been associated with the partitioning of drugs into phospholipid hydrocarbon chains [1, 16-18]. A lowering in the T_M of DMPC would have potentially promoted the formation of a more fluid phase bilayer. As drug injections were performed at 100µl/min for 2 minutes, it is difficult to comment on the magnitude of this effect during this time period. However, as there was a significant increase in response for concentrations between 750 and 1500µM, this may be indicative of an increase in membrane fluidity. An increase in membrane fluidity of DMPC vesicles through the electrostatic and hydrophobic interactions of CPZ may have promoted a more flexible process for drug binding and, as a result, contributed to the observed increase in SPR response, consistent with our results showing greater binding in more fluid membranes. In contrast, effects of CPZ on the T_M of DOPC surfaces are not expected to change the fluidity as DOPC has a T_M of -20°C, and it is therefore evident that these bilayers were in a complete liquid phase at 25°C.

With the aid of AFM, the effect of CPZ on the molecular organisation of DMPC SPBs has been investigated. Reinforcing previous SPR results, CPZ affected membrane fluidity, whereby time-lapse AFM images recorded the disappearance of DMPC gel domains. To confirm the change in morphology of DMPC, SPBs were initially scanned for 45 minutes prior to drug injection. As consecutive scanning of the AFM tip can potentially alter image contrast or create artificial features, it was important to assess the stability of the phospholipid bilayer. As previously noted, DMPC has a T_M of 23°C and it was important to determine any phase changes due to fluctuations in ambient room temperature. As experiments were constantly performed at ~23°C, the immiscibility between gel and fluid phases was observed, this also being consistent with previous reports (Fig 4) [4, 5, 15]. Over the duration of 45 minutes, continuous scanning of a 2µm area did not cause any significant changes on surface morphology.

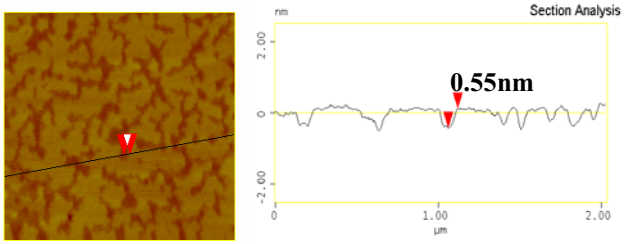

Figure 4. AFM images of DMPC on mica. Section analysis of fluid and gel domains, representative of 0.55nm.

After the injection of 125µM CPZ, time-lapsed AFM images at the same location were recorded. A series of topographic images were recorded at 10, 27, 35, 45, 61, and 70 minutes after CPZ injection (Fig 5). The moment the cantilever tip began to scan the surface; it was evident that the morphology of the bilayer was strongly affected. Over the duration of imaging there was a progressive decrease in the size of gel domains until they had completely disappeared. Interestingly, a defect in the bilayer was also observed throughout the course of imaging.

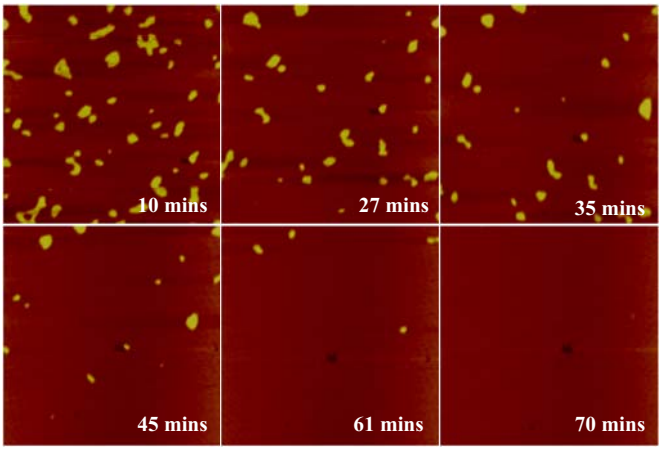

Figure 4. AFM height images (2.0 x 2.0 µm; z-scale: 5nm) of a DMPC bilayer recorded in a solution of 125µM CPZ at increasing incubation times.

Previously referred to as the 'first process' in relation to SPR data, we believe the observed affect was attributable to the electrostatic and hydrophobic interactions of CPZ in the lipid bilayer [19]. As a result, the drug potentially disrupts the interactions between the lipid polar headgroups and interfacial domains, affecting the T_M of the lipid bilayer as the phospholipids became further disordered and possess more motional freedom [19, 20]. In comparison to SPR data, the effect of CPZ on the T_M of DMPC was observed at a much lower concentration, however, it also important to once again establish that the interaction observed using SPR was during a much shorter time period, typically 2 minutes.

A 5µm topographical image was recorded at the end of the time-dependent experiment. It was revealed that other locations on the surface also demonstrated a complete liquid crystalline phase (Fig 5a). An increased number of defects in the bilayer were also observed. Cross-section analysis of these defects (Fig. 5b) revealed they were equivalent to the upper monolayer of a liquid crystalline bilayer (~1.8nm) [4].

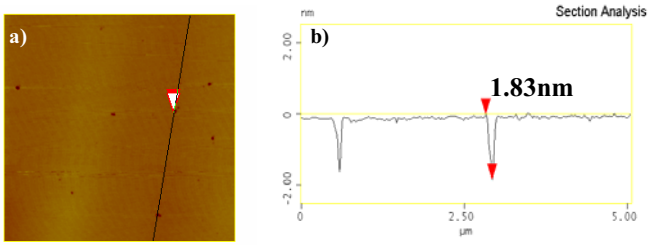

Figure 5. (a) AFM height image (5.0 x 5.0 µm; z-scale: 5nm) of a DMPC bilayer recorded in solution of 125µM CPZ and (b) section analysis of defects.

These bilayer defects are associated with a drug induced disruption. Electron cryo-microscopy studies have also proposed that a drug from the same family as CPZ disrupts the bilayer by perturbation of the local organisation of phospholipids [21]. In the current study, it also postulated that the second process represents intercalation of each drug, forming a drug/phospholipid complex [22, 23] as drugs did not dissociate completely from the membrane as evidenced by the residual response in SPR following exposure to the drug. It has been proposed that formation of drug/phospholipid complexes mimics phospholipidosis [14, 19, 23], a type of lipid storage disorder [24] which could give rise to the observed defects.

SPR and AFM have proven to be effective tools for the analysis of drug-phospholipid bilayer interactions. Extending previous research, it has been shown that CPZ binding involves two discrete processes. An effect of membrane fluidity on drug binding was also demonstrated, such that phospholipid bilayers that exhibit a complete liquid phase bilayer permit greater drug partitioning, and hence greater drug uptake. The effect of CPZ on the T_M of component phospholipids was also observed, indicative of the complementary effects associated with membrane partitioning of drugs. These experiments show that the methods used in the current study are potentially useful for the evaluation of drug-membrane binding interactions of new drug candidates and for providing insights into the processes that contribute to drug-membrane binding.

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