

β -Blockers: their self-assembly and interaction with model membranes

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List of Acronyms

ACMW	Air contrast match water	69, 167, 212
An	Analyser	72, 165
BAM	Brewster angle microscopy	46, 163, 211
C	Compensator	72
CAC	Critical aggregation concentration	86, 113
CMC	Critical micelle concentration	28, 134, 189
CoEK	Co-operative equal K model	100
CPP	Critical packing parameter	30
DM	Dimyristoyl-	46
DOPC	1,2-dioleoyl-sn-glycero-3-phosphocholine	168
DP	Dipalmitoyl-	46
DPR	R-(+)-propranolol hydrochloride	59, 137
DS	Disteroyl-	46
DSC	Differential scattering calorimetry	54
EK	Dimerization/equal K model	100
H-P	Hayter-Penfold	94, 137
LA _s	Local anaesthetics	39
LC	Liquid condensed phase	52, 214
LE	Liquid expanded phase	52
LPR	S-(-)-propranolol hydrochloride	60, 138
MD	Molecular dynamics simulation	45, 95, 135, 162
N_{agg}	Aggregation number	106, 142
NPT	Number of particles, pressure and temperature	60
NR	Neutron reflectometry	45, 157, 191, 205
NSAIDs	Non-steroidal anti-inflammatory drugs	37
NVT	Number of particles, volume and temperature	60

PBC	Periodic boundary conditions	60
PC	Phosphatidylcholine	46
PE	Phosphatidylethanolamine	46
PI	Phosphatidylinositol	46
Po	Polariser	72, 165
PO	1-palmitoyl-2-oleoyl-	46
POPC	1-palmitoyl-2-oleoyl-glycero-3-phosphocholine	163, 208
POPG	1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt)	163
propHCl	Propranolol hydrochloride	157
PS	Phosphatidylserine	46
<i>R</i>	Neutron reflectivity	72, 211
SANS	Small angle neutron scattering	57, 86, 135
SAXS	Small angle X-ray scattering	54
SDS	Sodium dodecyl sulfate	65, 91
SLD	Scattering length density	43, 56, 93, 137, 167, 211
SNR	Specular neutron reflectometry	66
SM	Sphingomyelin	46
SP	Solvent penetration	69
SPC	Simple point charge	59
SPC/E	SPC with average polarization	59
TIP3P	Transferable Intramolecular Potential with 3 Points	59
TOF	Time-of-flight	56
<i>Q</i>	Scattering vector	57, 93, 211
QWP	Quarter wave plate	72

Abstract

Many drug molecules contain hydrophobic and hydrophilic moieties in their structure, and are therefore amphiphilic. They can self-associate to form small, micelle-like aggregates when their concentrations in aqueous solution exceed their respective critical micelle concentrations. As a result, an amphiphilic drug may exhibit different therapeutic effects, depending upon whether the drug is in the aggregated form or not. In addition, the interaction of an amphiphilic drug with lipid membrane plays an important role in its therapeutic behaviour, due to the different interactions with phospholipids. The current work focuses on the amphiphilic drug, propranolol hydrochloride, its self-assembly in aqueous solutions and its interaction with model biomembranes, composed of 16:0-18:1 phosphocholine, in the absence and presence of 16:0-18:1 phosphoglycerol and cholesterol, respectively.

Single/multi-contrast small angle neutron scattering (SANS), ^1H NMR spectroscopy (NMR) and UV/vis absorption spectroscopy, with complementary all atom molecular dynamics (MD) simulations have been used to resolve the self-association of propranolol hydrochloride. While Langmuir trough measurements in combination with optical reflection techniques, namely ellipsometry, Brewster angle microscopy (BAM) and neutron reflectometry have been applied to shed light on the drug-membrane interaction.

The results from SANS, NMR and MD simulations suggest that the self-assembly of (racemic) propranolol hydrochloride is concentration-dependent, with pre-micellar aggregation being observed at concentrations well below the CMC. The S-(-) enantiomer, studied using SANS, was found to form slightly larger aggregates than the racemic mixture. Within the temperature range studied using single-contrast SANS (293 – 415 K), an increase of temperature was observed to oppose to self-association of the drug. The aggregates of propranolol hydrochloride were found to contain a dry core of aromatic rings and a solvated shell comprising of side chains with the CH- π interaction between the naphthalene rings playing an important role in the self-association of the drug in water. In contrast, the addition of electrolyte promotes the self-association of the propranolol hydrochloride and alters the nature of the interaction occurring between the aromatic rings from CH- π to π - π interaction.

A biomembrane interaction study demonstrated that the interaction of propranolol hydrochloride with model biomembranes is a complex process, causing immediate lipid loss and lipid phase

separation, with the results indicating that, at equilibrium, more drug molecules than lipids molecules are present at the interface. The precise number and conformation of molecules at the interface was found to largely depend on the physical state of the membrane, *i.e.* starting surface pressure and lipid composition, as well as the drug concentration in the subphase. Ellipsometry and BAM experiments showed that the addition of POPG (30 mol%) or cholesterol (30 mol% or 46 mol%) to POPC membranes, affected the drug penetration at the interface by altering the heterogeneity of the membrane. Low Q neutron reflection measurements were important in revealing an immediate loss of lipid from the interface upon exposure to drug. The presence of a physiological concentration of sodium chloride strengthened the interaction between the drug and the membrane, with propranolol hydrochloride penetrating the lipid chain layer over time.

Taken together, these findings unravel the molecular processes propranolol hydrochloride undergoes when delivered *in vivo*, and the insights will help improve the delivery of the drug for its clinical use.

Key words: amphiphile, propranolol hydrochloride, self-association, core-shell structure, drug-membrane interaction, air-water interface, multi-contrast neutron scattering/reflectometry techniques

Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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Publications

1. Yan, Y., Shen, Y., Hind, J., Tellam, J., Edkins, K., Campbell, R.A., Barlow, D.J., Leach, A.G., Lawrence, M.J., Self-assembly of propranolol hydrochloride in water: understanding mesostructures through experiment and simulation at different length scales. (*To submit*)
2. Yan, Y., Heenan, R., Campbell, R.A., Barlow, D.J., Leach, A.G., Lawrence, M.J., Self-assembly of propranolol hydrochloride: a small angle neutron scattering study. (*To submit*)
3. Yan, Y., Hind, J., Tellam, J., Campbell, R.A., Lawrence, M.J., Molecular view of the interaction between the amphiphilic drug propranolol hydrochloride and model lipid membranes. (*To submit*)
4. Yan, Y., Winnall, S., Hind, J., Tellam, J., Campbell, R.A., Lawrence, M.J., A mechanistic study of the interaction of model lipid membranes containing cholesterol with the amphiphilic drug propranolol hydrochloride at the air/water interface. (*Completed drafting*)
5. Barlow, D.J., Yan, Y., Ishkhanyan, H., Allen, D.T., Saaka, Y., Leach, A., Lorenz, C.D., Lawrence, M.J., Equivalent ellipsoid modelling of the atomic structures of micelles allows for more detailed comparison of the findings from molecular dynamics and small angle scattering experiments. *Nanoscale*. (*Under review*)

1 Introduction

1.1 Amphiphilic drug

1.1.1 Conventional surfactants

Surfactants (or surface-active agents) are molecules that are composed of two parts, one hydrophobic (term tails) and hydrophilic (called head-groups/heads). There are several classes of surfactant which are identified by the nature of their head-group: *i.e.* non-ionic surfactants and ionic surfactants, the latter of which is often further sub-divided into three groups, namely anionic, cationic and zwitterionic surfactants (Figure 1.1).

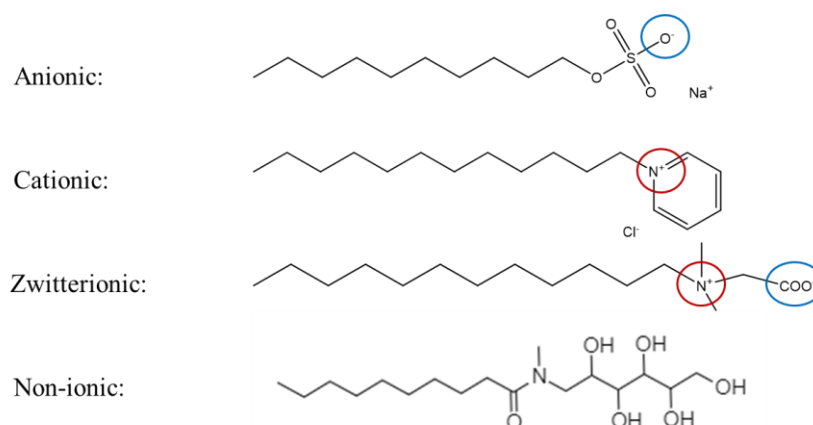


Figure 1.1 Examples of different types of surfactants

Surfactants exhibit surface activity in that they adsorb at interfaces including the air-water surface and self-associate in the bulk aqueous solution when their concentration exceeds the critical micelle concentration (CMC). This surface behaviour is a consequence of the amphiphilic nature of the surfactants. For example, the surfactant tail is excluded from the aqueous solution due to the hydrophobic effect, which results in the tail being orientated in the air, causing a decrease of surface tension. The extent of surface activity of a surfactant is determined by its structure, the temperature and the presence of any electrolyte. The surface activity increases as the length of hydrophobic tail increases. ^[1]

At an interface (surface) there is an equilibrium created in the system between the amount of surfactant molecules at the interface and in the aqueous solution. As the concentration of surfactant increases, the equilibrium tends to increase the amount of surfactant adsorbed at surface, and when the surface is almost saturated with surfactant molecules, the surfactant molecules start to self-assemble, forming various types of aggregates in the bulk, most commonly micelles, cylindrical aggregates or bilayers^[2]. The concentration at which the surface is (almost) full of surfactant/the surfactant molecules start to aggregate is called the CMC. The surface excess is defined as the amount of surfactant molecules adsorbed at the surface per unit area, in the unit of mol/m². It can be calculated from the Gibbs' equation:

$$d\gamma = -\sum \Gamma_i d\mu_i \quad (1.1)$$

where γ is the surface tension of solvent, Γ_i is the surface excess of the corresponding i component, μ_i is the chemical potential of the component. For solution comprising of a single surfactant, the Gibbs' equation can be expressed as:

$$\Gamma = -\frac{1}{2.303RT} \left(\frac{d\gamma}{d \log c} \right) \quad (1.2)$$

where c is the concentration of the surfactant.

In a surface tension measurement, as the concentration of surfactant approaches the CMC, the surface excess reaches a constant value such that the slope $\frac{d\gamma}{d \log c}$ is constant, and the surface is effectively 'saturated' by surfactant molecules. As Figure 1.2 shows there is a diffusion layer located between the surface layer and the bulk in which the concentration of surfactant is slightly less than that in the bulk. Just below the CMC (the linear part of the surface tension curve, Figure 1.3), although the surface excess remains constant with the increase of bulk concentration, once the surface is saturated with surfactant, the concentration in the diffusion layer slightly increases due to an increased surfactant concentration in the bulk, thereby decreasing the surface tension.^[1]

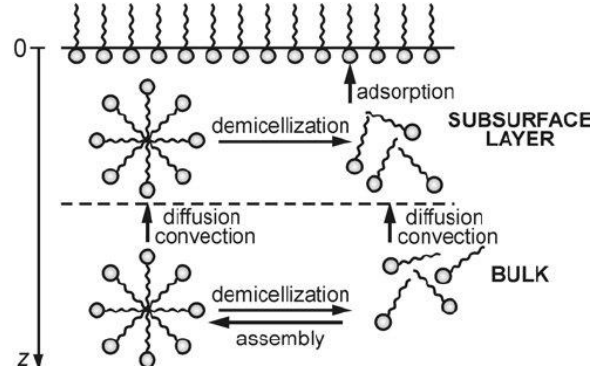


Figure 1.2 Process of surfactant adsorption in aqueous solution, where a sub-surface layer exists between surface and bulk. Adapted from reference [3].

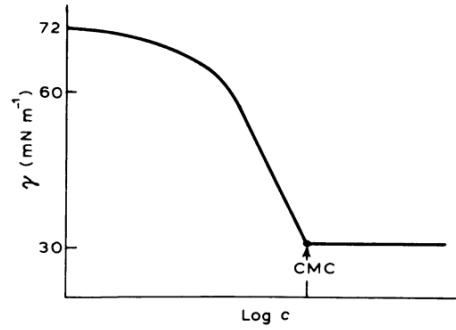


Figure 1.3 Schematic plot of surface tension (γ) against the logarithm of concentration (c). Figure adapted from reference [1].

The formation of micelles in aqueous solution is affected by several factors including the structure of the surfactant, the presence of additional electrolyte, the nature of any counterions, the temperature and pressure. For example, the CMC decreases as the length of hydrocarbon chain increases^[1]. Moreover, the molecular structure of the surfactant determines the shape of the aggregates formed. According to the critical packing parameter (CPP)^[4]:

$$CPP = \frac{V}{a_0 l_c} \quad (1.3)$$

where V is the effective volume of the hydrocarbon chain, a_0 the optimum surface area of the head-group and l_c the critical chain length, which is equal or slightly less than the fully extended alkyl chain length.^[5] When the $CPP < 1$, the overall shape of the surfactant is shown as a (normal) cone, with the precise nature of the aggregates being formed depending upon the value of the CPP. When the $CPP < \frac{1}{3}$ spherical micelles are formed, while cylindrical micelles are seen when $\frac{1}{3} < CPP < \frac{1}{2}$, while vesicles or flexible bilayers and lamellar phases are formed when $\frac{1}{2} < CPP < 1$, and

$CPP \approx 1$, respectively. When $P > 1$, the surfactant forms inverted micelles (Figure 1.4).^[6] The CPP is therefore a useful parameter to predict the preferred shape of the aggregates formed by a particular surfactant in aqueous solution.

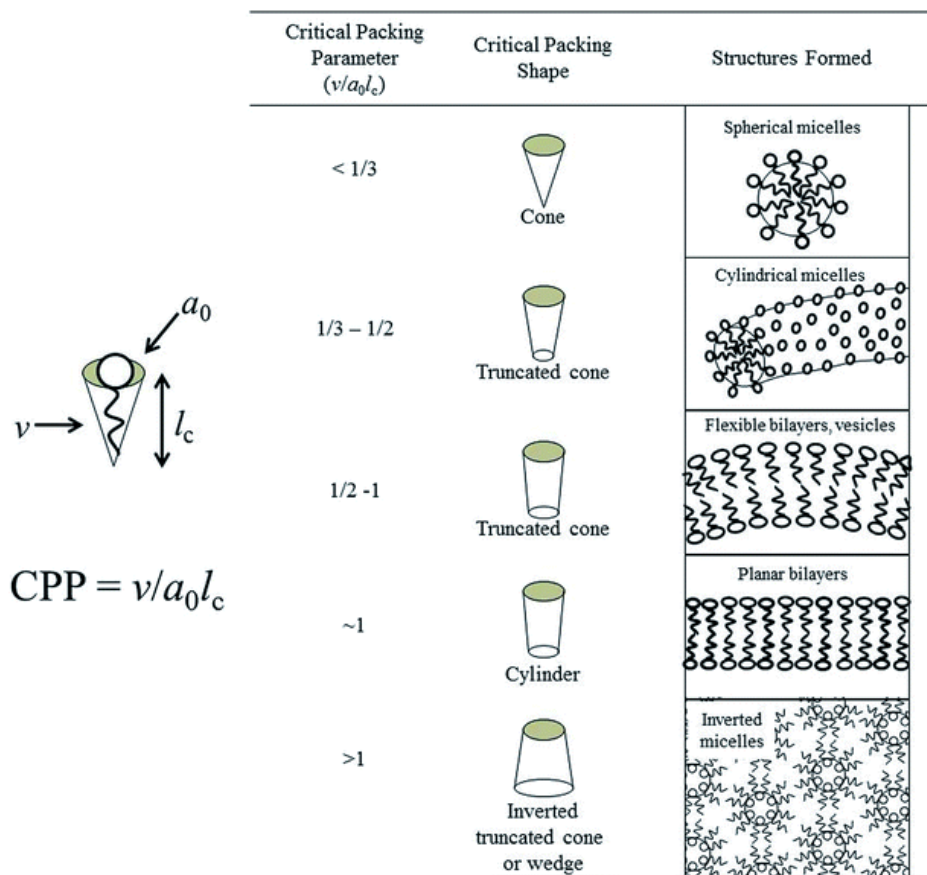


Figure 1.4 Packing shapes and CPP of surfactants in aqueous solution. Adapted from reference [6].

1.1.2 Surface active drug – amphiphilic drug

Drugs with a structure that contains both distinct hydrophobic and hydrophilic groups are likely to be surface-active. There are plenty of commercial drugs that possess an amphiphilic structure including phenothiazine tranquillisers, *e.g.* promazine hydrochloride, analgesics *e.g.* codeine phosphate and antibiotics *e.g.* penicillin G^[1], just like traditional surfactants such as sodium dodecyl sulfate, and consequently show similar properties in that they can adsorb at the air/water interface and self-assemble at concentrations above their CMC. However, the micelles formed by drugs tend to be smaller when compared to those obtained with traditional surfactants, largely because of their more complex molecular structure, particularly of their hydrophobic region. Although not a drug, bile

salts/acids^[7, 8] have a similar steroidal structure to many drugs. Like traditional surfactants, the 3-dimensional structure of a bile salt can be divided into two parts – one part hydrophobic and the other hydrophilic (Figure 1.5). However, unlike traditional surfactants, the hydrophobic and hydrophilic regions in bile salts are on either side of the steroidal ring structure – the concave side of the steroidal ring on which the hydroxyl groups are expressed is the hydrophilic region, while the other side, the convex side, is the hydrophobic region.

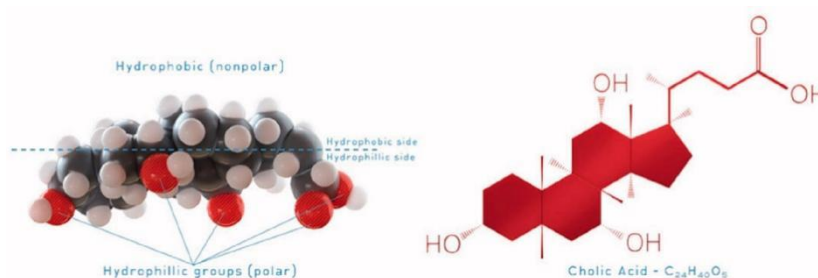


Figure 1.5 Structure of bile acid – cholic acid and its 3-dimensional conformer. Adapted from reference [9].

Furthermore, in some special cases, (very small) ‘micellar-like’ structures can be formed at concentrations below the CMC in a process known as pre-micellization^[10]. Studies performed by Attwood^[11] show that some drugs, such as promethazine exhibit a first ‘CMC’ (c_1) after which the stepwise pre-micellization of drug occurs. A second CMC (c_2) occurs at higher concentrations indicating the restructure of the aggregates, *e.g.* above the second CMC, the aggregate of promethazine is reported to undergo a sphere-to-rod shape transition. Dicloxacillin and flucloxacillin have also been reported to exhibit a second critical concentration as evidenced by NMR and light scattering experiments. It has been suggested that the c_2 , which is seen as an inflection point in the light scattering curve at high concentrations, may relate to a structural rearrangement of the aggregate or an increase in the size of the aggregates.^[12]

Whether a drug can self-aggregate or not largely depends on its structure. For example, hydrotropes, known for their ability to increase the aqueous solubility of poorly soluble solutes, have an amphiphilic structure but generally do not form micelles due to their very small hydrophobic region.^[13, 14] Aspirin (Figure 1.6), a commonly used non-steroidal anti-inflammatory drug, is hydrotropic in that it has a ‘small’ hydrophobic region, too small to form aggregates in aqueous solution. A relatively recent study, investigating the differences in the self-association of a series of sodium p-n-alkylbenzoates with different alkyl chain lengths (Figure 1.7), has argued that there is no

clear distinction between a hydrotrope and a surfactant, as upon increasing hydrophobic chain length, the hydrotropic molecules start to behave more like classical surfactants exhibiting a clear detectable CMC and definite micelles formed in solution.^[14]

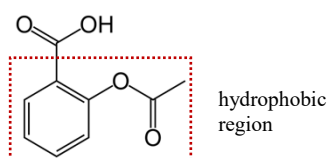


Figure 1.6 Structure of aspirin, hydrophobic part highlighted.

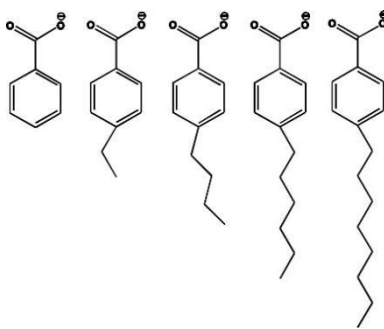


Figure 1.7 Structures of sodium p-n-alkylbenzoates with different n ($-CH_2-$). Adapted from reference [14].

The self-association of drugs depends largely on the temperature, pH, spatial factors, ionic strength and concentration of drug.^[10] Attwood and colleagues^[15, 16] studied the effect of temperature and the existence of electrolyte on the association of antidepressant drugs and of phenothiazine, respectively. These temperature studies indicated that the effect of temperature on the self-association of drugs can vary depending on whether an increase in temperature has a negative or a positive effect on the aggregation. As for the influence of electrolytes, there is often an increase observed in the size of micelles formed by charged drugs upon increasing the concentration of electrolyte.^[17] Other studies determining the effect of pH on the micellization of drugs show that the micellar size is likely to increase when the pH of a cationic amphiphilic drug solution increases. In contrast, any change in the pH of the solution, makes little difference to the micellization of non-ionic (uncharged) drugs.^[18]

The aggregation/self-association process is likely to involve non-covalent interactions including hydrogen bonding, London dispersion forces and hydrophobic effects. A thermodynamic study of the

association of a group of amphiphilic penicillin drugs has shown that the formation of aggregates can be attributed to enthalpy changes in the system.^[19] Furthermore, a study of entropy and enthalpy confirmed the spontaneity of the aggregation process.^[2]

1.1.3 Choice of optimum amphiphilic drugs to study

Amphiphilic drugs are a large group of molecules containing molecules commonly used for a variety of treatments. In the present study four categories of drugs were considered for investigation, namely tricyclic antidepressants, non-steroidal anti-inflammatory drugs, local anaesthetics and β -blockers. All four groups contain molecules that have been proven by earlier studies to possess surface activity and can self-assemble in bulk aqueous solution. Within each group, examples of drugs have been selected to represent the category to try and understand the structure-function relationship of their aggregation behaviour. To decide which drug/group of drugs to examine in the current study, several aspects of each of the four groups were compared, namely their structure, the availability (or otherwise) of deuterated forms for neutron experiments and their costs.

a. Tricyclic antidepressants

Tricyclic antidepressants (TCAs) are routinely used to treat depression, disorders of mood, anxiety as well as chronic pain. However, as TCAs possess cardiovascular and neurological toxicity, TCAs need to be very carefully used to avoid overdosing and, therefore, poisoning. Since most TCAs possess a long half-life, once a day administration of a dose of a TCA is likely to be suitable for most patients. As the major effect of antidepressants is to block the re-uptake of the transporters, serotonin and norepinephrine, which are located at presynaptic nerve endings, leading to an increase in the concentration of neurotransmitters in the synaptic cleft. As serotonin and norepinephrine have been proved to connected closely with anxiety and depression, activation of these neurotransmitters may play a positive role in mental disorders^[20-22].

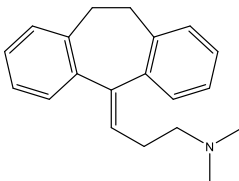
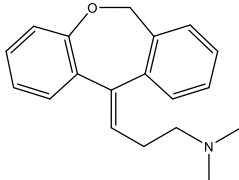
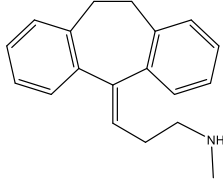
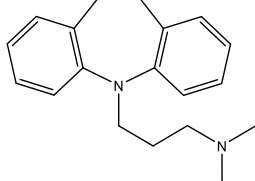
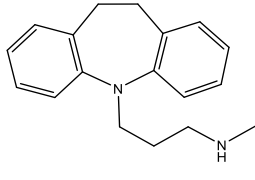
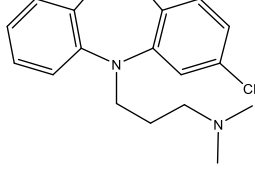
TCAs possess three rings within their structure which act as the hydrophobic moiety along with an acyl chain possessing a terminal dimethylamine group that can be protonated, which is the hydrophilic head region (Table 1.1). There are two major sub-groups of TCAs which are distinguished by the structure of their rings, one sub-group contains dibenzo-cycloheptadiene which is present in

drugs such as amitriptyline, cyclobenzaprine, doxepin and nortriptyline while the other sub-group consists of dibenzazepines, and drugs such as imipramine, clomipramine and desipramine. Both groups exhibit amphiphilic properties which means that they can self-assemble in solution as supported by several previous studies. For example, amitriptyline possesses a CMC of 3.6×10^{-2} M and forms aggregates containing approximately 7 monomers. Indeed, from the studies performed thus far, the aggregation numbers of TCAs are generally in the range 7-8, except for nortriptyline and clomipramine, which form aggregates of 4 and 6 monomers, respectively^[1, 23-28]. A study carried by Taboada *et al.* studied the self-association of TCAs from both groups of molecules, namely amitriptyline, doxepin, nortriptyline and desipramine in the presence of different concentration of salt. The CMC of all systems examined were determined using static light scattering, illustrating that for heterocyclic TCAs such as doxepin and imipramine, the existence of an oxygen and nitrogen on one of the rings shifts the properties of the ring to more hydrophilic, thereby increasing the amphiphilicity of these drugs^[29]. Interestingly, there is no clear evidence of the self-assembly of cyclobenzaprine in aqueous solution.

The potential use of TCAs in structure activity-function relationships

The variation of structure seen with the TCAs is relatively small, making it a good group of molecules on which to perform structure-function study. When compared to amitriptyline the shifts in the structure of the tricyclic antidepressants are slight, such as the transfer a carbon atom into oxygen (doxepin) and loss of a methyl group (nortriptyline), transfer of dibenzo-cycloheptadiene to dibenzazepines, *i.e.* imipramine, and the addition of halogen atoms, clomipramine. Due to this fact, the effects of structure on the aggregation behaviour of TCAs may not be large enough to be easily discernible in the present study. Therefore, it was decided that, in the current project, it was not appropriate to perform a structure-activity relationship study on the behaviour of TCAs.

Table 1.1 Structure and CMC of representative tricyclic antidepressants.

Name	Structure	CMC (mM)
Amitriptyline		36 ^[1]
Doxepin		67 ^[24]
Nortriptyline		23 ^[1]
Imipramine		47 ^[23]
Desipramine		49 ^[1]
Clomipramine		22 ^[1]

b. Non-steroidal anti-inflammatory drugs (NSAIDs)

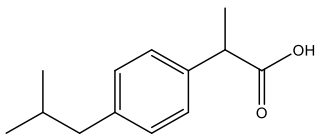
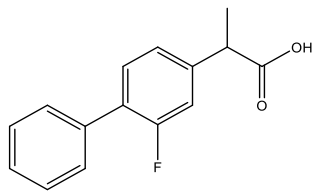
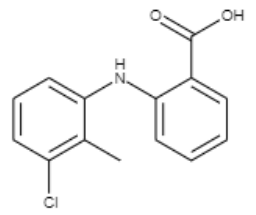
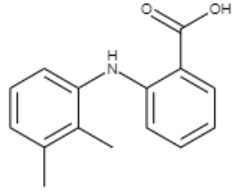
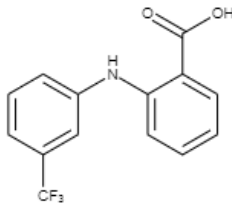
NSAIDs is an acronym for a group of non-steroidal anti-inflammatory drugs that are used to treat both chronic and acute pain, fever, coagulation and inflammation. Paracetamol, one of the most widely used NSAIDs, was used as early treatment during the COVID19 pandemic.^[30] The most well-known example of a NSAID is aspirin, which is clinically used to prevent blood clots and reduce pain, admittedly at different doses. However, the side effects of NSAIDs on the gastrointestinal tract are strong because the drugs prevent the clotting of blood and increase the possibility of bleeding. As a result, most NSAIDs are administered as enteric coated tablets after food to reduce the possibility of bleeding. In addition, the cardiovascular toxicity of NSAIDs means that these drugs are not recommended for patients with a history of heart-attacks.

The mechanism of NSAIDs actions in body has been extensively studied. Specifically, there are a group of cellular enzymes called cyclooxygenases (namely COX-1 and COX-2) that are involved in the formation of blood clots and inflammatory reactions. Most NSAIDs are non-selective COX-1 and COX-2 inhibitors, interacting with the enzymes to inhibit their activity, reducing the synthesis of prostaglandins, an inflammatory factor, to relieve the pain and prevent the formation of blood clots. It is reported that while COX-1 is the major enzyme in normal tissues, COX-2 is mainly distributed in inflammatory tissues. Selective COX-2 inhibitors seem to express no harm to the gastrointestinal tract^[31], and consequently there has been much interest in designing selective COX-2 inhibitors.

Here the self-association of 6 structural related NSAIDs is discussed: these are diclofenac sodium, tolafenamic acid, mefenamic acid, flufenamic acid, ibuprofen acid and flurbiprofen acid. Tavano and colleagues^[32] found that diclofenac sodium is likely to form vesicles in solution both with and without cholesterol. This finding suggests that a new way to deliver diclofenac sodium would be in the form of vesicles, thereby improving the drug loading of the delivery vehicle. Abe^[33] studied the organisation of ibuprofen and flurbiprofen in liquids using MD simulations, and found that hydrogen bonding is a major factor in determining the aggregation of these drugs, specifically the carboxylic group present in these molecules explains why the most commonly observed aggregates with these molecules are dimers. However, for tolafenamic, mefenamic and flufenamic, while evidence of micellization under specific circumstances has been reported, further studies are needed to study their self-association in aqueous solution to fully understand their aggregation behaviour. For example, mefenamic acid is solubilised in micelles and forms supramolecular complexes with

surfactants^[34, 35], while tolfenamic and flufenamic are reported to self-assemble to form crystals mainly via π - π stacking, and on a mild steel surface, respectively.^[36, 37] Only ibuprofen has been reported to possess a CMC of 179.5 mM,^[122] while no CMCs have been reported for the other NSAIDs listed in Table 1.2.

Table 1.2 Structure of commonly used NSAIDs.

Name	Structure
Ibuprofen	
Flurbiprofen	
Tolfenamic	
Mefenamic	
Flufenamic	

The potential use of NSAIDs in structure activity-function relationships

The problem with performing structure activity relations using the NSAIDs is that the structures does not vary systematically. For example, ibuprofen and flurbiprofen are considerably different from the other molecules in the group, while in comparison tolfenamic, mefenamic and flufenamic vary only slightly in structure, *i.e.* the structures of mefenamic and tolfenamic are very similar, varying in only one substituent group $-R_3$. Therefore, NSAIDs are not ideal choice for structure-functional study.

c. Local anaesthetics

Local anaesthetics (LAs) are clinically used to relieve acute and chronic pain, and in surgery as preoperative anaesthesia, especially in dental surgeries. Due to the cardiovascular toxicity and poor solubility of the LAs, they are often administrated with DMSO or other solvents, frequently in the form of a cream, gel, liquid and spray. Transdermal delivery is also a widely used as a means of inducing surface anaesthesia.

LAs temporarily inhibit the excitatory conduction of cell membranes in a way that the rate of depolarization and repolarization is decreased. This effect is achieved by reversibly blocking the sodium channels present on the neuron membranes, reducing the influx of sodium ions. Therefore, the membrane potential cannot reach the critical value needed to generate an action potential and is seen as a temporary disruption of excited conduction. However, as the excitatory conduction is similar both in central nerve system and peripheral nerves, there is a potential effect on the heart and central nervous system.

Most LAs can be sub-divided into one of two classes based on their structure, namely the amino esters and the amino amides. The typical LA structures are composed of three moieties - a planar ring, an alkyl chain and either an amino ester or an amide group with a terminal tertiary amine. The planar ring and alkyl chain comprise the hydrophobic part of the amphiphilic LAs. Here both amino esters and amino amides are discussed, specifically tetracaine, stadacaine, (butoxucaine), dibucaine, (cinchocaine) and procaine. From a study using total intensity light scattering and photon correlation spectroscopy, it was established that procaine forms small aggregates, dibucaine bigger aggregates of an aggregation number of 9 or 15, while tetracaine, exhibits an aggregation number of 7^[23, 38]. The aggregates of stadacaine appeared to be larger containing 17 monomers.^[23] The self-association of the LAs is thought to influence their pharmacological activity including their permeability and

diffusion by enhancing the binding of the hydrophobic moiety to the membrane, as the driving force for drugs crossing the cell membrane is the intermolecular drug transfer property.^[38-40]

The potential use of LAs in structure activity-function relationships

Unfortunately, the structures of the four LAs known to self-assemble in aqueous solution are not closely related to each other, for example dibucaine (Figure 1.8, right) contains an amino amide while the other three drugs contain an amino ester. Furthermore, the hydrophobic regions of the four local anaesthetics are not sufficiently different in a systematic way to warrant their study.

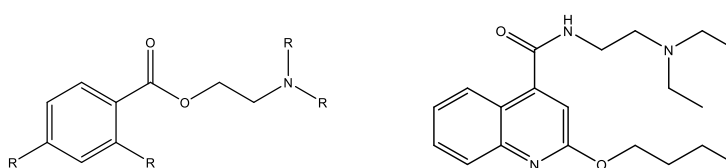


Figure 1.8 Left: general structure of amino ester local anaesthetics. Right: structure of dibucaine.

d. β -Blockers

β -blockers are commonly used drugs because of their cardiovascular protective effects, their ability to cure abnormal heart rhythms and to protect the heart from myocardial infarction occurring after a first heart attack. They are also used to treat hypertension, for which the first choice of drug is generally a calcium channel blocker or a thiazide-type diuretic.^[41] As their name suggests, β -blockers 'block' the activity of β -adrenaline receptors that are located widely throughout the body, and include the heart muscles, the airways, smooth muscle and kidneys, etc. Indeed, the action of β -blockers on these various tissues, is opposite to that seen with norepinephrine and epinephrine which cause an increase in blood pressure as well as the relaxation of smooth muscle. β -blockers, on the other hand, slow down the heart rate, decrease cardiac output and hence lower blood pressure, as well as causing inotropic effects on the bronchi.^[42]

Most β -blockers contain in their structure hydrophobic, aromatic groups and a hydrophilic, hydroxyl moiety which contribute to the drug's surface activity. Based on their aqueous solubility, β -blockers can be divided into two groups, namely the hydrophilic β -blockers, such as acebutolol and sotalol, and the hydrophobic β -blockers which include propranolol. A high hydrophobicity of a β -blocker

leads to its better bioavailability, and because of the fact that they are more likely to pass the blood-brain barrier^[43], a higher degree of central nervous system effects. In contrast, hydrophilic beta blockers exhibit a longer half-life than their hydrophobic counterparts, as well as exhibiting a lower metabolic rate because the hydrophobic beta blockers, like propranolol, are mostly metabolised in liver, undergoing a high first-pass metabolism.^[44] Some hydrophilic beta blockers also exhibit a significant first-pass metabolism, resulting in a variable bioavailability. For example, sotalol exhibits an intestinal absorption of 90-100%, while metoprolol and timolol exhibit a value of approximately 50%.^[45] As a result, most β -blockers are administrated at a high dose.

Depending upon their selectivity towards β_1 - and β_2 -receptors, β -blockers are divided into two groups, namely the selective β -blockers and the non-selective β -blockers. It is known that β_1 -receptors are mainly distributed mainly in the heart, while β_2 -receptors are found in organs where they cause a smooth muscle relaxation. As a result, selective β -blockers have effect on either cardio or bronchial muscle dependent upon whether they interact with β_1 - or β_2 -receptors.^[46] The β -blocker, propranolol, is a non-selective β -blocker which is widely used to treat hypertension and cardiac dysrhythmias. Because of its non-selective nature its use can cause bronchial side effects, such as bronchoconstriction thereby limiting its application in asthmatic patients. In contrast, metoprolol a β_1 -selective blocker, exhibits a lower risk of bronchial side effects.^[46] In the current study, several structurally related β -blockers, namely propranolol, atenolol, metoprolol, pindolol, acebutolol, oxprenolol and sotalol, were considered for study.

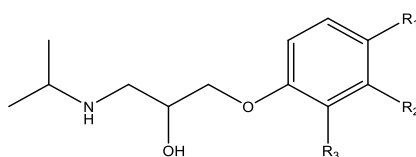


Figure 1.9 Basic structure of β -blockers.

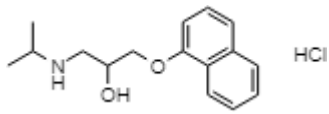
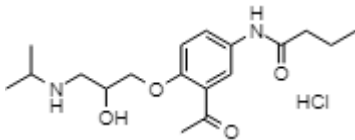
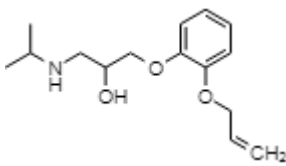
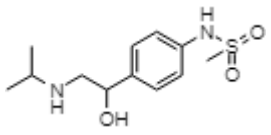
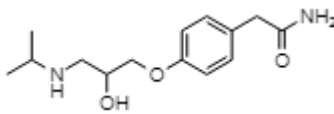
There are many research reports of the surface activity and aggregation behaviour of β -blocker drugs. The aggregation number of propranolol (as the hydrochloride salt) has been reported to be in the range 10-12, with a CMC in the order of 1.0×10^{-1} M.^[23, 47-49] A thermodynamic study has indicated that of propranolol has an aggregation number of 10 and follows a model of low aggregation number which assumes the conductivity of monomeric propranolol and its aggregates is linear as a function

of concentration and that at high aggregation numbers, the effective charge on the aggregates is far below their aggregation number.^[48] The same study demonstrated that both entropy and enthalpy during self-association decreased linearly as temperature increased. Furthermore, the free energy changes show first a smooth decrease and then a levelling off as temperature increases. In contrast, the variation of propranolol's CMC follows the shape of a curve upon increasing temperature. At temperatures below 295K, the predominant driving force of aggregation is considered to be the hydrophobic effect, while at temperatures above 295 K, London dispersion forces play a major role. Zuriaga *et al* ^[47] reports a study on the aggregation of atenolol, metoprolol and acebutolol which showed that, the intermolecular forces between the drug molecules, as well as the drugs' interaction with water, determines the nature of the drug's aggregation. It has been reported that the self-association of acebutolol is mainly driven by London-dispersion forces, in conjunction with negative free energy changes, and a decrease in both entropy and enthalpy as the temperature increases^[50]. Evidence for the nature of the aggregation of oxprenolol and sotalol in the form of hydrochloride salts are provided as well^[1, 51]. However, there is no clear description in the literature of the self-assembly of pindolol in aqueous solution. As the structure of pindolol is not that different from that of propranolol, we assume here that there is a strong possibility that pindolol self-associates in aqueous solution.

The potential use of β -blocker drugs in structure activity-function relationships

Compared with other three groups of drugs reviewed here, the β -blockers are more likely to be well suited for the study of their structure-activity relationships because their molecular structures are systemically related (Table 1.3).

Table 1.3 Structures of selected β -blockers and their CMCs.

Name	Structure	CMC (mM)
Propranolol HCl		95 ^[1]
Acebutolol HCl		170 ^[1]
Oxprenolol HCl		170 (with 0.5 M NaCl) ^[1]
Sotalol HCl		180 (with 0.5 M NaCl) ^[1]
Atenolol		22.9 ^[47]

As neutron experiments were planned as part of the present study, the neutron scattering length densities of representative β -blockers were calculated. In brief, the neutron scattering length density (SLD) can be considered as the ‘neutron’ refractive index of a material and is calculated from the sum of neutron scattering lengths of each atom, and considering the molecular volume of the material. Since deuterium (^2H) has a much larger and positive neutron scattering length compared to the smaller and negative value of hydrogen (^1H), exploitation of isotopic contrast during the study was also considered as it would allow for the study of more variants of the system under examination allowing more detailed information to be obtained. For example, the SLD of protiated propranolol hydrochloride was determined to be $1.22 \times 10^{-6} \text{ \AA}^{-2}$, while the SLD of the ring-deuterated form (d_7) is $3.1 \times 10^{-6} \text{ \AA}^{-2}$. This difference provides a good (‘neutron’) contrast between protiated and deuterated versions of the propranolol molecules, making propranolol an excellent candidate for neutron scattering experiments.

As described above, β -blockers were selected for study because (1) the self-association in bulk solution of β -blockers has been experimentally proven, (2) the systematic change in the nature of their structure makes them ideal for structure-activity studies and (3) the fact that β -blockers are readily available in deuterated forms make them ideally suited to neutron experiments.

In the present study, the basic drug, propranolol hydrochloride (Table 1.3) was selected for a detailed study of its surface and aggregation behaviour. In addition, other β -blockers, namely the structurally related atenolol and acebutolol hydrochloride, were also selected for subsequent study. The aqueous solubility of the selected 3 drugs follows the order: atenolol < propranolol HCl < acebutolol HCl^[45, 52]; note that atenolol is in the form of a free base. In the present study, the surface activity and self-assembly of the three β -blockers were studied using both surface techniques, including surface tension, and bulk properties using small angle neutron scattering in combination with contrast variation and complimented by molecular dynamics simulations.

1.1.4 Interactions between drugs and phospholipid membranes

The surface activity of an amphiphilic drug means that, because of the hydrophobic effect, it can adsorb to interfaces. In addition, the word *surfadrug*, which stems from the surface-active nature of an amphiphilic drug, has also been used to describe such molecules. Upon the addition of a surface-active drug to the body, it will encounter lipid bilayers/cell membranes. Studies^[1, 53, 54] have shown that an amphiphilic drug can self-associate and interact with biological membranes in a similar way to a classical surfactant.

As is well known, β -blockers exhibit their pharmacological activity by inhibiting β -adrenoceptors, resulting in a hypotensive and a preservation effect on the cardiovascular system. In addition, propranolol and timolol have been discovered to lower intraocular pressure and have been used in varieties of eye drop formulae for the treatment of glaucoma. However, studies have proposed that some β -blockers exhibit a local anaesthetic activity as well as a non-specific cardiac depression that is considered to originate from a membrane-stabilising effect.^[55, 56] Another study has indicated that the antiarrhythmic, local anaesthetic and membrane stabilising activity of β -blockers involves their interaction with membranes, and that this interaction is increased with the hydrophobic nature of the drug.^[57] It has been proposed that surface active drugs can either generate a liquid membrane

on a surface,^[58] or else in combination with membrane lipids, modify the properties of a biological membrane influencing access to active sites. To support this hypothesis, a study has shown that β -blockers can inhibit sodium influx, resulting in a membrane-stabilising activity. The membrane-stabilising activity is increased with an increase in the hydrophobicity of the β -blockers.^[58] In addition to the membrane stabilising action of β -blockers, Liu *et al.* have also observed an antioxidant effect resulting from the interaction between β -blockers and membranes. It has been discovered that β -blockers can interact with phospholipids to form a complex which can preserve the phospholipids being oxidized, therefore perform a membrane preservation effect.^[59] Therefore, it is inevitable that any drug-membrane interaction may play an important role in the pharmacokinetic behaviour of the drug, namely the adsorption, distribution, metabolism and elimination of the drug, making the study on the drug-membrane interactions of high importance.

There are some studies revealing the membrane interactions of β -blockers. A thermodynamic study shows that the partitioning of four selected β -blockers into membranes of a saturated lipid called DMPC leads to a minimization of surface free energy, accompanied by a condensing effect on lipids. However, the four drugs interact differently with the DMPC membrane according to their different hydrophobicity.^[60] Another study reported that the interaction of propranolol with vesicles composed of the unsaturated lipid, POPC, occurs in such a way that the hydroxyl groups of propranolol are located at the region of phospholipid head-group, while the terminal methyl groups of propranolol are positioned near the phospholipid acyl chains, the naphthalene ring is located close to the lipid carboxyl groups.^[61] In this study, molecular dynamics simulations (MD) were performed as complementary to fluorescent experiments to gain an overall insight into the interaction. Similarly, a study on six β -blockers passing across bilayers of POPC, using MD simulations, indicated that hydrogen bonds between β -blockers and water molecules located in POPC bilayer are the main forces in the drug-membrane interaction. Coulomb interactions were also found to play an important role in determining the interaction of drugs with membranes. It was also found that the interaction with the membrane is dependent on the β -blocker's lipophilicity.^[62] In the studies mentioned above, several techniques were applied, in order to shed light on the location of β -blockers in phospholipid bilayers. In some cases, MD simulations were applied in combination with experimental techniques to provide information on the free energy profile of the interaction in combination with images at the molecular level. While there are limitations in that most studies based on the differences before and after the interaction, the kinetics of interaction are a mystery to date. Meanwhile, though many

studies have revealed the location of drug in lipids, the structure of the whole drug-lipid system after the interaction either in the lateral or in longitudinal direction is unclear. Therefore, in this research, some advanced techniques, *i.e.* ellipsometry, Brewster angle microscopy (BAM) and neutron reflectometry (NR), were applied to explore the process of the drug-membrane interaction. Ellipsometry in combination of Langmuir trough is able to provide the information on the changes in surface amount and surface pressure, which reflects the orientation of lipids and the formation of any domains. Any changes in lipid domains could be visualized using BAM. Kinetic neutron reflection measurements at low- Q range were performed to obtain the changes in the amount of both lipids and drugs during the first few minutes of the interaction, where an interesting up-down-up peak was observed in surface pressure data. The structure of the drug-lipid system after the interaction has reached equilibrium were obtained from neutron reflection measurements across the full- Q range.

1.2 Model lipid membranes

1.2.1 Composition of human cell membrane

Human cell membranes consist of multiple components embedded in a fluid bilayer, composed mainly of phospholipids, sterols, proteins and polysaccharides. Phospholipids, which make up the backbone of the cell membrane, are amphiphilic structures containing a hydrophilic head, typically a phosphorylcholine, and hydrophobic tails comprised of alkyl chains. Due to the hydrophobic effect, the lipid molecules are solvated and oriented in such a way that the polar heads are orientated towards the external aqueous medium while on the interior of the bilayer are the hydrophobic tails^[63]. Phospholipids are named according to both the structure of their head-group and the nature and length of their tails. As shown in Figure 1.10, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are zwitterionic, while phosphatidylserine (PS) and phosphatidylinositol (PI) are negatively charged. The carbohydrate chains of lipids are named by the lengths and the degree of saturation, *e.g.* dimyristoyl- (DM) represents two saturated chain length of 14 carbon atoms each, sometimes written as 14:0, and analogues with longer saturated chains include dipalmitoyl- (DP; 16:0) and distearyl- (DS; 18:0). Similarly, 1-palmitoyl-2-oleoyl- (PO) has two different chains, C_{16} and C_{18} , where the C_{18} chain contains one double bond, and can be written as 16:0, 18:1, respectively.^[64] Sterols, generally cholesterol in human cell membranes, help to stabilize

and regulate the fluidity of the phospholipid membrane.^[65] There are several different ways in which proteins are orientated in a cell membrane, mainly depending upon the different role the protein plays. For example, transmembrane proteins normally act as transporters for ions like Na^+ . In contrast, proteins attached on one side of the bilayer typically act as a membrane receptor, while embedded proteins mainly act as enzymes. In addition, polysaccharides may be associated with membrane proteins being located on the outer leaflet of the bilayer, taking part in the process of enzyme recognition.

Furthermore, the composition of the cell membrane can be very different depending on its function in different tissues. For example, the erythrocyte cell membrane is composed of 60% phospholipids, namely PC, PE, sphingomyelin (SM) and some negatively charged lipids like PS and PI, 30% of cholesterol and 10% of glycolipids, such as sphingosine and present in the outer leaflet^[66]. In contrast, while the neural soma membrane contains 57% of phospholipids, mainly PC, PE and small amount of SM, it contains only 15% of cholesterol and about 5% galactolipids^[67]. In general, the most common phospholipids in mammalian cell membranes are PC and PE, which are present at between 12 to 48% and 13 to 28%, respectively of the cell membrane^[68].

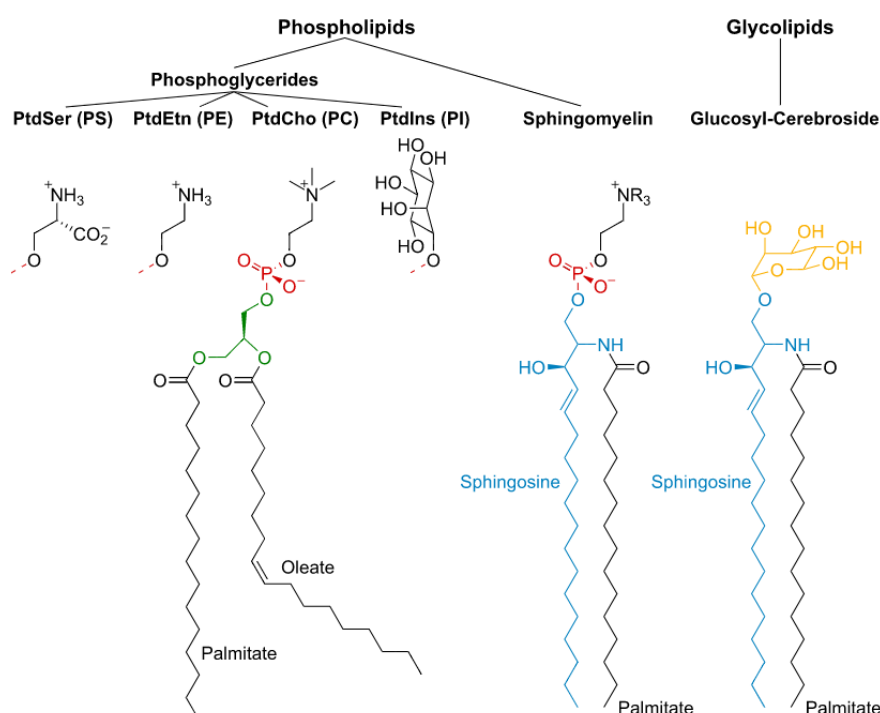


Figure 1.10 Structure of different phospholipids distinguished by heads and glycolipids. Adapted from https://commons.wikimedia.org/wiki/File:Membrane_Lipids.svg.

1.2.2 Application of model lipid membranes

As the complexity of natural occurring membranes makes it difficult to measure their properties, an artificial membrane containing well defined components often provides a more suitable model platform for researchers^[63]. Artificial membranes tend to exhibit a higher electrical resistance than natural cell membranes, although the value obtained for an artificial membrane is still within the range of values found for natural membranes. This leads to the conclusion that an artificial membrane composed of pure phosphatidylcholine may be a good substitute for a natural membrane.^[63] Consequently, therefore, double-chain phospholipids are widely used to form model biomembranes for research purposes.

There are several different types of commonly used model membranes including, bilayers, vesicles and monolayers (at the air/water interface). When the lipid concentration exceeds its CMC, double chained phospholipids are likely to form vesicles while single-chain phospholipids form micelles.^[69, 70] In aqueous solution, double-chain phospholipids are driven by the hydrophobic effect to form bilayers, a consequence of the critical packing parameter of a phospholipid being close to unity.

Small changes in the properties of membranes, such as membrane composition, thickness, fluidity and phase transition, etc., can make a significant difference to the biological properties of a cell.^[71] A special structure within a cell membrane that plays a very important role in the functioning of a biological membrane and the proteins bedded therein is the lipid raft, which is a region of highly ordered saturated phospholipids and enriched in cholesterol, and generally contains the membrane protein, within a sea of less/unsaturated lipids^[69, 72]. Further, the structure of a phospholipid can influence the properties of the model membrane it is used to make. Lu *et al.* investigated the properties of synthetic vesicles composed of different lipids on both sides of the bilayer, (*i.e.* asymmetric bilayers) and found that the length of the lipid acyl chain affects the properties of membrane, leading to the conclusion that bilayers composed of lipids with different alkyl length chains may exhibit a higher expansion modulus than symmetric bilayers.^[73]

Model membranes can be useful in the development of improved drug delivery vehicles. It is known that drugs follow a complicated process to be absorbed and subsequently utilized by the target cells. Although most drugs target membrane receptors, the interaction between drug and membrane may have a significant effect on the pharmacokinetic properties of the drug in the body, *i.e.* the drug's

absorption, distribution, metabolism and elimination. As a result, the ability of a drug to interact with a biological membrane plays an important role in the bioavailability of a drug.

There are two traditional approaches by which a molecule, including a drug molecule, may cross a cell membrane, namely passive translocation, including free diffusion and facilitated diffusion, and active transport. For small and lipophilic drugs, the preferred mechanism of cell uptake is free diffusion, which is driven by osmotic pressure and is therefore largely dependent on drug concentration. For large, hydrophilic drug molecules, active transport is the most likely mechanism by which they cross a cell membrane.^[74] For amphiphilic drugs, the approach by which these molecules cross the membrane is more complicated. The precise nature of how amphiphilic drugs interact with and cross biological membranes needs further study.

Numerous studies have been performed to shed light on the drug-membrane interaction. Interactions between drugs and lipids include non-covalent interactions, electrostatic interactions, Van der Waals forces, hydrogen bonds and hydrophobic interactions.^[75] Drugs are assumed to insert into the lipid membrane, causing a series of drug-induced changes in membrane properties such as lipid reorientation, lipid loss, bilayer disruption^[23, 76, 77], vesicle or micelle formation^[74], lipid flip-flop^[23] and lipidosis^[78], etc. Lipidosis is defined as the over accumulation of lipids occurring in lipid bilayers due to the drug-induced lipid metabolism disruption and solubilisation.^[79] Indeed, a study has shown that bilayer disruption is more likely to occur when the drug:lipid ratio is high, under such circumstances surface-active drugs can remove lipid components from the membrane when the surface is saturated with amphiphilic drug and when the bulk concentration reaches the CMC.^[23]

As lipid layers are always inhomogeneous, drugs assemble within the membrane heterogeneously, leading to high local drug concentrations. A study performed by Niga and co-workers^[70] shows that the drug propofol interacts with model phospholipid membranes at the air/water interface. The lipids DSPC, DPPC and DMPC enhance the fluidity of the membrane, resulting in propofol preferring to interact with lipid disordered regions rather than ordered lipids regions. Moreover, the effect of a drug molecule on the stability of lipid membranes, that is whether it disrupts or improves the ordering of the lipids, depends upon the drug's structure.^[80] For example, Giuffrida *et al.*^[81] investigated the interaction between a series of naproxen prodrugs and phospholipid bilayers, and found that the naproxen prodrugs cause a destabilisation of the bilayer when inserted into the membrane, while the degree of interaction of the prodrugs into the phospholipid bilayers is higher

than that seen with the parent naproxen molecule. As consequence, increasing the length of the lipoamino acidic side chain of a naproxen prodrug leads to an enhancement in the affinity of the naproxen prodrug for the phospholipid membrane. In contrast, research into the interaction of cationic drugs (such as doxorubicin, vinorelbine and topotecan) with polymeric phospholipid micelles (poly(ethylene glycol)–phosphatidylethanolamine) demonstrates that micelles loaded with cationic drug exhibit a higher stability than empty ones, predominately due to electrostatic effects promoting the association between drug-polymer.^[82] Changes in the structure of a drug can play a divisive role in its interaction with a biological membrane. For example, research into the amphiphilic drug, artepillin C and its interaction with a model membrane indicates that the prenylated groups bonded to cinnamic acid are the major contributor of the drug's interaction with a biological membrane, since the large volume of a prenylated group can significantly alter the membrane. Furthermore, the presence of a prenylated group is important in the amphiphilic properties of artepillin C.^[83]

The mechanism(s) of drug intercalation into a lipid membrane is diverse and can be largely attributed to the drug structure. For instance, tricyclic antidepressants possess a three-ring hydrophobic region and a short aliphatic chain terminated with a hydrophilic head-group. When in a biomembrane, the three-ring structure of a tricyclic antidepressant is orientated into the lipid acyl chain region with the polar head located near the lipid polar groups. The interactions involved in this process include Coulomb interactions, van der Waals forces, ion-induced dipole interactions along with the hydrophobic effect.^[75, 80, 84] Local anaesthetics have a common structure being composed of three moieties, namely an aromatic ring, an intermediate chain which together comprise the hydrophobic part, and an amino terminus. It has been determined that local anaesthetics interact with biological membranes to increase the fluidity of the membrane. When interacting with biological membranes, local anaesthetics undergo a process which results in the phospholipid molecules liberating water molecules bonded to themselves by inter-molecular hydrogens^[85]. Baciú and colleagues^[74] noticed that cationic amphiphilic drugs cross a phospholipid bilayer by a unique mechanism in that drug molecules insert into the bilayer from one side, causing the phospholipid molecules to degrade by converting from a double-chain molecule to a single-chain lipid molecule and a free fatty acid. The mechanism by which process occurs may be attributed to the protonation at physiological pH of cationic amphiphilic drugs, which results in the acid hydrolysis of membrane phospholipids. The resulting mono (lyso) chain lipid molecules and the drug molecules form mixed micelles, which are disengaged from the other side of the phospholipid bilayer. As drugs in mixed micelles separate from

the biological membrane, they can be transported to next biological membrane where they will undergo the same process. It can be concluded from the above studies that amphiphilic drugs can interact with biological membranes in a variety of complicated ways.

1.2.3 Methods of producing cell membrane

As described above, the most used model biological membranes are vesicles, lipid bilayers, lipid monolayers. However, all models have their pros and cons. For example, vesicles are comprised of closed spherical bilayers which are ideal for research into model membranes as they do not require the presence of a substrate for their preparation. Spherical vesicles can be produced in several different ways. Generally, the lipid is dissolved in an organic solvent, the solvent is then removed, possibly by freeze-drying, after which the lipid is finally suspended in aqueous buffer to form the vesicles.^[86] As a consequence of their method of preparation, only symmetrical bilayers can be formed.

Lipid bilayers are normally produced on solid substrates using one of two different approaches, *i.e.* vesicle fusion and Langmuir-Blodgett deposition.^[87] In brief, vesicle fusion is used to make vesicles collapse on a solid substrate to form bilayer on its surface. The bilayer can be stabilized by electrostatic interactions between lipid head-group and the oppositely charged substrates. When using Langmuir-Blodgett deposition to produce a bilayer, a single monolayer is first produced on a solid substrate, with a second monolayer being subsequently deposited on top to form the bilayer, as shown in Figure 1.11. Langmuir-Blodgett bilayers can be asymmetric in each leaflet, thereby mimicking the real cell membrane. However, with bilayers formed on a solid substrate, it is difficult to achieve a full coverage of the substrate by the bilayer, coupled with the fact that there is not much aqueous phase on the side of the bilayer deposited on the substrate, hence the effects of the substrate on the bilayer such as electrostatic attraction or repulsion are nonnegligible in the majority of studies.

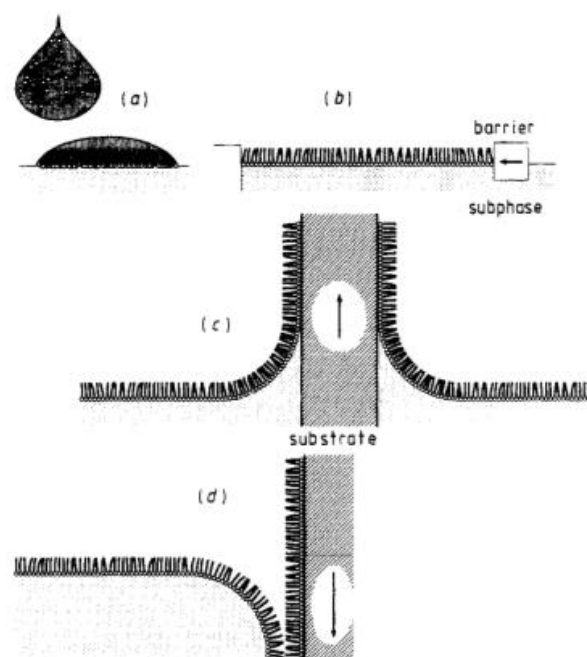


Figure 1.11 Steps in the Langmuir-Blodgett technique: (a) spread lipid on air/water interface; (b) compress surface area to make stable monolayer; (c) transfer the first monolayer to solid substrate; (d) subsequent downstroke to make the second leaflet. Adapted from reference [88].

In contrast, phospholipid monolayers are readily produced at the air/water interface by spreading a lipid, dispersed in chloroform or methanol, on the surface of the aqueous phase. Though the monolayer only mimics a single leaflet of cell membrane, it is a fluid membrane that has full coverage of the surface, without any substrate effects. A monolayer formed at the air/water surface can be affected by the surface pressure (lateral compression) of the monolayer, the length of the hydrocarbon chains of the lipid and the nature of the lipid head-group amongst other things. For example, as the surface pressure increases, the lipid layer changes from a disordered gas phase to condensed solid phase.^[69] When the area of per lipid molecule is large (that is greater than $120 \text{ \AA}^2/\text{molecule}$), the lipid molecules at the air/water interface are in a gaseous state. Upon compression, the area occupied by each lipid molecule decreases, and the lipid monolayers transformed into the liquid expanded (LE) phase, in which the lipid molecules are randomly oriented and fluid in nature. The transition from gas phase to LE refers to the lift off area (A_0) of surface pressure from zero in lipid isotherm (Figure 1.12).^[89] Upon further compression, the lipid molecules undergo a reorientation process into a more ordered phase termed the liquid condensed (LC) phase, which corresponds to the linear region, with the steepest slope, in surface pressure – area ($\pi - A$). Extrapolation of this

slope to zero pressure yields a value known as the limiting area (A_{lim}), representing the area of a single lipid molecule in condensed monolayer (Figure 1.12).^[89, 90] For some lipids such as DPPC, the transition from LE to LC involves a coexistence stage at room temperature, in which the surface pressure remains roughly constant while the area/lipid molecule keeps decreasing.^[89]

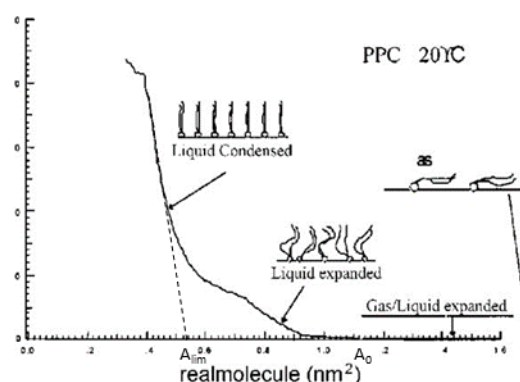


Figure 1.12 Lipid isotherm of DPPC at room temperature. Modified from reference [91].

In the present study, we chose to use a lipid monolayer on air/water interface, since it is the easiest way to produce a stable model membrane, and ideal for investigating the dynamic process of drug-membrane interaction using Langmuir trough.

2 Techniques

2.1 Traditional techniques

It is not uncommon for a drug to exhibit amphiphilicity, and while its amphiphilic behaviour may be slightly different than that exhibited by classical surfactants, it is possible to characterise an amphiphilic drug using the same techniques that have been applied to surfactants. There are a wide variety of experimental techniques that have been applied to examine both the self-assembly of drugs in bulk solution and their surface activity at a surface or interface. For example, static and dynamic light scattering and small angle X-ray scattering (SAXS) have been widely used to establish the size, structure and interaction between any aggregates formed. Furthermore, ^1H NMR dilution experiments have been used to determine the CMC of self-assembled particles^[92]; differential scattering calorimetry (DSC) has been valuable in providing thermodynamic information on self-association process^[81]; conductivity measurements have enabled the CMC of drugs to be estimated.

In many situations, the results of two or more techniques are combined to obtain more detailed and/or accurate results. For example, Attwood and co-workers^[17] studied the self-association of verapamil, a calcium channel blocker used to treat hypertension, using both light scattering and ^1H NMR spectroscopy coupled with a mass action model to study the concentration dependence of the aggregation. In another study, calorimetry was used to investigate the self-association of a range of amphiphilic drugs at much lower concentrations than is possible using light-scattering.^[15] A study on the self-association of penicillin in electrolyte solution using light-scattering in combination with NMR spectroscopy showed the existence of a second CMC.^[12] Although both techniques recorded the presence of a second CMC, the two techniques suggested quite different mechanisms for its formation.

As most drugs generally exhibit their pharmacological activity inside the target cell, it is of importance to investigate the mechanism of drug-biological membrane interactions. Fluorescence spectroscopy is a useful technique to resolve structural and dynamic information on the interaction of antidepressants with liposomes at the cellular/molecular level, by labelling the liposomes with a fluorescent compound and measuring the fluorescence intensity and decay time.^[93] Santos and co-workers^[78] applied solid-state ^{31}P and ^{14}N NMR techniques to investigate how antidepressants affect changes in the phospholipid head-groups present in the biological membranes. Unfortunately,

however, it is only possible to apply NMR techniques to a few isotopes such as ^2H , ^{13}C , ^{15}N , ^{31}P , ^{19}F and ^{17}O . DSC is also a widely used method to study how some amphiphilic molecules interact with biological membranes, often being used in conjunction with NMR spectroscopy and/or SAXS to better understand the measured interactions. A study performed by Bulavin and colleagues^[94] used DSC in combination with SAXS to investigate the change in the enthalpy and phase transition temperature induced by the presence of drug in a lipid bilayer. Unfortunately, both DSC and NMR have the experimental limitation of requiring a relatively high concentrations of sample to be examined. To avoid material depletion, Fox and Harris^[80] applied in conjunction with DSC and NMR, confocal Raman microscopy to investigate the changes in lipid vesicle structures due to the interaction with tricyclic antidepressants as it can be used at a lower, *i.e.* micromolar, concentrations.

2.2 Techniques to study bulk properties

2.2.1 Neutron scattering theory

There are many reasons to use neutrons for the study of the aggregation of molecules in bulk solution: (1) unlike electrons or photons, neutrons are neutral and do not possess oscillating magnetic fields, and are therefore unlikely to be affected by the orbiting electrons present in the atoms; (2) neutrons interact with atomic nuclei via the very short-range strong nuclear force and thus penetrate matter much more deeply than X-rays; (3) although not pertinent for this study, neutrons are able to interact with electrons in magnetic fields via spin-based, dipole-dipole interactions, allowing their wide application as an atomic level probe for the study of magnetism.^[95]

Neutrons interacting with a nucleus can be scattered either coherently or incoherently. The neutron scattering length (b) describes the coherent scattering, which depends on the composition of nucleus and the orientation of the nucleus to the spin of the neutrons, and is therefore isotope specific.^[95] The value of the coherent scattering length of an atom, can be either negative or positive indicating repulsion and attraction, and can be obtained via the NIST web-site.^[96]

The scattering cross section (σ) of an atom is proportional to its scattering length (b):

$$\sigma = 4\pi|b|^2 \quad (2.1)$$

The neutron scattering of a material can be affected by its size, shape and composition. The neutron scattering length density (SLD) of a material is used to quantify its neutron scattering. The neutron SLD is defined as the quotient of the total scattering lengths of all the atoms and the molecular volume (V_m) of the material, represented as ρ :

$$\rho = \frac{\sum_i^n b_i}{V_m} \quad (2.2)$$

where b_i is the coherent scattering length of the i atom in a molecule has n atoms. V_m can be calculated from the physical density of the material.

Since the scattering length of deuterium at 6.671 fm is much higher, and of opposite sign, than that of hydrogen at -3.739 fm, it is possible to use a methodology called isotopic contrast variation whereby (partially) deuterated materials are used to highlight different moieties/regions of the molecule/particle being studied, to obtain detailed structural information. In addition, by adjusting the ratio of D and H in the system under study, it is possible to match the SLD of some components/regions with air or solvent making them invisible to neutrons.

In a reactor neutron source, such as at the Institut Laue-Langevin or the ILL (Grenoble, France), the neutrons are produced by fission reaction, normally using a highly enriched uranium fuel element. At a spallation neutron source, like that at ISIS (Oxford, UK), neutrons are produced using an accelerator consisting of a linear accelerator and a synchrotron or cyclotron and focusing the beam on a heavy metal target, to produce, at ISIS, a high energy, pulsed beam of neutrons, with a repetition rate of 50 Hz. A moderator is used to cool down the energy of the neutron beam to yield neutrons of the required wavelength range. Due to the range of wavelengths present, a time-of-flight (TOF) method is used to monitor neutron wavelength using the de Broglie equation:

$$\lambda = \frac{h}{mv} \quad (2.3)$$

where λ is the wavelength of the neutrons, h is the Planck's constant, m is the mass and v is the velocity of the neutrons, the wavelength is inversely proportional to the velocity of neutrons. Over the fixed path length from the moderator to target, choppers are used to select the flight time of the fastest and the slowest neutrons produced within one pulse, thereby selecting the range of neutron wavelengths to be in a certain range.

2.2.2 Small angle neutron scattering

Small angle neutron scattering (SANS) is an advanced method to study the nanostructure of materials. A range of nanostructural information can be obtained using SANS, including size, shape, molecular weight and aggregation number as well as the sub-structure of the material at a length-scale of 1 – 400 nm.

In time-of-flight methodology, although the angle of the neutron beam is fixed, the wide range of neutron wavelengths present provide a wide range of scattering vectors (Q) to be studied. When studying a large sized structure, where a low Q -range ($|Q| \leq 1 \text{ nm}^{-1}$) is needed, a long sample-detector path length is required. Figure 2.1 shows a schematic of a SANS beam-line. First, a chopper allows the selection of a neutron beam of defined wavelengths, which is used for the subsequent experiments. Once the beam has interacted with, and been scattered by, the sample it is measured on a 2-D detector at an angle of 2θ , which is known as the scattering angle. As:

$$2\theta = \tan^{-1} \frac{R}{L_2} \quad (2.4)$$

where R is the radius of detector and L_2 is the sample-detector distance (Figure 2.1). The scattering vector, Q , is therefore defined as:

$$Q = |k_f - k_i| = \frac{4\pi \sin \theta}{\lambda} \quad (2.5)$$

where k_i and k_f represent incident and scattered intensity, λ is the neutron wavelength, as shown in Figure 2.2.

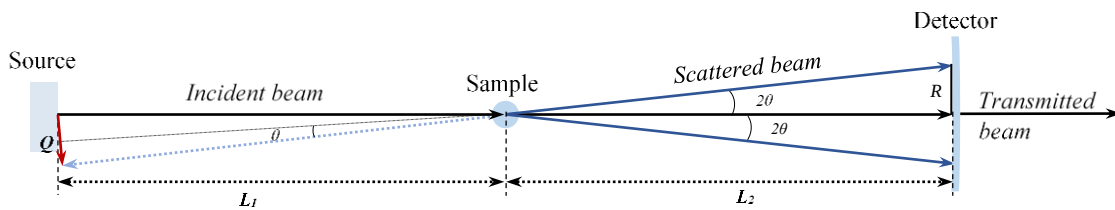


Figure 2.1 Illustration of the SANS beamline, where the L_1 and L_2 are distance from source to sample and distance from sample to detector, respectively.

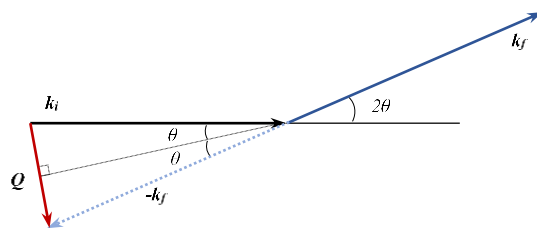


Figure 2.2 Diagram of elastic scattering of neutrons, $|k_i| = |k_f|$.

In order to obtain the optimal Q resolution, the distance between the source and sample (L_1) should be equal to the distance between the sample and detector (L_2) (Figure 2.1). For particles of different sizes, as illustrated in Figure 2.3, the coherent scattering of smaller particles result in a larger scattering angle, therefore a higher Q , while the larger particles result in smaller scattering angle and lower Q . It is important to note that not all the neutrons are scattered by the sample, in fact only a small proportion are scattered, as the majority are transmitted straight through the sample (without interacting with an atom) to the main detector where there is a beam stop to stop the main neutron beam hitting the detector and damaging it due to the high intensity of neutrons. The detector typically consists of a series of ^3He gas tubes. Just located behind the main detector is another, second detector which is used to measure the transmission of the sample and corresponding solvent.^[97]

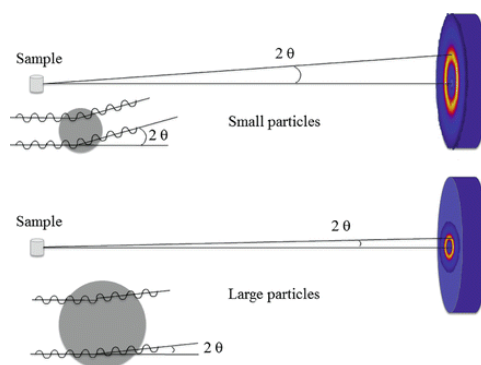


Figure 2.3 Coherent scattering of two particles of different size. Adapted from reference [98].

A particular advantage of the TOF methodology to measure neutron scattering is, that it provides a wide Q range in a single measurement, which improves the efficiency of measurement significantly and allows determination of sample kinetics. Using a reactor source might yield a higher intensity of neutrons but frequently requires the use of two L_2 distances to obtain a similarly wide Q range.

In the present study, SANS measurements have been performed to probe the behaviour of several beta blockers, specifically propranolol hydrochloride, atenolol and acebutolol hydrochloride, in bulk solution, determining the properties of their aggregates, including their shape, size, aggregation number and some internal structures.

2.2.3 All-atom molecular dynamics simulations

All-atom MD simulations is a modern technique, which exploits computational simulations to provide details of structure and inter-/intra-molecular interactions at atomic level, and is usually complementary to experiment or applied to guide research. The history of MD simulations trace back to the late 1950s, when Alder and Wainwright used MD simulations to study the behaviour of a system described as hard spheres.^[99] In the past ten years, MD simulations have become increasingly popular among biochemical researchers, accompanied with rapid developments in computational techniques. In brief, as atoms and molecules are not static but in constant motion, MD simulations predict the movement of atoms and molecules in a fixed (usually short) period of time. Given the positions of each atom in the system of interest, the trajectories of atoms/molecules can be calculated using Newton's laws of motion, taking account of all the interactions with every other atom (usually limited to atoms within a particular distance cut-off).^[100] The interactions can be divided into non-bonded interactions, such as Lennard-Jones, Coulomb, and bonded interactions, covalent bond-stretching and angle bending, etc. Both the inter- and intra-interactions determine the motions of atoms/molecules. The trajectories produced from a simulation are 3-dimensional, atomic level structures and can be visualised using specific software, enabled vision to atomic level structures.

There are several models commonly used for water that use different concepts from quantum mechanics, molecular mechanics and experimental results^[101], including TIP3P (Transferable Interatomic Potential with 3 Points), SPC (Simple Point Charge), SPC/E (SPC with average polarization), etc. In this case we used the SPC model, which was developed by Berendsen and colleagues using an idealised tetrahedral shape, *i.e.* the angle of H–O–H bonds is 109.3°. One advantage of the SPC model is that it can save a lot of time when simulating a fully hydrated system with thousands of water molecules, due to its rigid structure with the smallest possible number of

point charges.^[102] Periodic boundary conditions (PBC) are employed to minimize the effect of the boundary of the boxes. The concept is to surround the simulation box with copies of itself, therefore producing an infinite space with no boundaries.^[103] A force field is generally a set of equations and parameters that have been developed to calculate potential energies and forces. A range of force fields with slightly different parameters and equations were developed to address different objectives and to provide differing representations of each system.

The program used in the present study is Gromacs version 2018.4.^[103] The first step is to build a simulation box of the system of interest. In the present study, a 15×15×15 nm box was filled with SPC water molecules along with a defined amount of drug molecules (the two propranolol isomers LPR and DPR are present at a 1:1 ratio) and an equal amount of chloride molecules that are randomly distributed throughout the box (by replacing solvent molecules), the number of molecules of each species in the simulation box either in water or in saline were listed in Table 2.1 and Table 2.2, respectively. In the present study, we tried two different force fields. At first the simulations were performed under CHARMM force field, which is one of the most common used force fields in protein studies. However, the drug molecules in CHARMM force field form very unstable aggregates, *i.e.* some drug molecules come close to each other to form an aggregate then fall apart within 1 ps, which makes no physical sense. As a result, a force field GROMOS 53A6 were applied in this study, which is designed to reproduce the thermodynamic properties of small polar molecules and hydration free enthalpies in water.^[104]

The simulation boxes were energy minimized to get rid of unusual steric clashes and geometry. The reason for running energy minimization is that if the starting point of the system is far from equilibrium, the simulation may fail due to the huge forces. For a multi-molecular system, such as a protein-ligand system, the potential energy can be regarded as a landscape, where only one lowest point exists, known as the global minimum, with many alternative local minima sometimes including some that are very close in energy to the global minimum. However, currently it is unrealistic to find the global minimum directly within a practical period of time, therefore in Gromacs, a method of finding the “*nearest local minimum*” has been developed, by moving down the local gradient to look for the next reachable minimum.^[103] Then the simulation box is equilibrated at constant NVT and then NPT, respectively for 20 ns each. NVT is equilibration using a constant Number of particles, Volume and Temperature, while NPT is to keep a constant Number of particles, at constant Pressure

(rather than volume) and Temperature. Finally, the systems were put into production runs for a total of 60 ns, which produces the trajectory of atoms/molecules that carries the information of the motions of atoms and molecules. To make sure the systems were well equilibrated, the radial distribution function was calculated for the two isomers LPR and DPR respectively, to see if the two isomers were evenly distributed in the system. The well equilibrated final 20 ns of the production run were used for *gmx pairdist* analysis, which calculates the distance between every pair of the drug molecules in the system. A cut-off distance of 0.45 nm was used as it was assumed that molecules separated by a distance of 0.45 nm or greater were not in the same aggregate. As a result, the aggregates were built up by looking for all the surrounding molecules within 0.45 nm, to make a first layer of the aggregate, then searching for neighbour molecules of the first layer within the cut off distance to build the second layer, in total 6 layers were built, to make sure no molecules were missed from inclusion within the aggregate.

Table 2.1 Number of each species in the simulation box under different drug concentrations in water.

Concentration (mM)	Water molecules	Propranolol molecules	Chloride molecules
200	103035	406	406
170	103884	344	344
150	104454	304	304
130	105004	264	264
110	105592	222	222
90	106177	182	182
70	106711	142	142
50	107248	102	102
30	107859	60	60
10	108385	20	20

Table 2.2 Number of each species in the simulation box under different drug concentrations in saline.

Concentration (mM)	Water molecules	Propranolol molecules	Chloride molecules	Sodium molecules
200	102337	406	719	313
170	103190	344	657	313
150	103750	304	617	313
130	104309	264	577	313
110	104837	222	535	313
90	105485	182	495	313
70	105963	142	455	313
50	106504	102	415	313
10	107646	20	333	313

2.2.4 UV/vis spectroscopy

UV/vis spectroscopy is commonly used as a quantitative identification technique because the position and intensity of the measured peak(s) in the absorption spectra depends largely on the structure of the chemical being measured in that environment. When a chemical exposed to UV or visible light, the electrons are excited and move from a lower energy orbital to higher anti-bonding energy orbital as a consequence of the absorbance of the UV/visible light. The difference in energy between low and high orbitals corresponds to the wavelength of light being absorbed. In general, there are 4 types of electron transition relevant to the structure of a chemical, namely $\sigma \rightarrow \sigma^*$, $n \rightarrow \sigma^*$, $\pi \rightarrow \pi^*$, $n \rightarrow \pi^*$,^[105] as shown in Figure 2.4. The energy needed for these transitions follows the order: $\sigma \rightarrow \sigma^* > n \rightarrow \sigma^* > \pi \rightarrow \pi^* > n \rightarrow \pi^*$. The maximum absorbance that occurs at a specific wavelength is indicative of different types of electron transition and which can be used to aid in identifying the structure of the chemicals.

**Figure 2.4** The electron transition between different orbitals.

In addition, the relationship between UV absorbance and the concentration of a chemical is linear at a certain wavelength, leading to the ability to quantitatively analyse the chemical using the Beer-Lambert law:

$$A = \log_{10} \frac{I_0}{I} = \epsilon c L \quad (2.6)$$

where A is the absorbance being measured, I_0 and I are the intensities of the incident and transmitted light after passage through the sample, ϵ is the molar absorptivity of the specific material, c is the concentration and L is the path length of the cuvette.

In present study, UV spectroscopy was used to determine the solubility of propranolol in a range of aqueous solvents by measuring the absorbance of saturated drug solutions. The solvents used in the present study were water, D₂O, 0.154 M sodium chloride (in H₂O and D₂O), 0.3 M sodium chloride (in H₂O and D₂O) and 0.5 M sodium chloride (in H₂O and D₂O). For propranolol, the maximum absorption wavelength was determined to be 288.7 nm, in agreement with the value of 290 nm quoted in the literature.^[106] The standard curve was made using solutions of 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.08, 0.1 mM propranolol in water.

Samples were prepared by first adding excess powdered drug into 1 ml of aqueous solvent, making sure that there are always some undissolved drug present. For each solvent type, 10 samples were prepared, half of which were kept at room temperature on a circular rotator, while the others were kept at 37°C in an incubator under constant vibration. To make sure the samples were well equilibrated with drug, they were measured twice so that there was a several-day gap between the first and the second measurements. If the two measurements were comparable, equilibrium were assumed to have been reached, while if the second measurement is higher than the first, equilibrium was assumed not to have been reached and the sample was put on to equilibrate for longer. Once equilibrated, the samples were centrifuged for 5 minutes at 185.6 × g before took an aliquot of the supernatant was taken for UV measurement. Before measurement the aliquot needed to dilute 1000- to 3000-fold to fall in the linear range for absorbance. The absorbance measured for the diluted sample was converted to concentration using a standard calibration curve.

2.3 Techniques for surface study

2.3.1 Surface tension

It is known that, due to their specific structure, amphiphilic drugs can adsorb at the air/water interface. Therefore, techniques used to investigate surfactant behaviour at the air/water interface can be used to examine the interfacial properties of drug molecules. For instance, as the surface tension undergoes a decrease with an increase in the surface-active agent molecules adsorbed at the surface, surface tensiometry is widely used to measure the surface properties of drug solutions. The term surface tension refers to the attraction between molecules in the surface, which generates a tendency to reduce the area of the surface. As amphiphilic agents experience a positive adsorption at the surface, the concentration of drug molecules is higher at the surface than in the bulk, consequently due to the difference in molecular density between bulk phase and surface, the surface tension and free energy of the surface changes. The surface excess (Γ) is defined as the difference between drug molecules adsorbed at surface and in the interior/(bulk) of the solution.^[1] Surface excess is a parameter for assessing the surface-activity of amphiphilic drugs, as it reflects the ability of a drug to adsorb at the surface in order to lower its free energy. There are several different methods to measure the surface tension of liquids and solutions including (a) shape measurements by calculating the parameters of gravity acting on generated bubbles or droplets, (b) force measurements using Du Noüy ring tensiometry or Wilhelmy plate tensiometry, and (c) bubble pressure tensiometry by generating bubbles in the solution bulk phase and measuring their internal pressure.^[107] Du Noüy ring tensiometry is used to measure surface tension by measuring the force to pull, from the surface, a platinum ring slightly submerged in the surface.^[108] Wilhelmy plate tensiometry is used to calculate the wetting force when a wetted platinum plate is placed perpendicular in the surface, assuming that complete wetting occurs on the plate (Figure 2.5). However, to ensure that the plate is well wetted, a flame is used to clean and dry the plate. It should be noted however, that in some cases, the plate is made of filter paper, in which case the plate is often degreased by soaking in an organic solvent such as chloroform or hexane.^[109, 110] Among the techniques described above, the simplest and most accurate method is the Wilhelmy plate tensiometer which is widely used in research these days.

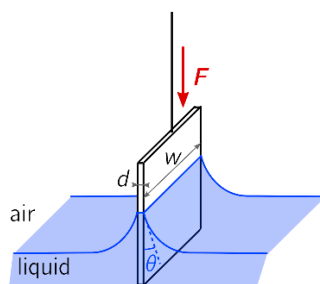


Figure 2.5 Illustration of Wilhelmy plate method of determining surface tension.
https://en.wikipedia.org/wiki/Wilhelmy_plate

The measured force F on the plate can be used to calculate the surface tension (γ):

$$\gamma = \frac{F}{(2w + 2d) \cos \theta} \quad (2.7)$$

where θ is the contact angle between the plate and liquid, w is the width and d is the thickness of the plate, hence the term $(2w + 2d)$ represents the length of the plate that contact with liquid phase.^[110]

In the present work, the surface tension of propranolol at a range of concentrations was measured using Wilhelmy plate method, and the data were recorded every 15 seconds until it reached equilibrium, the last 10 points were taken for the average surface tension at each concentration. However, at concentrations close to the CMC, it took a lot longer to reach equilibrium than at the other concentrations measured. When measuring the surface tension of propranolol in water there is a dip in the surface tension curve at the CMC. A similar phenomenon has been previously reported for sodium dodecyl sulfate (SDS) where the minimum at around the CMC is known to originate from the presence in SDS of surface-active impurities.^[110] As propranolol is reported to be unstable under light, producing 1-naphthol, *N*-acetylpropranolol and *N*-formylpropranolol (Figure 2.6)^[112], it was hypothesized that propranolol was also likely to contain a surface active contaminant, most probably naphthol. As a consequence, propranolol was purified prior to measurement in an attempt to obtain a more accurate assessment of CMC.

As 1-naphthol dissolves in ethyl acetate, ethyl ether and chloroform, while propranolol does not^[111], each of these three solvents were used to wash the same volume of drug solution, three times. The resulting surface tension curves (Figure 2.7) suggested that chloroform acts as the most efficient solvent to eliminate surface active impurities and was therefore used in subsequent studies.

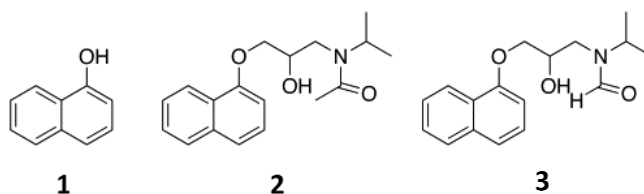


Figure 2.6 Structures of the photodegradation products of propranolol hydrochloride, (1) 1-naphthol, (2) *N*-acetylpropranolol, (3) *N*-formylpropranolol. Adapted from reference [112].

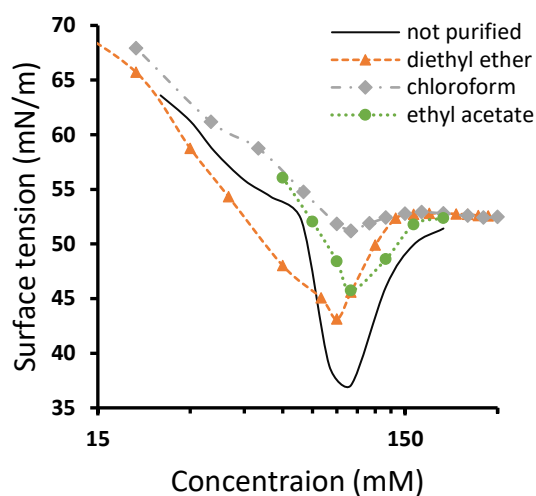


Figure 2.7 Surface tension curves of propranolol purified using different solvents, concentration plotted as $\log_{10}$.

2.3.2 Neutron reflectometry

Specular neutron reflectivity is a technique that enables the determination of the neutron SLD profile perpendicular to the plane of a surface or an interface, allowing information to be obtained on the concentration or composition of the surface. Length scales in the order of 5-5000 Å are characteristically probed.

Specular neutron reflectometry (SNR) determines the reflectivity of a neutron beam from a sample at a reflection angle the same as the angle of the incident neutron beam. The reflectivity, R , is defined as the ratio of the number of neutrons reflected to those in the incident beam:

$$R = r^2 = \left[\frac{q - (q^2 - q_c^2)^{1/2}}{q + (q^2 - q_c^2)^{1/2}} \right]^2 \quad (2.8)$$

where q is the wave transfer vector ($q = 2k_i \sin \theta$).

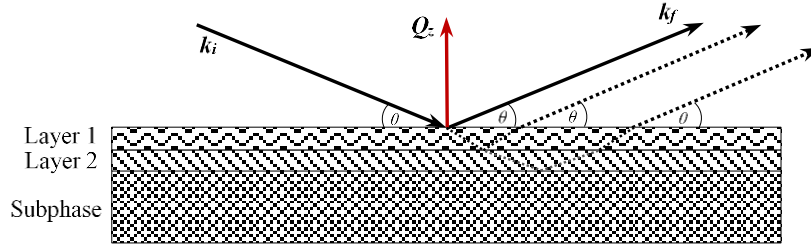


Figure 2.8 Momentum transfer (Q_z) is shown to be perpendicular to the sample.

The momentum transfer, Q_z , is defined as the change in momentum of the neutrons after their reflection off the sample with respect to the sample normal, where the subscript z represents the direction perpendicular to the surface (Figure 2.8). For bare substrate or very sharp interfaces, the reflectivity can be described by:

$$R(Q) \approx \frac{16\pi^2 \beta_s^2}{Q_z^4} \quad (2.9)$$

where β_s is the SLD.

When scattered by a sharp interface or single material, the reflectivity falls as a function of Q_z^4 , an effect which depends on the structure/composition of the surface. A reflectivity profile is defined as a plot of R as a function of Q_z , in which when Q_z approaches 0, $R \leq 1$. For a monolayer at the air/water interface, the system can be considered as air/monolayer and monolayer/water interfaces. Reflection at the top interface is always accompanied by refraction of the transmitted beam, which leads to a second reflection occurring at the bottom interface.

There is a phase shift in the wavelength between the second reflected beam and the first incident beam, which can be attributed to the difference in refractive index (n) of the two different phases. According to Snell's law of refraction:

$$\sin\theta_1 n_1 = \sin\theta_2 n_2 \quad (2.10)$$

where θ_1 and θ_2 are the incident angle and refracted angle, n is the refractive index of the respective medium. In the case of neutrons, the refractive index is related to SLD of the material.

Total reflection ($R = 1$) will occur when the beam incident moves from air to a material with a lower refractive index (or higher SLD) at an incident angle below a critical angle, which means below a critical value of Q_z . When reflected off a thin film or multiple layers, the reflectivity will not decrease smoothly as a function of Q_z^4 , and Keissig fringes will appear within a repeat Q_z range (ΔQ_z) in proportion to the SLD depth profile ($d\beta/dz$). The SLD profile is the product of Fourier transform of the reflectivity profile, obtained via modelling and fitting of the reflectivity profile. Information about the surface structures, its thickness, roughness and hydration of the interface, therefore, can be obtained from the co-refined modelling of the neutron reflectivity profiles recorded in multiple contrasts.

There are two different instrumental approaches to obtaining a reflectivity profile, using monochromatic reflectometer or a TOF reflectometer. The former type of instrument records data over a wide range of incident angles using a fixed wavelength. The second instrument uses a wide range of wavelengths and one or more narrow incident angles. For individual experiments, the TOF approach may be a better choice, as it results in recording a wide Q in a single measurement. Indeed, measurement of the whole Q_z range simultaneously allows the study of the kinetics of a sample.

Figure 2.9 shows a basis schematic of the main components of a TOF reflectometer. The monitors are low efficiency detectors that are used to normalize the detector signals, while detectors themselves are of high efficiency. The beam from the neutron source passes through a monitor and slits that collimate the beam. Then one or more frame overlap mirrors select the required wavelengths of neutrons by removing too long and/or too short wavelengths of neutrons. The super mirror is used to change the incident angle (θ_i) for systems such as the air/water interfaces where the sample cannot be changed (angled) to suit the incident beam. In specular reflection, the incident angle is equal to the reflected angle (θ_r), while in off-specular reflection the two angles are different. The detector is located at an angle of $2\theta_{\text{det}}$.^[113, 114]

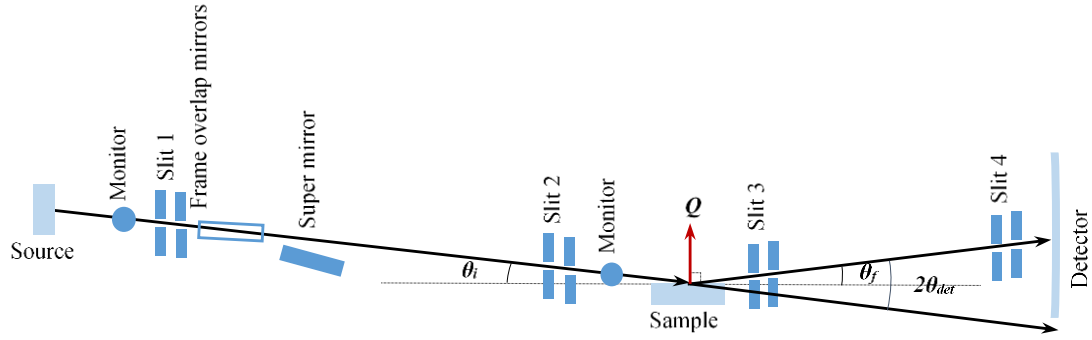


Figure 2.9 The main components of TOF reflectometer on a pulsed neutron source, *e.g.* ISIS.

In a surfactant system at the air/liquid interface, the surface excess (Γ) for a single component, can be obtained from measurement using:

$$\Gamma = \frac{\beta_s d \phi_i}{b_i N_A} \quad (2.11)$$

where β_s is the SLD of the component, d is the thickness of the layer, ϕ_i is the volume fraction of the component in the layer, b_i is the scattering length of the component, N_A is Avogadro's number. The value of the surface excess is therefore determined by β_s and d . When performing model fitting therefore β_s and/or d have to be fitted. In addition, a parameter accounting for the solvent penetration of the layer (SP) in % also need to be considered, which is related to ϕ_i . Note that the fitted β_s is the average value of all the components in the layer.

Since SNR is sensitive to the difference in nuclei, isotopic substitution can be used to resolve the composition and structure of a material of interest. Therefore, selectively deuterated materials are frequently used in SNR, due to the big difference in scattering length between ^1H and ^2H , -3.739 fm and 6.671 fm respectively. Air contrast matched water (ACMW) is also frequently used to make the solvent 'invisible' in SNR experiments. ACMW has a scattering length density that equals that of air and is prepared by adding 8.1% v/v D_2O to H_2O . A recent study of a polymer-surfactant mixture on the air/water interface has exploited several different isotopic contrasts, namely, *d*-SDS/ACMW, *h*-SDS/ D_2O , *d*-SDS/ D_2O and *h*-SDS/ACMW.^[115] Different isotopic contrasts can be applied to highlight the components of interest or to make component invisible to neutrons by mixing *h*- and *d*- materials.

Through simultaneous fitting of the multi-contrast data the complex structure of the system can be resolved.

Low Q analysis is an accurate and easy approach to determine the surface excess of a single component. The reflectivity of a thin layer of the component on ACMW in the low Q region is directly related to its surface excess, *i.e.* the product of β_s and d ^[115]:

$$\beta_s d = \Gamma b N_A \quad (2.12)$$

The structural details of the thin layer are seen in the data obtained at higher Q yet are virtually independent of the data in the low Q range. Determining only one scattering angle at low Q , can take only a few seconds, much quicker than the full Q -range measurement. As a result, a short scan at very low Q can be used to investigate the kinetics of a process.^[115] For a two-component system, the product $\beta_s d$ can be expressed as the sum of the two components:

$$\beta_s d = N_A(\Gamma_1 b_1 + \Gamma_2 b_2) \quad (2.13)$$

The analysis of a given component in a two-component system normally involves two measurements with different contrast in the interfacial layer, for example, measurements for a given component in both protiated and deuterated forms, both in ACMW and with the other component protiated. Hence the surface excess of the given component can be resolved from the difference in the product $\beta_s d$ of the two measurements, while the surface excess of the other component is resolved from scattering of the more contrast-matched measurement above the background. In the present study, this method was used to determine drug-membrane interactions.

In this work, two TOF reflectometers were used applied to investigate the kinetics of the interaction of the drug with a lipid membrane as well as the structure of the resulting drug/lipid system.

2.3.3 Langmuir trough

A Langmuir trough is a piece of commonly used apparatus to produce monolayers of water-insoluble amphiphilic materials such as lipids at the air/water interface. The trough is normally equipped with one or two barriers that can be moved across the interface to change its interfacial area. After

spreading a solution of the amphiphilic material of interest in a volatile liquid onto the interface, it is possible to compress or expand the resulting monolayer of amphiphilic molecules by moving the barriers to change its interfacial area. The surface pressure of the amphiphilic monolayer is constantly monitored during its compression and expansion. Surface pressure is defined as the difference in the surface tension of the pure sub-phase and the surface tension of the monolayer^[1]. A Wilhelmy plate is generally used to measure the surface tension of the amphiphilic monolayer which is then converted using the surface tension of pure water to surface pressure.

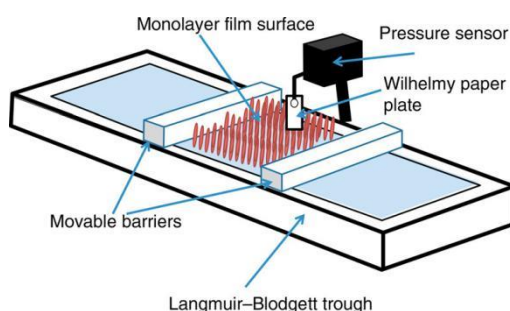


Figure 2.10 Diagram of a Langmuir trough with lipid monolayer. Adapted from reference [107].

2.3.4 Ellipsometry and Brewster angle microscopy

Ellipsometry is a surface technique that uses polarized light to measure the properties of thin films at an interface both precisely and quickly. Compared with neutron reflectometry, ellipsometry is a faster and simpler way to investigate film properties with high precision and sensitivity. Indeed, it is often used in collaboration with the SNR to obtain comprehensive information about the interfacial layers of interest.

Ellipsometry involves directing a beam of polarized light, typically a laser, onto the surface film/monolayer and using a detector to measure its reflection off the sample at the same angle as the angle of the incident light. The incident light beam can be considered as being linearly polarised in two directions, s-polarisation and p-polarisation. The incident plane is defined as the plane in which the incident light and the reflected light lie, s-polarisation is oriented perpendicular to the incident plane, while the p-polarised light is oriented parallel to the incident plane.

The Fresnel equation (or coefficient) describes the reflectivity (R) of the light in terms of the ratio of the reflected and transmitted beam to that incident, given by:

$$R = \left| \frac{n_1 - n_2}{n_1 + n_2} \right|^2 \quad (2.14)$$

where n_1 and n_2 are the refractive index of the incident and refracted phase, respectively. Furthermore, the Fresnel coefficient can distinguish between the s-and p-polarisation of the light source, and are referred to as R_s and R_p , respectively.

Figure 2.11 shows a schematic of an ellipsometer. As can be seen on the left-hand side of the schematic there is a laser light source on the far end of the incident arm. Next on the arm is a quarter wave plate (QWP), a polariser (P_o) and a compensator (C). When the laser light passes through a quarter wave plate it becomes circularly polarised, however when the laser has passed through the polariser it becomes linearly polarised with a specific orientation. This linearly polarised light then passes through a compensator, which converts the light into an elliptical polarisation, after which the elliptically polarised light falls onto the sample. Once the light is reflected off the sample, it passes through a second polariser, known as an analyser (A_n) contained on the reflection arm, after which the light is measured by a detector. The principle of the null ellipsometer is that according to the optical properties of the interface, there is a specific elliptical polarisation tuned by the polariser P_o that will reflect from the sample to produce linearly polarised light, which can then be extinguished to zero by a specific orientation of the analyser A_n . Practically, the machine works by rotating both P_o and A_n until no light is detected, and for this reason the instrument is often called a ‘null ellipsometer’.

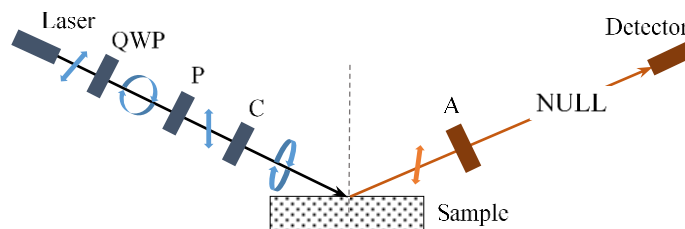


Figure 2.11 The basic components and laser path of an ellipsometer.

The ellipsometric measurement is based on the changes in polarization of the reflected light, which refers to two indexes, namely the amplitude ratio (Ψ) and the phase shift (Δ). Ψ and Δ are determined by the angles of the polariser P and the analyser A. When the polariser and analyser are under nulling conditions, Ψ and Δ are given by:

$$\Psi = A \quad (2.15)$$

$$\Delta = 2P + \frac{\pi}{2} \quad (2.16)$$

As there are two phases at any fixed angle of the compensator, $A_1 = -A_3$, $P_3 = P_1 + \frac{\pi}{2}$, Ψ and Δ are described as:

$$\Psi = \frac{A_1 - A_3}{2} = A \quad (2.17)$$

$$\Delta = P_1 + P_3 = 2P + \frac{\pi}{2} \quad (2.18)$$

In an ideal case *i.e.* where there is no surface roughness, the relationship between Ψ and Δ can be described as the ratio of p-polarised reflection to s-polarised reflection, a value which is called the coefficient ellipticity (ξ), and is given by:

$$\xi = \frac{r_p}{r_s} = \tan \Psi e^{i\Delta} \quad (2.19)$$

where $\tan \Psi$ is the amplitude ratio after reflection. As Δ is the phase shift in wavelength, the thickness of a film (d) at an interface can be calculated from Δ , on the basis of some model assumptions. The density of the measured thin film is denoted by ρ . Indeed, the change in Δ of a given measurement can be considered as the extra optical path length that the light transmits as it refracts then reflects through a film present at the interface (Figure 2.12).

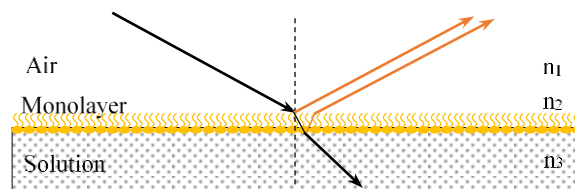


Figure 2.12 The path of light when refracts and reflects though an amphiphilic monolayer.

The incident angle used for ellipsometry experiments is close to a specific angle called Brewster angle, where the p-polarised incident light is totally refracted at an interface. At the air-water interface, the Brewster angle is at 53.1° for visible light, around which the change in Δ for a given thin film at interface reaches a maximum, while the value of Ψ is relatively insensitive for thin, non-absorbing interfacial layers. The surface excess can be obtained from the data obtained by modelling the interaction of light with samples of differing optical properties. However, since the surface excess is related to the product of ρ and d , it is not possible to calculate the value of both ρ and d from the surface excess without making assumptions about the nature of the layer itself. In contrast, at the solid-liquid interface, for example silicon or silica in contact with a liquid, the maximum changes observed in both in Ψ and Δ are observed near the Brewster angle, making it possible to obtain both ρ and d from the results, which helps to resolve the structure at the interface.

To date, ellipsometry has been applied to the study of surface properties. For example, Howland and colleagues^[71] have studied the physical properties of phospholipid membranes using imaging ellipsometry, in which the detector is a CCD camera. This team demonstrated that imaging ellipsometry can provide clear insight into the lateral details of the layer, which overcomes the limitations of SNR and other techniques. In yet other studies, because ellipsometry alone cannot provide information about composition coupled with the fact that measurements do not need isotopic contrast, it is used in conjunction with NR as a pre-screening method to support the results of SNR and other surface sciences techniques.^[117, 118]

Δ_{delta} is used for analysis, which is the measured Δ of the thin film minus that of pure water. For a fluid film on air/water interface, when the interface is laterally heterogeneous (on the micrometre scale), *e.g.* existence of highly ordered lipid domains, the Δ_{delta} is likely to be very fluctuated, resulted from the signal changes of the heterogeneous regions pass in and out of the laser beam.^[119, 120]

BAM is an imaging technique that is based on the same principles as ellipsometry, but with BAM the incident angle is exactly at the Brewster angle. Since no p-polarised light is reflected at Brewster angle for pure water, only when material such as a thin layer is present at the surface of a liquid is light strongly reflected. The brightness of the BAM images is directly related to a combination of the thickness and other optical properties of the film. Anisotropy of lipid molecules in a monolayer, *i.e.* condensed phase domains, results in differences in its refractive index normal to and in the plane of

the interface, and the reflectivity of light as a result is generally enhanced, resulting in brighter regions of the images acquired.^[121] As a consequence, insight can be gained into any lateral structures present at the surface through use of a CCD camera.

In the present study, both ellipsometry and BAM were used in combination of with Langmuir trough to produce a lipid monolayer at the air/water interface below which the drug in solution was injected into the sub-phase. For the ellipsometric measurements, changes in Δ and Ψ were recorded, while for BAM images of the surface were occasionally taken during the interaction of drug and lipid.

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3 Self-assembly of propranolol hydrochloride in water: understanding mesostructures through experiment and simulation at different length scales

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3.1 Abstract

Small amphiphilic and hydrophobic drug molecules, such as the beta blocker propranolol hydrochloride, self-assemble into supramolecular nanostructures that can affect, amongst other things, their interaction with cellular structures and their ability to reach intracellular targets. There is a strong need for research to understand the conditions under which drugs self-assemble to understand the physiological implications of this behaviour. A combination of UV/vis absorption spectroscopy, ¹H NMR spectroscopy and contrast variation small angle neutron scattering (SANS) experiments coupled with full atom molecular dynamics simulations, have been used here to determine the size, morphology, internal structure, and concentration behaviour of the micelles formed by propranolol hydrochloride. Taken together, the insight gained from these studies allowed a detailed picture of the solution behaviour of propranolol hydrochloride to be gained. A limited aggregation occurred in the concentration range 0.15 – 5 mM indicative a critical aggregation concentration (CAC) *i.e.* the concentration at which pre-micellar aggregates consisting of a few propranolol hydrochloride molecules form. Surface tension measurements, ¹H NMR, contrast variation SANS, and MD simulations all indicated the presence of a CMC at a concentration of around 100 mM. However, the aggregates formed by propranolol hydrochloride at the CMC are not classical micelles such as seen with surfactants like sodium dodecyl sulfate but are small and polydisperse, consisting of a core of naphthalene rings, surrounded by a shell of solvated side-chains. The results show the power of using multiple techniques to understand the self-assembly of drug molecules such as propranolol hydrochloride. It is clear that propranolol hydrochloride exhibits limited self-association at low concentrations such as might be experienced after intravenous injection and so its aggregation will influence the behaviour of the drug in the body.

3.2 Introduction

Nanoscale characterisation of the self-associated species formed by amphiphilic pharmaceuticals in aqueous solution carries relevance across their entire journey from development through to manufacture and use. Knowing the supramolecular architectures of the self-associated species and understanding the mechanism(s) of their formation, is relevant not only as regards formulation of the drug products as medicines, but also potentially to their bioavailability, activity, and clinical side effects. More widely, such knowledge will contribute too to our understanding of the molecular mechanism(s) leading to solute nucleation and crystallisation,^[1] and it will thus provide insight to guide the design of preparation protocols employed in chemicals manufacture, and be pertinent, therefore, not just to pharmaceuticals, but also to dyes, agrochemicals, and foodstuffs.

The first studies of self-association of pharmaceuticals were focussed on the antipsychotic phenothiazines.^[2-4] Experiments performed using a combination of NMR, conductivity, viscosity, and light scattering measurements showed that these drugs form micellar aggregates, with aggregation numbers of 8 – 12, and critical micelle concentrations (CMCs) in the millimolar range. Subsequent research, as reviewed by Schreier *et al.*,^[5] showed that a great many other drugs show similar behaviour, this list including numerous examples of local anaesthetics, analgesics, antibiotics, anti-histamines, and anti-hypertensive agents. As found for the phenothiazines, these drugs are shown to have CMCs typically in the range 10 – 100 mM, with aggregation numbers of the order of 4 – 20.

For those drugs that possess anionic or cationic groups and exhibit poor aqueous solubility, it is common practice to use the device of salt formation as a means to improve drug solubilisation, but if the drug of interest also self-associates in solution, this will likely affect the pK_a of the ionisable group, and this in turn will lead to non-ideality of salt solubility,^[6] confounding the formulation of the required solid dosage form. Moreover, Abramov *et al.*^[7] have shown, by means of crystal growth morphology simulations, that the solution phase aggregation of self-associating drugs can adversely affect the kinetics of their crystallisation, so that crystals are not formed (as desired) with the drug in its thermodynamically most stable polymorphic form but with the drug instead crystallising as a metastable polymorph. Transformation of the metastable polymorph to the more stable form either in the scale-up for manufacturing or over the course of the marketed product shelf-life will necessitate a costly reformulation – as proved necessary for GSK's Zantac, Abbott Laboratories' Norvir, and USB's Neupro.^[8]

Issues also arise for liquid formulations, such as ophthalmic preparations, wherein the micelles or aggregates formed by self-associating drugs solubilise sparingly soluble additives in the preparations, thereby reducing their physical and/or microbiological stability.^[9]

While the circulating concentrations of self-associating drugs required to achieve therapeutic effect will generally be significantly lower than their CMC, it is nevertheless likely that local concentrations of the drugs might exceed their CMC, and in this event, their self-association is likely to have adverse consequences on their rate of passage across membranes and into cells. Such a consequence was demonstrated by Khan *et al.*^[10] who showed that self-association leads to a reduced cellular bioavailability of DNA minor groove-binding terbenzimidazoles. Likewise, Seelig *et al.*^[11] showed a link between the CMC of self-associating drugs and their capacity to penetrate the blood-brain barrier, and Inacio *et al.*^[12] showed that the kinetics of (passive) transport of tetracaine across both artificial membranes and pig skin showed significant variation as a function of the drug's aggregation state.

In addition, if the local concentration of a self-associating drug in the vicinity of a cell membrane rises sufficiently high, such that partitioning of the drug into the membrane leads to a locally high drug:lipid ratio, there is the further possibility that the drug might cause lipid flip-flop, solubilisation, or non-bilayer phase formation,^[13] and the resulting membrane disruption might then manifest as clinical side effects.

Even within the drug discovery stage of pharmaceutical development, there are complications posed by the phenomenon of molecular self-association. Studies by Owen *et al.*^[14] have shown that self-association can lead to reduced activity when measured in cell-based assays, and studies by Feng *et al.*^[15] and Seidler *et al.*^[16] clearly show that target promiscuity and a high incidence of false-positives in high-throughput-screening against soluble protein targets can often be attributed to the presence of aggregates formed through the self-association of lead compounds.

Whereas the prevalence and consequences of drug self-association are well-reported in the literature, there are very few studies as yet performed that have provided experimental detail on the structure of these small micellar aggregates at the atomic scale (*e.g.*, Perez-Villar *et al.*^[17]), and only a few studies reported that explore their behaviour in computer simulation (*e.g.*, Kozłowska *et al.*^[18]). Indeed, as regards the latter, the studies performed to date have served to reveal a number of key

challenges. The first of these is the selection of starting geometry for the simulation. This choice can be critical in determining whether appropriate structures are sampled. It has often been necessary to use experimental observations to impose constraints on the starting geometry (*e.g.* NMR-derived distance constraints) or to use computational protocols (such as annealing-based strategies to greatly speed up sampling) in order to provoke aggregation.^[19] These tackle the problem that some aggregates, especially highly ordered ones, form over timescales inaccessible to most simulation protocols.^[20] A second challenge is the selection of an appropriate parameterisation for the solute and for water^[21] both of which have important influences on the outcome but, as in many other areas, tensioning of computational observation against experimental observables provides a powerful means to validate any computational results.

In the work reported here, we sought to rectify these various deficiencies, using a combination of UV/vis absorption spectroscopy, ¹H NMR spectroscopy and contrast variation SANS experiments coupled with full atom molecular dynamics simulations, to determine the size, morphology, internal structure, and dynamic behaviour of the micelles formed through self-association of the beta blocker drug, propranolol hydrochloride. This important drug is a widely used for the treatment of high blood pressure, hyperthyroidism and in some cardiac arrhythmia scenarios as well for prevention of migraines. Studies of the type performed here are important because while a number of small, amphiphilic drug molecules such as propranolol hydrochloride have been reported to self-assemble into supramolecular nanostructures, it is not understood the impact their aggregation and the concentration as which is occurs has on the biological performance of active compounds.

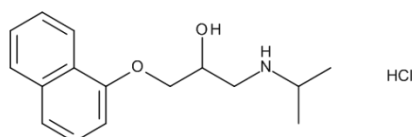


Figure 3.1 Molecular structure of propranolol hydrochloride.

3.3 Materials

(±)-Propranolol hydrochloride (99.9 %) was purchased from Alfa Aesar. (±)-*d*₇(ring-deuterated)-propranolol hydrochloride was obtained as the salt (±)-*d*₇(ring-deuterated)-propranolol

hydrochloride (88%- d_7) synthesised by the ISIS Deuteration Facility, Rutherford-Appleton Laboratory, STFC, Didcot, Oxford (Supplementary Information, Figure SI - 3.1). Deuterium oxide (D_2O , 99.9 atom % D), dichloromethane (DCM, $\geq 99.8\%$) and sodium chloride (NaCl, 99.9 %) were obtained from Sigma Aldrich, while chloroform ($CHCl_3 \geq 99.8\%$) was supplied by Fisher Scientific. Milli-Q (ultra-pure) water, with the resistivity of 18.2 m Ω .cm, was obtained from a Suez pure water system.

Purification of propranolol hydrochloride

Propranolol hydrochloride is reported to be unstable under light, yielding 1-naphthol, *N*-acetylpropranolol and *N*-formylpropranolol (Supplementary Information, Figure SI - 3.2). To purify propranolol hydrochloride, it was dissolved in water at a concentration of 200 mM, washed three times with an equal volume of chloroform, after which the propranolol hydrochloride-containing aqueous solution was freeze dried at 216 K and 0.48 mbar for at least 12 hours (Christ Beta 2-8 LSCbasic Freeze Dryer, Wem, UK) to yield a solid (designated here as 'purified' propranolol hydrochloride) which was used to prepare propranolol hydrochloride solutions for surface tension and SANS experiments immediately after purification. Purification was assessed by surface tension measurements (Supplementary Information, Figure SI - 3.3).

3.4 Methods

3.4.1 Solubility measurements

The solubility of (protiated) propranolol hydrochloride in H_2O and D_2O was determined at room temperature (295 ± 2 K) and at 310 ± 1 K in quadruplicate. An excess of propranolol hydrochloride was added to H_2O/D_2O contained in a microfuge tube which was then sealed, protected from light, and mechanically agitated on a rotating wheel for 10 days at the required temperature, after which time the samples contained in the microfuge tubes were centrifuged at $185.6 \times g$ (Beckman Microfuge 16, Beckman Coulter, High Wycombe, UK) for 5 minutes to sediment any excess propranolol hydrochloride. A 10 μ L aliquot of the resulting supernatant was diluted 2000-fold using the appropriate aqueous solvent. The amount of propranolol hydrochloride dissolved was established by measuring the UV absorbance of the solution at the wavelength of the maximum

absorbance,^[22] at a resolution of 1 nm using a PerkinElmer Lambda 365 UV/vis spectrometer (Waltham, USA) (Supplementary Information Figures SI - 3.4 and SI - 3.5).

Table 3.1 Solubility (mM) of propranolol hydrochloride in H₂O and D₂O at 295 K and 310 K.

<i>Temperature (K)</i>	<i>H₂O</i>	<i>D₂O</i>
295 ± 2	252 ± 7	133 ± 4
310 ± 1	474 ± 14	375 ± 7

3.4.2 Surface tension measurements

Surface tension measurements (EzPi, Kibron, Helsinki, Finland) were performed using the Wilhelmy plate method (inert metal alloy), to determine the surface activity of propranolol hydrochloride (either as purchased or specifically purified) in aqueous solution at room temperature. Propranolol hydrochloride (300 mM) was dissolved in ultrapure water (surface tension of 72.2 mN m⁻¹ at ambient) and its surface tension measured as a function of concentration. Surface tension was recorded every 15 seconds until it reached equilibrium, and the last 10 points were used to determine the average surface tension at each concentration. At propranolol hydrochloride concentrations of 150 – 300 mM surface tension measurements took 30 min, at concentration around the CMC (50 – 150 mM) measurements took 1 h, while at concentrations of less than 50 mM measurements took about 20 min.

Significantly, the surface tension of the ‘as purchased’ propranolol hydrochloride displayed a distinct minimum (comparable to that seen with sodium dodecyl sulfate (SDS) contaminated with its surface-active degradation product, dodecanol). As with SDS, where contaminant dodecanol can be removed through purification,^[23] the process of purification of propranolol hydrochloride largely removed the minimum which was attributed here to the presence of surface active 1-naphthol^[24] used in the synthesis of propranolol hydrochloride.^[25] The purification of propranolol hydrochloride in the present study was successfully performed by washing the saturated aqueous propranolol hydrochloride solution three times with an equivalent volume of chloroform (Supplementary Information, Figure SI - 3.3).

3.4.3 UV/vis absorption spectroscopy

The UV spectra of propranolol hydrochloride in H₂O at ten concentrations over the range 0.011 mM – 0.45 mM were measured at room temperature in a 10 mm path length cell over the wavelength range from 200 – 350 nm, at a resolution of 1 nm, using a PerkinElmer Lambda 365 UV/vis Spectrometer (Waltham, USA). In order to compare the effect of concentration on the spectra, the data were normalised to an absorbance value of 1 at a wavelength of 214 nm.

3.4.4 ¹H NMR spectroscopy

¹H NMR measurements of various concentrations of propranolol hydrochloride solution in D₂O over the range 2.5 mM – 180 mM were made at room temperature over the range -4 to 16 ppm at a resolution of 0.003 ppm using a Bruker Avance-III HD 500 spectrometer (Karlsruhe, Germany). The 180 mM stock solution was made using bath sonication at supersaturation state. DCM, chemical shift of 5.33 ppm, was used as external standard. Analysis of the NMR data was performed using MestreNova 14.2 (Mestrelab Research, Spain).

3.4.5 Contrast variation small angle neutron scattering

Small angle neutron scattering studies were performed on the SANS2D beam line at the ISIS pulsed neutron source (ISIS, Rutherford-Appleton Laboratory, STFC, Didcot, UK). The pattern of scattering of neutrons after passage through the sample was recorded on a 96.5 x 96.5 cm² two-dimensional detector at a distance of 2.4 m from the sample. As SANS2D utilizes neutrons with wavelengths in the range 2 - 14 Å, the geometry yielded a scattering vector, $Q = 4\pi\sin(\theta)/\lambda$ in the range of $0.0045 \leq Q \leq 0.4 \text{ Å}^{-1}$. All samples were measured in disc-shaped quartz cuvettes (Hellma UK Ltd) of 1 mm or 2 mm path length (depending upon the deuterium content of the solvent) using a 12 mm diameter neutron beam and measured at $298 \pm 0.1 \text{ K}$. Comparisons with scattering from a partially deuterated polystyrene standard allowed determination of the absolute scattering cross-section of the sample (in units of cm⁻¹), with an error of approximately $\pm 2 \%$. After correction of the SANS data due to

instrumental factors, the scattering intensity ($I(Q)$) of the sample, was calculated as a function of scattering vector (Q) after subtraction of the scattering intensity due to the solvent.

Stock solutions of protiated and partially deuterated d_7 -propranolol hydrochloride in H_2O (h - or d_7 -propranolol hydrochloride/ H_2O) and in D_2O (h - or d_7 -propranolol hydrochloride/ D_2O) were prepared and diluted with the appropriate aqueous solvent to form solutions of concentration in the range 110 mM – 200 mM. With the exception of 200 mM propranolol hydrochloride, where it was not possible to prepare deuterated d_7 -propranolol hydrochloride in D_2O at room temperature due to the lower solubility of the partially deuterated form of the drug in D_2O , 4 isotopic contrasts of propranolol hydrochloride were measured, namely h - or d_7 -propranolol hydrochloride in H_2O and h - or d_7 -propranolol hydrochloride in D_2O . At propranolol hydrochloride concentrations of 60, 70 and 90 mM, only 2 contrasts (namely h -propranolol hydrochloride/ D_2O and d_7 -propranolol hydrochloride/ H_2O) were measured as the scattering from the other two contrasts was too low. A similar series of freshly purified protiated h -propranolol hydrochloride in D_2O solutions were prepared in order to determine whether there was any difference in the resultant scattering behaviour, and here only 1 ‘contrast’ was studied. Significantly, the scattering and the resultant aggregation numbers of the aggregates for the ‘as purchased’ and ‘purified’ propranolol hydrochloride were virtually identical (Supplementary Information, Figure SI - 3.6 and Supplementary Information, Table SI - 3.1) and the suggesting that the likely presence of surface active 1-naphthol had no effect on the bulk aggregation of propranolol hydrochloride and so the ‘as purchased’ propranolol hydrochloride was used for all remaining SANS studies. Unfortunately, it did not prove possible to correct the SANS data in the present study for the CMC as it was not possible to measure a concentration of propranolol hydrochloride low enough to detect only the presence of monomers.

Scattering intensity is described in terms of a form factor, $P(Q)$, and a structure factor, $S(Q)$:

$$I(Q) \propto \frac{N}{V} \langle \Delta\rho \rangle^2 V_p^2 P(Q) S(Q) \quad (3.1)$$

where N is the number of particles; V is the total volume of the system and V_p is the volume of a particle; $\Delta\rho$ is the difference in scattering length density (SLD) of the particle and the solvent. The term $\frac{N}{V} \langle \Delta\rho \rangle^2 V_p^2$ can be used to calculate the molecular weight of the particle. In the present study, a variety of form factors [$P(Q)$] were investigated including triaxial ellipsoids of uniform scattering length density, and core-shell spherical, prolate, and oblate spheroids, together with structure

factors $[S(Q)]$ of the Hayter-Penfold (H-P) and hard sphere models for charged^[26, 27] and uncharged aggregates.^[28] Model-fitting of the data was performed using the program SasView.^[29] The 3 or 4 contrasts measured for each propranolol hydrochloride concentration were modelled simultaneously (over the Q range 0.02 to 0.5 $\text{\AA}^{-1}$) using the SasView package^[29] to obtain the best fit to the data. When modelling the data, a number of parameters were fixed namely, the SLD of the solvent (H_2O or D_2O) and the SLD of the propranolol hydrochloride and, for the core-shell models, the SLD of the particle core. For the Hayter-Penfold $S(Q)$, the dielectric constant of the solvent and temperature were fixed.

The chemical structures of propranolol hydrochloride and its apolar and polar moieties are shown in Figure 3.2, and the volumes, atomic scattering lengths, and SLDs of these species are given in Table 3.2.

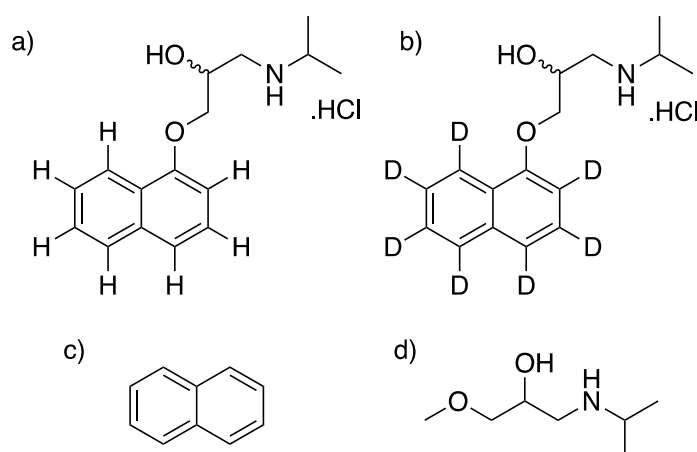


Figure 3.2 Molecular structures of protiated (h-) (a) and deuterated (d-) (b) propranolol hydrochloride and the fragments (c): naphthalene ring and (d): 1-oxy-3-propan-2-ylamino)propan-2-ol side chain.

Table 3.2 Scattering lengths, molecular volumes and scattering length densities of the molecular constituents of the solvent and drug studied.

<i>Molecule/Fragment</i>	<i>Scattering length ($10^{-5}\text{\AA}$)</i>	<i>Molecular volume ($\text{\AA}^3$)</i>	<i>Scattering length density ($10^{-6}\text{\AA}^{-2}$)</i>
<i>Naphthalene ring (protiated)</i>	40.28	183 [†]	2.20
<i>Naphthalene ring (d₇, 88% D)</i>	113.16	183 [†]	6.18
<i>Propranolol HCl side chain</i>	9.32	264	0.35
<i>Propranolol HCl (protiated)</i>	54.63	447 [†]	1.22
<i>Propranolol HCl (d₇, 88%D)</i>	127.50	447 [†]	2.65
<i>H₂O</i>		30	-0.56
<i>D₂O</i>		30	6.38

[†] volumes calculated from density and molecular weight data (PubChem, 2021)

3.4.6 Molecular dynamics simulations

All atom molecular dynamics (MD) simulations of the aggregation of propranolol hydrochloride in aqueous solution were performed using GROMACS 2018.4.^[30] Spontaneous aggregation was sought and thus no initial geometric constraints or structuring were imposed. However, initial simulations in which distributions of each of the two enantiomers of propranolol were added sequentially revealed inhomogeneities that likely reflected the initial conditions. To avoid this issue, the initial distribution was constructed using the GMX insert-molecules protocol with a molecular structure including a racemic pair (LPR and DPR) of molecules present in a 1:1 ratio. The initial box size was 15 nm × 15 nm × 15 nm. Subsequently, water was added using GMX solvate with default settings. Finally, GMX insert-molecules was used again in order to add a number of chloride ions equal to the number of molecules of propranolol in the simulation box, with the added chloride ions replacing solvent molecules. The GROMOS 53A6 forcefield was used with parameters for the propranolol molecules obtained from PRODRG version AA180301.0717.^[31] Water was modelled using the SPC model.^[32]

The simulation box was first energy minimised using the steepest descent optimisation in GROMACS with default settings (emtol, 10 kJ/mol and emstep, 0.01 nm). The resulting geometry was then subject to an initial NVT equilibration of 200 ps duration and a timestep of 0.001 ps. The Verlet cutoff scheme with cubic periodic boundary conditions was employed for defining neighbours. The particle mesh Ewald and potential-shift-Verlet scheme were employed for electrostatics and van der Waals interactions. The Nose-Hoover method for temperature coupling with a 0.2 ps time constant and reference temperature of 300 K was employed. This was followed by a 200 ps NPT simulation that was then used to create a starting geometry for NPT ensemble production runs, each of 20 ns duration. The NPT simulations used a timestep of 0.001 ps, the Verlet cutoff scheme, and cubic periodic boundary conditions for defining neighbours. The PME and potential-shift-Verlet scheme were employed for electrostatics and van der Waals. The Nose-Hoover method for temperature coupling with a 0.2 ps time constant and a reference temperature of 300 K was employed. Pressure coupling used the Parrinello-Rahman method with a time constant of 2 ps, compressibility of $4.5 \times 10^{-5} \text{ bar}^{-1}$ and a pressure of 1 bar. The variation in the average aggregate size was followed during the simulations. Typically, during the first and second 20 ns runs this was seen to increase and then, during the third run, equilibrium was achieved. Hence for most concentrations, three 20 ns simulations were performed. The lowest (10 mM) concentration system achieved equilibrium in the

first run and so only two runs were required. The equilibration is described in more detail in the results section.

The target propranolol hydrochloride concentrations were simulated at 10 concentrations in the range 10 – 200 mM (Table 3.3). In order to construct a racemic system, even numbers of propranolol hydrochloride molecules needed to be included in the simulation box and as a consequence small changes in box volume were found to occur and so there were slight differences between the final concentrations and the target concentrations. The numbers of propranolol hydrochloride molecules used in each case, the resulting box size, and hence the final drug concentrations are shown in Table 3.3, along with the numbers of water molecules in each of the simulated systems.

Table 3.3 Description of the simulated systems of propranolol hydrochloride in water: for each system, the number of propranolol hydrochloride and water molecules are presented.

Target propranolol hydrochloride concentration (mM)	Number of propranolol hydrochloride molecules per box	Final box dimensions (nm)	Final propranolol hydrochloride concentration (mM)	Number of solvent (H₂O) molecules per box
200	406	14.926	202.7	103035
170	344	14.929	171.7	103884
150	304	14.926	151.8	104454
130	264	14.936	131.6	105004
110	222	14.926	110.8	105592
90	182	14.941	90.6	106177
70	142	14.925	70.9	106711
50	102	14.938	50.8	107248
30	60	14.932	29.9	107859
10	20	14.938	10	108385

3.4.7 Computation of $I(Q)$ versus Q profiles from atomistic molecular dynamics models

The scattering form factors for the individual drug aggregates found in the MD trajectory, were calculated using the SasCalc routine^[33] as provided in the SASSIE program^[34] and accessed through the SASSIE-web utility.^[35, 36] The scattering form factors for the individual aggregates, calculated with a converged number of Golden vectors, were computed for the protiated drug in D₂O, sampling over the Q -range, 0.02 Å⁻¹ to 0.56 Å⁻¹, at intervals of 0.02 Å⁻¹, and with $I(0)$ arbitrarily set at 1.0. The Cartesian co-ordinates for the aggregates were input to SasCalc in Protein Databank format.^[37] The

SasCalc-predicted scattering profiles for the aggregates were then model-fitted in SasView,^[29] using the in-built triaxial ellipsoid model, with the scale factor, particle dimensions, and the background freely optimised, and with the scattering length density for the drug fixed at $2.2 \times 10^{-6} \text{ \AA}^{-2}$, and that for the solvent fixed as $6.38 \times 10^{-6} \text{ \AA}^{-2}$.

3.5 Results

Initial surface tension measurements yielded a CMC of 92.4 mM for the purified propranolol hydrochloride (Supplementary Information, Figure SI - 3.1). The value obtained is slightly lower than that determined by static light scattering and NMR (*viz.*, 124 mM and 108 mM, respectively),^[38] and also lower than the values determined through conductivity measurements of 131 mM.^[39] The apparent discrepancy between reported values is thought to be due in part to different physical properties being used to determine the CMC coupled with the fact that the CMC is in fact a narrow range of concentrations.^[40]

Here a combination of analytical techniques, namely UV and ^1H NMR spectroscopy and SANS have been used alongside atomistic MD simulations to probe the nature of the self-assembly of propranolol hydrochloride over a wide concentration range, spanning 0.011 mM – 200 mM. In order to cover this range of concentrations, UV spectroscopy was used to probe concentrations below the experimentally determined CMC of 92.4 mM, while ^1H NMR studies and SANS in combination with isotopic contrast variation, and atomistic MD simulations extended the concentrations studied from below to above the CMC.

3.5.1 UV/vis titration studies

UV/vis absorption spectroscopy and dilution were used to study concentrations of propranolol hydrochloride ranging from 0.15 mM down to 0.011 mM (Figure 3.3) – a concentration much lower than the observed CMC. There is no significant band shift of the E band and the B band observable indicating that there is no self-assembly seen at concentrations up to and including 0.15 mM.

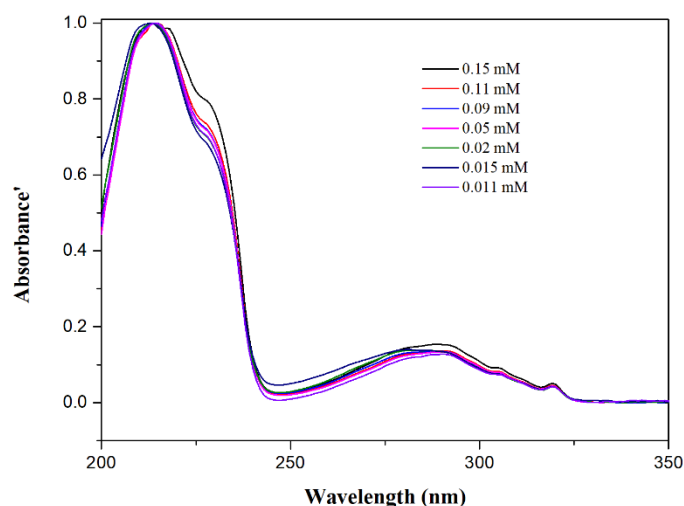


Figure 3.3 UV Titration of propranolol hydrochloride in water at room temperature. The UV spectra were normalised to an absorbance of 1 at a wavelength of 214 nm.

3.5.2 ^1H NMR studies

To probe the aggregation of propranolol hydrochloride in solution at higher concentrations, ^1H NMR spectra were recorded as a function of concentration over the range 2.5 mM – 180 mM (Figure 3.4). For aggregators, the proton signals of the molecule are likely to show concentration-dependent chemical shifts, while for non-aggregators the shifts are expected to be concentration-independent.^[41]

For the solutions of propranolol hydrochloride studied here, all of the observed ^1H NMR signals were shifted downfield upon dilution, and this is consistent with a change in intermolecular interactions stemming from the formation or breaking of aggregates in solution. Moreover, the observed chemical shifts are seen to change continuously over the whole range of titration, suggesting that the drug self-associates in a continuous manner. The magnitude of the changes in the proton chemical shifts, however, are seen to vary in a non-linear manner as a function of propranolol hydrochloride concentration (Figure 3.5), with the higher concentrations showing the larger changes in shift. Processing of the ^1H NMR data following the methodology detailed by Ruso *et al.*^[38] (plotting the second derivative of the shift changes as a function of concentration) yielded a CMC of 90-95 mM (Supplementary Information Figure SI - 3.7), which is consistent with the CMC estimated through surface tension measurements (*vide supra*).

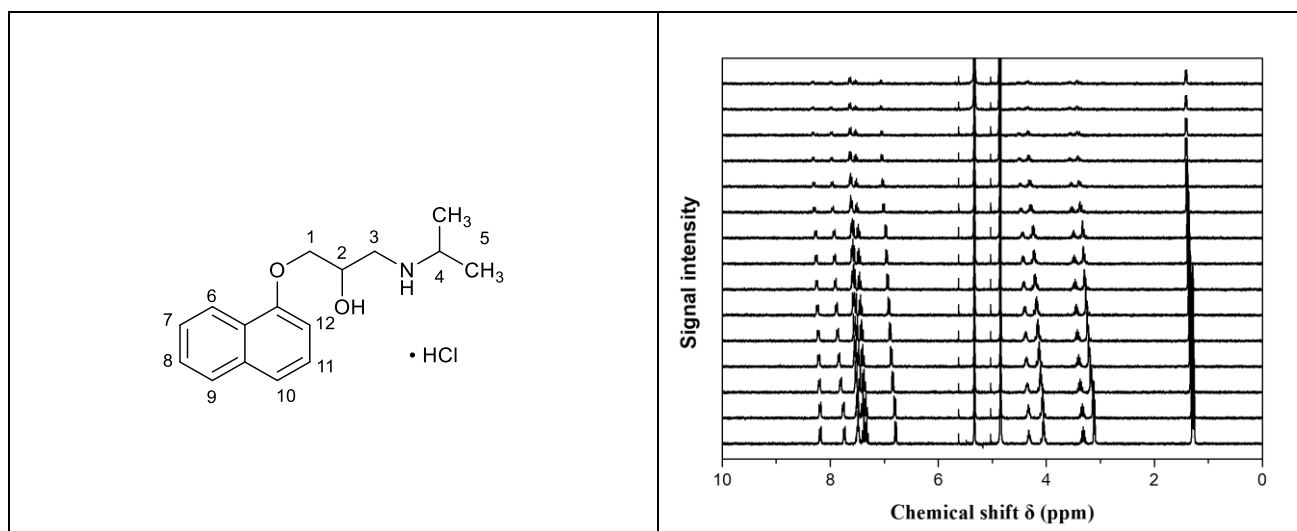
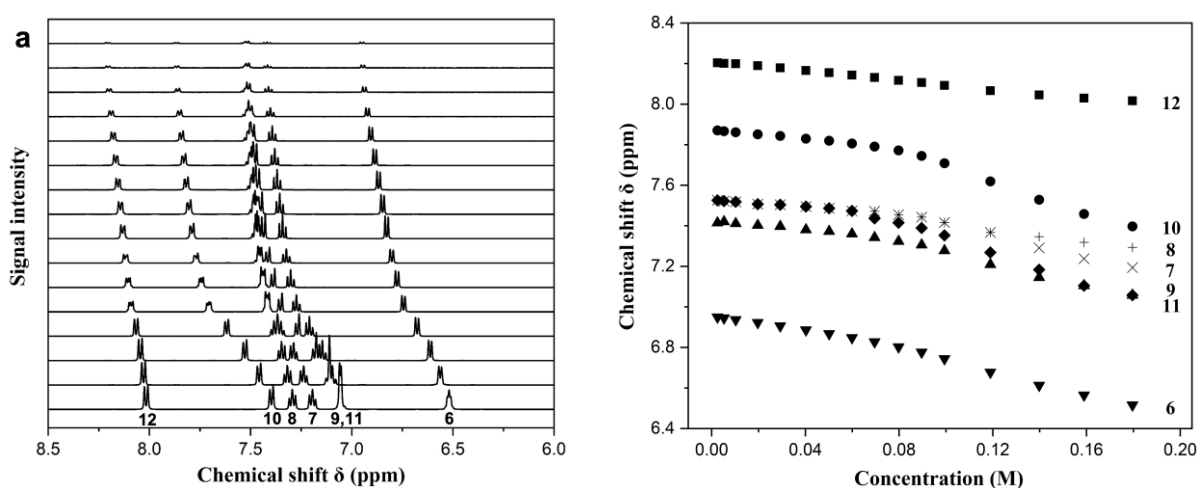


Figure 3.4 (a) Chemical structure of propranolol hydrochloride with protons labelled; (b) ^1H NMR spectra of propranolol hydrochloride in D_2O at a concentration of 180 mM (bottom) through 160, 140, 120, 100, 90, 80, 70, 60, 50, 40, 30, 20, 10, 5 to 2.5 mM (top). The peak at around 4.7 ppm is the peak due to H_2O .

The concentration-dependent changes in the chemical shifts of the protons on the drug's aliphatic side chain and naphthyl ring system are shown in detail in Figure 3.5(a). Comparison of the magnitudes of the changes in shift seen for the various protons, reveals that those on the naphthyl moiety change most upon dilution, and this would suggest that the aggregation of the drug in solution likely involves close intermolecular interaction between the aromatic rings. Since it is also apparent that H_9 and H_{10} show bigger changes in chemical shift than the other aromatic protons, it is concluded that these two protons are the ones that are most affected by the drug's aggregation, and given their position on the unsubstituted long edge of the naphthyl ring system, it seems likely that the self-association of the drug involves significant aromatic edge-to-face ($\text{CH}-\pi$) interaction.^[42, 43]

As with the aromatic protons, the aliphatic side-chain protons also show significant concentration-dependent changes in their chemical shifts, and this would indicate that – in addition to the aromatic $\text{CH}-\pi$ interactions – the self-association of the drug may also involve intermolecular interactions between the side-chains, *e.g.*, through hydrogen bonding, and/or that there are changes in the shifts resulting from changes in the pattern of side-chain hydration. The splitting pattern of the side-chain proton signals is strongly influenced by the stereocentre on carbon 2,^[44] generating an ABMX spin system.^[45] This results in two different chemical shifts for the two protons in positions 1 and 3, which overlap to varying extents due to different shifts arising upon dilution. To gauge this, an iterative spin simulation using the algorithm implemented in TopSpin (Bruker, Karlsruhe, Germany) was performed

on those five protons (Figure 3.6). The shape and position of the simulated peaks are similar to those obtained experimentally (Figure 3.5(b)). The coupling constants do not change much with all these five chemical shifts and change monotonically with concentration, though to slightly different extents (Table 3.4). The signals of the methyl protons in the isopropyl group (Figure 3.5(c)) show two doublets moving closer towards overlap with decreasing concentration. The two doublets suggest that the two CH₃ groups are not equivalent.^[46] The difference in methyl groups may be a consequence of the diastereotopic influence from the stereocentre, causing different magnetic environments of propranolol hydrochloride molecules,^[46] even though the chiral center has considerable distance from the methyl groups. Another reason for these differences may be that the formation of aggregates causes steric hindrance and hinders the free rotation of the isopropyl group.^[46] As the drug aggregates tend to grow larger with concentration (*vide infra*), the steric hindrance tends then to be larger, thereby contributing even more to the non-equivalence of the methyl groups. Two binding models, namely the EK (dimerization/equal K) and CoEK (co-operative equal K) models,^[47] (Table 3.5) were used to model the self-aggregation of propranolol hydrochloride based on the ¹H NMR dilution data using Bindfit.^[48] The EK model, is a dimerisation model used to describe a continuous aggregation for which additional aggregation proceeds with the same aggregation constant as the initial dimerisation. For the CoEK model, further aggregation is co-operative, either positive or negative to the initial dimersation, with different association constants.^[49]



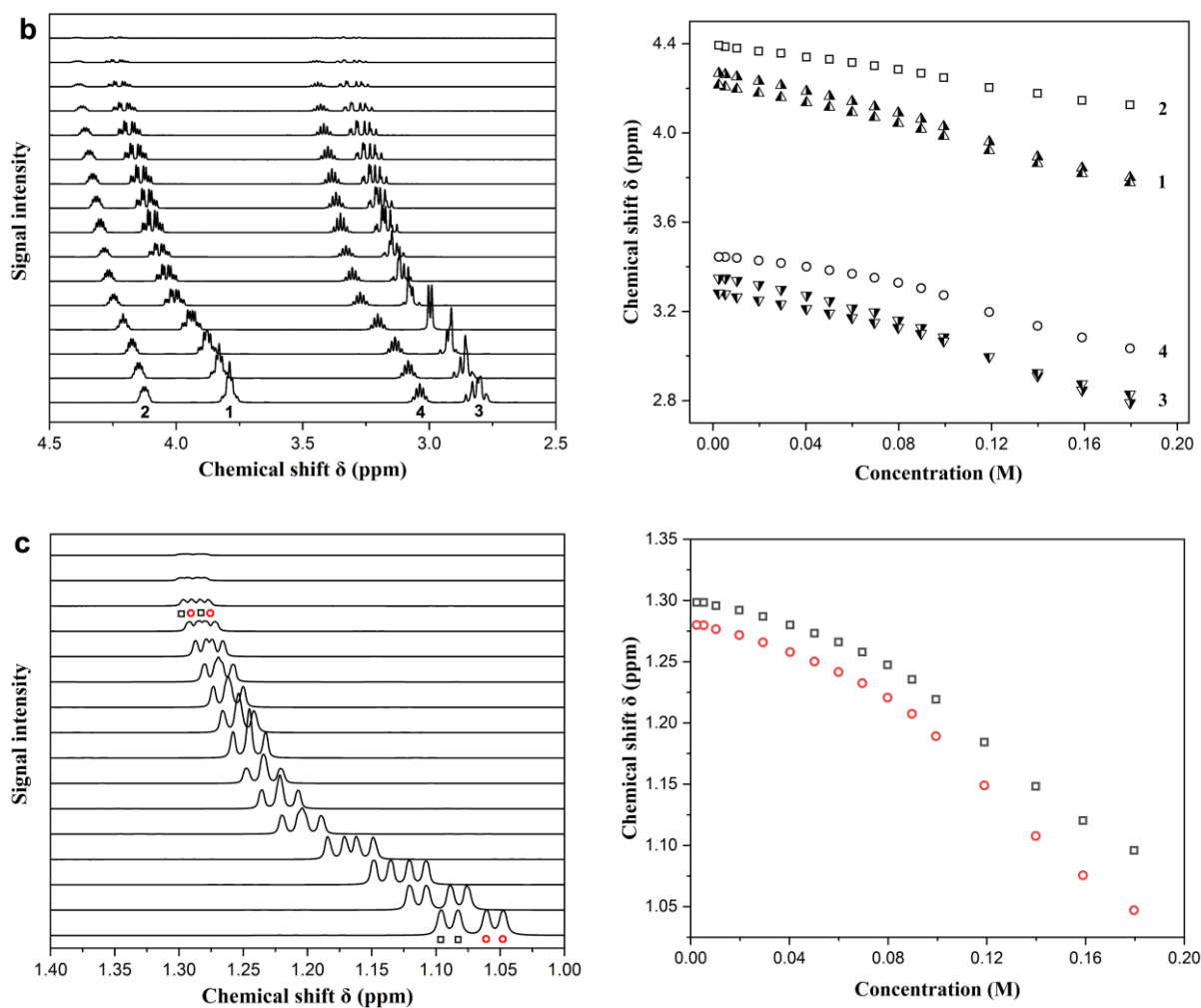


Figure 3.5 ^1H NMR spectra and proton positions of propranolol hydrochloride as a function of concentration in D_2O in (a) the naphthalene ring protons 6 to 12, (b) aliphatic chain protons 1 to 4 and (c) methyl protons in the isopropyl group (the two doublets are distinguished by colour).

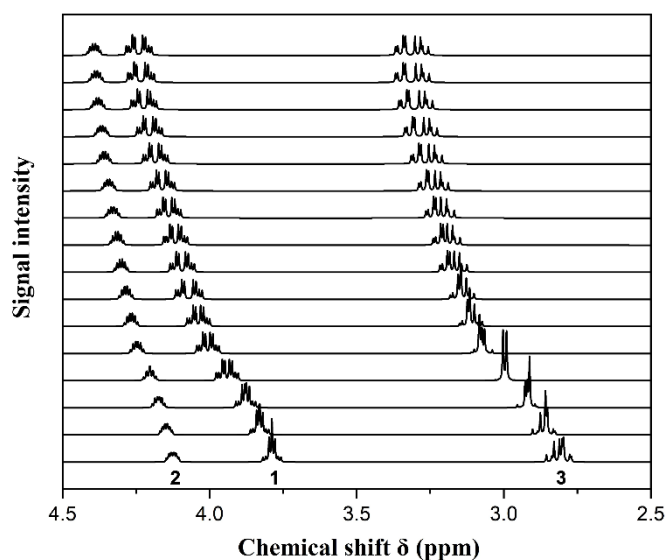


Figure 3.6 Simulated peaks of protons 1,2 and 3 using spin simulation of the ABMXY spin system.

Table 3.4 Simulated coupling constants of ABMXY spin system of protons 1, 2 and 3 (A, B, C, D and E stand for proton 3 in low field, proton 3 in high field, proton 2, proton 1 in low field and proton 1 in high field, respectively).

Spin system	AB	AM	BM	MX	MY	XY
Coupling constants	13.1	2-3.5	9.5-9.8	4.2-4.3	5.0-5.1	10.2-10.3

Table 3.5 Binding constants and calculated ΔG based on different binding models.

Binding model	$K_{dimer} (M^{-1})$	ρ	$\Delta G_{dimer} (KJ/mol)$
EK Model	-0.89 ± 0.01	-	-
CoEK Model	6.67 ± 0.15	0.07 ± 0.01	-4.62 ± 0.05

EK = dimerization/equal K mathematical and CoEK = co-operative equal K models

Of the two models, the CoEK model was found to give the better fit, resulting in a positive binding constant and smaller error. The self-aggregation constant K_{dimer} is low at $6.67 \pm 0.15 M^{-1}$, which gives a corresponding Gibbs free binding energy ΔG_{dimer} of -4.62 ± 0.05 kJ/mol. This energy is only just above the kinetic energy of the solution at room temperature ($K_T = -3.66$ kJ/mol). The cooperative factor ρ is much smaller than 1, and the calculated further association constant K_E and Gibbs free binding energy ΔG_E are $95.3 \pm 15.9 M^{-1}$ and -11.1 ± 0.4 kJ/mol, respectively. These results indicate that the appearance of the propranolol hydrochloride dimer promotes further aggregation of propranolol hydrochloride to a large extent and the further self-aggregation of propranolol hydrochloride becomes much stronger after dimerisation.

To further explore the aggregation of propranolol hydrochloride, 2D ^1H NOESY NMR experiments were performed at concentrations of 50 mM and 180 mM, which are below and above the CMC, respectively (Figure 3.7). NOESY NMR spectra show inter- and intramolecular interactions of up to 5 Å distance as cross-peaks off the diagonal of the spectrum.^[50] The spectrum of the 180 mM propranolol hydrochloride solution shows several significant cross peaks, of which intramolecular interactions extracted from the single crystal X-ray structure^[51] are labelled in black. For the cross peaks labelled in red, namely the proton pairs 6 and 9/11, and 8 and 12, the intramolecular distance is larger than 5 Å and the appearance of cross peaks suggests the intermolecular interactions of propranolol molecules through their naphthalene ring. This result is consistent with the large changes seen in the chemical shifts for the protons on the naphthalene ring caused by dilution. When the concentration is low, at 50 mM, the cross peaks labelled in red still exist (Figure 3.7(b)), suggesting that the interactions *via* the naphthyl ring persist even to lower concentration.

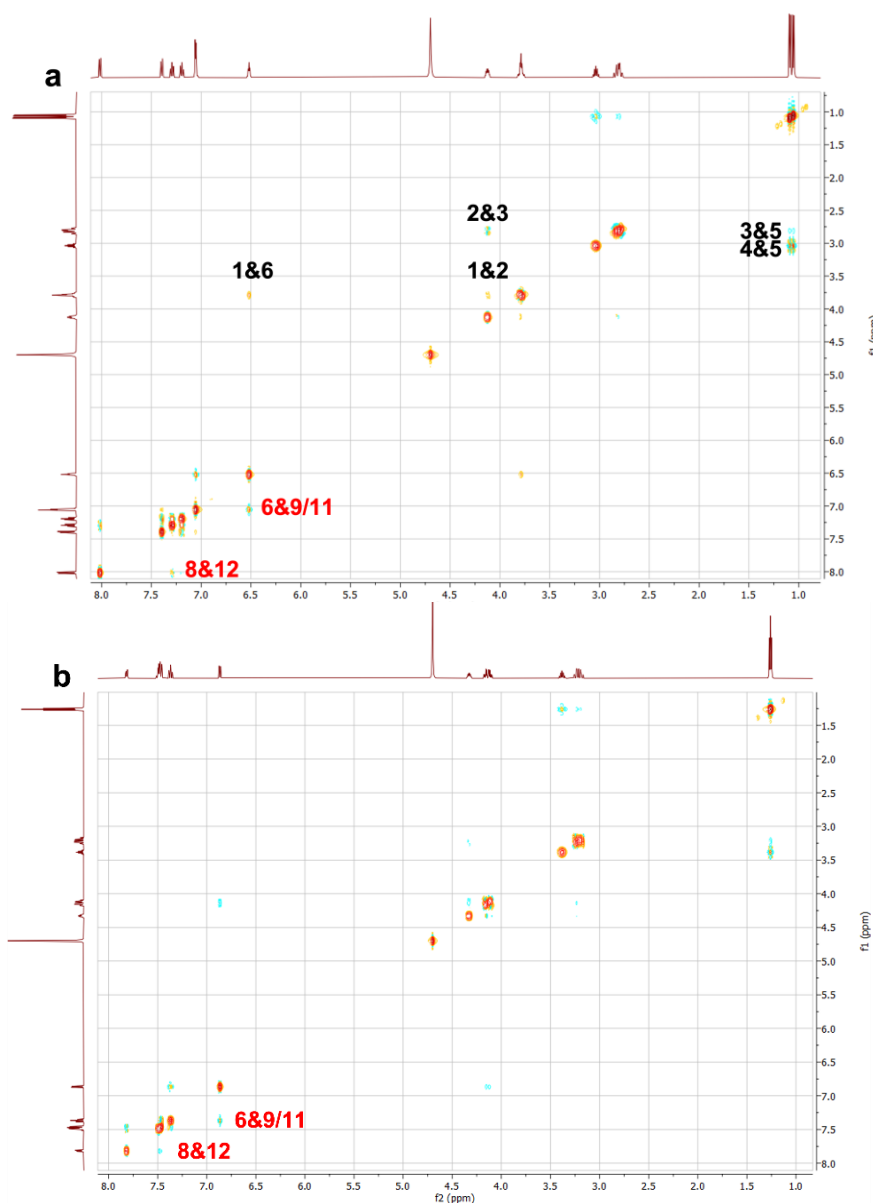


Figure 3.7 2D NOESY NMR of propranolol hydrochloride with concentration of (a) 180 mM and (b) 50 mM.

3.5.3 Contrast variation small angle neutron scattering studies

Of the various form factors and structure factors considered in model-fitting the measured SANS profiles, the best fits to the data were obtained using a core-shell, prolate ellipsoid form factor, convoluted with a Hayter-Penfold structure factor. The values of the final fitted parameters are summarised in Table 3.6, and the measured SANS data together with the corresponding best fits are shown in Figure 3.8. These fits, and the associated parameters, were obtained assuming that the core

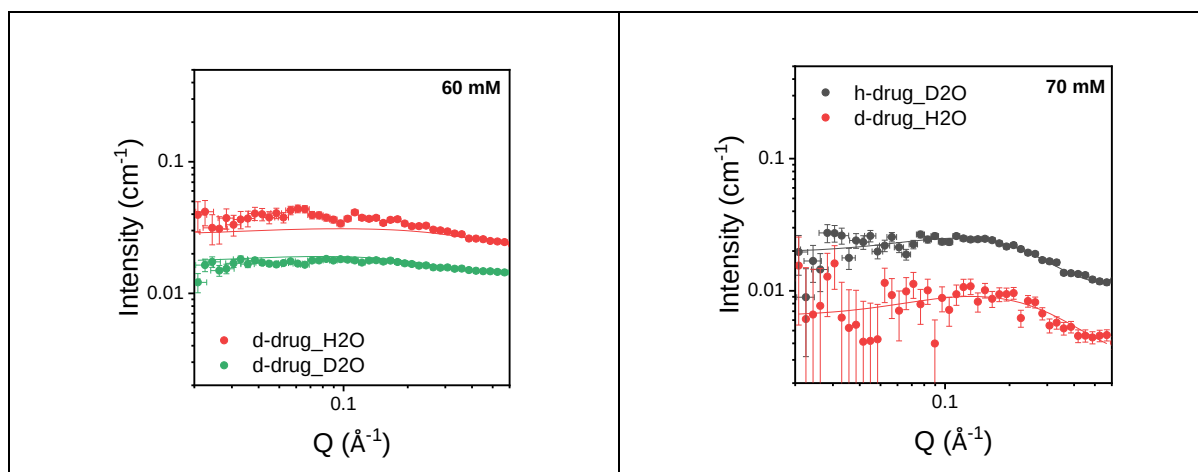
of the aggregates contained only dry naphthyl moieties; modelling performed wherein the SLD of the cores was freely optimised resulted in physically unrealistic models and/or poorer quality fits.

Table 3.6 Structural parameters obtained through simultaneous fits of a prolate core-shell model to the multiple sets of SANS data obtained for aqueous dispersions of propranolol hydrochloride at 298 ± 0.1 K.

Propranolol Hydrochloride Concentration (mM)	Radius Equatorial Core (± 0.04 Å)	Radius Polar Core (± 0.04 Å)	Shell Thickness (± 0.04 Å)	Charge (± 0.5 e)	Core Volume (Å ³)	Hydration of Shell* (vol fraction)	Aggregation Number (N_{agg}) [†]
200	6.7	18.8	5.7	4.3	3560	0.70	19
170	5.9	16.5	5.7	3.0	2405	0.71	13
150	5.9	15.6	5.4	2.8	2245	0.73	12
130	5.2	11.4	5.0	2.5	1293	0.76	7
110	4.4	9.9	4.7	1.8	822	0.79	4
90	4.1	9.0	3.0	1.3	627	0.78	3
70	3.5	8.5	3.4	2.4	429	0.72	2
60	3.5	7.0	3.5	1.0	358	0.75	2

[†] Aggregation numbers were obtained by dividing the aggregates core volumes by the volume of the drug's naphthyl moiety.

**h*-propranolol hydrochloride in D₂O was used to obtain an estimate of shell hydration.



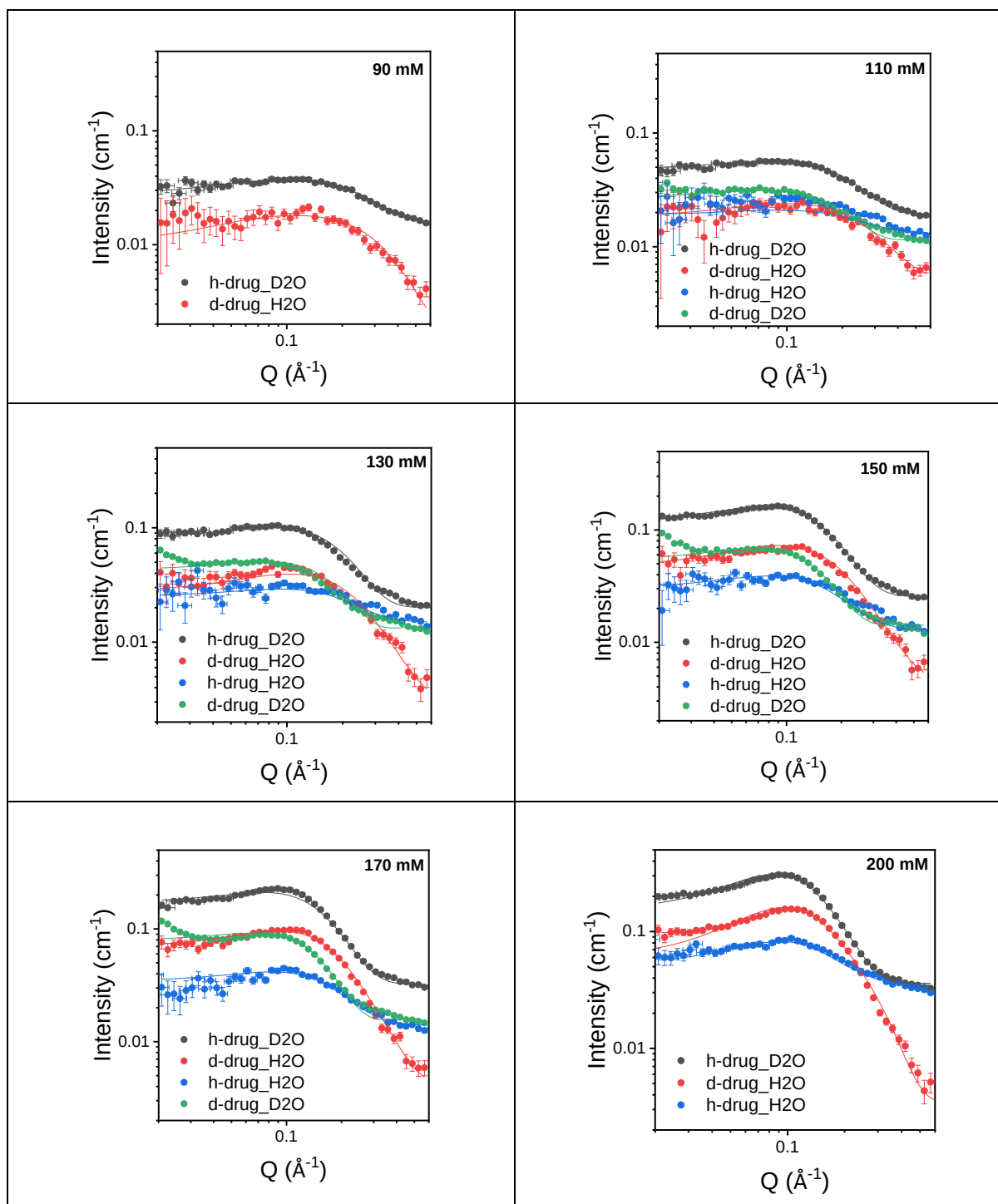


Figure 3.8 SANS data (points) at 298 ± 0.1 K and best constrained fits (solid line) obtained assuming the presence of a core-shell prolate ellipsoid for propranolol hydrochloride concentrations in the range 60 – 200 mM. Propranolol hydrochloride concentrations of 200 mM - 3 contrasts obtained for (*h*-propranolol hydrochloride in D₂O (black), *d*-propranolol hydrochloride in H₂O (red) and *h*-propranolol hydrochloride in H₂O (blue)), propranolol hydrochloride concentrations in the range 110 – 170 mM - 4 contrasts (*h*-propranolol hydrochloride in D₂O (black), *d*-propranolol hydrochloride in H₂O (red) *h*-propranolol hydrochloride in H₂O (blue) and *d*-propranolol hydrochloride in D₂O (green)) and propranolol hydrochloride of concentrations in the range 60 – 90 mM - 2 contrasts for 60 mM – *d*-propranolol hydrochloride in H₂O (red) and *d*-propranolol hydrochloride in D₂O (green) and for 70 and 90 mM – *h*-propranolol hydrochloride in D₂O (black) and *d*-propranolol hydrochloride in H₂O (red).

With the multiple contrast measurements made possible by the availability of both the protiated propranolol hydrochloride and the d_7 -(ring deuterated) drug, each of which could be dispersed in H₂O or D₂O, the constrained simultaneous model-fitting that was performed was used to provide detailed information on the interior structure of the aggregates, due to the differences in SLD between the aggregate core, shell, and solvent.

The mean aggregation numbers (N_{agg}) of the aggregates were determined from the fitted volumes of their cores, simply by dividing these volumes by the volume of a single naphthyl moiety:

$$N_{agg} = (\frac{4}{3}\pi R_e^2 R_p) / MV_n \quad (3.2)$$

where R_e is the equatorial radius of the aggregate core, R_p is the polar radius of the core, MV_n is the molecular volume of a single naphthalene ring. As shown in Table 4, the aggregation numbers of the aggregates are found to be concentration-dependent, with progressively larger aggregates formed with increasing drug concentration. The fact that the SANS data are modelled assuming aggregates present over all of the measured concentrations, once again indicates that self-association of the drug is continuous.

When the aggregation number of the aggregates is plotted as a function of reciprocal concentration, the best fit lines for the low and high concentration parts of the curve intersect to give an estimate of the CMC as 111 mM (Figure 3.9), and while this is somewhat higher than the values determined on the basis of the surface tension and ¹H NMR measurements detailed here, the results are at least comparable, and the SANS-derived value is also close to the values previously determined by others.^[40, 41]

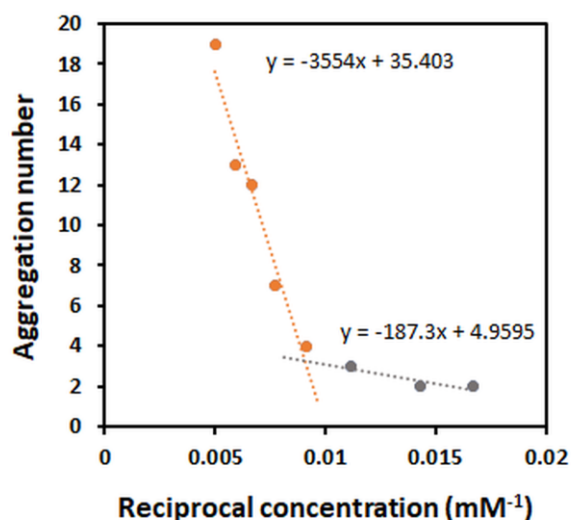


Figure 3.9 Concentration-dependent changes in the calculated propranolol hydrochloride aggregation number (N_{agg}) derived from the core volumes of the drug particles determined via core-shell ellipsoid model-fitting of the SANS data.

As for the shell that surrounds the aggregate core, within which the charged polar side-chains of the drug molecules are assumed to reside, the fitted thickness is found to vary with drug concentration over the range 3.5 Å – 6 Å (Table 3.6), with the thickness greater for the larger aggregates formed at the higher concentrations. From the fitted SLD and volume of this layer – determined here by considering only the values got for the *h*-propranolol hydrochloride/D₂O and *d*-propranolol hydrochloride/H₂O contrasts (where the contrast between the chains and solvents is greater and the error in the calculations thus minimized) we get an estimate of the level of hydration of the drug side-chains. As might be expected, we find that the side-chains in the smaller aggregates (formed at low concentration) are more heavily hydrated than those in the larger aggregates (formed at higher concentration), with ca. 21 and 14 H₂O per drug molecule, for the 60 mM and 200 mM dispersions, respectively. This concentration-dependent difference in the level of the side-chain hydration is consistent with the concentration-dependent changes in the chemical shifts seen for the protons on the side-chains (*vide supra*).

3.5.4 Molecular dynamics simulations

The MD simulations performed with the propranolol hydrochloride concentration varied over the range 10 mM to 200 mM show that there are aggregates formed in all of the systems, but with the

ensembles in each case exhibiting a distribution of sizes and dimensions, and with the aggregates becoming generally larger and involving more drug molecules as the concentration is increased (*cf.* Figure 3.10, and Supplementary Information Figure SI - 3.8). The aggregation numbers of the aggregates seen in the various MD ensembles range from 2 up to 20, and they thus span the same range as those obtained via model-fitting of the SANS data.

It is clear from the MD simulations that a significant proportion of the propranolol hydrochloride molecules exist in a monomeric state. At concentrations of 10, 30, 50 and 70 mM, the percentage of the monomeric species decreases from 73% to 58% to 43% and finally to 34%, while at concentrations of 90 mM and above, the percentage of propranolol hydrochloride molecules in the monomeric state stays within the range 23-14%. The variation in frequency of the various sized aggregates follows a similar trend. At propranolol hydrochloride concentrations of 90 mM and above the number of the larger sized aggregates increases and so the distribution moves to larger aggregate sizes.

When the atomic co-ordinates of the MD-modelled aggregates are used to compute an equivalent ellipsoid description of their size and shape, using the methodology reported in a recent work of Barlow and coworkers^[52] (Figure 3.11), their geometries are found to approximate to a mixture of near spherical, triaxial, oblate, and prolate ellipsoids (Figure 3.12 and Supplementary Information Figure SI - 3.9), and this is supported by a SasView-modelling of their SasCalc-derived scattering form factors (Supplementary Information Figure SI - 3.10). Such calculations of the equivalent ellipsoidal geometries allow that a representative mean geometry can be computed for the MD ensembles, and this can then be compared against the geometry modelled by fitting to the SANS data, where the time- and space-averaged scattering of the particles is weighted by particle volume fraction. When the volume-weighted mean aggregation numbers for the MD ensembles seen at each concentration are calculated in this way, the values are generally lower than those obtained through model-fitting of the SANS data, with the aggregation numbers for the 200 mM system, for example, obtained as 12 vs. 19. This difference can in part perhaps be attributed to the restricted size of the MD ensembles, but it will also undoubtedly reflect the very different timescales of the MD and SANS measurements, the former covering only nanoseconds, and the latter, typically an hour or more. We note, however, that when the volume-weighted mean aggregation numbers of the MD-modelled micelles are plotted as a function of reciprocal concentration, the best fit lines for the low and high concentration parts

of the curve intersect to give a very rough estimate of the CMC as 82 mM (Figure 3.13), which is rather lower than our NMR- and SANS-derived CMC values but of the same order.

From the equivalent ellipsoid approximation of the MD-modelled micelles,^[52] we are also able to determine the extent and composition of their water-free cores, and the thickness and composition of their surrounding shell layers (Figure 3.11). As regards the aggregates' cores, we find that these contain just the drug's naphthyl moieties (Figure 3.11), and they thus have a calculated SLD in the range $1.7 \times 10^{-6} \text{ \AA}^{-2} - 2.3 \times 10^{-6} \text{ \AA}^{-2}$, and their composition thus accords well with that assumed in model-fitting the SANS profiles, where the core SLD was best taken as $2.2 \times 10^{-6} \text{ \AA}^{-2}$. We find too that the dimensions and composition of the shell of the MD-modelled micelles are consistent with those found from model-fitting of the SANS data (Table 3.4), with the mean thickness and SLD computed for the 200 mM system, for example, obtained as 6.6 Å vs. 5.7 Å, and $3.6 \times 10^{-6} \text{ \AA}^{-2}$ vs. $4.5 \times 10^{-6} \text{ \AA}^{-2}$, respectively. The calculated volume fraction of solvent within the MD-modelled aggregate shells ranges from 0.4 to 0.64, with a mean of 0.54, and this compares very well with the range of 0.3 – 0.7 determined from the SANS modelling (Table 3.4).

Moreover, if we take the volume calculated for the shell of each MD-modelled micelle together with the aggregation number of the particle, we can, in addition, calculate the volume of water in the layer, and from this we can then determine the level of hydration of the drug's charged polar side-chains. In the 200 mM system, such calculations give a mean level of hydration of 16 H₂O per drug molecule, and this compares very favourably with the 14 H₂O per molecule obtained from the SANS modelling.

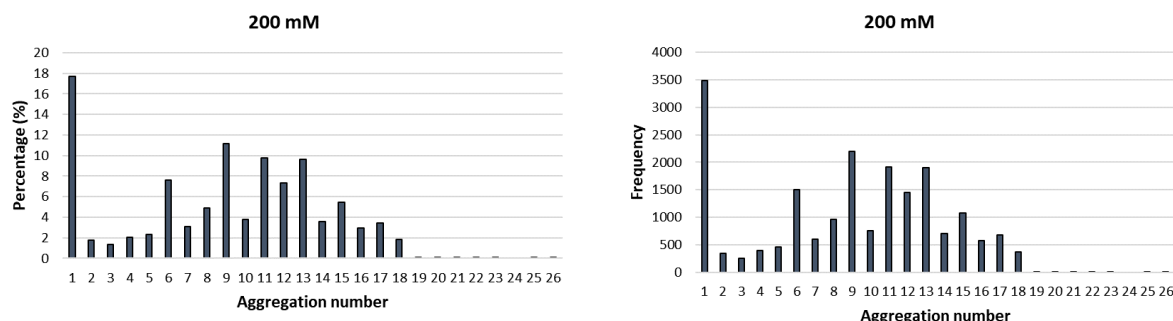
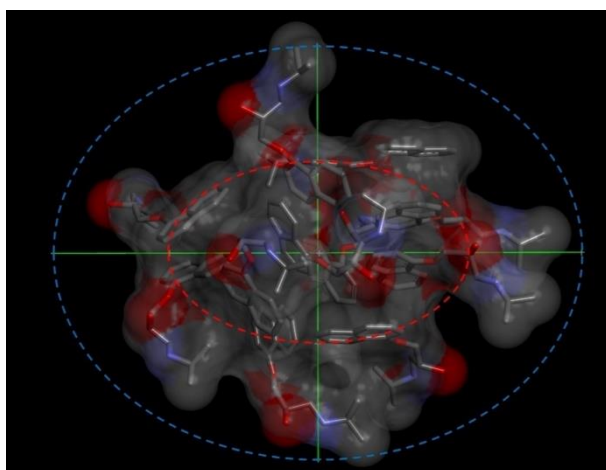


Figure 3.10 The variation in the percentage and frequency of the aggregates as a function of aggregation number (N_{agg}) when equilibrated at 200 mM propranolol hydrochloride.

Calculation of the atomic solvent accessible surface areas for the free drug monomers and the MD-modelled micelles, shows that the fraction of the combined monomer surface area that is buried on formation of the aggregates is consistent across all the various simulations (Figure 3.14), with ca. 567 Å² of surface area buried per molecule. While it is encouraging to see that a given size of aggregate shows the same internal architecture regardless of whether it is formed in a low concentration ensemble or a higher concentration one, this finding is somewhat surprising in light of the fact that all of the experimental measurements performed here show that the drug has a critical micelle concentration, so that we might, therefore, have expected to see a break or discontinuity in the buried surface area plot. In addition, if we extrapolate a binding free energy from the buried surface area increment,^[53] the resulting ΔG of ca. 50 kJ.mol⁻¹, seems very high when compared against the free energies estimated from our NMR studies.

What is clear from a molecular graphics inspection of the MD-modelled aggregates, however, is that the naphthyl groups within the core of the structures show numerous CH- π aromatic interactions – just as was deduced from the ¹H-NMR shift measurements. These inspections also reveal an absence of intermolecular hydrogen bonding between the side-chains of the self-associated drug molecules – indicating that the concentration-dependent changes in the chemical shifts seen for the side-chain protons likely result only because of changes in the pattern of hydration of the groups that arise because of the concomitant changes in the sizes and shapes of the aggregates.



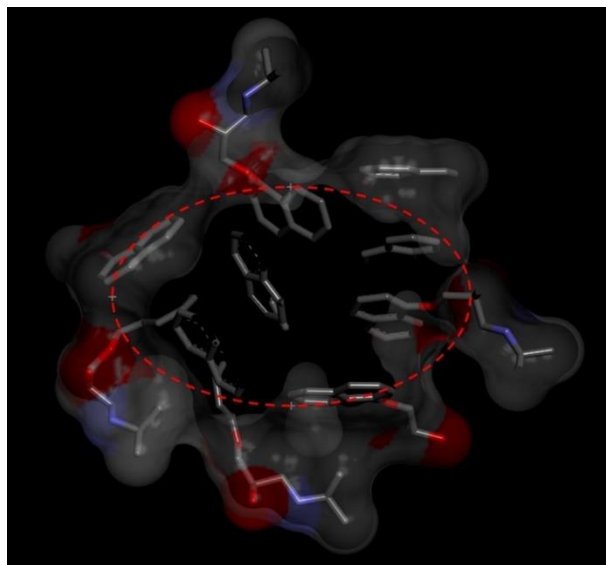


Figure 3.11 Upper panel: Atomic structure of an MD-modelled propranolol hydrochloride micelle (having an aggregation number of 11), overlaid with a semi-transparent rendering of its solvent accessible surface (colour coded by atom type; with C, grey, N, blue, O, red), and showing the semi-axes and outer perimeter of the equivalent ellipsoid – scaled so as to enclose 95% of the atoms (blue), together with the ellipsoidal outer perimeter of the water-free particle core (red). Lower panel: Cross-sectional view of the same micelle as above, overlaid with a semi-transparent rendering of its solvent accessible surface (colour coded as in the upper figure), and showing the calculated ellipsoidal boundary defining the water-free core of the particle – and showing the core contents of the drugs' naphthyl moieties.

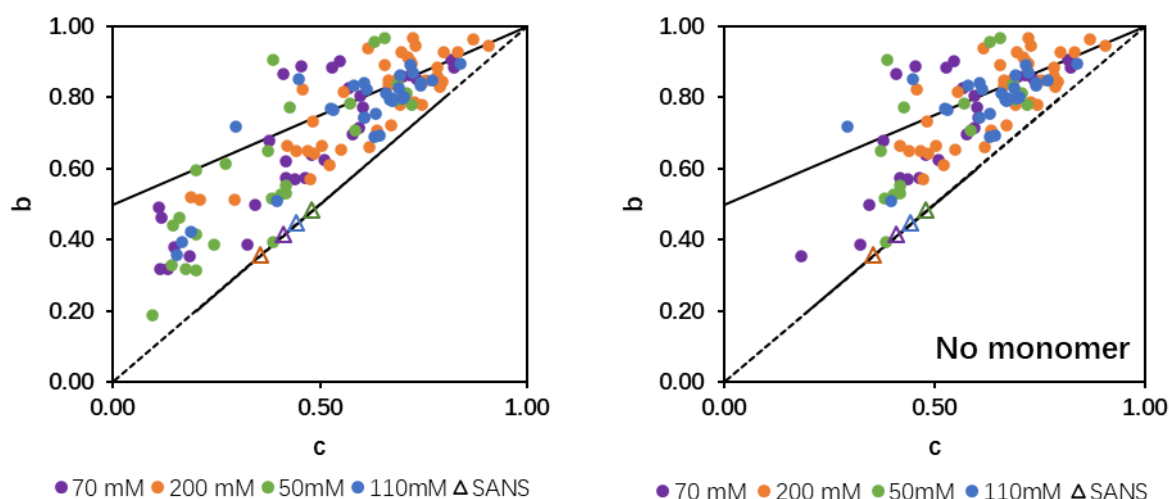


Figure 3.12 Semi-axes dimensions of the MD-modelled propranolol hydrochloride micelles approximated as their equivalent ellipsoids (data plotted as filled circles, coloured by drug concentration), with each of the shorter semi-axes, b and c , normalised with respect to the longest axis, a . Aggregates that plot with (b, c) clustering close to $(1.0, 1.0)$ are roughly spherical. Those that plot above the top diagonal (with b at or close to 1.0 and $c \leq 0.5$) are oblate, and those that plot above the main diagonal and below the top diagonal (with b and c both ≤ 0.5) are prolate. For comparison, the normalised semi-axes of the aggregates modelled on the basis of fits to the SANS data are plotted as triangles.

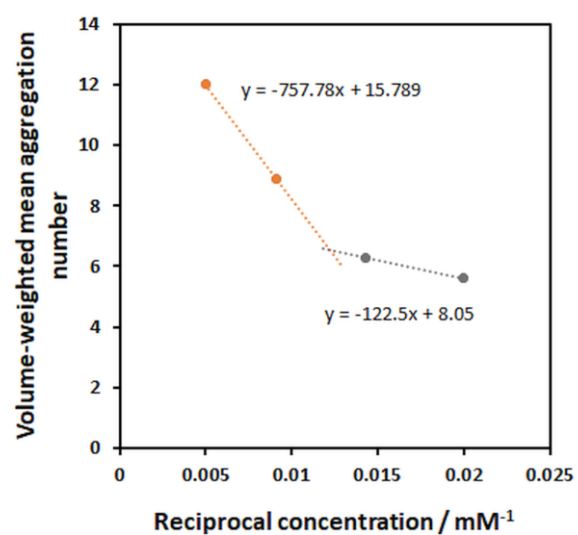


Figure 3.13 Concentration-dependent changes in the volume-weighted mean micelle aggregation number calculated from the equivalent ellipsoid volumes of the drug particles modelled in the MD simulations.

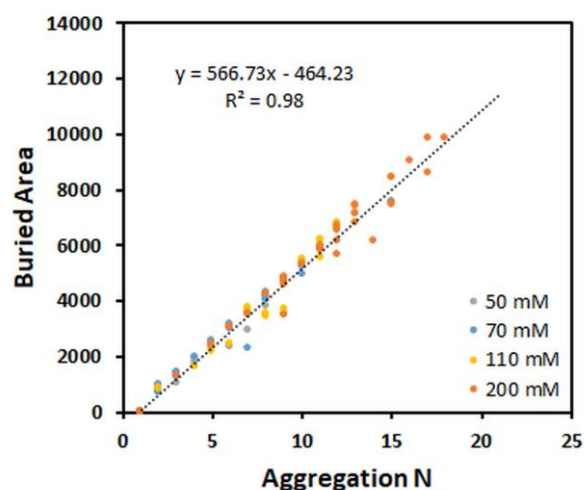


Figure 3.14 Buried solvent accessible surface area plotted against micelle aggregation number for the aggregates seen in the MD simulations, with symbols colour-coded according to the concentration of drug in the system.

3.6 Discussion and conclusions

Taken together the insight gained from the various techniques used here allow a detailed picture of the solution behaviour of propranolol hydrochloride to be obtained. In terms of aggregation, while UV/vis titration studies suggest that no self-assembly occurs at concentrations of up to and including 0.15 mM, the ^1H NMR spectroscopy studies indicate a very limited aggregation of propranolol hydrochloride at concentrations as low as 5 mM and possibly at even lower. Contrast variation SANS indicated that aggregates, in the form of dimers, were present at propranolol hydrochloride concentrations of 60 and 70 mM, lower concentrations were not tested, while MD simulations supported the presence of very limited aggregates at drug concentrations as low as 10 mM. The very low aggregation numbers seen under these low concentration conditions suggest the presence of a critical aggregation concentration (CAC) *i.e.* the concentration at which pre-micellar aggregates consisting of a few propranolol hydrochloride molecules form.

As the drug concentration increased, a continuous aggregation of propranolol hydrochloride was seen, with all the techniques studied indicating a break in the aggregation number against concentration indicative of a CMC of around 90-95 mM (^1H NMR) 111 mM (SANS) and 82 mM (MD). Note that the surface tension measurements were unable to detect a CAC, yielding only a CMC of 92.4 mM, a value in agreement with the data obtained from the other techniques. The variation in the value of the CMC obtained with the various techniques in the present study can be attributed to

a variety of reasons including the fact that the CMC is not a single concentration but rather a narrow range of concentrations^[40] coupled with the fact that the different measurement techniques are sensitive to different physical properties of the system.^[40] The range of propranolol hydrochloride concentrations studied using a particular technique may also have an impact on the calculated CMC.

Significantly the aggregates formed by propranolol hydrochloride at the CMC are not classical micelles seen with traditional surfactants such as sodium dodecyl sulfate where one species of micelle composed of a large number of surfactant molecules are seen, rather the aggregates formed by propranolol hydrochloride are small and polydisperse. Interestingly, the MD-simulations showed the presence of a considerable number of monomeric drug molecules which slightly decreased in number at concentrations above the CMC. Note that it is not possible to detect monomers with SANS or surface tension measurements.

Information about the molecular packing of the propranolol hydrochloride aggregates was obtained from the ¹H NMR and MD simulation studies, both of which indicate that the core of the aggregates contains naphthyl rings, which reassuringly agrees with the SANS modelling where the best fit to the data is obtained when the aggregate core is composed solely of naphthyl rings. Furthermore, the ¹H NMR and MD simulations indicate that the naphthalene rings interact via CH- π aromatic interactions. The results of the 2D ¹H NOESY NMR experiments indicate that the interactions *via* the naphthyl ring persist to drug concentrations as low as 50 mM (lower concentrations not tested) - below the measured CMC, suggesting that the CH- π aromatic interactions are important in the formation of dimers of propranolol hydrochloride. NMR studies also recorded significant concentration-dependent changes in the chemical shifts of the aliphatic side-chain protons the self-association of the drug which are likely to be due to changes in the pattern of side-chain hydration rather than intermolecular hydrogen bonding involving the side chains, as inspection of the aggregate structure obtained by MD simulation yield no evidence of intermolecular hydrogen bonding involving the side chains.

Depending on the techniques used, the mean size and the geometry of the aggregates vary due to the different properties used for their calculation and the polydispersity of aggregates. Although the sizes of the aggregates vary with the techniques used, variations in size between the different techniques are in good agreement. For example, while the MD models are approximated as triaxial ellipsoids, their calculated dimensions are close to those obtained through modelling of the SANS

profiles - and the dimensions obtained through triaxial ellipsoid model fits of the SANS data accord well.

Statements:

The SANS experiments were performed by Yixuan Yan supported by instrument scientist Dr. Najet Mahmoudi. The data analysis of the SANS experiments was undertaken by Yixuan Yan, in conjunction with Professor Jayne Lawrence and Dr. David Barlow. MD simulations and data analysis were performed by Dr. Andrew Leach, Dr. David Barlow and Yixuan Yan. The SASSIE modelling was done by Dr. Dave Barlow. The ^1H NMR experiments and data analysis were performed by Yichun Shen and Dr. Katharina Edkins. The deuterated drug was synthesised by Jasmine Hind and Dr. James Tellam. The project was conceived by Professor Jayne Lawrence and supervised by Dr. Richard Campbell and Professor Jayne Lawrence.

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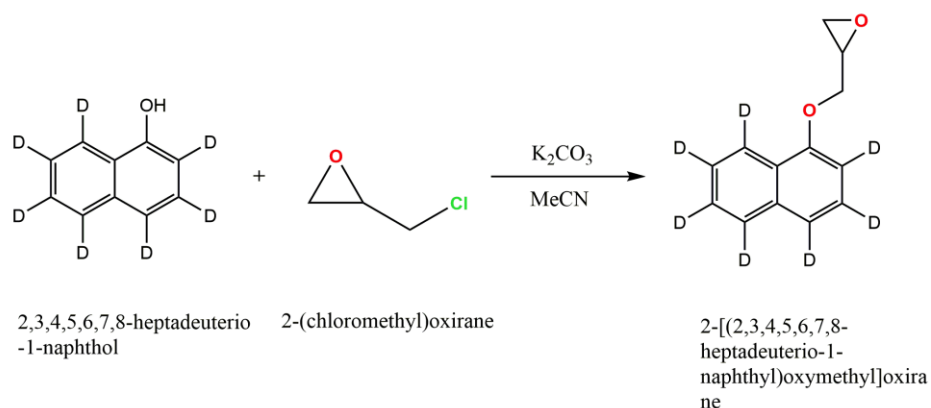
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Supplementary information

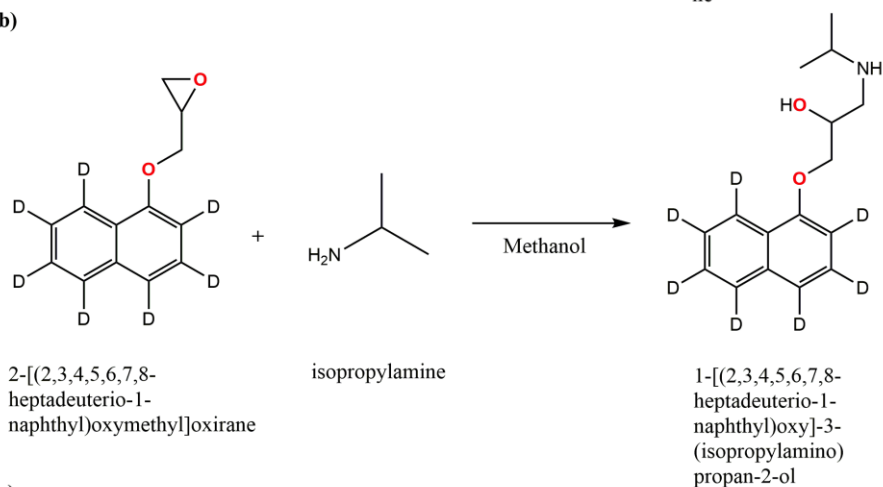
SI-1 Synthesis of d_7 -propranolol hydrochloride

d_7 propranolol HCl was synthesised by a 3-step procedure (Figure SI - 3.1). In the first reaction (Figure SI - 3.1(a)) the side chain was added to fully deuterated naphthol (2,3,4,5,6,7,8-heptadeuterio-1-naphthol) via the Williamson ether synthesis using 2-(chloromethyl)oxirane, The product of this reaction, 2-[(2,3,4,5,6,7,8-heptadeuterio-1-naphthyl)oxymethyl]oxirane, was reacted with isopropylamine to add an isopropylamine group to the terminal oxirane moiety thereby preparing d_7 -propranolol (Figure SI - 3.1 (b)). Finally, the free base was acidified to prepare the required d_7 -propranolol hydrochloride salt (Figure SI - 3.1 (c)).

(a)



(b)



(c)

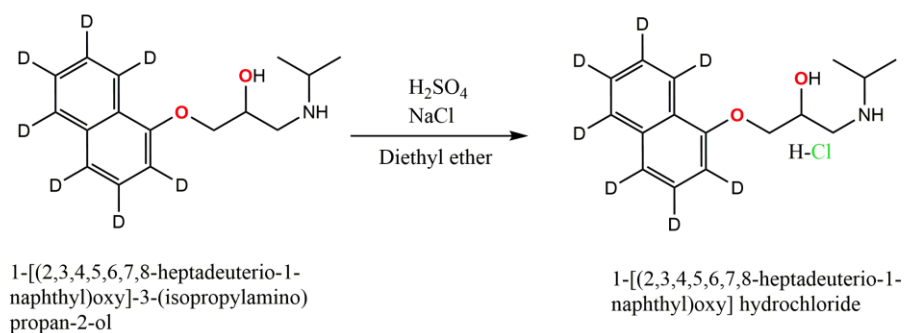


Figure SI - 3.1 Schematic synthesis of d_7 -propranolol hydrochloride.

SI-2 Purification of propranolol hydrochloride

Propranolol hydrochloride is reported to be unstable under exposure to light, yielding 1-naphthol, *N*-acetylpropranolol and *N*-formylpropranolol (Figure SI - 3.2). Therefore, it was hypothesized that the reason for the ‘dip’ observed in the surface tension measurements around the CMC was the presence of a surface-active contaminant in propranolol hydrochloride as purchased (Figure SI - 3.2), most

probably naphthol as it has been reported to exhibit surface active behaviour.^[24] As a consequence, the purification of propranolol hydrochloride was attempted to obtain a more accurate assessment of its CMC.

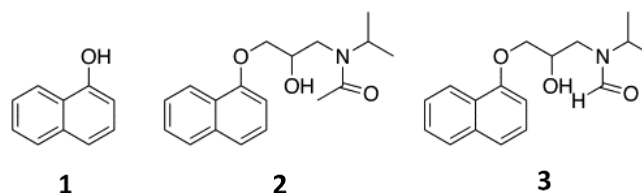


Figure SI - 3.2 Structures of the photodegradation products of propranolol hydrochloride, (1) 1-naphthol, (2) *N*-acetylpropranolol, (3) *N*-formylpropranolol.^[25]

As 1-naphthol dissolves in ethyl acetate, ethyl ether and chloroform, while propranolol hydrochloride does not, each of these three solvents were used to wash the same volume of drug solution, three times. The resulting surface tension curves (Figure SI - 3.3) suggested that chloroform acts as the most efficient solvent to eliminate surface active impurities and was therefore used in subsequent purification studies. The surface tension of propranolol hydrochloride was measured immediately after purification with the propranolol hydrochloride being stored in the dark to reduce degradation.

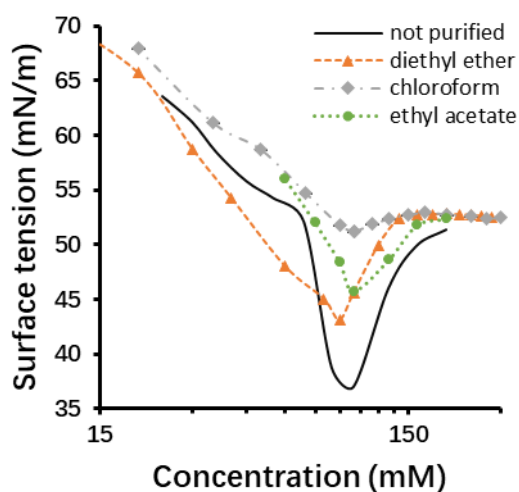


Figure SI - 3.3 Variation in surface tension as a function of propranolol hydrochloride concentration, 'as purchased' and purified using different organic solvents at ambient temperature.

SI-3 UV/vis absorption spectroscopy

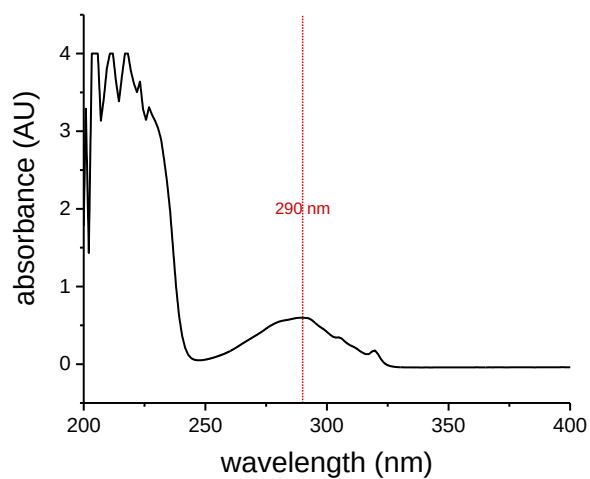


Figure SI - 3.4 Wavelength scan of propranolol hydrochloride from 400 to 200 nm, the maximum absorbance is at 290 nm.

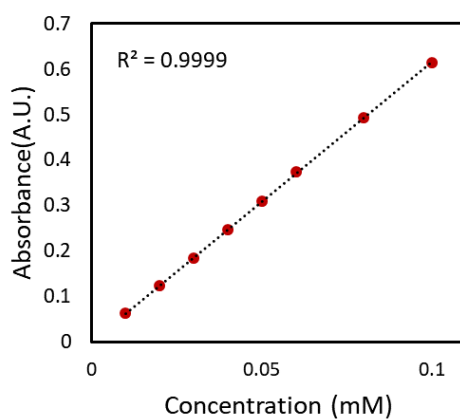


Figure SI - 3.5 Standard curve of propranolol hydrochloride absorbance measured at 290 nm against propranolol hydrochloride concentration (mM).

SI-4 Comparison of the small angle neutron scattering of propranolol hydrochloride 'as purchased' and as 'purified'

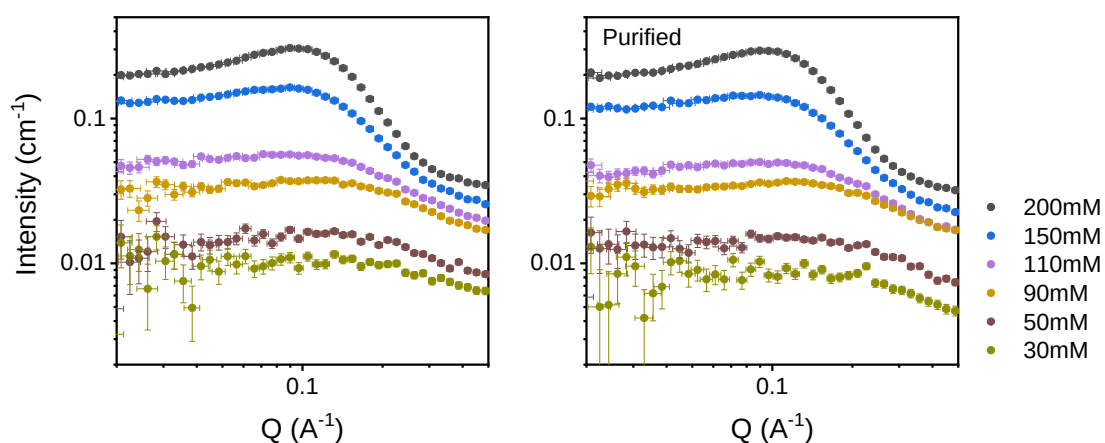


Figure SI - 3.6 The variation in neutron scattering intensity with scattering vector for unpurified (left hand side curve) and purified (left hand side curve) *h*-propranolol hydrochloride dissolved in D₂O, measured at 298 ± 0.1 K.

Table SI - 3.1 Structural parameters obtained through fits of a prolate core-shell model to the SANS data obtained for aqueous dispersions of purified *h*-propranolol hydrochloride, measured at 298 ± 0.1 K.

Drug Concentration (mM)	Radius Equatorial Core (Å)	Radius Polar Core (Å)	Shell Thickness (Å)	Charge (e)	Volume of Core (Å ³)	Hydration of Shell (vol fraction)	Aggregation Number (<i>N_a</i>)
200	6.73	18.51	5.60	4.96	3506	0.71	19
150	5.99	16.25	5.21	3.81	2443	0.75	13
110	4.86	9.14	3.97	2.38	905	0.77	5
90	5.20	11.43	4.99	2.25	646	0.77	4
50	4.45	9.93	4.72	1.04	430	0.80	2
30	4.07	9.04	3.01	1.22	290	0.77	2

SI-5 Determination of propranolol hydrochloride CMC using ¹H NMR spectroscopy

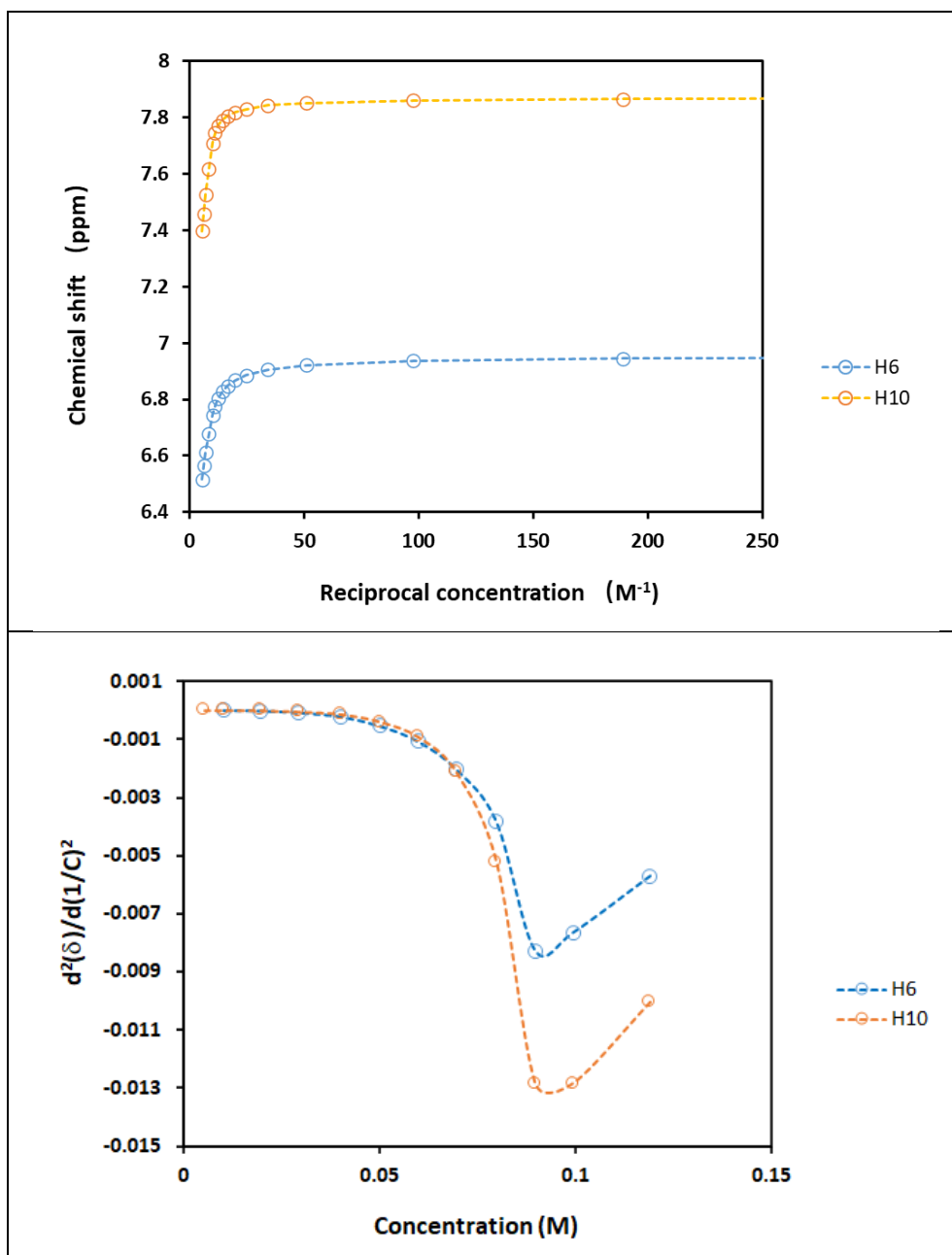
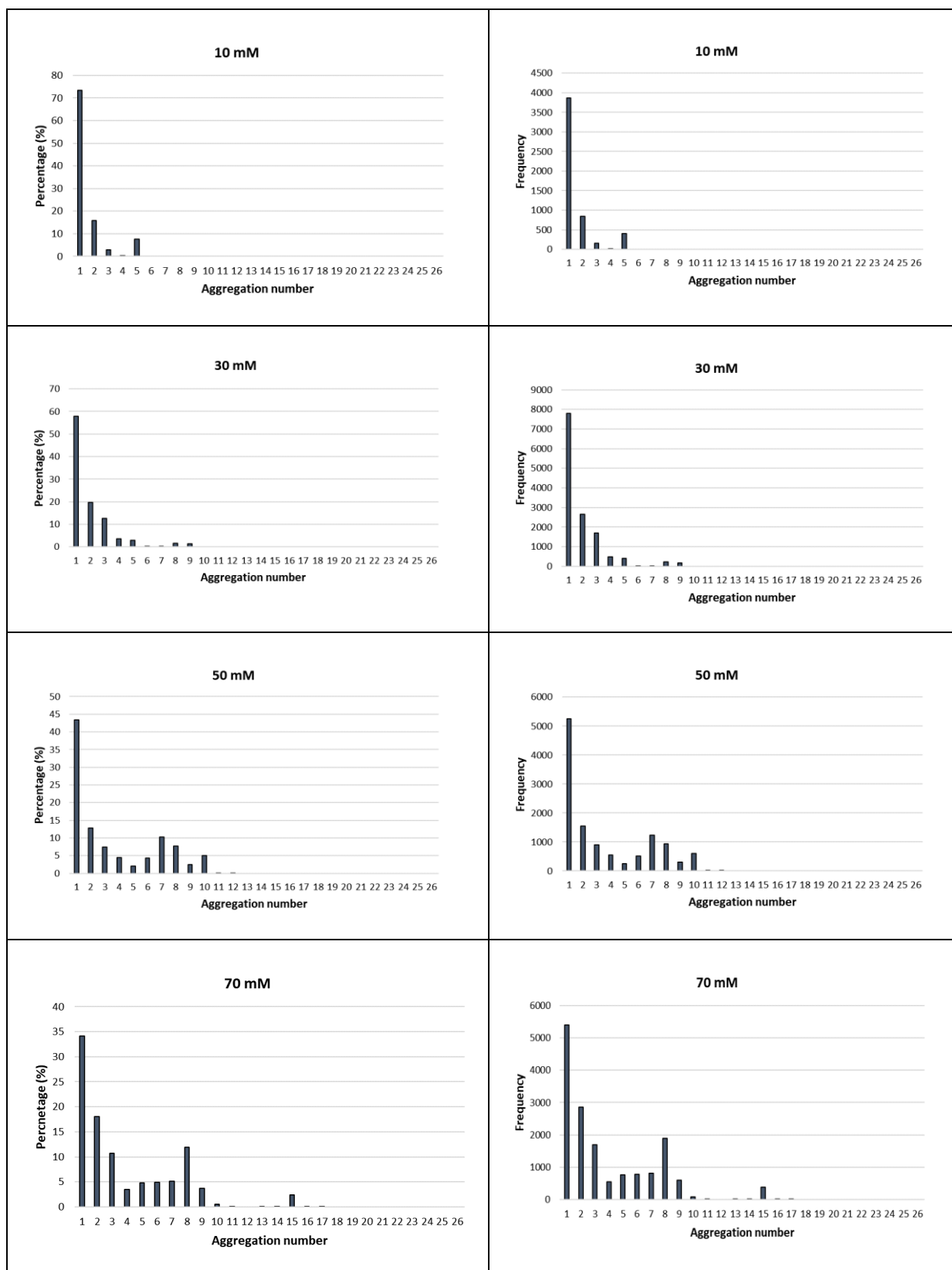
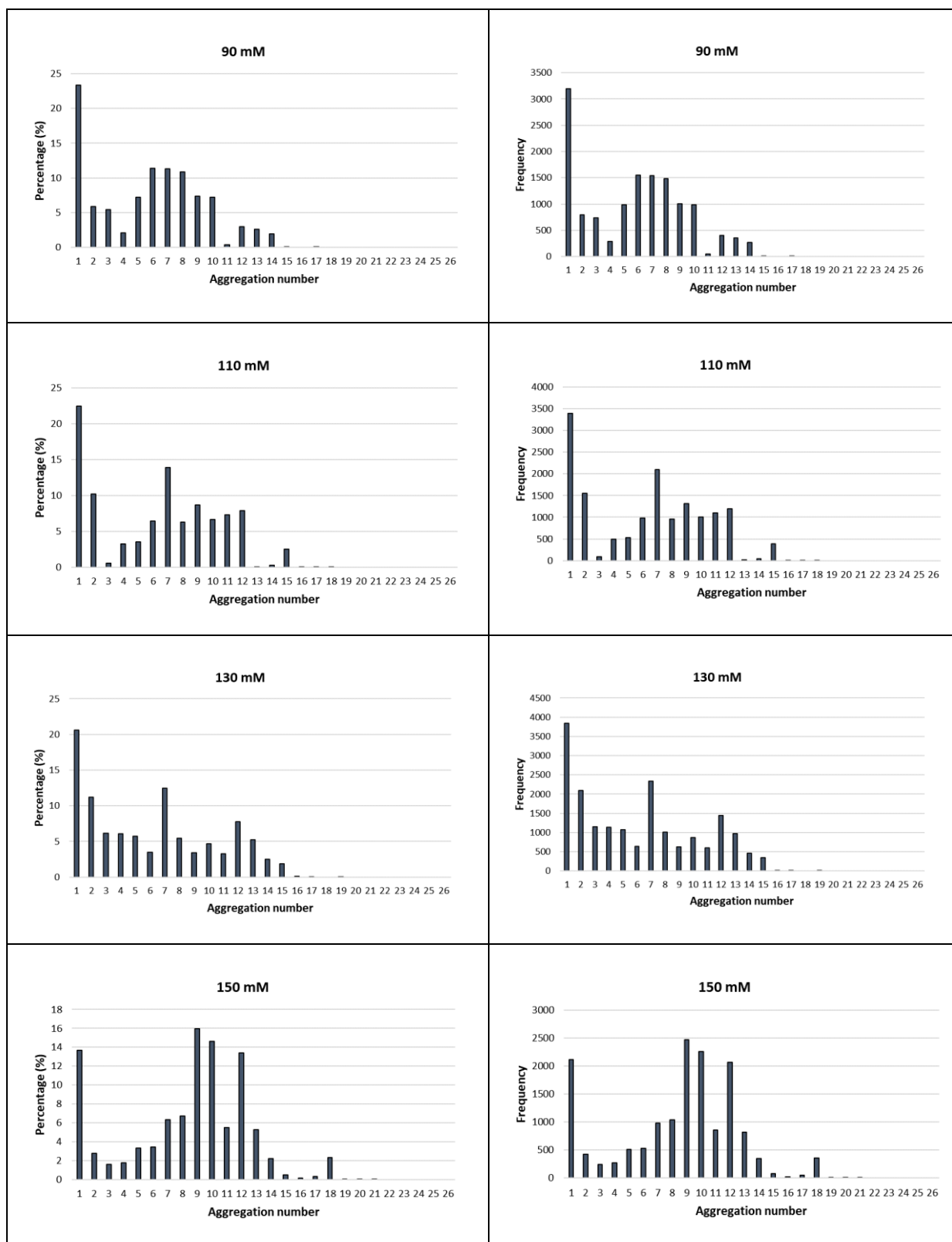


Figure SI - 3.7 Variation in the magnitude of the shift change for H6 and H10 as a function of reciprocal concentration (upper curve) and in the second derivative of the shift changes for H6 and H10 as a function of concentration (lower curve).

SI-6 Percentage and frequency of the aggregates of varying size at propranolol hydrochloride concentrations (10 – 200 mM) as determined using all atom MD simulations





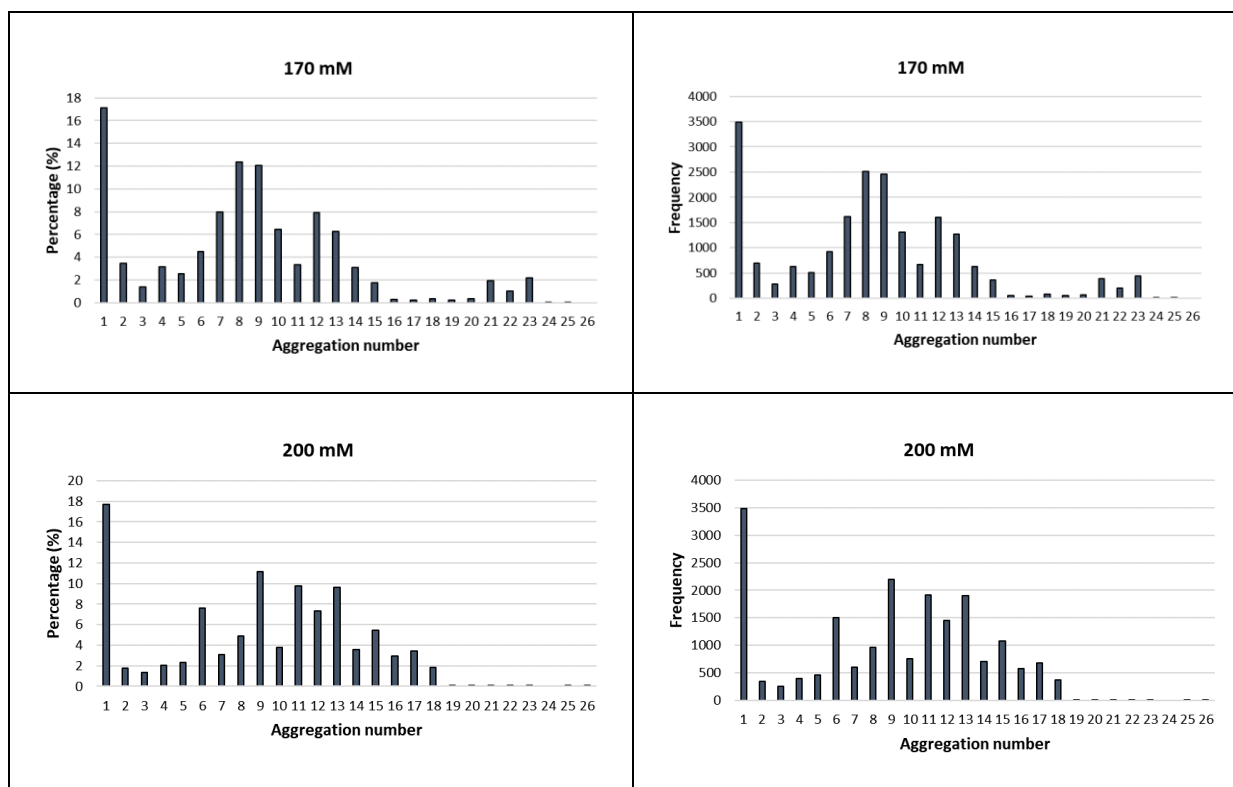
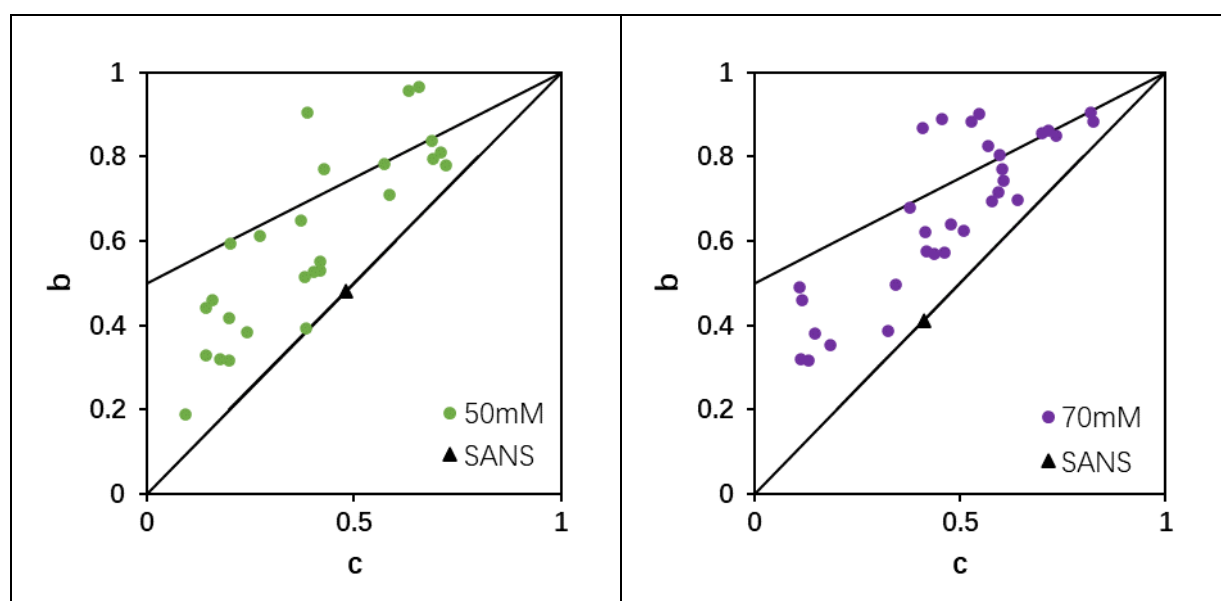


Figure SI - 3.8 The variation in the percentage and frequency of the aggregates of varying size as a function of aggregation number (N_{agg}) when equilibrated at a range of propranolol hydrochloride concentrations.

SI-7 Geometries of MD-modelled aggregates as equivalent ellipsoids



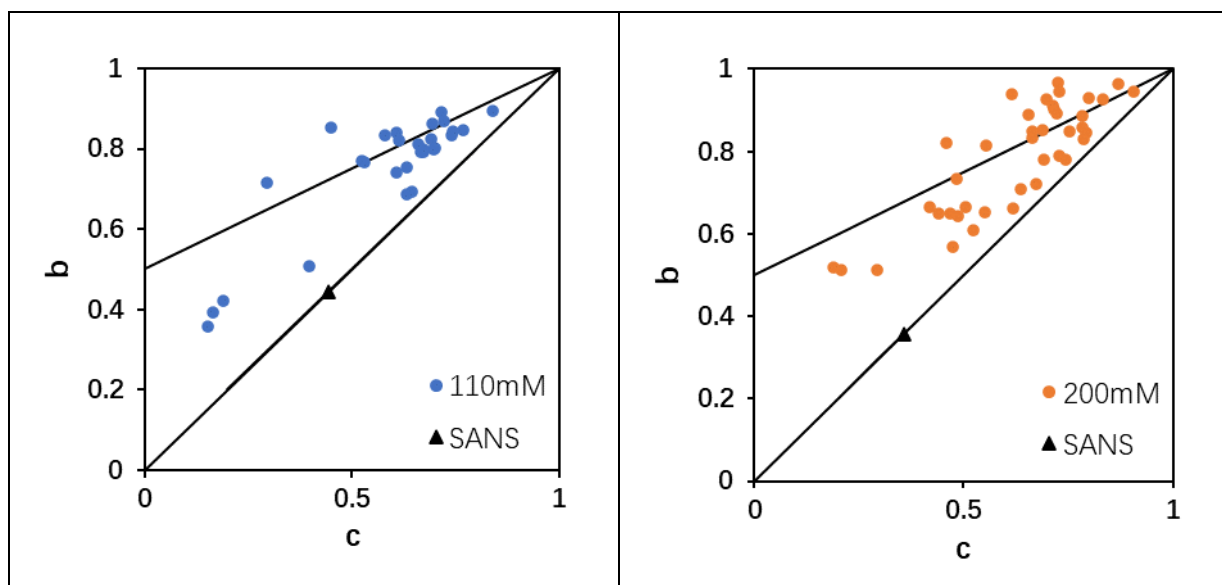


Figure SI - 3.9 Semi-axes dimensions of the MD-modelled propranolol hydrochloride micelles at concentrations of 50, 80, 110, 200 mM, approximated as their equivalent ellipsoids (data plotted as filled circles, coloured by drug concentration), with each of the shorter semi-axes, b and c , normalised with respect to the longest axis, a . Aggregates that plot with (b, c) clustering close to $(1.0, 1.0)$ are roughly spherical. Those that plot above the top diagonal (with b at or close to 1.0 and $c \leq 0.5$) are oblate, and those that plot above the main diagonal and below the top diagonal (with b and c both ≤ 0.5) are prolate. For comparison, the normalised semi-axes of the aggregates modelled on the basis of fits to the SANS data are plotted as triangles.

SI-8 SasView-modelling of their SasCalc-derived scattering form factors

Investigating the variation in aggregation number of Table SI - 3.2, together with their model-fitted semi-axes dimensions (a , b , and c) determined through SasView modelling of their SasCalc-computed form factors. The data for the aggregates whose scattering form factors are modelled assuming exactly prolate ellipsoidal geometry are shown in bold and highlighted.

Table SI - 3.2 Measured dimensions of aggregates obtained when modelling the 200 mM propranolol hydrochloride system.

Propanolol HCl Aggregation Number	Ellipsoid Semi-Axes Dimensions (Å)		
	a	B	c
8	14.9	10.4	7.9
9	15.9	11.0	8.2
9	14.4	10.2	9.1
9	15.7	10.8	8.0
9	14.5	9.9	8.8
9	15.8	11.8	7.1
10	15.0	11.8	8.4
10	15.5	11.0	8.0
11	16.1	12.2	7.9
11	19.4	12.8	7.6
11	17.8	11.2	7.5
11	15.2	10.8	9.8
12	16.4	11.1	9.4
12	16.5	11.5	9.7
12	16.0	10.6	10.6
12	15.2	14.7	8.5
12	16.0	10.5	10.5
13	16.0	12.4	9.9
13	17.5	10.8	10.8
13	15.3	11.5	10.0
14	15.6	15.6	8.3
14	16.2	13.0	10.1
14	16.0	11.3	11.3
15	15.0	11.1	11.1
15	16.0	11.3	11.3
15	17.2	11.4	11.4
15	18.2	13.3	10.7
16	17.6	13.0	9.8
17	18.3	13.2	10.8
17	18.5	13.6	9.8

The smallest aggregates, having $8 \leq N_{agg} \leq 10$, have SasCalc-computed form factors that are modelled assuming a triaxial ellipsoidal geometry, with dimensions, $a \neq b \neq c$. The larger aggregates, however, that have $N_{agg} \geq 12$, have SasCalc-computed form factors that are modelled assuming a more closely prolate geometry, with $c \approx b \neq a$, $a > c$. Several of the largest aggregates indeed have computed form factors that are fully consistent with a prolate ellipsoidal geometry, having $c = b \neq a$, $a > c$ (Figure SI - 3.10). Given that the measured SANS profiles for the propranolol dispersions will be weighted towards the larger aggregates (that account for a higher volume fraction of the sample), the model-fitted SasCalc predictions of the small angle scattering arising from the aggregates found in the equilibrated MD snapshot are thus found to be gratifyingly consistent with the population average determined by experiment.

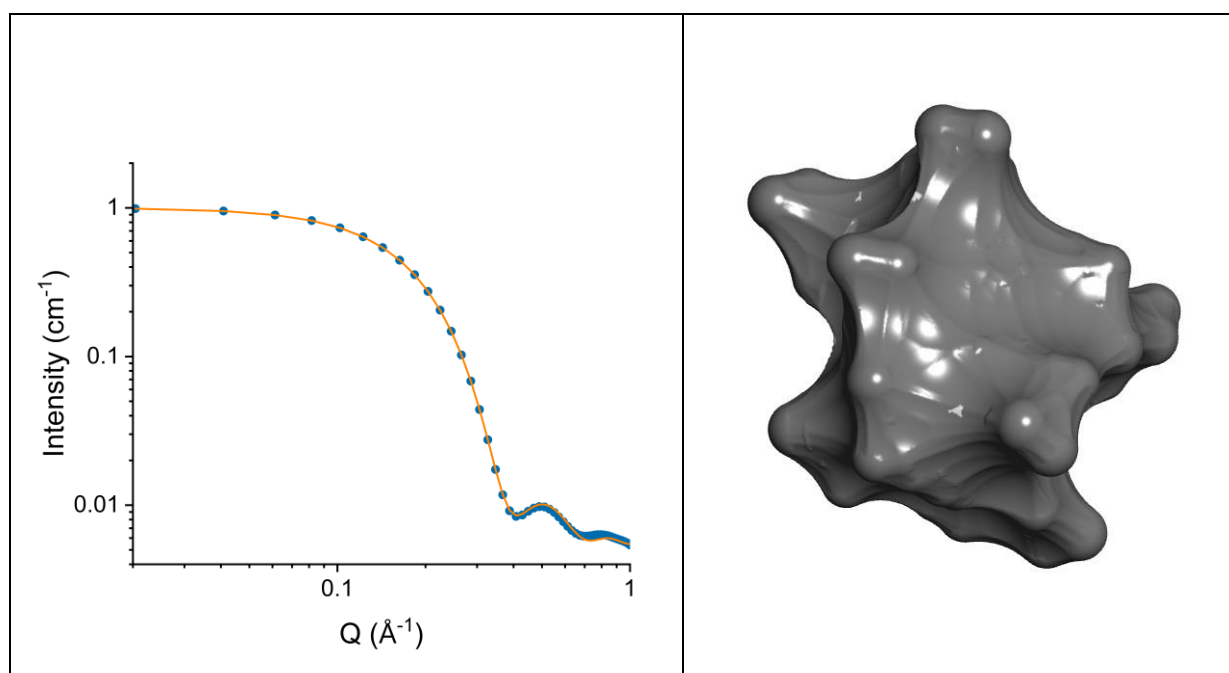


Figure SI - 3.10 SasCalc computed form factor and SasView model fitted curve (left hand panel) for one of the propranolol aggregates (with $N_{agg} = 14$) seen in the equilibrated MD snapshot (right hand panel). The scattering form factor is modelled with the aggregate approximated as a prolate ellipsoid with dimensions, $11.3 \text{ \AA} \times 11.3 \text{ \AA} \times 16.0 \text{ \AA}$. The aggregate is shown enclosed within its solvent-accessible surface, visualised using Accelrys Discovery Studio Visualizer 4.0.

4 Self-assembly of propranolol hydrochloride: a small angle neutron scattering study

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4.1 Abstract

The amphiphilic nature of many commercial drugs results in their self-assembly to form small aggregates in aqueous solution which may influence the drug's therapeutic activity. As with classical surfactants, the self-assembly of an amphiphilic drug may be affected by a range of environmental factors as well as the drug's structure. The aim of the current study was to explore the self-association behaviour of the amphiphilic drug, propranolol hydrochloride, under a range of conditions, namely concentration, temperature, the presence of electrolyte and the effect of enantiomeric form. Small angle neutron scattering in combination of all-atom molecular dynamics simulations have been used to obtain, at the molecular level, information on the size, shape, aggregation number and average surface charge of the aggregates under a range of conditions. While the propranolol hydrochloride aggregates are shown to grow as a function of concentration, temperature plays a negative effect on their self-association, with aggregates decreasing in size with an increase in temperature. In contrast, the addition of 0.154 M NaCl promotes the growth of the aggregates, with the drug altering its orientation within the aggregate. The two enantiomers, S-(-) and R-(+), exhibited slight differences in their aggregation behaviour that may relate to the lattice packing of the crystal. The results of this work emphasize the significance of understanding the self-association of propranolol hydrochloride in the clinical use of the drug.

4.2 Introduction

The possession of an amphiphilic nature is not uncommon amongst commercially used non-steroidal anti-inflammatory, antidepressants, antibiotics and cardiovascular drugs. Although the word amphiphile is frequently used to refer to a surfactant, many drug molecules similarly contain hydrophobic and hydrophilic moieties.^[1] In common with surfactants, amphiphilic drugs exhibit surface active behaviour and self-associate in aqueous solution when the amphiphile exceeds a certain value, known as the critical micelle concentration (CMC) to form “micelle-like” structures. For example, the anti-allergic drug, cromolyn sodium, has been shown to form, in aqueous solution, different aggregate types depending upon concentration.^[2, 3] Similarly, the anti-hypertensive drug, trimetaphan, camphorsulphonate, was found to self-associate in a stepwise manner producing polydisperse aggregates.^[4]

In contrast to classical surfactants, amphiphilic drug molecules tend to contain smaller hydrophobic regions, often composed of aromatic or heterocyclic rings together with a short side chain, explaining the smaller and more polydisperse nature of the aggregates they form.^[1] However, despite their reduced tendency towards surface activity and aggregation, their amphiphilic behaviour is considered to play an important role in moderating the apparent solubility, permeability and bioavailability of a drug, influencing its therapeutic activities. For example, the self-association of terbenzimidazoles has been reported to modulate the compound's bioavailability, with terbenzimidazoles exhibiting an enhanced propensity for self-association and a large aggregate size leading to a reduced cellular bioavailability.^[5]

In addition to surfactant structure, the CMC and self-association of a drug can be influenced by environmental factors such as concentration, temperature, presence of electrolyte, pH and solvent.^[1] For example, many drugs including the antibiotic tetracycline, exhibit a concentration dependent self-association,^[6] while the nature of the aggregates formed by some drugs including the anti-allergic drug, cromolyn sodium, are determined by their concentration.^[2, 3] Further, an increase in temperature resulted in an increase in the CMC of the antihypertensive drug, propranolol hydrochloride,^[7] while the self-association of the antibiotic, tetracycline was reported at a lower concentration in the presence of salt.^[6]

Propranolol hydrochloride is an antihypertensive agent widely used clinically for the treatment of hypertension and, because of its ability to lower intraocular pressure after topical administration, glaucoma.^[8] The structure of propranolol hydrochloride is amphiphilic containing a hydrophobic naphthalene ring and hydrophilic amine and hydroxyl groups (Figure 4.1) and has been reported to self-assemble in aqueous solution at a concentration (or CMC) of 108 mM in water and 69 mM in the presence 0.2 M KCl.^[9] Additionally, as propranolol hydrochloride contains a chiral carbon resulting in the existence of enantiomers, the structure and the stability of the aggregates formed by the two propranolol hydrochloride isomers may differ. For example, the RR dimer of the antitumor drug, imexon, was reported to be less stable than RS the dimer.^[10]

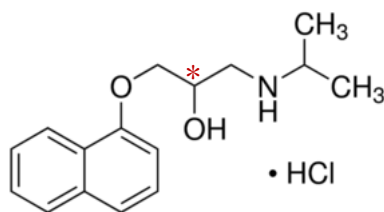


Figure 4.1 Structure of propranolol hydrochloride, chiral carbon highlighted with star.

In the present study, we investigated how the variation in temperature and electrolyte composition (including 0.154 M sodium chloride to mimic the ionic strength of blood) as well as enantiomer type influences the self-assembly of propranolol HCl. Although previous research has investigated the aggregation behaviour of propranolol HCl, using a range of techniques, including dynamic and static light scattering,^[11] conductivity^[7] NMR^[12] and surface tension,^[13] none of these techniques allow the study of the propranolol HCl aggregates at the molecular level. In order to investigate the self-association process and the structure of resulting aggregates at the molecular level, we have used small angle neutron scattering (SANS) and all-atom molecular dynamics simulations (MD). The use of these two techniques have allowed us to gain an insight into the inter- or intra- molecular interactions that control the self-association of propranolol HCl.

4.3 Materials

Protiated ($\pm$)-propranolol hydrochloride (99.9 %) was obtained from either Alfa Aesar or Thermo Fisher Scientific. (R)-(+)-propranolol hydrochloride ($\geq 98\%$, TLC), (S)-(-)-propranolol hydrochloride ($\geq 98\%$, TLC), sodium chloride ($\geq 99.5\%$, AT) and deuterium oxide (D_2O) (99.9 atom % D) were purchased from Sigma Aldrich. Ring deuterated free base ($\pm$)- d_7 -propranolol (98% D) was provided by CDN Isotopes and was acidified into hydrochloride salt for small angle neutron scattering samples measured at 310 K. Unless otherwise stated, all chemicals were of the highest grade available and were used as supplied. Ultrapure water (resistivity of 18.2 m Ω cm) was obtained from Suez pure water system.

4.4 Methods

4.4.1 Small Angle Neutron Scattering

SANS experiments were performed on the instrument SANS2D at the ISIS Neutron and Muon Source (Rutherford-Appleton Laboratory, Didcot, UK). SANS2D is a time of flight instrument which uses neutrons of wavelength (λ) in the range 2 – 14 Å. In the geometry used in the present study, two 96.5 x 96.5 cm² detectors of 5 mm resolution were placed at distances of 2.4 and 4 m from the sample to yield a momentum transfer, Q , (where $Q = (4\pi/\lambda) \sin(\theta/2)$, and θ is the scattering angle) in the range of $0.005 \leq Q \leq 1 \text{ Å}^{-1}$. In all cases the samples were placed in circular ‘banjo’ cells (Hellma UK Ltd, Southend on Sea, UK) of 1 mm or 2 mm thickness depending upon the solvent, H_2O or D_2O respectively, while a 12 mm diameter neutron beam was used throughout.

The SANS of protiated propranolol hydrochloride in either D_2O or D_2O containing 0.154 mM NaCl were measured at a range of temperatures (293, 298, 307, 310, 315 and 318 K) and concentrations (in the range 30-200 mM), while samples of (R)-(+)- propranolol hydrochloride and (S)-(-)- propranolol hydrochloride in D_2O were measured at concentrations of 150, 130 and 110 mM were measured at 310 K. In all cases the samples were initially bath sonicated for at least 40 minutes to ensure clarity of the samples (Supplementary Information, Table SI-4.1). The raw SANS data obtained from the experiments were processed using wavelength-dependent corrections to allow for the incident spectrum, detector efficiencies, and measured sample transmissions.^[14] The SANS data were then

put on an absolute scale using the scattering from a standard sample (comprising a solid blend of protiated and perdeuterated polystyrene) in accordance with established procedures and expressed as intensity (I) as a function of Q .

The neutron scattering intensity can be described in terms of a form factor, $P(Q)$, and a structure factor, $S(Q)$:

$$I(Q) \propto \frac{N}{V} \langle \Delta\rho \rangle^2 V_p^2 P(Q) S(Q) \quad (4.1)$$

where N is the number of particles; V is the total volume of the system and V_p is the volume of a particle; $\Delta\rho$ is the difference in the scattering length density (SLD) of the particle and the solvent, calculated from the neutron scattering lengths^[15] and physical density of the material.

In the present study, a variety of form [$P(Q)$] and structure [$S(Q)$] factors were investigated including core-shell spheroids coupled with either the Hayter-Penfold (H-P) or the hard sphere models for uncharged aggregates.^[16 – 18] When modelling the SANS data, a number of parameters were fixed, namely the SLD and dielectric constant (relative permittivity) of the solvent, the volume fraction of the drug and the ratio of the thickness of the polar to the equatorial shell (where the assumption was made that the thickness of the shell was constant).

To minimize the effect of systematic errors from large error bars at low Q and noisy backgrounds at high Q , the SANS data were fitted within a defined Q range in the SASView program.^[19] For (racemic) *h*-propranolol hydrochloride in D₂O, the fitted Q range was 0.02 – 0.6 Å⁻¹ while for the (racemic) *h*-propranolol hydrochloride in D₂O containing 0.154M NaCl was fitted over the whole Q range of 0.005 – 1 Å⁻¹ with the low Q range (0.005-0.5 Å⁻¹) being fitted separately than the wide angle scattering (0.5 – 1 Å⁻¹) which due to the significant upturn observed at high Q was fitted using the Gaussian peak available in SASView. Multi-contrast systems of (racemic) propranolol hydrochloride were fitted over the Q range 0.01 – 1 Å⁻¹. The R/S- propranolol hydrochloride data were fitted over the Q range 0.02 – 1 Å⁻¹.

Table 4.1 Neutron scattering length, molecular volume and neutron scattering length density of molecules and fragments.

Molecule/Fragment	Neutron scattering length, b ($10^{-5}\text{\AA}$)	Molecular volume, M_v ($\text{\AA}^3$)	Scattering length density, ρ ($10^{-6}\text{\AA}^{-2}$)
Naphthalene ring ($-\text{C}_{10}\text{H}_7$)	40.28	183	2.20
Naphthalene ring (deuterated) ($-\text{C}_{10}\text{D}_7$) (98%D)	113.16	183	6.18
Propranolol hydrochloride aliphatic chain ($-\text{C}_6\text{H}_{15}\text{ClNO}_2$)	9.32	264	0.35
Propranolol hydrochloride whole molecule protiated ($\text{C}_{16}\text{H}_{22}\text{ClNO}_2$)	54.63	447	1.22
Propranolol hydrochloride whole molecule (ring deuterated) ($\text{C}_{16}\text{H}_{15}\text{D}_7\text{ClNO}_2$) (98%D)	127.50	447	2.82
Sodium chloride (NaCl)	13.21	45	2.93
H_2O	-1.68	30	-0.56
D_2O	19.14	30	6.35
0.154 M sodium chloride in D_2O	-	-	6.21

Neutron scattering length density of a molecule/fragment can be calculated by dividing the sum of the scattering length of all atoms in the molecule/fragment by the molecular volume of the molecule/fragment, $\rho = \frac{\sum b_i}{M_v}$

The scattering length density of the solvent (H_2O or D_2O), propranolol hydrochloride (protiated and ring deuterated forms) and the constituent fragments of propranolol are given in Table 4.1, along with the scattering length and molecular volumes used to calculate these values.

4.4.2 Molecular dynamics simulations

Full atom molecular dynamics simulations of propranolol hydrochloride were performed using GROMACS 2018.4^[20] using the GROMOS 53A6 force field^[21] with parameters for the propranolol molecules obtained from PRODRG version AA180301.0717^[22] to study the aggregation of propranolol hydrochloride under a range of conditions including temperature, electrolyte (specifically NaCl) and enantiomer type. For simulations of 200 mM (racemic) propranolol hydrochloride, 406 propranolol hydrochloride molecules (*i.e.* 203 R-(+)-propranolol hydrochloride (DPR) and 203 S-(-)-propranolol hydrochloride (LPR), were inserted randomly into a $15 \times 15 \times 15$ nm box using the GMX insert-molecules protocol that was subsequently filled with SPC water molecules using GMX solvate. Lastly, a number of chloride ions equal to the number of molecules of propranolol in the simulation box, with the added chloride ions replacing solvent molecules using GMX insert-molecules. To investigate

the effect of temperature, simulations were run at a pressure of 1 atm and temperatures of either 293 K, 298 K, 300 K, 310 K or 315 K. The effect of 0.154 M NaCl on the aggregation of propranolol hydrochloride at each temperature was studied by the addition of 313 NaCl molecules. When studying the effect of enantiomer type at 300 K, 200 mM of LPR was simulated as opposed to 200 mM of racemic propranolol hydrochloride.

The various systems were equilibrated for 200 ps of NVT and 200 ps of NPT ensemble simulation, followed by 60 ns of NPT production run. The well equilibrated final 20 ns of production run were used for analysis of the simulations. *pairdist* analyses (GROMACS) were performed to calculate the shortest distance between any atom in every pair of propranolol molecules in the system, with a cut off distance of 0.45 nm, *i.e.* any molecules separated by distance above 0.45 nm were not considered as being in the same aggregate. The aggregates were built up by taking each molecule of propranolol in turn and identifying all molecules within 0.45 nm of that molecule. This list of molecules is then used to identify all further molecules within 0.45 nm of any of them to form a second shell of the aggregate. A further five shells are then identified. Each other molecule is then used as the starting seed for aggregate formation. The set of aggregate structures is obtained by sorting the set of clusters built up for all of the molecules in the simulation to remove duplicates.

4.5 Results

4.5.1 Effect of temperature in the absence of electrolyte

The SANS of protiated (*h*-)propranolol hydrochloride in D₂O, measured over a range of concentrations (30 – 200 mM) and range of temperatures (298, 307 and 315 K) was best fitted using a core-shell, prolate ellipsoid form factor, convoluted with a Hayter-Penfold structure factor. The values of the parameters used to obtain the best fit to the data are summarised in Table 4.2, while the recorded SANS data, along with the corresponding best fits to the data are shown in Figure 4.2, with the exception of 30 mM *h*-propranolol hydrochloride where it did not prove possible to satisfactorily fit the neutron scattering data, possibly due to very low scattering. In addition, a study was performed at 293 K using a limited range of *h*-propranolol hydrochloride concentrations (Table 4.2). The scattering data and the best fit to the data obtained for the more limited range of

propranolol hydrochloride concentrations at 293 K are given in the Supplementary Information, Table SI-4.2. In all cases the model used to obtain the best fit to the SANS data assumed that the core of aggregates were composed solely of the naphthalene rings of propranolol hydrochloride while the surrounding hydrophilic shell was composed of the molecule's solvated side chains.

It should be noted that a small number of other propranolol hydrochloride 'neutron' contrasts were measured at 315 K, namely partially (ring) deuterated (*d*-)propranolol hydrochloride in either D₂O or H₂O and protiated (*h*-)propranolol hydrochloride in H₂O. In cases where one or more of these additional contrasts were measured, the SANS data obtained was simultaneously analysed with the corresponding data for *h*-propranolol hydrochloride in D₂O. The parameters used to obtain the best fit to this set of SANS data are shown in Supplementary Information, Table SI-4.1. Significantly, the parameters used to obtain the simultaneous fits are comparable to those obtained from the analysis of *h*-propranolol hydrochloride in D₂O alone, therefore giving weight to the results obtained using a single contrast.

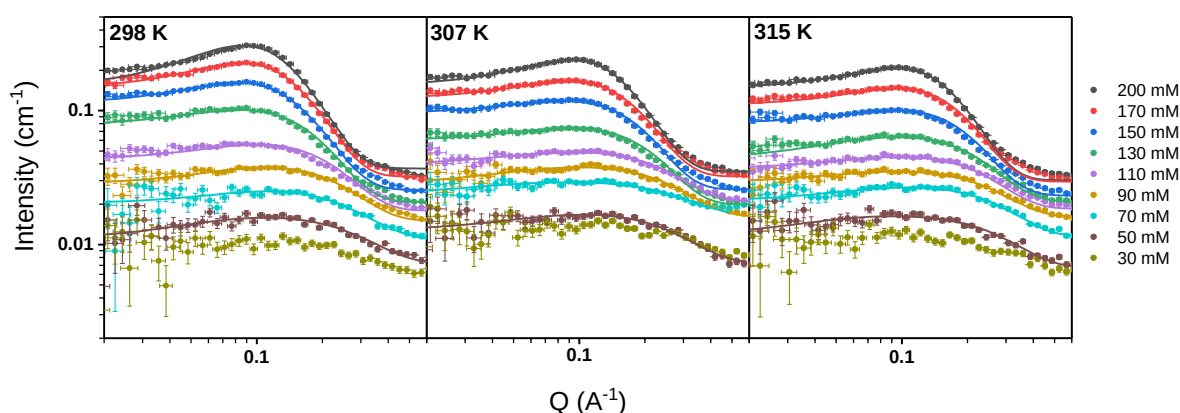


Figure 4.2 The SANS data (filled circles) obtained for varying concentrations (30 – 200 mM) of *h*-propranolol hydrochloride in D₂O expressed as a function of neutron scattering intensity plotted against momentum transfer (Q) and the best fits (solid lines) obtained assuming a core-shell prolate ellipsoid form factor combined with the Hayter-Penfold structure factor at either (a) 298 K; (b) 307 K or (c) 315 K. Note that it did not prove possible to satisfactorily model the SANS data obtained for the 30 mM *h*-propranolol hydrochloride in D₂O.

The shape of the SANS curve, including the peak seen at $\sim 0.1 \text{ \AA}^{-2}$, obtained for *h*-propranolol hydrochloride in D₂O is comparable to that seen for the micelles formed by charged surfactants such as sodium dodecyl sulfate^[23] and is a consequence of the charged nature of the propranolol hydrochloride aggregates. It is noticeable, however, that the intensity of the peak seen at $\sim 0.1 \text{ \AA}^{-2}$

was seen to reduce as temperature increased suggesting that the aggregates are tending to become less charged as a function of temperature. The analysis of the SANS data in Table 4.2 confirms that the average charge on the aggregates tends to decrease with an increase in temperature.

Table 4.2 Best fit parameters for the SANS data of *h*-propranolol hydrochloride in D₂O at 293 K, 298 K, 307 K and 315 K using the ellipsoid form factor and associated Hayter-Penfold structure factor model.

Concentration (mM)	Equatorial radius core (Å)	Polar radius core (Å)	Volume of core (Å ³)	Thickness of shell (Å)	Charge (e)	Charge/drug molecule (e)	Hydration of shell	Aggregation number (<i>N_{agg}</i>)
293 K								
200	7.3	20.5	4558	4.9	7.0	0.28	0.69	25
150	6.4	17.6	3016	4.7	4.8	0.29	0.71	16
110	5.1	14.3	1537	3.8	1.9	0.22	0.74	8
90	4.1	11.3	805	2.8	1.6	0.36	0.76	4
50	3.2	8.5	361	3.0	1.3	0.66	0.78	2
298 K								
200	6.7	18.8	3569	5.6	4.3	0.22	0.71	20
170	6.3	17.1	2840	5.5	4.1	0.27	0.71	16
150	6.0	16.7	2560	5.3	3.8	0.27	0.74	14
130	5.5	12.9	1624	5.0	2.7	0.31	0.75	9
110	4.9	9.5	960	4.0	2.6	0.49	0.76	5
90	4.5	7.8	643	3.9	2.4	0.69	0.77	4
70	4.1	9.1	640	2.7	2.3	0.65	0.79	3
50	3.7	7.7	442	2.7	1.8	0.74	0.75	2
307 K								
200	6.5	16.5	2883	5.2	4.3	0.27	0.72	16
170	6.0	14.0	2107	5.1	3.3	0.29	0.74	12
150	5.6	12.1	1581	5.1	2.7	0.31	0.74	9
130	5.0	9.8	1016	4.6	2.3	0.41	0.74	6
110	4.4	7.9	654	3.8	1.5	0.41	0.76	4
90	4.4	8.0	636	3.2	1.4	0.40	0.76	3
70	4.0	7.3	484	3.3	1.1	0.42	0.81	3
50	3.8	6.0	359	3.6	1.9	0.99	0.74	2
315 K								
200	6.2	15.7	2553	5.1	4.1	0.29	0.73	14
170	5.8	12.8	1795	5.0	3.5	0.36	0.73	10
150	5.4	11.6	1394	4.6	3.0	0.39	0.74	8
130	4.8	10.7	1044	4.0	1.8	0.32	0.79	6
110	4.5	7.9	685	4.3	1.7	0.47	0.80	4
90	4.4	7.2	577	4.0	1.5	0.47	0.80	3
70	4.5	6.1	517	3.2	1.7	0.59	0.76	3
50	4.2	5.8	431	3.4	1.3	0.57	0.77	2

Best fit parameters were not obtained for 30 mM propranolol hydrochloride.

N were obtained by dividing the micelle core volumes by the volume of the drug's naphthalene moiety.

Furthermore, from Table 4.2 it can be seen that propranolol hydrochloride forms aggregates at all concentrations down to 50 mM, it was not possible to fit the data for 30 mM propranolol hydrochloride. It is clear, however, that the aggregation is not typical of the micelles formed by traditional surfactants in that the aggregates formed at low drug concentrations (50 – 70 mM) are very small ($N_{agg} = 2 - 3$) and gradually increase in size to a maximum size of 25 at 200 mM propranolol hydrochloride at 293 K. Furthermore, the aggregates are seen to reduce in size with increasing concentration – most likely a consequence of the higher Brownian motion of the propranolol hydrochloride molecules at higher temperatures. It is worth noting that the concentrations in Table 4.2 are not corrected for the CMC of the propranolol hydrochloride, because although surface tension measurements yielded a CMC of approximately 92.4 mM, it is clear that propranolol hydrochloride, aggregation occurred, albeit limited, at concentrations of 50 mM or even lower, making the choice of the concentration to use for CMC correction difficult.

The aggregation number (N_{agg}) of the propranolol hydrochloride aggregates was established by dividing the volume of the core obtained from the SANS modelling (Table 4.2) by the volume of a naphthalene ring (Table 4.1). The variation in the resulting aggregation number with both the concentration and temperature is shown in Table 4.2 and Figure 4.3. With reference to the aggregation numbers obtained at 298 K, at concentrations below 110 mM, the increase in aggregate size with propranolol hydrochloride concentration is slow while after ~ 110 mM the aggregation number increases more quickly but still in an approximately linear fashion to the highest drug concentration of 200 mM measured. A similar trend in the variation of aggregation number with drug concentration was seen at the other temperatures studied. The concentration at which the break in aggregation number (Table 4.3), *i.e.* the intersects of the linear fits to the reciprocal of concentration, occurs increases with temperature from ~ 94 mM at 293 K to ~ 109 mM at 298 K and ~ 114 mM at 307 – 315 K – a result which correlates with the reduction in aggregation number observed with increasing temperature.

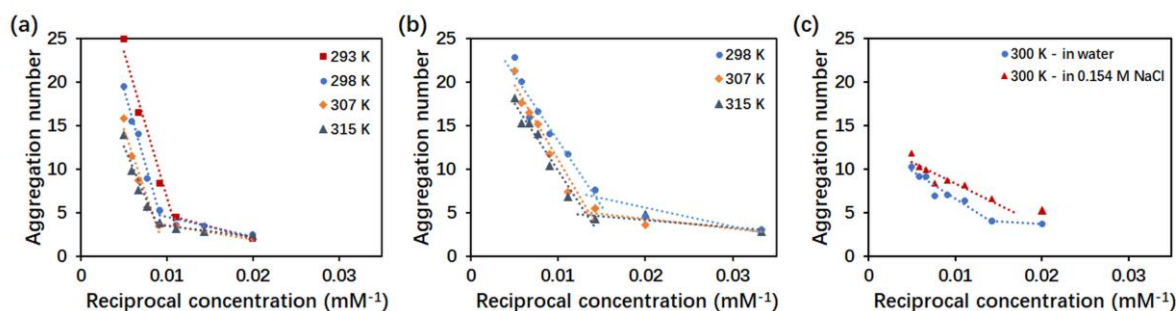


Figure 4.3 Calculated aggregation number (N_{agg}) from the parameters obtained for the best fit of the SANS data (a and b) using core-shell ellipsoid model and calculated from MD simulation results at different temperatures (c). a: propranolol hydrochloride dispersed in D_2O ; b: propranolol hydrochloride dispersed in D_2O containing 0.154 M sodium chloride. c: propranolol hydrochloride dispersed both in water and in 0.154 M sodium chloride. Dashed lines are the linear fits.^[30]

Table 4.3 Concentrations (mM) at the break point of aggregation number of propranolol hydrochloride obtained from SANS data.

	293 K	298 K	307 K	315 K
In D_2O	93.6	109.4	115.4	113.8
In D_2O containing 0.154 M NaCl	/	69.8	73.7	75.2

Reassuringly, all atom molecular dynamic simulations confirmed the model used by the SANS data in that the core of the propranolol hydrochloride micelle was comprised solely of naphthalene rings surrounded by a shell of the solvated side chains. The larger aggregates occasionally include a side chain that is partially among the naphthalene rings and this is in contrast to the aggregates in the absence of the added NaCl.

It is clear from Figure 4.4, which shows a plot of the mole fraction of the aggregates as a function of aggregation number for a 200 mM aqueous propranolol hydrochloride dispersion, that the aggregates formed by propranolol hydrochloride are very polydisperse in nature. In this context, it should be noted that the mean aggregate size obtained from molecular dynamic simulations are a number average, while the aggregation numbers calculated from the SANS data are a volume mean weighted. As a result it would be expected that due to the polydispersity of the system the mean aggregate sizes obtained from SANS will be larger than those obtained from the MD simulations, as shown in Figure 4.3 (c). (results of other concentrations are listed in Supplementary information SI-4)

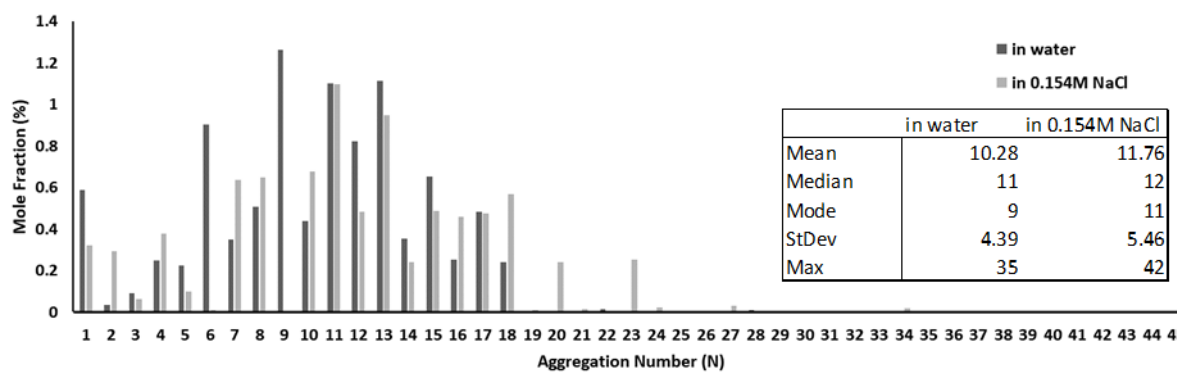


Figure 4.4 Comparison of the size distribution of aggregates calculated from all-atomistic MD simulation of 200 mM propranolol hydrochloride in the absence and presence of 0.154 M NaCl at 300 K. Insert gives a statistical summary of the data.

4.5.2 Effect of temperature in the presence of electrolyte

An aqueous solution of 0.154 M sodium chloride was used as a simple mimic of blood which contains Na^+ , K^+ , Ca^{2+} , Mg^{2+} , Cl^- and HCO_3^- ions and has an ionic strength of about 0.15 M.^[24] The variation in the SANS scattering of propranolol hydrochloride dissolved in D_2O containing 0.154 M sodium chloride as a function of temperature shown in Figure 4.5 shows a similar trend to that obtained in the absence of electrolyte given in Figure 4.2 in that aggregate size decreases with temperature, due to an increase in Brownian motion with increasing temperature. It is particularly noticeable from Figure 4.5 that there is an up-turn in the scattering data at high Q . To the authors knowledge this up-turn has not been reported previously. It proved possible to successfully model the whole Q range for all concentrations using the prolate ellipsoid model consisting of a core of dry naphthalene rings and a shell of solvated side chains coupled with the Hayter-Penfold structure factor elaborated to include a Gaussian function. The upturns seen at high Q in Figure 4.5 are taken to be the leading edge of a wide-angle peak - centred around $1 \text{ \AA}^{-1}$ (and corresponding to spacings of the order of $6 \text{ \AA}$) - most likely arising because of interactions between the aromatic moieties of the aggregated drug molecules, as seen, for example in surfactant/perfume aggregates.^[25] However, due to an absence in the up-turn of the scattering at high Q , it only proved possible to fit the SANS data for propranolol hydrochloride at 50 and 30 mM using ellipsoid form factor and associated Hayter-Penfold structure factor model, suggesting that either the naphthalene rings were too far apart for their interaction to be seen and/or alternately there is a change in packing seen at low drug concentrations. The best fits obtained to the SANS data in the presence of 0.154 M NaCl using the ellipsoid form factor and

associated Hayter-Penfold structure factor model along with the fit to the Gaussian function are shown on Figure 4.5 as solid lines, while the parameters used to obtain these best fits are given in Table 4.4.

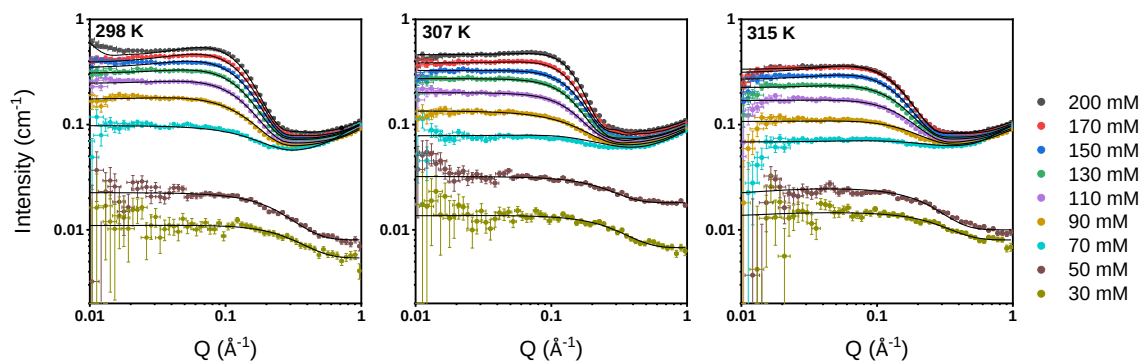


Figure 4.5 The SANS data (points) expressed as neutron scattering intensity plotted as a function of momentum transfer (Q) and the best fits (solid lines) obtained assuming a core-shell prolate ellipsoid form factor coupled with the Hayter-Penfold structure factor and elaborated to include a Gaussian function to fit the up-turn at high Q to varying concentrations of propranolol hydrochloride at either 298, 307 and 315 K in D_2O containing 154 M sodium chloride.

The shape of the SANS curves recorded in the presence of 0.154 M NaCl were lacking the peak seen at about $0.1 \text{ \AA}^{-2}$ in the SANS data obtained with propranolol hydrochloride in D_2O . This difference is a consequence of the increased ionic strength of the aqueous solution reducing the ionisation of propranolol hydrochloride in solution resulting in a reduced repulsion between charges on the two adjacent drug molecules, allowing the formation of larger aggregates. The increased aggregation number of the aggregates formed by propranolol hydrochloride in the presence of 0.154 M NaCl can be seen in Table 4.4 and Figure 4.3(b). It is noticed that the concentration that corresponds to the break of aggregation number has decreased from 109 mM to 70 mM at 298 K with the addition of 0.154 M NaCl, a result which is consistent with the surface tension data obtained at room temperature (supplementary information SI-2). It should be noted that despite the absence of a peak in the scattering curves observed in the presence of 0.154 M NaCl it was not possible to fit that scattering data using the hard sphere model assuming that there is no charge on the aggregates.

There are some noticeable differences between the propranolol hydrochloride aggregates formed in the absence and presence of 0.154 M NaCl. For example, at 298 K, the higher propranolol hydrochloride concentrations in the presence of 0.154 M NaCl possessed a thicker shell of $\sim 7 \text{ \AA}$ as

opposed to $\sim 5 \text{ \AA}$ in its absence, the shell possessed was very slightly lower solvent $\sim 65\%$ versus 71% . As the drug concentration decreased to $\sim 70 \text{ mM}$ in the presence of 0.154 M NaCl and 110 mM in its absence there was a decrease in the thickness of the shell which further decreased with decreasing propranolol hydrochloride concentration, suggesting a re-orientation of the aggregates. Similar observations were made at different temperatures and roughly comparable concentrations.

Table 4.4 Fitted parameters for the SANS data of propranolol hydrochloride in D_2O containing 0.154 M sodium chloride at 298 K , 307 K and 315 K using ellipsoid form factor and associated Hayter-Penfold structure factor model and elaborated to include a Gaussian function to fit the up-turn at high Q .

Concentration (mM)	Equatorial radius core ($\text{\AA}$)	Polar radius core ($\text{\AA}$)	Volume of core ($\text{\AA}^3$)	Thickness of shell ($\text{\AA}$)	Charge (e)	Charge/drug molecule (e)	Hydration of shell	Aggregation number (N)	Distance between naphthalene rings ($\text{\AA}$)
298 K									
200	7.2	19.0	4166	7.1	4.6	0.20	0.64	23	5.1
170	7.1	17.2	3661	7.1	4.0	0.20	0.63	20	5.2
150	7.1	14.9	2916	7.2	4.3	0.27	0.63	16	5.7
130	7.0	15.1	3033	7.3	4.5	0.27	0.66	17	4.7
110	6.8	13.4	2656	7.5	4.8	0.34	0.69	14	4.8
90	6.3	12.8	2139	7.3	3.7	0.31	0.73	12	4.4
70	5.5	11.0	1383	6.1	0.0	0.00	0.79	8	5.6
50	4.2	11.4	829	2.2	0.0	0.00	0.78	5	-
30	4.0	8.4	557	1.6	0.9	0.30	0.79	3	-
307 K									
200	7.2	18.0	3897	6.5	5.3	0.25	0.65	21	5.3
170	7.0	15.7	3209	7.0	4.8	0.27	0.67	18	5.5
150	6.9	15.2	3022	7.9	4.0	0.24	0.68	17	5.4
130	6.7	15.0	2774	6.8	3.1	0.21	0.68	15	5.2
110	6.3	12.9	2139	6.8	1.3	0.11	0.69	12	5.4
90	5.8	11.5	1337	6.7	0.0	0.00	0.70	7	5.2
70	5.0	9.8	1004	5.7	0.0	0.00	0.71	6	5.2
50	4.0	9.1	646	3.2	0.0	0.00	0.77	4	-
30	4.0	8.5	560	1.9	0.3	0.10	0.77	3	-
315 K									
200	7.0	16.3	3327	7.4	4.1	0.23	0.73	18	5.7
170	6.8	14.4	2792	6.9	4.0	0.26	0.70	15	5.2
150	6.7	14.9	2786	6.9	3.8	0.25	0.70	15	5.8
130	6.5	14.7	2575	6.6	3.3	0.24	0.72	14	5.5
110	6.0	12.6	1899	6.4	2.6	0.25	0.73	10	5.5
90	5.2	10.9	1235	6.1	1.8	0.27	0.78	7	5.5
70	4.6	8.7	767	4.5	0.7	0.16	0.79	4	5.4
50	4.5	10.3	869	2.9	0.0	0.00	0.80	5	-
30	4.8	8.6	511	2.4	0.0	0.00	0.79	3	-

All data are fitted with ellipsoid core_shell model with H&P $S(Q)$ at $Q = 0.02\text{--}0.5 \text{ \AA}^{-1}$, other data were also fitted with wide angle peak at $Q = 0.5\text{--}1 \text{ \AA}^{-1}$

4.5.3 Effect of optical isomerism on the aggregation of propranolol hydrochloride

Propranolol hydrochloride contains a chiral carbon, making the molecule optically active (Figure 4.1). Although the racemic mixture is used clinically, the two enantiomers have been reported to exhibit slightly different therapeutic effects. For example, S-(-)-propranolol hydrochloride exhibits a higher affinity for β -receptors than R-(+)-propranolol hydrochloride, and as a consequence plays a major role reducing blood pressure.^[26] In contrast, the R-(+) enantiomer is known improve the bioavailability of the S-(-) enantiomer when in the form of a racemate.

To investigate whether the self-assembly of the individual S-(-) and R-(+) propranolol hydrochloride enantiomers varies from that of the racemate, the individual enantiomers were dissolved in D₂O at concentrations of 110, 130 and 150 mM and their SANS determined at 310 K. The higher temperature was used here, due to the lower solubility (*i.e.* less than 110 mM) at room temperature of the single enantiomers.^[26] The difference in solubility between enantiomers and racemate may related to the stability of crystal lattice of racemate and enantiomers, because a study of both racemic and scalemic forms demonstrated that although the crystal packing of racemic and scalemic propranolol hydrochloride are similar, the racemic crystal seems less stable than the enantio-pure crystal.^[27]

The SANS results, which are shown in Figure 4.6 along with the best fit to the data obtained using the same model as that used for racemic propranolol hydrochloride, namely a prolate ellipsoid with a dry core composed of naphthalene rings and a hydrated shell consisting of aliphatic chains, along with the Hayter-Penfold structure factor. The fitted parameters of the shape and sizes of the aggregates were listed in the Table 4.5. Compared with racemic form that measured at 310 K, the single enantiomers tend to have very similar aggregates formed in solutions. The S-(-) enantiomer is likely to form slightly larger aggregates than the racemate at same concentration, while the aggregates of R-(+) are slightly smaller than that of racemate.

MD simulations of pure enantiomer LPR, *i.e.* S-(-) dispersed in water at a propranolol hydrochloride concentration of 200 mM has been performed, however little difference was observed when the data was compared with that of the racemic form. (results in the supplementary information SI-5)

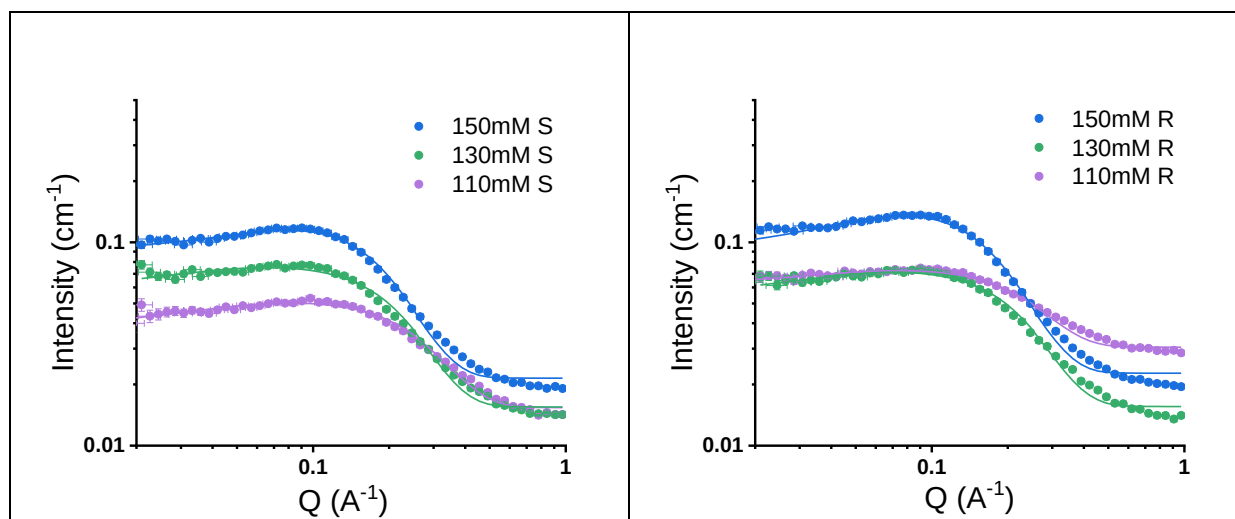


Figure 4.6 Neutron scattering intensity of S-(-)-propranolol hydrochloride (left) and R-(+)-propranolol hydrochloride (right) plotted as a function of the scattering vector Q , measured at 310 K. The solid lines are the models of experimental data fitted with prolate ellipsoid that consist of dry shell and wetted shell.

Table 4.5 Fitted parameters of S-(-)-propranolol hydrochloride and R-(+)- propranolol hydrochloride SANS data at 310 K.

Concentration (mM)		Temperature (K)	Volume core (Å ³)	Thickness shell (Å)	Charge (e)	Hydration of shell	Aggregation number (N)
150	S-(-)	310	1816	4.20	2.15	0.7	10
150	R-(+)	310	1848	4.55	2.02	0.7	10
150	racemate	307	1581	5.11	2.67	0.7	9
130	S-(-)	310	1078	4.45	1.71	0.7	6
130	R-(+)	310	829	4.26	1.66	0.7	5
130	racemate	307	1016	4.65	2.29	0.7	6
110	S-(-)	310	750	2.64	1.39	0.7	4
110	R-(+)	310	652	3.17	1.53	0.7	4
110	racemate	307	654	3.84	1.47	0.8	4

4.6 Discussion and Conclusion

The self-association of the amphiphilic drug, propranolol hydrochloride, under a range different experimental conditions, namely concentration, temperature, ionic strength and the optical conformation, has been studied. SANS in combination with contrast variation has allowed any changes in the size and shape of the aggregates to be elucidated at a molecular level. A core-shell ellipsoid model was used for analysing the data obtained using multicontrast SANS exploiting both protiated and ring-deuterated propranolol hydrochloride dispersed in H₂O and D₂O (Supplementary Information, SI-3). The all-atom MD simulations has been used in the present study as

complementary to the SANS results. It is concluded from these studies that the self-association of propranolol hydrochloride in water is concentration dependent. Increasing temperature was seen to play a negative effect on the self-assembly of the drug, regardless of the presence or otherwise of 0.154 M NaCl. In contrast, the presence of 0.154 M NaCl promotes the aggregation of propranolol hydrochloride as evidenced by an increase in aggregate size and a decrease in the CMC. Finally, the S-(-) and R-(+) isomers behave slightly differently in respect to self-association in that the S-(-) isomer tends to form slightly larger aggregates compared with R-(+) isomer and the racemate.

The effect of temperature (range 293 – 315 K) on the aggregation of propranolol hydrochloride was studied both in the absence and presence of 0.154 M sodium chloride. It is observed that, regardless of the presence or otherwise of electrolyte, aggregate size decreased with an increase of temperature, suggesting an antagonistic effect of temperature on the self-association of the drug. Slightly more complex temperature effects have been reported for conventional ionic surfactants in that an increase of temperature may first lower the CMC of the ionic surfactant, followed by an increase in CMC with a further temperature increase. The minimum value of CMC recorded for the anionic surfactant, sodium dodecyl sulfate, was observed in the temperature range 293 – 303 K,^[1, 28] while the minimum CMC measured for the cationic surfactant, *N*-dodecylpyridinium chloride, was observed to be around 298 K.^[28] An increase in temperature below the temperature at which the minimum CMC occurs, encourages micellization, while an increase in temperature above that at which the minimum CMC occurs, opposes micellization. The self-association of ionic surfactants is considered to be a balance between the hydrophobic effect driving aggregation and electrostatic interactions opposing aggregation. In the present work, the temperature range examined seems to fall within the latter range, that is it is above the temperature at which the minimum in CMC occurs, as the self-association of the drug is discouraged. An increase in temperature will contribute to a weakened hydrophobic effect while at the same time strengthening the electrostatic repulsions between drug molecules. An enhanced Brownian motion of both the drug and water at higher temperatures contributes to a reduction in the structuring of water molecules (or the water “cage”) surrounding the hydrophobic moieties, and as a consequence less energy is required to break the water “cage”. As the free energy of a system is determined by the balance between enthalpy and entropy, a small change in entropy can result in enthalpy dominating at higher temperatures, causing an increase in the free energy required for self-association.^[29] Additionally, at higher temperatures,

the electrostatic repulsion between molecules increases,^[28] resulting in smaller sized aggregates at higher temperatures.

Sodium chloride (0.154 M) was added to make the solution physiological ionic strength, whereupon both the SANS and MD results yielded a significant change in the size and charge of the propranolol hydrochloride aggregates. The average charge for each drug molecule when in the form of an aggregate was slightly decreased compared with that seen in water, leading to a reduction in the intermolecular-interaction peak. Another noticeable change in the presence of salt is the increase in the thickness of the hydrated shell. It is proposed that the hydrated side-chains of the drug that make up the shell have reoriented in the presence of NaCl, allowing for the closer packing of the side-chains due to a decreased electrostatic repulsion between drug molecules. An interesting observation is the upturn in the SANS data at high Q , assumed to be a leading edge of the wide scattering peak. Fitting of these peaks resulted in a corresponding distance of 4 – 6 Å, which suggested a possible π - π interaction between the naphthalene rings. Our previous work performed in the absence of sodium chloride indicated that the interaction between the naphthalene rings was via a CH- π rather than a π - π interaction,^[30] hence it can be concluded that the nature of the interaction between the ring is altered by the addition of sodium chloride.

The aggregation of S-(-) and R-(+) enantiomers were studied at 310 K. The SANS results suggested that there are slight differences between the two enantiomers in terms of their average size and aggregate geometry. Specifically, the S-(-) enantiomer tends to form slightly larger aggregates than the R-(+) enantiomer, which forms aggregates of similar size to that recorded for the racemates. Both enantiomers have much smaller apparent aqueous solubility than the racemate,^[26] as a result of the different crystal packing of the enantiomers and racemate. It is deduced therefore that the crystal packing may play an important role in the self-association of propranolol hydrochloride in water. As a consequence, the drug molecules in aggregates composed of enantiomer only may be in a different orientation to the aggregates formed by the racemates, for example the shell thickness of the aggregates formed by the racemates were thicker than that of the enantiomers.

The different variables, which have been studied in detail at the molecular scale, have differing effects on the self-association of propranolol hydrochloride. It has been suggested that the self-association of a drug may modify its therapeutic activity, partly because the number of the monomeric drug molecules have decreased due to their self-association. Although the concentration

of drug used in this study is much higher than its usual concentration in plasma, the current study has shown that the presence of electrolyte can increase self-association significantly, which means that self-association in the biological environment is possible.

Statements:

The SANS experiments were performed by Yixuan Yan supported by the instrument scientist Dr. Najet Mahmoudi. Data analysis was undertaken by Yixuan Yan with the support of Professor Jayne Lawrence and Dr. David Barlow. The MD simulations and data analysis were performed by Dr. Andrew Leach, Dr. David Barlow and Yixuan Yan. The project was conceived by Professor Jayne Lawrence and whole work is supervised by Dr. Richard Campbell and Professor Jayne Lawrence.

4.7 Acknowledgement

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Supplementary information

SI-1 Solubility measurements

The solubility of (*h*-)propranolol hydrochloride in H₂O and D₂O was measured in quadruplicate at ambient (295 ± 1 K) and at 310 ± 1 K. An excess of drug was added to the solvent in a microfuge tube which was then sealed and covered with foil to protect from light, and then agitated at the required temperature on a wheel for 10 days, after which time the samples were centrifuged at 185.6 × g (Beckman Microfuge 16, Beckman Coulter, High Wycombe, UK) for 5 minutes to sediment any excess (unsolubilised) drug. An aliquot (10 µL) of the supernatant was diluted 2000-fold with the appropriate solvent. The amount of drug dissolved was determined by measuring the UV absorbance of the diluted supernatant at the wavelength of its maximum absorbance^[31] and at a resolution of 1 nm using a PerkinElmer Lambda 365 UV/VIS spectrometer (Waltham, USA).

Table SI - 4.1 Solubility of propranolol hydrochloride in water, D₂O and saline (dissolved in H₂O and D₂O) determined using UV/vis spectroscopy at room temperature and 310 K, respectively.

Solubility (mM)	Water	D ₂ O	Saline/H ₂ O	Saline/D ₂ O
Room temperature	252 ± 7*	133 ± 4*	67 ± 1	42 ± 2
310 K	474 ± 14*	375 ± 7*	325 ± 10	119 ± 3

*reference [30]

Propranolol hydrochloride solutions used for SANS experiments were examined at drug concentrations of up to 200 mM in either D₂O or D₂O containing 0.154 M NaCl. These systems exceed the apparent solubility obtained from UV/vis spectroscopy measurements (Table SI - 4.1), which suggests that the systems were probably in a supersaturated state. These supersaturated solutions were used to probe the aggregation behaviour of propranolol hydrochloride at higher concentrations because otherwise due to the very low apparent solubility of propranolol hydrochloride in D₂O and D₂O containing 0.154 M NaCl, the concentration range studied would have been very limited.

The supersaturated samples were prepared by ‘dissolving’ the powdered drug in solvent using an ultrasonic water bath for at least 40 min at room temperature. The samples were checked for clarity and homogeneity before and after the SANS measurements. Propranolol hydrochloride is reported to have relatively larger metastable zone such that the difference between the supersaturated

temperature and the cooling temperature at which the crystals start to form by nucleation is comparatively large.^[32] As a consequence, we assume the supersaturated samples were stable during the measurement period.

The effect of temperature also plays a significant role in increasing the solubility of propranolol hydrochloride. The solubility of propranolol hydrochloride in pure water, D₂O and 0.154 M NaCl in H₂O or D₂O) at 295 ± 1 K and 310 ± 0.2 K were measured using UV/vis spectroscopy. The results shown in Supplementary Information, Table SI – 4.1 indicate that the solubility of propranolol hydrochloride significantly increases as the temperature increased which is considered to be due to an endothermic reaction, where the energy needed to break the bonds in the solid drug overcame the heat given off from the dissolving process.^[33]

SI-2 Surface tension data of propranolol hydrochloride in 0.154 M sodium chloride

Surface tension measurements on a series of aqueous propranolol hydrochloride solutions containing 0.154 M were recorded to determine the CMC of propranolol hydrochloride (Figure SI - 4.1). For comparative purposes, the surface tension values for the corresponding aqueous propranolol hydrochloride solutions are plotted on the same figure. The CMC recorded in the presence of electrolyte has decreased to approximately 57 mM from a value of 92.4 mM in water, a decrease in CMC which accords with the increase in aggregate size in the presence of electrolyte recorded from the SANS experiments.

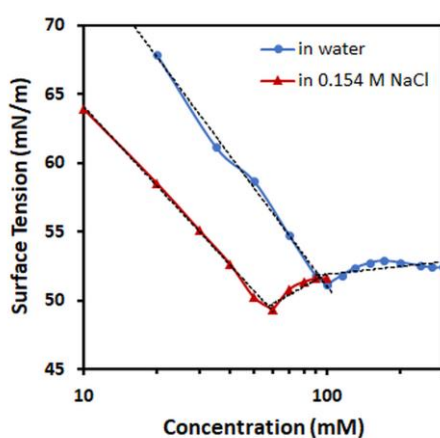


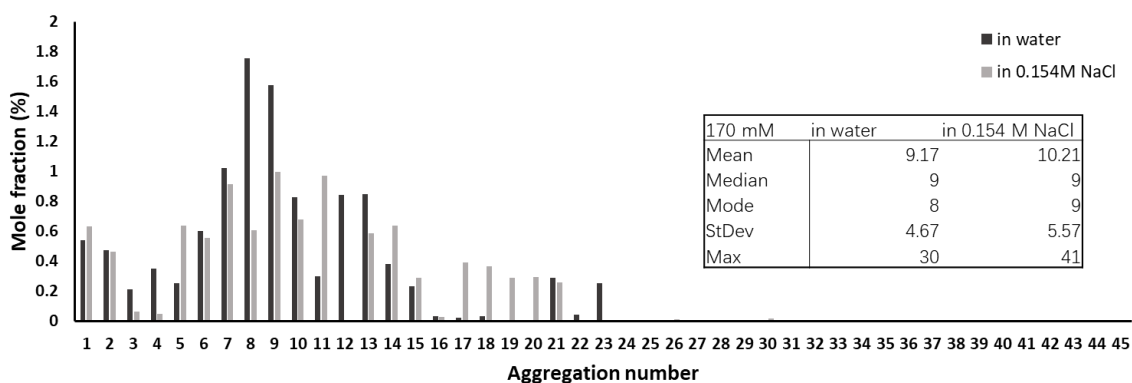
Figure SI - 4.1 Variation in surface tension of propranolol hydrochloride in water and in an aqueous 0.154 M sodium chloride solution, concentration expressed in terms of log₁₀.

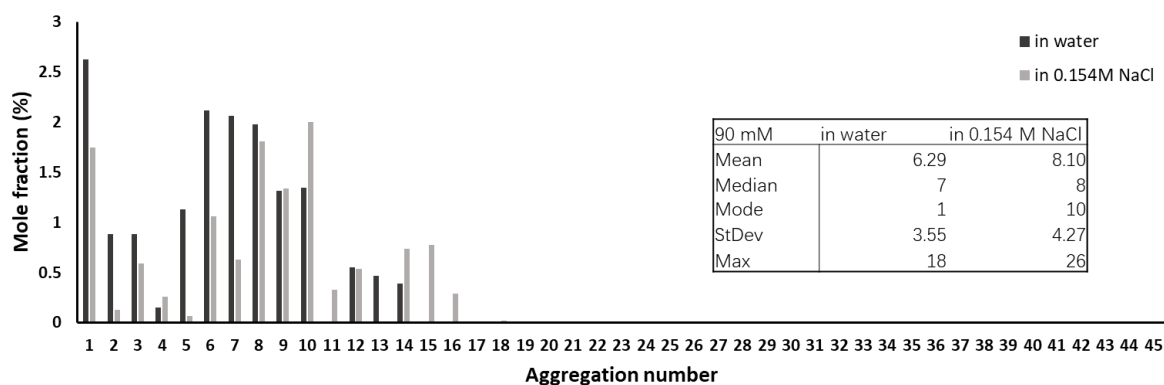
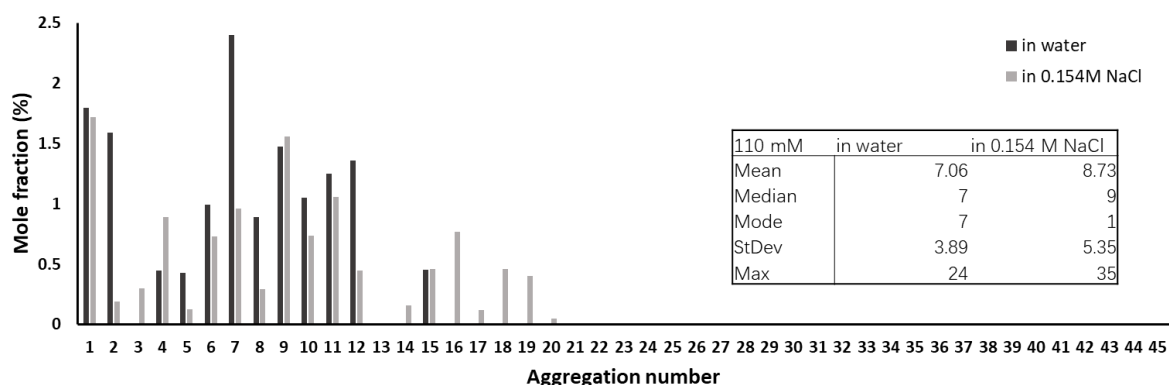
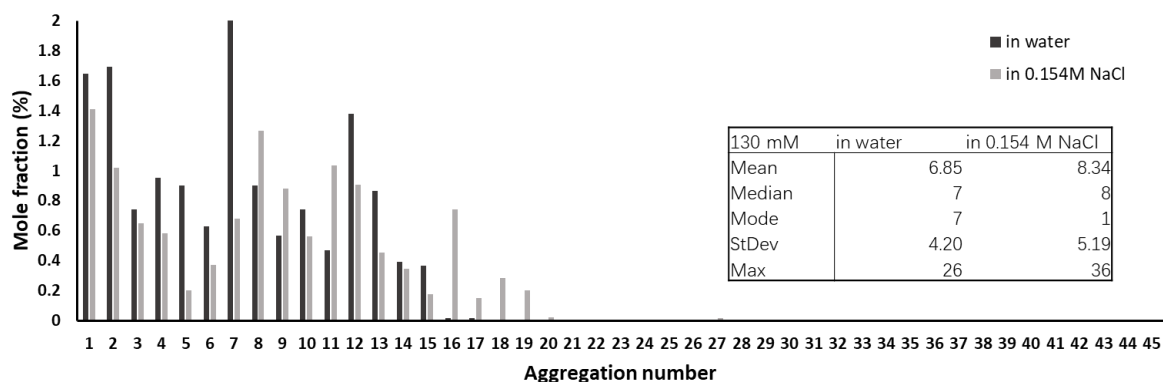
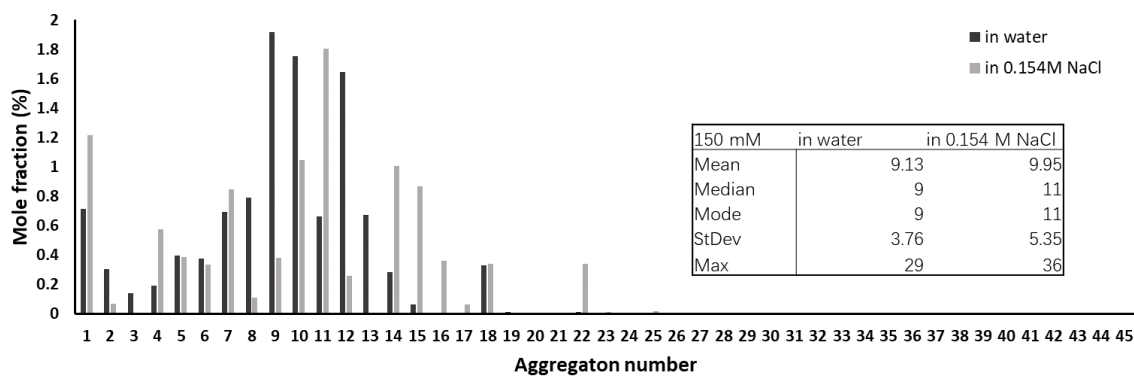
SI-3 SANS data of various isotropic contrast of propranolol hydrochloride

Table SI - 4.2 Fitted parameters for the SANS data of various isotropic contrast of propranolol hydrochloride (propHCl) in aqueous solvent at 310 K.

Contrast (mM)	Equatorial radius core (Å)	Polar radius core (Å)	Volume core (Å ³)	Thickness shell (Å)	Charge (e)	Hydration of shell	Aggregation number (N_{agg})
200 mM							
<i>h</i> -propHCl/D ₂ O	5.7	21.1	2843	4.3	4.3	0.67	15
<i>d</i> -propHCl/H ₂ O	5.7	21.1	2843	4.3	4.3	0.43	15
170 mM							
<i>h</i> -propHCl/D ₂ O	5.7	16.1	2219	4.6	4.2	0.72	12
<i>h</i> -propHCl/H ₂ O	5.7	16.1	2219	4.6	4.2	0.19	12
<i>d</i> -propHCl/H ₂ O	5.7	16.1	2219	4.6	4.2	0.50	12
<i>d</i> -propHCl/D ₂ O	5.7	16.1	2219	4.6	4.2	0.73	12
150 mM							
<i>h</i> -propHCl/D ₂ O	5.4	13.1	1599	4.7	4.0	0.73	9
<i>h</i> -propHCl/H ₂ O	5.4	13.1	1599	4.7	4.0	0.26	9
<i>d</i> -propHCl/H ₂ O	5.4	13.1	1599	4.7	4.0	0.66	9

SI-4 MD simulations of propranolol hydrochloride at different concentrations





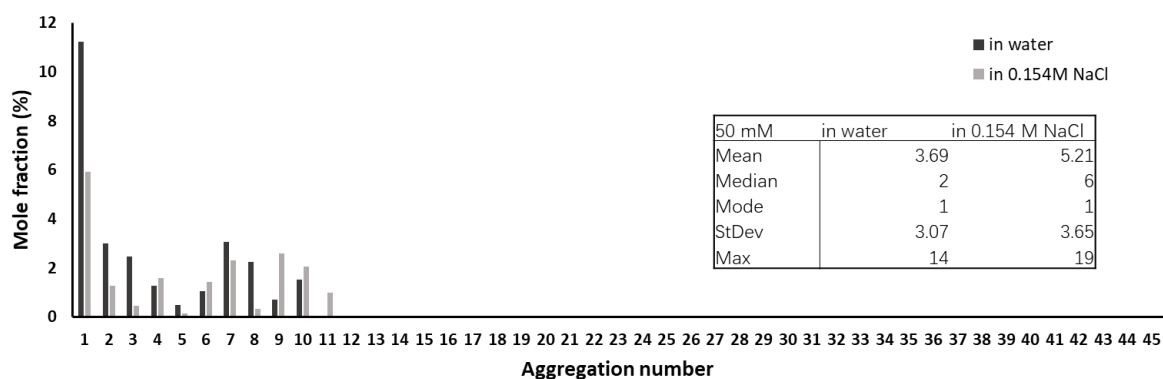
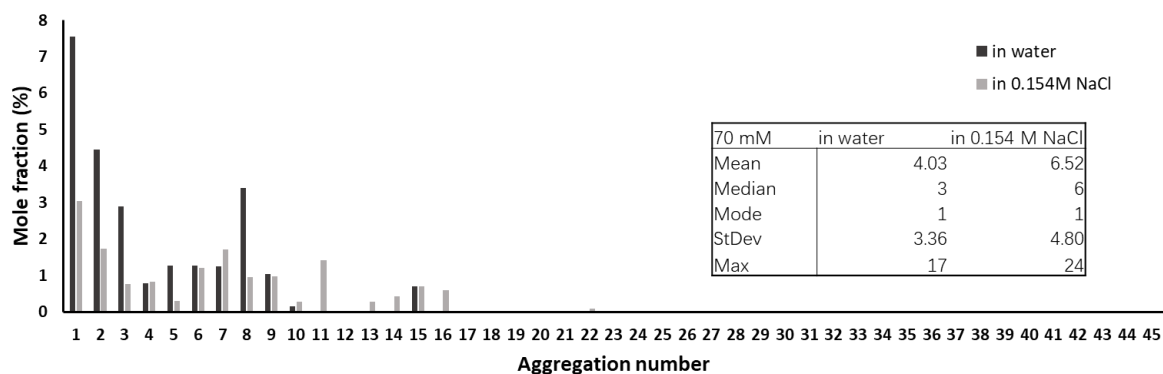


Figure SI - 4.2 Comparison of the size distribution of aggregates calculated from all-atomistic MD simulation of propranolol hydrochloride at concentrations range from 50 mM to 170 mM, in the absence and presence of 0.154 M NaCl at 300 K. Insert gives a statistical summary of the data.

SI-5 MD simulations of pure enantiomer LPR

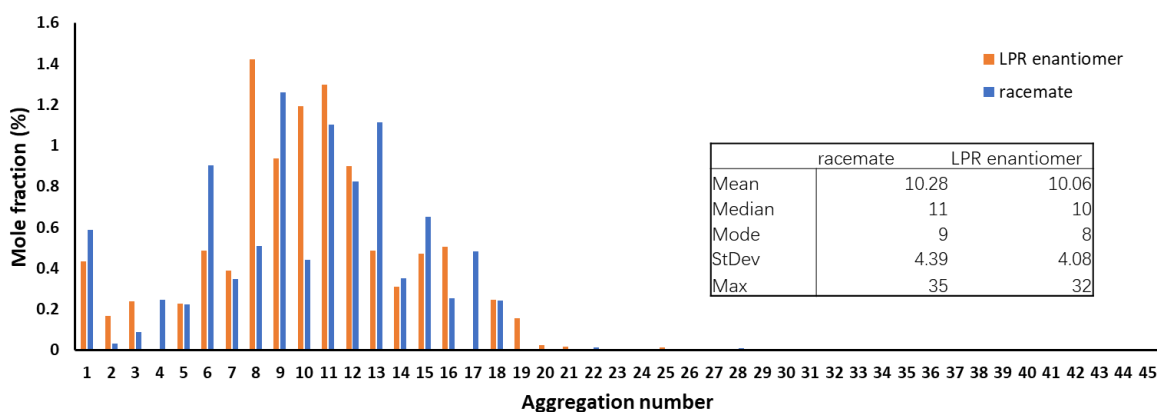


Figure SI - 4.3 Comparison of the size distribution of aggregates calculated from all-atomistic MD simulation of 200 mM propranolol hydrochloride of racemate and LPR enantiomer at 300 K, respectively. Insert gives a statistical summary of the data.

5 Molecular view of the interaction between the amphiphilic drug propranolol hydrochloride and model lipid membranes

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5.1 Abstract

There is a strong need for new research on the role of cell membrane interactions in the physiological activity of small amphiphilic drugs. Propranolol hydrochloride is a beta blocker that is used clinically to treat hypertension, abnormal heart rhythms and glaucoma, yet its membrane stabilising and side effects remain poorly understood. The aim of the present work is to resolve in detail the nature of membrane interactions of propranolol hydrochloride in terms of progressive changes in lipid domain morphology, the favoured structural location of drug, any changes in drug orientation, and the extent of any lipid loss with respect to the membrane composition, starting surface pressure and drug concentration. Using the experimental platform of lipid monolayers on a Langmuir trough, the study is executed through the application of a powerful suite of advanced reflectometry techniques, namely ellipsometry, Brewster angle microscopy and neutron reflectometry. It was found that both the extent of drug interaction and the extent of lipid loss from the membranes are strongly related to the starting surface pressure and therefore the packing and heterogeneity of the membrane. Surprisingly, all studied systems ended up with more drug than lipid molecules in the membrane as the systems reached steady state, even though the drug concentrations used were close to those used clinically. While it was expected the presence of negatively charged lipid may enhance the interaction, the opposite was discovered as the interaction was suppressed. The complexity of the parallel interaction processes resolved in the present work is astounding and provides important new structural information about the possible origin of anaesthetic side effects of a small amphiphilic drug.

5.2 Introduction

Many clinically used small drugs have an amphiphilic structure, including local anaesthetics, antidepressants, antibiotics and β -blockers.^[1] It is important to recognise that their amphiphilic structures enable the drug's resulting surface activity and aggregation behaviour as well as driving their interaction with cell membranes and other molecules in the wider biological environment such as albumin. Cell membranes themselves are primarily composed of amphiphilic lipid molecules, consequently making the interaction of amphiphilic drugs with a lipid membrane highly likely. As a result of their interaction with a cell membrane, an amphiphilic drug may alter the conformation of the membrane, for example, resulting in membrane fusion, phase separation, lipid flip-flop and lipid loss.^[2] Still more work is required to elucidate the implications of a drug's amphiphilic nature on its ability to affect these processes in terms of drug-lipid interactions.^[3]

Of particular interest in the present study is the small amphiphilic drug, propranolol hydrochloride, which has an amphiphilic structure that is composed of a hydrophobic naphthalene ring and a side chain with two hydrophilic centres (Figure 5.1). Propranolol hydrochloride is clinically used to treat hypertension, abnormal heart rhythms and glaucoma. It acts on β -adrenaline receptors to block their activity, and therefore exhibits inverse effects to epinephrine and norepinephrine.^[4] The drug, however, has also been proposed to exhibit local anaesthetic effects, is considered to be related to electrophysiological effects,^[5] and exhibits membrane stabilising effects,^[6] suggesting significance to the interactions of propranolol not only with β -receptors but also with cell membranes.

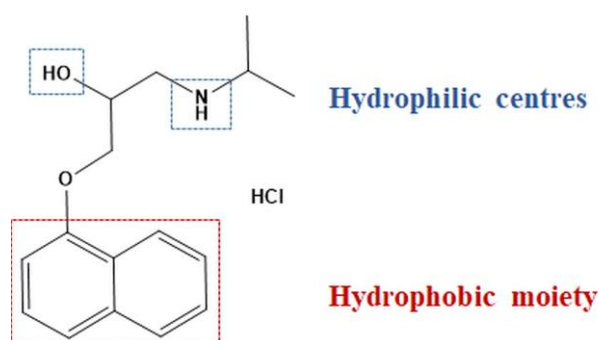


Figure 5.1 Chemical structure of propranolol hydrochloride. The hydrophobic and hydrophilic centres are labelled with red and blue boxes, respectively.

There is some experimental evidence concerning the interaction between propranolol hydrochloride and model lipid membranes. For example, Herbette and co-workers determined the location of propranolol hydrochloride and two other beta-blockers in model lipid membranes using X-ray and neutron diffraction, and related the results to possible clinical effects.^[7] De Carlo studies the interaction of the drug with model liposomes using electron cryo-microscopy to conclude that the drug disrupts the bilayer by disturbing the local lipid organisation.^[8] Also, Först and co-workers studied the interaction of propranolol with fluorescently labelled vesicles and with bilayers via molecular dynamic (MD) simulations, illustrating that the conformation of the lipid membrane is perturbed as a result of its interaction with the drug, and the MD results gave an estimation of the orientation of propranolol in the membranes.^[9]

There are various experimental platforms to model cell membranes that have been widely applied in studies that focus on different system properties, such as vesicles (in the bulk), planar supported bilayers (usually at a solid/liquid interface) and monolayers (at the air/water interface).^[10] Both vesicles and bilayers have bilayer structures that well mimic cell membranes, and the Langmuir-Blodgett preparation method even enables models of asymmetric bilayers to be prepared.^[11] Monolayers prepared on a Langmuir trough are the simplest model that mimics a single leaflet of the fluid bilayer structure. Although such a platform precludes studies on transmembrane and translocation processes, it is the easiest approach to produce fluid model membranes for interaction studies, achieve full lipid coverage without defects, eliminate unwanted electrostatic interactions from solid substrates, and importantly allow fine control of the surface pressure.^[12]

The aim of this work is to resolve the mechanism of interaction at the molecular level of propranolol hydrochloride with lipid monolayers composed of either POPC and POPC/POPG (7:3 molar ratio) in terms of the interaction dynamics including gradual changes in lipid domain morphology, the favoured structural location of drug in the model membrane including its preferred orientation and the extent of any lipid loss. In addition to the charge of the model membrane, two further parameters of drug concentration and starting surface pressure are altered to understand more about factors influencing the interaction. The maximum effective plasma concentration of propranolol is above 100 ng/L,^[13] while the intravenous dose is up to 4.28 mg/mL (~ 15 mM).^[14] Drug concentrations of 9.4 mM and 18.8 mM were chosen as being close to the physiological range with the former mimicking slight dilution and the latter slight accumulation of a high local concentration. The human cell

membrane has an average area of 40–70 Å² per lipid molecule.^[15] One starting surface pressure of 25 mN/m was chosen as being within this physiological range for both the POPC and POPC/POPG (7:3 molar ratio) systems, while another starting surface pressure of 10 mN/m was chosen as allowing the extent of interaction to be resolved with the monolayer in the same liquid expanded phase yet with looser lipid packing.

The approach is application of optical reflection techniques ellipsometry and Brewster angle microscopy (BAM) as well as specular neutron reflectometry (NR) to study the drug-membrane interaction at a planar air/water interface.^[10] A Langmuir trough is used to prepare the lipid monolayers as well as to control and monitor the surface pressure.^[16] Ellipsometry provides information about the extent and rate of the drug interaction as well as an indication of the lateral heterogeneity of the membrane.^[17] BAM provides visual images of the lateral heterogeneity and changes in domain size as a result of the drug interaction.^[18] Finally, NR provides quantitative information about the steady state amount of drug that interacts with the different membranes, its location partitioned between the lipid chains and headgroups, and the extent to which any lipid is lost from the interface as a result.^[19] Together these techniques provide a powerful insight into how a small amphiphilic drug interacts with a lipid membrane yielding information important in understanding how a drug's amphiphilicity affects its biological interactions.

5.3 Materials

1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC, ≥ 99.0% TLC), 1-palmitoyl-*d*₃₁-2-oleoyl-sn-glycero-3-phosphocholine (*d*₃₁-POPC, ≥ 99.0% TLC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt) (POPG, ≥ 99.0% TLC) from Avanti Polar Lipids were purchased from Sigma Aldrich. Chloroform (≥ 99.8%) was purchased from Fisher Scientific. (±)-1-Isopropylamino-3-(1-naphthyloxy)-2-propanol hydrochloride (99%) was purchased from Alfa Aesar. (±)-*d*₇(ring-deuterated)-propranolol hydrochloride (84%-*d*₇) was synthesised by the ISIS Deuteration Facility, Rutherford-Appleton Laboratory, STFC, Didcot, Oxford. Deuterium oxide (D₂O, 99.9 atom% D) was purchased from Sigma Aldrich. Ultrapure water (Integra L, Suez Pure Water System) was used throughout and had a resistivity of 18.2 mΩ cm⁻¹.

5.4 Methods

5.4.1 Surface pressure measurements

The surface pressure (π) of a sample at the air/water interface is defined as the difference between the surface tension of pure water and that of the sample, which in a lipid monolayer is dependent on the average area per lipid molecule (A). Values of π of POPC and POPC/POPG (7:3 molar ratio) monolayers were measured on a Langmuir trough using a Wilhelmy plate made of filter paper. The Langmuir trough (Kibron, Finland) was made of Teflon with dimensions of 80 mm $\times$ 350 mm, giving a surface area of 280 cm². Two Delrin barriers, each had a width of 16 mm, were used to modulate the surface area of the trough and therefore the area of the lipid monolayer by moving them across the surface. Ultrapure water (160 ml) was used as the sub-phase. Once the value of π of ultrapure water was calibrated and confirmed to be stable, an aliquot of a stock solution of lipid in chloroform was added dropwise to the surface of the sub-phase using a Hamilton syringe. After ten minutes, when the chloroform was deemed to have evaporated the barriers were moved at a speed of 10 mm/min to compress the area over which the lipid was spread, while at the same time constantly measuring π . By knowing the amount of lipid added to the surface and the area over which it is spread it is possible to determine an area per lipid molecule, or in the case of a mixed lipid system the average area per lipid molecule, generally expressed in Å². The experiments were performed at 295 $\pm$ 1 K and were repeated at least twice to ensure reproducibility.

The relationship between π and A of a lipid monolayer is generally represented as a π – A isotherm. For lipid monolayer studies reported here, π – A isotherms were recorded by compressing the monolayer to 35 mN/m then expanding back to 0 mN/m. The compressibility modulus, C_s^{-1} , which is related to the phase of the lipid monolayer, was calculated by:

$$C_s^{-1} = -A \frac{d\pi}{dA} \quad (5.1)$$

where values of C_s^{-1} between 12.5 and 50 mN/m indicates that the lipid(s) comprising the monolayer are in a liquid expanded phase while values of between 100 and 250 mN/m correspond to lipid(s) in a liquid condensed phase.^[20]

For studies examining the interaction of drug with model lipid membrane, the lipid monolayer was compressed to a surface pressure of either 10 or 25 mN/m whereupon the barriers were held

constant in that position. Once the lipid monolayer was deemed to be stable, determined by monitoring the value of π , 10 ml of aqueous sub-phase was removed, and 10 ml of a concentrated propranolol hydrochloride solution (either 150 or 300 mM) was injected into the sub-phase, to prepare a sub-phase of either 9.4 or 18.8 mM propranolol hydrochloride. Values of π were then recorded for a few minutes prior to the injection of propranolol hydrochloride into the sub-phase and until 140 min after the interaction when steady state was approached.

5.4.2 Null ellipsometry

Ellipsometry is a reflection technique that uses a polarised laser beam to measure the phase shift (Δ) and amplitude ratio (ψ) occurring upon the reflection and refraction of a light beam subsequent to its interaction with a film at a surface.^[17] It is a powerful technique to investigate the properties of thin films. In an ellipsometer, a linearly polarised beam from a laser first passes through a quarter wave plate, a polariser (Po) and a compensator, in order to convert the linearly polarised beam to an elliptical polarised beam. After interacting with the surface, the reflected elliptical polarised beam becomes linearly polarised, and then nulled by an analyser (An). The phase shift (Δ) and amplitude ratio (ψ), are calculated from the angles Po and An by the following equations:

$$\psi = An \quad (5.2)$$

$$\Delta = 2Po + \frac{\pi}{2} \quad (5.3)$$

The reflectance ratio (ξ) of the p-polarisation and s-polarisation component of the reflected beam can be represented in terms of Δ and ψ :

$$\xi = \frac{r_p}{r_s} = \tan \psi e^{i\Delta} \quad (5.4)$$

In contrast to ellipsometric measurements at the solid/water interface where ψ and Δ can be modelled to yield a refractive index and thickness of the interfacial layer,^[21] for ellipsometric measurements at the air/water interface, performed at close to the Brewster angle of 53.1° where p-polarised light exhibits zero reflection (in the absence of surface roughness), ψ is found relatively insensitive to any changes in the properties of the thin film while Δ exhibits its maximum

sensitivity.^[22] Therefore, in the present study an incidence angle of 50° was used and only Δ was analysed. Here a quantity termed $\Delta\delta$ is used, according to:

$$\Delta\delta = \Delta_{\text{sample}} - \Delta_0 \quad (5.5)$$

where Δ_{sample} is the phase shift of the sample and Δ_0 is the phase shift for pure water. The value of $\Delta\delta$ is interpreted as an approximate measure of the amount of material (in the present study, the amount of lipid/(drug)) present at the interface,^[22] while fluctuations in the value of $\Delta\delta$ over time are used to infer the presence of lateral heterogeneity in the membrane and are possibly related to the phase of the lipid and/or to regions of drug binding that perturb the ellipsometric response by passing in and out of the $\sim 1 \text{ mm}^2$ probed area of the interface.^[23]

The ellipsometric data in the present study were recorded at $295 \pm 1 \text{ K}$ using an Accurion Nanofilm EP4 instrument (Goettingen, Germany) fitted with a blue diode laser of wavelength 489 nm at an incident angle of 50° together with the same Langmuir trough as described above to examine the drug-membrane interactions with respect to the starting surface pressure and drug concentration. At least two repeats were performed for each system under study. The standard deviation and frequency of the values of $\Delta\delta$ before and after the injection of propranolol hydrochloride are reported, the peak value of the shift and the value of its width are related to changes in the properties of the film resulting from the interaction of the drug with the lipid monolayer.

5.4.3 Brewster angle microscopy

BAM is an imaging technique to visualise the lateral morphology of films on the micrometre length scale. The use of p-polarised light at the Brewster angle, which has only a minimal reflectivity contribution from surface roughness at the bare air/water interface, means that the technique is sensitive to the amount of material at the interface. Specifically the images visualised can be used to obtain valuable information about domains of anisotropy of the lipid chains,^[24, 25] and, from the brightness of the images, the total amount of material, e.g. the extent and lateral heterogeneity of drug binding.^[26]

Using an Accurion Nanofilm EP4 instrument with the same Langmuir trough with an incident angle of 53.1° , BAM images were recorded at 295 ± 1 K to resolve changes in film morphology subsequent to and during drug-membrane interactions at different starting surface pressures and drug concentrations. The images obtained from BAM were optimised by constant gamma correction using a software Irfanview (Germany). Due to the incident angle 53.1° , the illumination of the laser is elliptical and therefore the BAM images need to be rescaled in length by a ratio of 1.66 to obtain to their original shape.^[18] For the POPC/drug system, at least two repeats were performed at each value of the starting π and propranolol hydrochloride concentration, while for the POPC/POPG/drug systems, at least one repeat was performed.

5.4.4 Neutron reflectometry

Neutron reflectometry is a powerful structural technique, which exploits the Å-scale wavelength of neutrons coupled with the fact that they are highly sensitive to replacement of a protium atom with a deuterium atom (the neutron scattering length of deuterium is 6.671 fm, much larger than and of opposite phase to that of protium, -3.739 fm^[27]). As a result, deuterated materials can be used as isotopic contrast agents to resolve the detailed structure of the systems of interest. In addition, when studying films at the air/water surface, in order to highlight the film, air contrast matched water (ACMW), composed of 8.1% v/v D₂O in H₂O, can be used to make the sub-phase “invisible” to neutrons. NR enables the structure and composition and the thickness of films of the magnitude on the Å-scale normal to the interface to be established. The scattering length density (SLD) can be regarded as the “effective” refractive index of a material to neutrons. It is calculated from the total scattering lengths of all atoms of the material coupled with its molecular volume:

$$\rho = \frac{\sum_i^n b_i}{V_m} \quad (5.6)$$

where ρ represents the SLD, b_i is the scattering length of the i atom in a molecule of n atoms, and V_m is the molecular volume of the molecule. The SLDs calculated for the different materials used in the present study are listed in Table 5.1. Analysis of the neutron reflectivity data allows the composition of a multilayer system to be calculated by modelling the data using an average SLD, a solvent penetration and a thickness of each layer assuming a given roughness.^[19] The resulting SLD

profile from modelling data recorded in different isotopic contrasts can be used to resolve the location of each component in the interface.

NR was used to investigate the structure and composition of the drug-membrane systems when approaching steady-state for both POPC and POPC/POPG (7:3 molar ratio). Generally four isotopic contrasts were measured for each system and condition, (1) *d*-lipid/*d*-drug/ACMW, (2) *d*-lipid/*d*-drug/D₂O, (3) *d*-lipid/*h*-drug/D₂O, (4) *h*-lipid/*d*-drug/ACMW, where '*d*-lipid' and '*d*-drug' refer to the deuterated forms of the components stated in the Materials section, and '*h*-lipid' and '*h*-drug' refer to the components without isotopic substitution. The latter contrast is particularly sensitive to the presence of drug. Reference measurements of the pure lipid monolayers in the absence of drug were recorded either in contrasts 1-3 or all four contrasts according to the time available. Unlike the surface pressure measurements in which drug was injected in the subphase, in the NR experiments, lipid was spread on drug solution directly, in a 238 mm × 50 mm trough (surface area = 119 cm²). In order to achieve the same value of *A* for starting surface pressures of 10 and 25 mN/m, the volume of lipid spreading solutions was pre-calibrated on pure water (Supporting Information; section SI-1). Work was also carried out to compare the resulting steady state surface pressures with measurements on a Langmuir trough, and the data were considered to be within acceptable agreement (Supporting Information; section SI-1). Preparation of the films in this way enabled the films steady state to be more rapidly achieved. As data were shown to reach steady state quickly, acquisition of the neutron data took place over 1–10 h after sample preparation. The experiments of each contrast were performed only once due to time constraints of the resource available.

Table 5.1 Scattering lengths, molecular volumes and SLDs of different materials used in the present study.

Molecule	Part	Scattering length (fm)		Molecular volume (Å ³)	SLD (× 10 ⁻⁶ Å ⁻²)	
POPC	Head (C ₁₀ H ₁₈ NO ₈)	60.07		344 ⁽¹⁾	1.75	
	Tail (C ₃₂ H ₆₄)	-26.62		937 ⁽¹⁾	-0.28	
<i>d</i> ₃₁ -POPC	Tail (C ₃₂ H ₃₃ D ₃₁)	296.09		937 ⁽¹⁾	3.16	
POPG	Head (C ₈ H ₁₂ O ₁₀ PNa)	71.46		291 ⁽²⁾	2.46	
Propranolol	<i>h</i> -Ring (C ₁₀ H ₇)	40.29		183 ⁽³⁾	2.20	
	<i>d</i> ₇ -Ring (C ₁₀ D ₇ , 84%D)	101.50		183 ⁽³⁾	5.55	
	Chain (C ₆ H ₁₅ NO ₂)	5.59 (in ACMW)	15.17 (in D ₂ O)	264 ⁽⁴⁾	0.21 (in ACMW)	0.57 (in D ₂ O)

⁽¹⁾ Data sourced from ref [28]. ⁽²⁾ Data sourced from ref [29]. ⁽³⁾ Sourced from PubChem database. ⁽⁴⁾ Calculated from a physical density prediction using the Percepta Platform from ACD Labs in ref [30].

Neutron reflectivity profiles of the reflectivity, R , with respect to the momentum transfer normal to the interface, Q_z , were recorded at 295 ± 1 K on the INTER reflectometer at the ISIS Pulsed Neutron and Muon Source (Harwell, UK) in time-of-flight mode at incident angles of 0.8° and 2.3° using a wavelength range of 1.5-17 Å. The data were analysed using the Motofit package in Igor 8,^[31] with the four isotopic contrasts of the same system being co-refined. The lipid monolayer was modelled as a two-layer system of acyl chains above solvated headgroups, since the SLDs of the components are very different (Table 5.1). The approach used was consistent with that detailed in a recent article on modelling such data,^[32] that is: (1) solvation of the headgroup was constrained from a calculation of the equivalent surface amounts of chains and headgroups to respect the molecular structure, (2) no acyl chain compaction was added given the liquid expanded nature of the lipid indicated by the π -A isotherms of the lipids, and (3) surface roughness was applied consistent with the presence of capillary waves. The principle to obtain the amount of drug in each layer was to calculate the average SLD of each layer with the addition of different volume fraction of drug (Supporting Information; section SI-2).

To resolve the position of propranolol in the membrane, several different models were examined, namely incorporating drug either wholly in the lipid chains, or wholly in the lipid headgroups, or split between both layers or else under the head groups in the third layer. Moreover, because propranolol is an amphiphilic drug, a partition model that divides the drug molecule into hydrophobic naphthalene rings and hydrophilic side chains was also applied, where an assumption was made that the naphthalene ring exists only in lipid chain region as a result of hydrophobic effects, yet the side chains may be present in either layer^[9] (Supporting Information; section SI-3). The optimum fits were chosen based on the minimum χ^2 that makes physical sense. The uncertainties stated are calculated based on 10% error of the χ^2 values.

The amount of each component in the multilayer system can be calculated from the fitted structure parameters. Surface excess (Γ) is the surface concentration of a given component at the air/water interface and can be calculated from the fitted thickness (d), solvation of the layer and the averaged SLD (ρ) of all components in the layer:

$$\Gamma = \frac{\rho}{b_i} \frac{d}{N_A} \phi_i \quad (5.7)$$

where ϕ_i is the volume fraction of the component in the layer, which can be calculated from the solvation; b_i is the scattering length of the component; N_A is the Avogadro's constant. Values of A were calculated from the surface excess of lipids.

5.5 Results and Discussion

5.5.1 POPC and POPC/POPG (7:3 molar ratio) model membranes

To aid in the interpretation of data from the interaction of propranolol hydrochloride with the model lipid monolayers, it was necessary first to characterize the monolayers. Measurements of π - A isotherms were acquired up to a maximum value of π of 35 mN m⁻¹ (Figure 5.2(a)), and the reciprocal of the compressibility modulus was calculated (insert Figure 5.2(a)). These data show that no phase transition is observed for either system, as the values of C_s^{-1} remain always below 100 mN/m.^[20] As a consequence, both POPC and POPC/POPG (7:3 molar ratio) monolayers are in the liquid expanded phase under the starting conditions of this study (namely a π of 10 and 25 mN/m). Furthermore, while the PC head group is zwitterionic, the PG head group possess a single negative charge, resulting in an increased electrostatic repulsion between the lipid heads in the mixed POPC/POPG (7:3 molar ratio) monolayer.^[33] As a consequence, the POPC/POPG (7:3 molar ratio) mixed monolayer exhibits lower values of π at a given average area per lipid molecule compared with the POPC monolayer.

BAM images were taken at π of 0, 10 and 25 mN/m during compression of the two monolayers to explore their lateral morphology (Figure 5.2(b)). POPC monolayers were observed not to form many lipid domains even at the higher π of 25 mN/m where only a few round domains of ~ 6 μ m in size are seen. (Figure 5.2(b)c). In contrast, upon compression the mixed POPC/POPG (7:3 molar ratio) monolayer was seen to form an increasing number of evenly distributed lipid domains. At π of 25 mN/m, the domains observed for the POPC/POPG (7:3 molar ratio) monolayer at ~ 12 μ m in size are larger than those seen for the POPC monolayer and are much more irregular shaped (Figure 5.2(b)f). It is clear therefore, that the presence of 30 mol% of POPG in a POPC monolayer promotes phase separation within the plane of the monolayer.^[34, 35]

The structure of the POPC and POPC/POPG (7:3 molar ratio) model membranes was determined at both 10 and 25 mN/m using NR. The neutron reflectivity data were analysed by dividing the monolayer into two sub-layers, namely a lipid chain region in contact with air above a solvated head group region in contact with water. The NR data of POPC and POPC/POPG (7:3 molar ratio) monolayers at π of 10 and 25 mN/m are shown in Figure 5.3, with SLD profiles shown as inserts, and a summary of parameters obtained from the fitting reported in Table 5.2. For both systems, an increase of π leads to an increase of the thickness of lipid chains layer, and a reduction in solvation of the head group layer. Both of these effects are attributed to a decrease in the average area per lipid molecule, as with increasing surface amount the lipid chains become thicker while the head group region excludes solvent. The mixed POPC/POPG monolayer is slightly thicker than the pure POPC monolayer, with a higher surface amount of lipid, which is consistent with the surface pressure data (lower A at a given value of π) and BAM images (tendency to phase separate forming ordered lipid domains) shown in Figure 5.2.

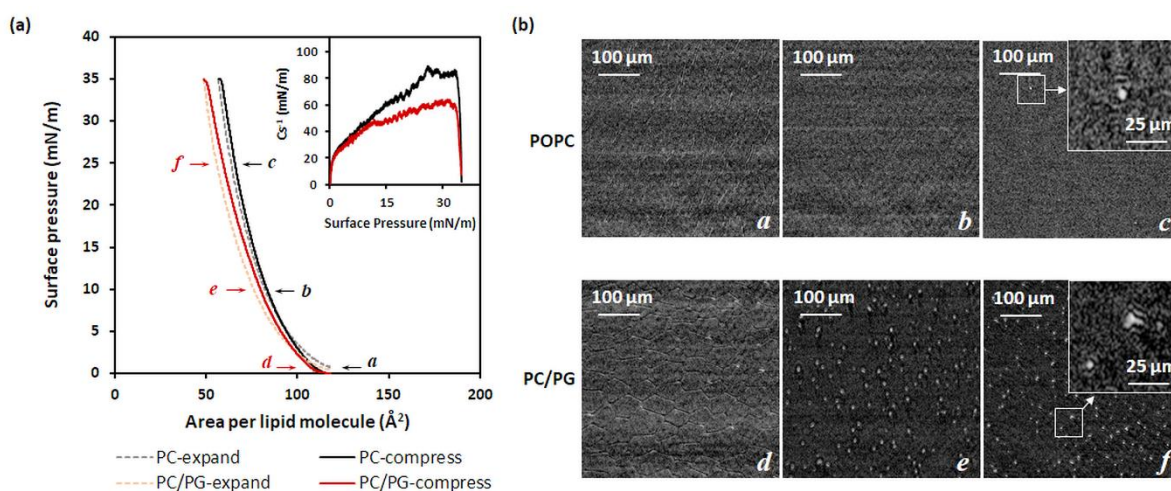
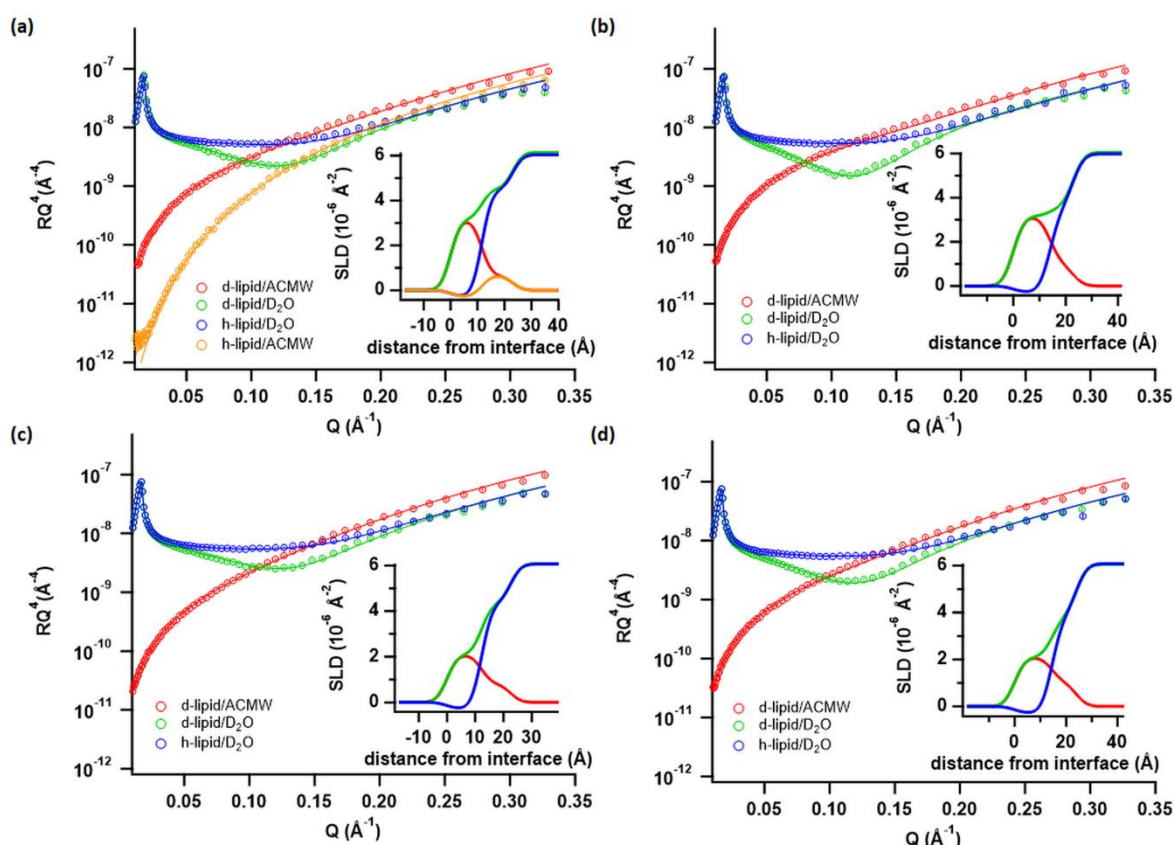


Figure 5.2 (a) Lipid isotherms of POPC and POPC/POPG (7:3 molar ratio) at room temperature. Insert: reciprocal of the compressibility modulus plotted against surface pressure; (b) BAM images of the (a-c) POPC and (d-f) POPC/POPG (7:3 molar ratio) monolayers recorded during compression at surface pressures of (a,d) 0, (b,e) 10 and (c,f) 25 mN/m, respectively.

Table 5.2 Fitted structural parameters of pure POPC and POPC/POPG (7:3 molar ratio) lipid monolayers.

System and starting surface pressure	Lipid chain thickness (Å)	Lipid head thickness (Å)	Solvation in head (vol%)	Surface excess of lipid ($\mu\text{mol}\cdot\text{m}^{-2}$)	Area per lipid molecule ($\text{\AA}^2$)
POPC at 10 mN/m	11.4 ± 0.5	9.4 ± 2.7	55 ± 13	2.02 ± 0.14	82.2 ± 6.2
POPC at 25 mN/m	14.1 ± 0.2	8.9 ± 1.3	42 ± 9	2.49 ± 0.04	66.6 ± 1.1
POPC/POPG at 10 mN/m	12.2 ± 0.3	10.3 ± 1.6	58 ± 6	2.17 ± 0.04	76.4 ± 1.6
POPC/POPG at 25 mN/m	14.6 ± 0.4	9.5 ± 1.6	46 ± 7	2.59 ± 0.08	64.2 ± 1.8

**Figure 5.3** Neutron reflectivity profiles of 3 or 4 isotopic contrasts of lipid monolayers: (a) POPC at 10 mN/m, (b) POPC at 25 mN/m, (c) POPC/POPG (7:3 molar ratio) at 10 mN/m and (d) POPC/POPG (7:3 molar ratio) at 25 mN/m and room temperature. The circles are experimental data, and the solid lines represent the best fit to the data. Fitted SLD profiles are shown as inserts.

For the lipid systems studied in the present work, namely POPC and POPC/POPG (7:3 molar ratio) values of the starting π of 25 and 10 mN/m were used. A value of π of 25 mN/m corresponds average areas per lipid molecule of 66 and 64 $\text{\AA}^2$ for the POPC and POPC/POPG (7:3 molar ratio) systems, respectively (Figure 5.2(a)). As the π of the outer leaflet of erythrocyte membrane is $\sim 30\text{--}35$ mN/m, corresponding to an area per lipid molecule of 40–70 $\text{\AA}^2$ for different membranes,^[15] the chosen value

falls within the physiological range. By contrast, a value of π of 10 mN/m corresponds to average areas per lipid molecule of 82 and 76 Å² for the POPC and POPC/POPG (7:3 molar ratio) systems, respectively (Figure 5.2(a)), which falls below the lateral pressure of physiological relevance but potentially allows additional insight into the specific nature of the drug-lipid interactions.

5.5.2 Interactions of propranolol hydrochloride with POPC model membranes

In the present study the lipid monolayers were compressed to the required π (either 10 or 25 mN/m) and held thereafter at constant surface area to ensure the stability of the lipid monolayer prior to the injection of sufficient propranolol hydrochloride into the aqueous sub-phase to produce a concentration of either 9.4 mM or 18.8 mM. Any changes observed in π of the film subsequent to the addition of propranolol hydrochloride into the aqueous sub-phase reflects the manner and extent to which drug interacts with the lipid monolayer at the air/water interface to lower the free energy of the system. Figure 5.4 shows the changes in π and $\Delta\delta$ from the ellipsometry after the injection of propranolol hydrochloride at starting values of π of 10 mN/m (left panels (a) and (c)) and 25 mN/m (right panels (b) and (d)).

First, we discuss the data with obtained with both propranolol hydrochloride concentrations at the lower starting π of 10 mN/m. The interaction of propranolol hydrochloride with the POPC monolayers is fast as there is no lag-phase in the changes observed in π (and $\Delta\delta$). Except for an immediate drop in π (discussed below) both the lower and higher propranolol hydrochloride concentrations induce a gradual and continual increase in π , which suggests an interaction of the drug, probably a penetration into the model membrane. At the lower propranolol hydrochloride concentration of 9.4 mM, the overall increase in π is such that after 140 min π is levelling off, having increased by 13 mN/m, making the final π to be 23 mN/m. At the higher propranolol hydrochloride concentration of 18.8 mM, the corresponding overall increase in π is 17 mN/m, and the final π is 32 mN/m, suggesting a greater penetration of drug into the model monolayer when its bulk concentration is higher. In both cases, however, there is a noticeable, and indeed unexpected, sharp drop in π after the injection of drug, followed by the slow but continuous increase in π over the 140 min measured period. As this initial drop in π was observed in every experiment that was performed, including all repeats, we preclude the effect to result from an experimental artifact. It seems plausible that the drop in π as a

result of the interaction between drug and model monolayer membrane may be related to lipid loss, resulting in an increase in the area per lipid molecule and therefore a reduction in π . Such an observation is not considered unreasonable in the light of the addition of the amphiphilic propranolol hydrochloride because of its ability to form mixed micelles with phospholipid. In fact, a similar observation has been recently reported upon the addition of the amphiphilic drug amitriptyline to solid supported bilayers of the phospholipid, 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC).^[36] Furthermore, the addition of a number of other drugs (including a peptide,^[37] an anaesthetic^[38] and anthracyclines^[39]) to a lipid monolayer have also been observed to induce lipid loss. However, a technique such as NR is necessary to validate such a hypothesis.

Further information about the extent of interaction (increase in $\Delta\delta$) as well as any increase in lateral heterogeneity of the membrane (onset of fluctuations) was gained from ellipsometry. The variation with time of the value of $\Delta\delta$ from the ellipsometric measurements is plotted in Figure 5.4(a), the same graph as the variation in π is recorded, to allow the results from both techniques to be correlated. The variation in the frequency of the $\Delta\delta$ values averaged over the 4 min prior to injection of propranolol hydrochloride (denoted 'before') and those averaged of 4 min prior to the end of the measurement period (denoted 'after') are shown in Figure 5.4(b). Although the variation in the $\Delta\delta$ values before the injection of the propranolol hydrochloride are slight, as expected given the same starting point for the two measurements, the fluctuations in the variation in $\Delta\delta$ after the injection are greater, particularly at the lower of the two drug concentrations (Figure 5.4(c) and Table 5.3). We attribute the increase in the fluctuations of $\Delta\delta$ to the drug inducing phase separation in the film and possibly the consequent preferential interaction of propranolol hydrochloride in one of the phase separated domains, such that over time different domains move in and out of the $\sim 1 \text{ mm}^2$ area of the air/water interface probed by the laser.

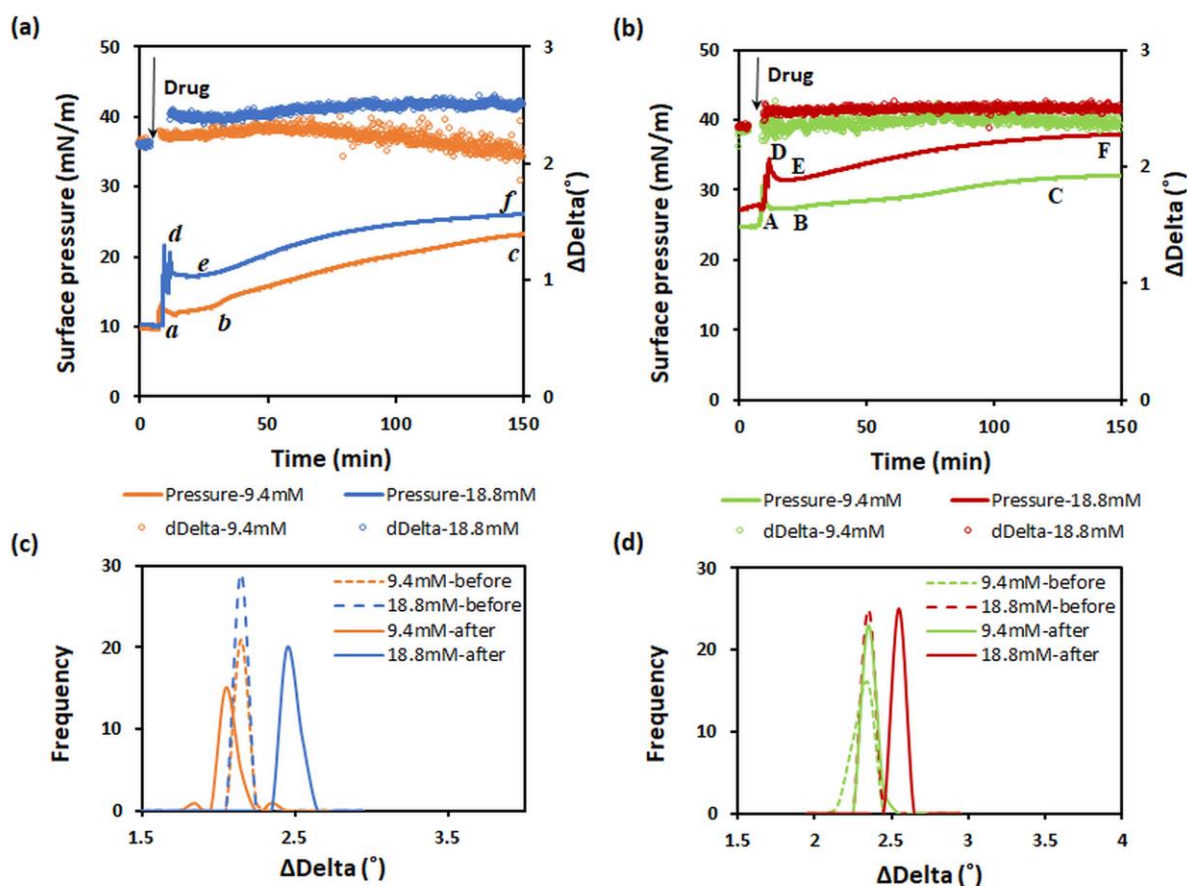


Figure 5.4 Variation in π and $\Delta\delta$ of POPC membranes after propranolol hydrochloride solution was injected into the sub-phase at two starting π : (a) 10 mN/m, (b) 25 mN/m and at room temperature. The final concentrations of propranolol were 9.4 mM (orange and green) and 18.8 mM (blue and red). The letters contained within panels (a) and (b) refer to the points when BAM images in Figure 5.5 were taken. The frequency distributions of $\Delta\delta$ before (dashed lines) and after (full lines) the interaction are shown for the two starting π : (c) 10 mN/m, (d) 25 mN/m.

Table 5.3 The mean value and standard deviation of $\Delta\delta$ obtained from the POPC membranes before* and after^ injection of propranolol hydrochloride into the aqueous sub-phase at room temperature.

Starting surface pressure (mN/m)	Propranolol hydrochloride concentration (mM)	Before		After	
		Mean (°)	Standard deviation (°)	Mean (°)	Standard deviation (°)
10	9.4	2.17	0.019	2.08	0.083
	18.8	2.15	0.014	2.49	0.022
25	9.4	2.30	0.035	2.36	0.029
	18.8	2.33	0.010	2.49	0.013

*4 min prior to injection of propranolol hydrochloride into the sub-phase; ^ 4 min prior to the end of the 150 min measurement period.

While the mean position of the peak in the frequency distribution curve for $\Delta\delta$ decreases slightly as a result of the interaction with 9.4 mM drug, the peak position increases for the analogous experiment with 18.8 mM drug. This increase in $\Delta\delta$ can be attributed to an overall increase in density of material at the air/water interface occurring as a result of a stronger drug-membrane in the experiment with the higher concentration of drug, an observation that is consistent with the greater increase in π ; on the other hand, we may speculate that the drug interaction is suppressed with respect to lipid loss in the experiment with the lower concentration of drug.

Next, we discuss the data with obtained with both propranolol hydrochloride concentrations when using the higher starting π of 25 mN/m. Here both the variation in π and $\Delta\delta$ exhibit similar trends to those seen when using a starting π of 10 mN/m in that the value of the steady state π is greater and the mean value of $\Delta\delta$ moves to a higher value for the experiments with the higher drug concentration. However, the drug-membrane interaction appears to be weaker for the starting π of 25 mN/m compared with those seen after the lower starting π of 10 mN/m, possibly due to the fact that the monolayer possesses a higher density of lipid packing, such that penetration of the drug into the monolayer is weakened. The final increase in π after approximately 140 min after injection is 7 mN/m (final π of 32 mN/m) at a drug concentration of 9.4 mM and 11 mN/m (final π of 36 mN/m) at a drug concentration of 18.8 mM; corresponding values of 13 and 17 mN/m, respectively, were observed for a starting π of 10 mN/m. A further indication of the presence of a weaker interaction with propranolol hydrochloride at the higher starting π comes from the observation that the shift in the peak of the frequency distribution of $\Delta\delta$ is less than that seen for the starting π of 10 mN/m, combined with a slight reduction in the standard deviation of the peak. For example, at the higher drug concentration, the standard deviation decreases from 0.022° for a starting π of 10 mN/m to 0.013° for a starting π of 25 mN/m. Based on these results, it is clear that the starting π of the POPC monolayer plays an important role in nature of the interaction of propranolol hydrochloride with the model membrane. It is proposed that propranolol hydrochloride induces an heterogeneity in the POPC membrane, with the drug seemingly to prefer interacting with the more expanded phase. Another technique such as NR is required to quantify the loss of lipid from the model membrane as well as the extent of interaction with drug.

BAM was used to image the lateral heterogeneity and changes in domain size as a result of the drug interaction for both drug concentrations and starting π values. As seen in Figure 5.5, POPC

monolayers are relatively homogeneous with only a few domains observed, even at a starting π of 25 mN/m.^[28] However, after the addition of propranolol hydrochloride, BAM images (Figure 5.5) taken at different time points (indicated in Figure 5.3) show that the presence of drug induced changes to the model membrane and that these changes differ over time. The left side images of each group of Figures 5.5(a),(d),(A)&(D) were taken immediately after the injection of drug has been performed, the images top centre of Figures 5.5(b),(e),(B)&(E) corresponded to the minimum π observed a few minutes after initiation of the interaction, while the right hand images at the top Figures 5.5(c),(f),(C)&(F) show the morphology of the system as it approaches steady state. The images at the top of Figures 5.5(a)–(c)&(A)–(C) are the higher drug concentration of 18.8 mM while the bottom images of Figures 5.5(d)–(f)&(D)–(F) are the lower drug concentration of 9.4 mM.

Regardless of the drug concentration used, Figures 5.5(a),(d),(A)&(D) show that, at the early stages of the interaction, there is no apparent difference in lateral morphology of the film. However, relatively soon after injection of propranolol hydrochloride (*i.e.* within 20 min), when π had reached its minimum value, (Figures 5.5(b),(e),(B)&(E)) a number of domains of ~ 10 μm had appeared. Although the appearance of these domains is not immediate, it follows that at least part of the amphiphilic drug molecules had penetrated into POPC monolayer, inducing the formation of condensed domains, possibly as a consequence of a local high drug concentration during injection. As the interaction progresses and π gradually increases towards a steady state value (Figures 5.5(c),(f),(C)&(F)), the number of domains reduces, demonstrating that their presence is short-lived, possibly linked with any ongoing lipid loss resulting a decrease in A . Interestingly, however, the size and shape of the remaining domains are roughly constant. Possible reasons for the disappearance of the domains over time is that any lipid lost from the membrane is an ongoing process that results in a change in the lipid phase or the distribution of the drug within the film becomes more uniform over time.

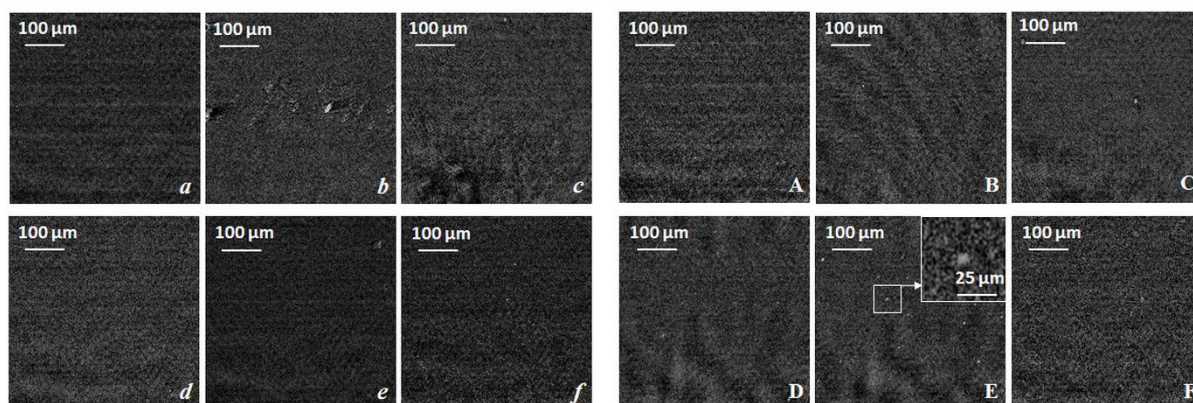


Figure 5.5 BAM images of the POPC/drug system during the interaction corresponding to the times denoted by the labels contained within the panels in Figures 5.4 (a) and (b). The starting surface pressure was 10 mN/m (a)–(f) or 25 mN/m (A)–(F). Propranolol hydrochloride concentration was 18.8 mM (a)–(c)&(A)–(C) or 9.4 mM (d)–(f)&(D)–(F).

NR was used to provide quantitative information about the amount of drug that interacts with the model POPC membranes at steady state, as well as its location and orientation together with amount of any lipid lost from the membrane as a result of the interaction with propranolol hydrochloride. Here, due to time constraints, only one propranolol hydrochloride concentration, namely 20 mM, was studied using NR at values of starting π of 10 and 25 mN/m. Figure 5.6 shows the reflectivity data obtained for the 4 isotropic contrasts studied along with the best model fits to the data; the inserts show the volume fraction profile of the various components of the interface as a function of their position in the interface. A summary of structural parameters obtained from the fit of the neutron data is reported in Table 5.4. Various models were attempted to fit the NR data by minimising the χ^2 value based on a number of stratified layers of defined composition (Supporting Information; section SI-2). The optimum fits for the POPC system with drug were obtained using the partition model, in which the monolayer was divided into two sub-layers (namely the lipid chain region and the solvated head group region each with defined parts of the drug molecules) to provide detailed information on the composition of each sub-layer and, significantly, the location and orientation of the drug within the model membranes. The propranolol hydrochloride molecule was divided up into 2 regions, namely the hydrophobic naphthalene ring portion and the hydrophilic cationic side chain (Figure 5.1).

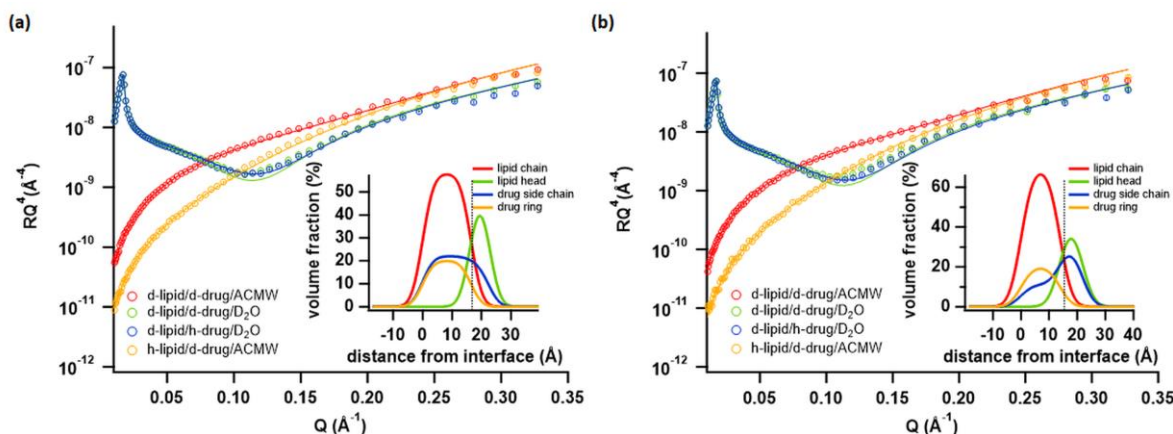


Figure 5.6 Neutron reflectivity data of the 4 isotopic contrasts (*d*-POPC/*d*-drug/ACMW (red); *d*-POPC/*d*-drug/D₂O (green); *d*-POPC/*h*-drug/D₂O (blue); *d*-POPC/*h*-drug/ACMW (orange)) recorded after steady state was achieved for the POPC/propranolol hydrochloride system at two different starting surface pressures: (a) 10 mN/m, (b) 25 mN/m. The circles are experimental data and the solid lines are the best fits to the data obtained using the partition model. Insert shows the volume fraction of each component as a function of position in the air/water interface.

Table 5.4 Fitted structural parameters of POPC monolayers following their interaction with propranolol hydrochloride as a result of the optimum model to fit a two-layer model* to neutron reflectivity data recorded in 4 isotopic contrasts.

Starting surface pressure (mN/m)	Lipid chain thickness (Å)	Lipid head thickness (Å)	Solvation in head (%)	Drug naphthalene ring in layer 1 (%)	Drug hydrophilic chain in layer 1 (%)	Drug hydrophilic chain in layer 2 (%)	Surface excess of lipid (μmol·m ⁻²)	Surface excess of drug (μmol·m ⁻²)	Area per lipid molecule (Å ²)
10	16.4 ± 0.3	6.0 ± 0.9	23 ± 7	20 ± 1.2	22 ± 1.3	19 ± 1.1	1.68 ± 0.03	2.98 ± 0.20	98.7 ± 2.1
25	13.9 ± 0.4	7.8 ± 1.0	23 ± 5	19.8 ± 3.2	11 ± 1.8	31.5 ± 5.1	1.71 ± 0.05	2.50 ± 0.40	97.2 ± 2.7

* The upper lipid chain region is noted here as 'layer 1' and the solvated lipid head group region is denoted here as 'layer 2'.

A comparison of the lipid surface excesses calculated for POPC before interaction with propranolol hydrochloride, 2.02 ± 0.14 and 2.49 ± 0.04 $\mu\text{mol}\cdot\text{m}^{-2}$ at a values of the starting π of 10 and 25 mN/m, respectively, with those after the interaction as the system approaches steady state, 1.68 ± 0.03 and 1.71 ± 0.05 $\mu\text{mol}\cdot\text{m}^{-2}$, provides definitive evidence that POPC is lost from the model membranes as a result of its interaction with the drug, as suggested by the π and ellipsometry measurements discussed above. It is noticeable that the loss of lipid for a starting π of 25 mN/m is much higher (~ 0.8 $\mu\text{mol}\cdot\text{m}^{-2}$) than the loss recorded for a starting π of 10 mN/m (~ 0.3 $\mu\text{mol}\cdot\text{m}^{-2}$). Interestingly, the final amounts of POPC present in the membrane as it approaches steady state are equivalent for the two values of starting π . Surprisingly, however the amount of drug interacting with the model membrane at starting π of 25 mN/m (2.50 ± 0.40 $\mu\text{mol}\cdot\text{m}^{-2}$) is less than that (2.98 ± 0.20 $\mu\text{mol}\cdot\text{m}^{-2}$)

for the lower starting π of 10 mN/m, supporting the hypothesis that at the higher π the interaction is weaker because the packing of POPC was greater.

Thanks to the selective deuteration of the drug-ring part of propranolol hydrochloride used in these measurements, the partition model that is able to distinguish the locations of the drug-ring and drug-side chains in the model membrane produced the best fitting results. The model has allowed us to resolve changes in orientation of the drug with respect to different experimental conditions. While it is a model assumption that all of the drug's naphthalene rings are located in the lipid chain region, as explained the Methods section, the number of drug-side-chains in each sub-layer of the membrane can be calculated from the respective volume fractions of the components and the thickness of each sub-layer under different experimental conditions. For a starting π of 10 mN/m, there are ~ 1.8 drug molecules per POPC molecule present in the model membrane and, significantly, 76 ± 7 % of the drug's side chains are located in the same region with the rest residing in the head group region. For the higher starting π , there are ~ 1.5 drug molecules per POPC molecule, and in this case, at 38 ± 9 %, significantly fewer of the drug's side chains are located in the lipid chain region than in the head group region. These results conclusively demonstrate a change in orientation of the drug with changing starting π . It follows that the resultant redistribution of drug's side chains is a consequence of the reorientation of drug molecules due to the higher packing of the lipid membrane at the starting π of 25 mN/m, there are less space left for drug molecules. Indeed, it was unexpected to observe that the surface amounts of drug molecules in the membrane for the higher starting π (lipid and drug: 1.71 ± 0.05 and 2.50 ± 0.40 $\mu\text{mol}\cdot\text{m}^{-2}$, respectively) were lower than those for the lower starting π (lipid and drug: 1.68 ± 0.03 and 2.98 ± 0.20 $\mu\text{mol}\cdot\text{m}^{-2}$, respectively). This observation may be explained by the observed change in orientation of the drug in the membrane under the two experimental conditions.

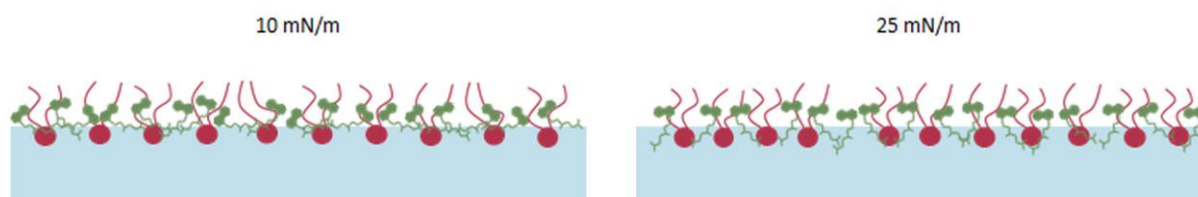


Figure 5.7 Schematic representation of the changes in steady state structure following the interaction of propranolol hydrochloride with POPC model membranes at two different starting surface pressures, 10 and 25 mN/m. POPC molecules are coloured magenta while drug molecules are coloured green.

In summary, the interaction of propranolol hydrochloride with model POPC membranes results in a number of interesting parallel processes and ongoing changes to the system, which have been resolved in detail, as follows:

- (1) there is immediate loss of lipid from the monolayer upon exposure to drug, with the amount of lipid loss is greater for the higher starting π and the higher drug concentration in the sub-phase;
- (2) there is the formation of transient lipid domains for a period, perhaps coincident with a maximum surface density of molecules in the membrane if lipid loss are ongoing over the measurement period; and
- (3) there is penetration of drug into the lipid membrane such that the naphthalene rings are present in the lipid chain region with the side chains present in both the lipid chain and head group region with both the total amount of drug and the amount of the parts of the drug present in each region depending upon the value of the starting π (Figure 5.7).

5.5.3 Interaction of propranolol hydrochloride with POPC/POPG (7:3 molar ratio) model membranes

The POPC/POPG (7:3 molar ratio) mixture was studied to investigate the effect of the presence of negatively charged lipid on the interaction of the cationic propranolol hydrochloride with a model membrane. As with the POPC monolayers, the POPC/POPG (7:3 molar ratio) mixtures were compressed to the required starting π (either 10 or 25 mN/m) and held at constant surface area to ensure the stability of the monolayers prior to injection of propranolol hydrochloride into the aqueous sub-phase to produce a final concentration of either 9.4 mM or 18.8 mM. Any changes observed in the π of the film after the addition of propranolol hydrochloride into the aqueous sub-phase are considered to reflect the manner and the extent to which drug interacts with the lipid monolayer. Figure 5.8 shows the changes in π and $\Delta\epsilon$ from ellipsometry after the injection of propranolol hydrochloride at values of the starting π of 10 mN/m (left panels (a) and (c)) and 25 mN/m (right panels (b) and (d)).

At a starting π of 10 mN/m, the variation in π with the POPC/POPG (7:3 molar ratio) monolayers follows a broadly similar trend to that seen with the POPC monolayers (Figure 5.8(a)), *i.e.*, a rapid increase, a slow decrease and then a slower increase. It is noteworthy, however, that although there was again a rapid interaction of the drug with the model monolayers post injection, the rate of the subsequent decrease in π is slower, with the extent of the decrease in π , particularly at the higher drug concentration of 18.8 mM, being less than that seen for the corresponding POPC/drug system, suggesting the loss of less lipid from the surface. For the mixed POPC/POPG (7:3 molar ratio) membranes after the initial decrease in π , π starts to continuously and very slowly increase (Figure 5.8(a)) with the increase in π commencing at ~ 30 and 10 min for sub-phase concentrations of 9.4 and 18.8 mM, respectively. Furthermore, the overall increase in π recorded 140 min after injection is less than that obtained for the POPC membranes, namely 9 mN/m (as opposed to 13 mN/m) giving a final π of 19 mN/m for 9.4 mM of drug, and 16 mN/m (as opposed to 17 mN/m) giving a final π of 26 mN/m for 18.8 mM drug. These results suggest a relatively weak interaction of the drug with the POPC/POPG (7:3 molar ratio) membranes. This observation was unexpected due to the oppositely charged nature of the drug and the POPG lipid component of the model membrane.

At a starting π of 25 mN/m (Figure 5.8(b)), an interesting effect was observed in that the shape of the variation in π over time after injection of propranolol hydrochloride changed significantly with the initial decrease in π , assumed to be due an extended period of lipid loss. Specifically, at a propranolol hydrochloride sub-phase concentration of 18.8 mM, this loss phase took over 50 min with only a 5 mN/m increase over the starting π of 25 mN/m recorded after 140 min (final π of 30 mN/m). Furthermore, when propranolol hydrochloride was injected into the sub-phase at a final concentration of 9.4 mM drug, no overall increase in π was observed at 140 min post injection. One possible reason for these observations is that the lipids in the mixed POPC/POPG (7:3 molar ratio) monolayers at 25 mN/m prior to the addition of drug are in a more condensed state than the POPC monolayers when present as sole lipid at the same π , as evidenced by the fact that the presence of 30 mol% of POPG in a POPC monolayer appears to promote the formation of lipid domains (Figure 5.2(b)). On the basis of these observations, it seems plausible that a significant role of interaction of propranolol hydrochloride with the mixed lipid membrane is to reorientate the lipids within the regions of domains explaining why there is relatively little change in π . This point will be elaborated in connection with the BAM and NR data below.

Ellipsometric measurements allowed further information to be gained on the extent of interaction with propranolol hydrochloride including the extent of lateral heterogeneity of the membrane. When compared to the position of the peaks in the frequency distribution of $\Delta\delta$ prior to injection of propranolol hydrochloride at 10 and 25 mN/m, the injection of propranolol hydrochloride resulted in increase of the mean values, with the largest shifts being observed for the lower starting π of 10 mN/m (Table 5.5). The peak shifts seen in the POPC/POPG/drug system are relatively large, when compared with those recorded for the POPC system, although interestingly there is little difference observed between the effects of the two drug concentrations in the sub-phase. For example, after the injection of propranolol hydrochloride at a starting π of 10 mN/m, the mean values of $\Delta\delta$ are 2.84° and 2.81° for 9.4 and 18.8 mM propranolol hydrochloride, respectively, suggesting that the concentration of drug has less of an effect on $\Delta\delta$ than the starting π . The standard deviation in the value of $\Delta\delta$ before and after interaction with propranolol hydrochloride are very similar to each other. As a consequence of this fact, coupled with the observation that the POPC/POPG (7:3 molar ratio) model membranes exhibit phase separation and are very heterogeneous prior to the addition of propranolol hydrochloride (Figure 5.2), it follows that the addition of drug does not induce the formation of further lipid domains but rather changes the orientation and packing of the lipids within these regions. This inference will be explored below through the application of BAM.

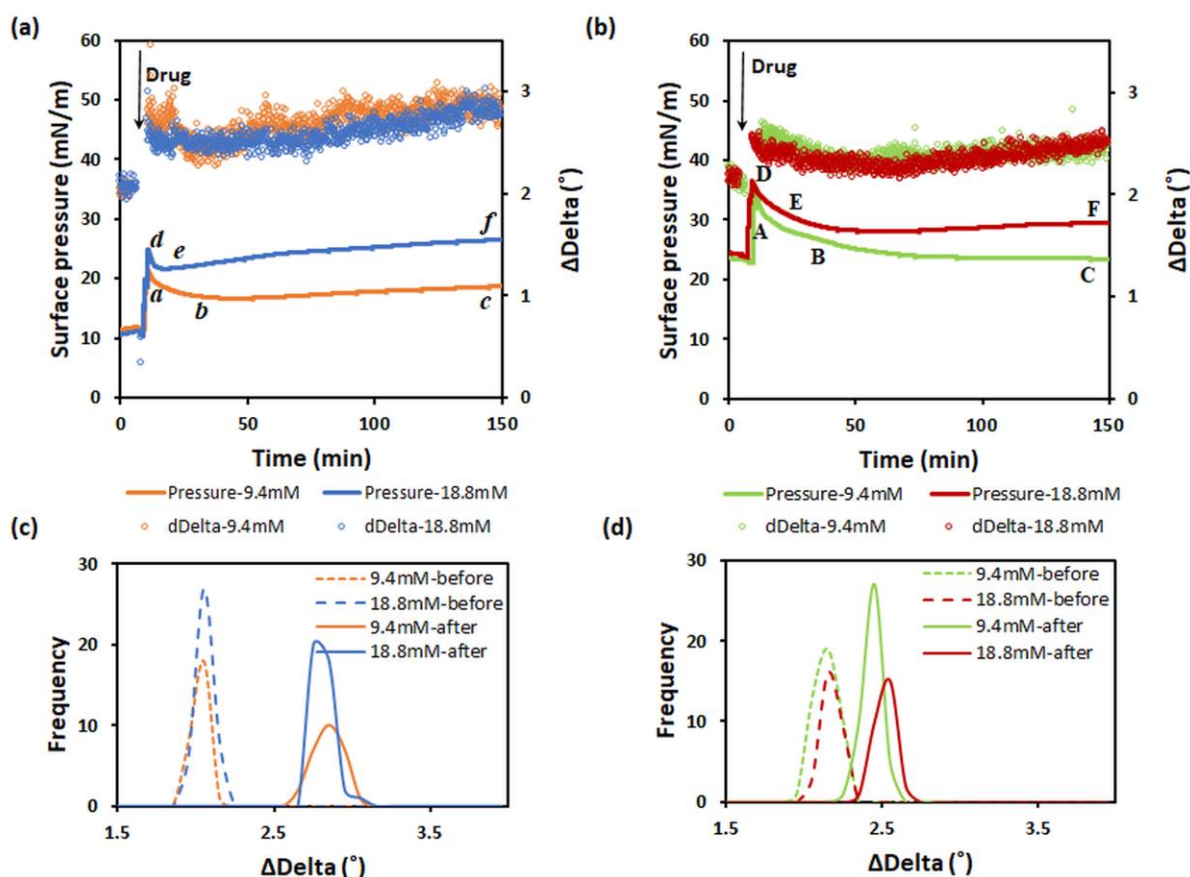


Figure 5.8 Variation in π and $\Delta\Delta$ of POPC/POPG (7:3 molar ratio) membranes after propranolol hydrochloride was injected into the sub-phase at two starting π : (a) 10 mN/m, (b) 25 mN/m and at room temperature. The final concentrations of propranolol were 9.4 mM (orange and green) and 18.8 mM (blue and red). The letters contained within panels (a) and (b) refer to the points when BAM images in Figure 5.5 were taken. The frequency distributions of $\Delta\Delta$ before (dashed lines) and after (full lines) the interaction are shown for the two starting π : (c) 10 mN/m, (d) 25 mN/m.

Table 5.5 The mean value and standard deviation of $\Delta\Delta$ obtained from the POPC/POPG (7:3 molar ratio) membranes before* and after^ injection of propranolol hydrochloride into the aqueous sub-phase at room temperature.

Starting surface pressure (mN/m)	Propranolol hydrochloride concentration (mM)	Before		After	
		Mean (°)	Standard deviation (°)	Mean (°)	Standard deviation (°)
10	9.4	2.03	0.048	2.84	0.094
	18.8	2.06	0.060	2.81	0.063
25	9.4	2.14	0.073	2.44	0.052
	18.8	2.16	0.049	2.50	0.050

*4 minutes prior to injection of propranolol hydrochloride into the sub-phase; ^4 minutes prior to end of the 150 min total measurement period.

BAM was used to obtain images of the lateral heterogeneity of the membranes and to determine any changes in domain size as a result of the interaction of propranolol hydrochloride with the POPC/POPG (7:3 molar ratio) membranes. The BAM images (Figures 5.9(a)–(f)) showed that over time, the lipid domains present at a starting π of 10 mN/m in the absence of drug, grew slightly in size and became more irregular in shape as a result of the drug interaction. Indeed, upon approaching steady state, most domains had merged to produce irregular shaped domains ranging in size from ~ 12 to ~ 22 μm (Figures 5.9(c)&(f)). The changes in domain shape observed here is considered to be a consequence of a decrease in line tension at the edges of the domains and anisotropy of the lipid molecules, as line tension acts to decrease the total boundary length of domains within a phase separated membrane.^[40] It is possible that the change in line tension results from a phase separation of a single lipid component to form the condensed domains. These BAM results taken with the very slow increase in π recorded in Figures 5.9(e)–(f) and Figures 5.9(b)–(c) are likely to result from the merger and rearrangement of the lipid domains, accompanied with small amount of drug penetration into the membranes. At the higher starting π of 25 mN/m, the corresponding BAM images (Figures 5.9(C)&(F)) showed a similar, but more significant, trend when compared with the effects seen at 10 mN/m. Indeed, some very large, irregular “worm-like” domains were observed, consistent with the relatively small increase in π surface pressure of POPC/POPG (7:3 molar ratio) system at this higher starting π , as inferred above.

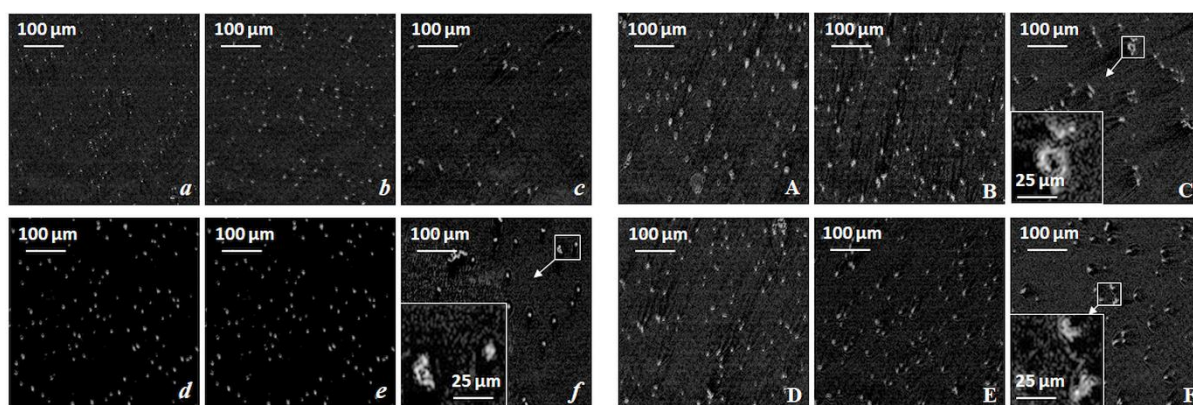


Figure 5.9 BAM images of the POPC/POPG (7:3 molar ratio) drug system during the interaction corresponding to the times denoted by the labels contained within the panels in Figures 3 (a) and (b). The starting surface pressure was 10 mN/m (a)–(f) or 25 mN/m (A)–(F). The propranolol hydrochloride concentration was 18.8 mM (a)–(c)&(A)–(C) or 9.4 mM (d)–(f)&(D)–(F).

Quantitative information about the interaction of propranolol hydrochloride with the model POPC/POPG (7:3 molar ratio) membranes at steady state, as well as the location and orientation of any drug associated with the membrane along with any loss of lipid from the interface, were obtained using NR. Figure 5.10 shows the neutron reflectivity data for the 4 isotropic contrasts of the POPC/POPG (7:3 molar ratio) membranes after interaction with drug along with the best fit curves obtained by simultaneously analysing the data. The inserts in Figure 5.10 show the volume fraction of the various components of the interface as a function of their position in the interface. The structural parameters used to obtain the best fit to the neutron data are reported in Table 5.6. As with the POPC system, the optimum fits of the neutron reflectivity data of the POPC/POPG (7:3 molar ratio) system after interaction with propranolol hydrochloride were obtained using the partition model.

From a comparison of the lipid surface excess calculated for POPC/POPG (7:3 molar ratio) before interaction with propranolol hydrochloride, namely 2.17 ± 0.04 and $2.59 \pm 0.08 \mu\text{mol}\cdot\text{m}^{-2}$ at values of the starting π of 10 and 25 mN/m, respectively, with that after, 2.19 ± 0.05 and $2.27 \pm 0.06 \mu\text{mol}\cdot\text{m}^{-2}$, it is clear that little lipid was lost from the model membranes, a result supported by the smaller variations in π and the higher final values of $\Delta\pi$ (Figure 5.7). Significantly, at a starting π of 10 mN/m no lipid was lost, while at the higher starting π of 25 mN/m, $\sim 0.3 \mu\text{mol}\cdot\text{m}^{-2}$ was lost. This result contrasts with that obtained with the POPC membranes, for which a lot more lipid was lost from the surface. Surprisingly, the amount of drug interacting with the model membrane at starting π of 25 mN/m at $2.57 \pm 0.23 \mu\text{mol}\cdot\text{m}^{-2}$ is comparable to that of $2.63 \pm 0.22 \mu\text{mol}\cdot\text{m}^{-2}$ recorded at the lower starting π of 10 mN/m. Overall the total amounts of lipid and drug at steady state are comparable for both values of the starting π , and the amounts are greater than that seen for the corresponding POPC systems. Interestingly, it can be concluded that the smaller increase in π seen is in the mixed lipid system is not a result of the loss of lipid from the membrane and/or a decreased penetration of drug into the membrane, but rather due to the presence of drug promoting the formation of condensed structures in the lipid membrane.

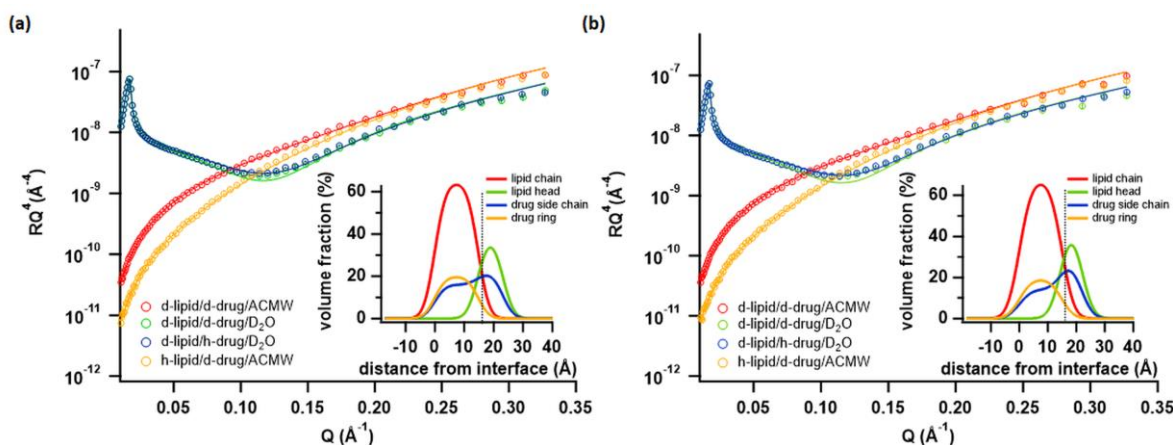


Figure 5.10 Neutron reflectivity data of the 4 isotopic contrasts (*d*-POPC/*d*-POPG/*d*-drug/ACMW (red); *d*-POPC/*d*-POPG/*d*-drug/D₂O (green); *d*-POPC/*d*-POPG/*h*-drug/D₂O (blue); *d*-POPC/*d*-POPG/*h*-drug/ACMW (orange)) recorded after steady state was achieved for the of POPC/POPG (7:3 molar ratio) membranes after propranolol hydrochloride injection at two different starting surface pressures: (a) 10 mN/m, (b) 25 mN/m. The circles are experimental data and the solid lines are the best fits to the data obtained using the partition model. Insert shows the volume fraction of each component as a function of position in the air/water interface.

Table 5.6 Fitted structural parameters of POPC/POPG (7:3 molar ratio) monolayers following their interaction with propranolol hydrochloride as a result of the optimum model to fit a two-layer model* to neutron reflectivity data recorded in 4 isotopic contrasts.

Starting surface pressure (mN/m)	Lipid chain thickness (Å)	Lipid head thickness (Å)	Solvation in head (%)	Drug naphthalene ring in layer 1 (%)	Drug hydrophilic chain in layer 1 (%)	Drug hydrophilic chain in layer 2 (%)	Surface excess of lipid (μmol·m ⁻²)	Surface excess of drug (μmol·m ⁻²)	Area per lipid molecule (Å ²)
10	14.7 ± 0.3	8.4 ± 0.9	38 ± 4	19.8 ± 1.7	16 ± 1.3	22 ± 1.9	2.19 ± 0.05	2.63 ± 0.22	75.8 ± 1.6
25	14.9 ± 0.3	6.6 ± 1.1	16 ± 9	19 ± 1.7	14 ± 1.3	30.5 ± 2.7	2.27 ± 0.06	2.57 ± 0.23	73.0 ± 1.7

* The upper lipid chain region is noted here as 'layer 1' and the solvated lipid head group region is denoted here as 'layer 2'.

At a starting π of 10 mN/m, all the naphthylene rings are present in the lipid chain region of the membrane while, in contrast, 56 ± 7 % of the side chains are present in the same region (Table 5.6). At the higher starting π , 51 ± 7 % of the drug's side chains are present in the lipid chain region, which within the uncertainty of the measurements is not significantly different. For a starting π of 10 mN/m, there are ~ 1.2 drug molecules per lipid molecule present in the model membrane. For the higher starting π , there are ~ 1.1 drug molecules per lipid molecule. The number of drug molecules in the POPC/POPG (7:3 molar ratio) membrane is less than the number in the POPC membrane, which is surprising because it had been expected that the negatively charged POPG would promote

interaction of the positively charge propranolol hydrochloride with the membrane. Here as the number of molecules in the membrane at the higher starting π (lipid and drug 2.19 ± 0.05 and $2.63 \pm 0.22 \mu\text{mol}\cdot\text{m}^{-2}$) were comparable to that seen at the lower starting π (lipid and drug 2.27 ± 0.06 and $2.57 \pm 0.23 \mu\text{mol}\cdot\text{m}^{-2}$), with the similar amount of drug in the two membranes consistent with there being no significant different in its orientation.

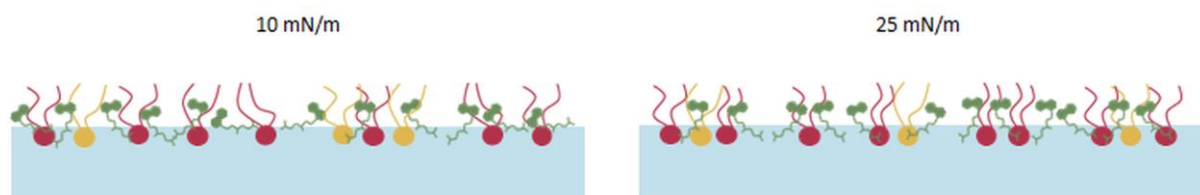


Figure 5.11 Schematic representation of the changes in steady state structure following the interaction of propranolol hydrochloride with POPC model membranes at two different starting surface pressures, 10 and 25 mN/m. POPC molecules are coloured red, POPG molecules are coloured yellow while drug molecules are coloured green.

In summary, the interaction of propranolol hydrochloride with model POPC/POPG (7:3 molar ratio) membranes also results in a number of interesting parallel processes and ongoing changes to the system, but they are distinct from the interactions with POPC membranes, as follows:

- (1) there is loss of lipid from the monolayer upon exposure to drug that is greater for the higher starting π and higher sub-phase drug concentration in the sub-phase, but the extents are much less than those seen with the POPC monolayers;
- (2) there is the formation of lipid domains, but their number and size are greater than the effects seen with the POPC monolayers; and
- (3) there is penetration of drug in the lipid membrane such that the naphthalene rings are present in the lipid chain region with the side chain present in both the lipid chain and head group region, but the amount present in each region depends is not significantly affected by the initial π , and the effect of drug concentration is much less significant than the effects seen with the POPC monolayers (Figure 5.11).

5.6 Conclusions

Novel drug-membrane interaction mechanisms of the beta-blocker propranolol hydrochloride have been elucidated in detail in the present work. The interaction is surprisingly complex with parallel processes resulting in ongoing changes, *e.g.* immediate lipid loss accompanied with drug penetration in different orientations combined with progressive changes to the lateral heterogeneity of the membrane. A combination of advanced reflectometry techniques combined with use of monolayers spread on a Langmuir trough allowed us to resolve various facets of the interactions over different length and time scales including non-monotonic changes in properties such as the surface pressure and domain morphology during the interaction and the structures formed as the system approaches steady state. Different aspects of the system were studied including effects of starting surface pressure (10 or 25 mN/m), drug concentration (9.4, 18.8 or 20 mM) and presence of negatively charged lipid in the membrane (POPC or POPC/POPG (7:3 molar ratio)).

Two intriguing outcomes of the research are discussed first. The use of NR demonstrated quantitatively the loss of lipid from the membranes as a result of the drug interaction in all cases studied, similar to conclusions made in other recent works involving interactions of small amphiphilic drugs with lipid monolayers.^[38, 39] These data help to explain the drop in the surface pressure data with time, and point to lipid solubilisation induced by drug. Similar effects were proposed with other small amphiphilic drugs, for example, amitriptyline is reported to solubilise lipid bilayer and form drug-lipid mixed micelles, the lipid loss was observed below the drug's critical micelle concentration (CMC).^[36] Another study demonstrated the ability of the base of propranolol to solubilise DMPC liposomes at different pH, which is largely depend on the phase state of the lipids, *i.e.* the solubilisation is much more pronounced in fluid phase.^[41] Moreover, our recent study on the self-assembly of propranolol hydrochloride has proved the pre-micellization of propranolol hydrochloride well below its CMC.^[42] It can be concluded that propranolol hydrochloride solubilises lipids from membranes upon their interactions probably resulting in drug-lipid mixed micelles.

Another outcome concerns results from experiments conducted using a different experimental methodologies: injecting drug solution under a lipid membrane (for study in the laboratory) versus spreading a lipid membrane on drug solution (predicated by requirements for conducting the NR

experiment at a large scale research facility). While a benefit of the former approach is the ability to resolve the dynamics of the interaction, a benefit of the latter is the faster reaching of steady state. Comparable measurements using the two methodologies showed that as the systems approached steady state, but the surface pressures were not identical. These results provide insight into the possibility that the nature of the lipid monolayer (*e.g.* its packing and presence of domains) with which the drug initially interacts may influence the extent of drug interaction or more likely the extent of lipid loss from the membrane, which intuitively would be an irreversible process given the macroscopic nature of the bulk solution in comparison with the membrane and the low bulk concentration of lipid sequestered into mixed micelles. Hence in either or both cases, the system approached steady state over the period of the measurements, but as a result of the system remaining in a kinetically-trapped state, this is not the same as equilibrium.

We now consider effects of the three experimental variables in turn. The starting surface pressure determines the packing and phase of the membrane prior to the drug interaction. While both types of monolayers are in liquid expanded phase at both 10 mN/m and 25 mN/m, the lateral heterogeneity of the membranes was observed to be different. At the higher starting surface pressure, there are slightly more condensed domains observed in both membranes while those in the mixed lipid membrane are larger and more irregularly shaped. The drug-membrane interactions are weaker (less drug penetration) at higher – more physiologically relevant – starting surface pressure, which we attribute to the more closely packed nature of the lipid molecules, and in the case of the mixed lipid membrane the presence of condensed phase domains. Indeed, surprisingly, equivalent amounts of lipid remained in the membrane as the systems approached steady state at the two starting surface pressures studied for both membrane compositions, supporting the conclusion made above that the initial state of the lipid in the membrane influences the resulting steady state properties.

Changes to the drug concentration are observed to have a stronger effect on the drug-membrane interaction for POPC than POPC/POPG (7:3 molar ratio) monolayers. An increase in the drug concentration results in an increase in the amount of the drug penetrated in POPC membrane, which contributes to increased surface pressure and membrane heterogeneity, as shown by BAM. Additionally, the NR data provided a surprising stoichiometry of more drug molecules than lipid in the resulting membrane for types of systems studied. It has been reported by Albertini *et al.*^[43] that when there are more propranolol hydrochloride molecules than lipid (drug:lipid ratio > 1), the

membrane undergo chain melting transition, and even become destroyed with drug: lipid ratio > 2.2 (not observed in our work). Similar effects have been reported that the interaction of the base of propranolol at its therapeutic concentration with human erythrocyte membranes where concentration-dependent changes of the morphology of red cells were demonstrated.^[44] As the drug concentration used in our NR experiments is only slightly above the intravenous dose used clinically, it can be concluded that in a physiological context, the drug may induce profound effects on cell membranes such as side effects for patients.

Fascinating observations were made about the interactions of propranolol hydrochloride with POPC/POPG (7:3 molar ratio) membranes. As propranolol hydrochloride is cationic in aqueous solution ($pK_a = 9.5^{[45]}$), the electrostatic effects with the polar head groups of lipids played a significant role in the drug-membrane interaction.^[46] However, the interaction of propranolol hydrochloride with the membrane containing 30 mol% of negatively charged POPG exhibited less pronounced effects (reduced drug penetration and lipid loss) compared with the pure POPC membrane. One possible reason is that the mixed lipid membrane exhibits phase separation with the presence of condensed domains prior to the interactions with drug. It has been proposed that mixed drug-surfactant micelles of propranolol hydrochloride with negatively charged surfactant show higher stability with cationic surfactant, due to the strong electrostatic attraction.^[47] Therefore, the reason of less lipid loss for the mixed lipid membrane compared with the pure POPC membrane may be related to the electrostatic attraction between drug molecule and domains of the charged lipid head groups in the membrane.

The results of this work have helped to improve the understanding of parallel processes involved in the interactions between the small amphiphilic drug propranolol hydrochloride and model lipid membranes, which may be related to the local anaesthetic side effect of the drug. However, human internal environment is much more complicated than the modelled system, the importance of physiological pH, plasma ionic strength and temperature to the drug-membrane interaction is nonnegligible. Further investigation of the interaction under physiological circumstances is necessary to fully understand the drug-membrane interaction.

Statements:

The NR experiments were performed by Dr. Maximilian Skoda. All the data analysis and other experiments were undertaken by Yixuan Yan. The deuterated drug was synthesised by Jasmine Hind and Dr. James Tellam. The project was conceived and supervised by Professor Jayne Lawrence and Dr. Richard Campbell.

5.7 Acknowledgement

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Supplementary information

SI-1 Calibration for neutron reflectivity experiments

The neutron reflectometry (NR) experiments were based on spreading a pre-determined amount of lipid solution on the surface of ultrapure water to obtain the same areas per lipid molecule as the experiments with starting surface pressures of 10 mN/m and 25 mN/m. The calibration was performed by spreading lipidic stock solution on water contained in the same troughs as those used for the NR experiments, with the surface pressure again being monitored using the Wilhelmy plate method. For each lipid composition, the calibration was repeated at least 3 times to ensure good reproducibility. The calibrated volume of each lipid solution is given in Table SI - 5.1. As a check, the average area per lipid molecule was calculated from a knowledge of the spread amount and the total area of the trough. The area per molecules calculated in this way is consistent with the data obtained from the lipid isotherm and from the values obtained from fitting the NR data of lipids (section 5.2).

Table SI - 5.1 Calibrated spreading volumes of lipid stock solution for NR experiments.

Lipid	Concentration of lipid (mg/mL)	Volume to 10 mN/m (μL)	Area/molecule at 10 mN/m ($\text{\AA}^2$)	Volume to 25 mN/m (μL)	Area/molecule at 25 mN/m ($\text{\AA}^2$)
POPC	0.5	34	88.4	43	75.1
POPC/POPG	0.5	40	74.8	49	61.1
d_{31} -POPC	0.5	43	72.7	52	60.2
d_{31} -POPC/POPG	0.5	46	66.9	55	56.0

The calibrated amount of lipid stock solutions was also spread on an aqueous solution of 20 mM propranolol hydrochloride (Figure SI – 5.1) contained in the same trough as being used for NR to compare with the surface pressures those obtained from the Langmuir trough experiments where a concentration solution of propranolol hydrochloride was injected under the lipid monolayer.

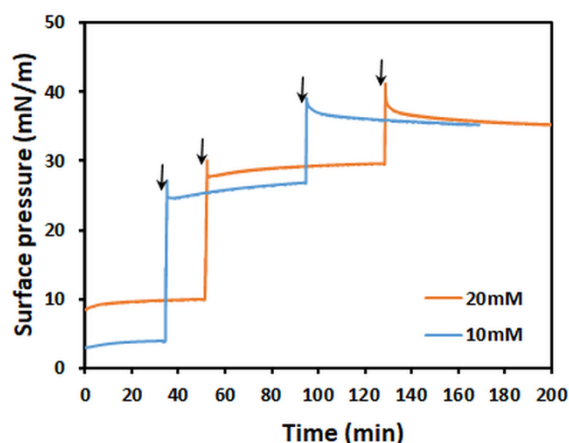


Figure SI - 5.1 Variation in surface pressure obtained by spreading POPC dropwise onto aqueous solutions of propranolol hydrochloride (either 20 mM or 10 mM) in the trough used for NR experiments. The arrows showed the spreading of POPC onto the surface of the aqueous drug solution.

The first noticeable difference from Figure SI - 5.1 is that the value of surface pressure tended to stabilise in the 'NR trough' much quicker than when injecting the concentrated drug solution below the lipid monolayer in the Langmuir trough.

The surface pressure of aqueous 20 mM propranolol hydrochloride solution is ~ 10 mN/m. The addition of 34 μ L of 0.5 mg/mL POPC solution to the surface of the trough was required to mimic the steady state value of surface pressure obtained using a POPC monolayer of a starting surface pressure of 10 mN/m. The surface pressure of the film recorded here was 29 mN/m which is similar but not the same as the measured surface pressure of 26 mN/m for the membrane obtained approaching steady state in a Langmuir trough after adding drug to the sub-phase.

A further 9 μ L of 0.5 mg/mL POPC solution was then added to the surface of the trough to mimic the starting surface pressure of 25 mN/m recorded in the Langmuir trough. In this instance the surface pressure increased to just above 40 mN/m and decreased to 35 mN/m by ~ 50 min after spreading, recording a very similar value in surface pressure to the 37 mN/m obtained in the Langmuir trough.

The surface pressure of aqueous 10 mM propranolol hydrochloride solution is ~ 4 mN/m. The addition of 34 μ L of 0.5 mg/mL POPC solution to the surface of the trough was required to mimic the steady state value of surface pressure obtained using a POPC monolayer of 10 mN/m starting surface pressure. The surface pressure of the film recorded here was 27 mN/m which compares to the

surface pressure of 23 mN/m for the film obtained at steady state in Langmuir trough after adding drug to the sub-phase.

A further 9 μ L of 0.5 mg/mL POPC solution was then added to the surface of the trough to mimic the starting surface pressure of 25 mN/m recorded in the Langmuir trough. In this instance the surface pressure increased to about 40 mN/m and decreased to 35 mN/m by $\sim$ 50 min after spreading, recording a very similar value in surface pressure to the 32 mN/m obtained in the Langmuir trough.

It was felt that, considering the slight increase of drug concentrations compared with those studied using the injection method, the surface pressures of the drug-lipid model membrane systems obtained using the above spreading approach were comparable enough to those obtained at steady state using the Langmuir trough to merit use of this more efficient methodology for valuable and limited NR beam time.

The decrease in surface pressure after the second spreading of lipid agrees with the results of the modelling of the NR data where the amounts of both the lipid and drug in the membrane decreased after the addition of the higher amount of POPC, and we attribute this result to the solubilisation of lipids induced by the drug-membrane interaction.

SI-2 Optimisation of the model used for analysis of the neutron reflectivity data

To obtain the best fit the NR data at the same time as using a realistic model, a range of different models were explored.

Initially propranolol hydrochloride was considered as a 'whole' molecule to obtain information on the location of propranolol in the membrane. Here, four models were applied to investigate the location of propranolol in the monolayer:

- (1) drug present only in the lipid head group layer;
- (2) drug present in the lipid head group layer and in an additional layer below the head group layer;
- (3) drug present in both the lipid head group layer and lipid chains layer;

(4) drug present only in the lipid chain layer.

Figure SI - 5.2 to Figure SI - 5.5 show the best fits and SLD profiles of the various neutron isotopic contrasts used obtaining models (1) – (4).

For the POPC/drug system, the data obtained using both surface pressures suggested that the optimum model (*i.e.* the minimum χ^2) was obtained using model (1) (Table SI - 5.2), *i.e.* drug only in lipid head group layer.

For the POPC/POPG/drug system, modelling the data suggested that model (3) with drug present in both the lipid head group and the lipid chain region of the membrane best represented the experimental NR data.

Table SI - 5.2 Comparison of χ^2 obtained for four different models of propranolol hydrochloride (20 mM) location in the lipid monolayers.

	POPC - 10 mN/m	POPC - 25 mN/m	POPC/POPG - 10 mN/m	POPC/POPG - 25 mN/m
Model 1	9.4	5.8	10.7	7.0
Model 2	27.1	5.8	10.8	6.7
Model 3	12.6	5.9	8.6	5.6
Model 4	29.7	11.2	11.0	8.0

Model 1: drug in lipid head group region; Model 2: drug in lipid head group region and additional sub-layer; Model 3: drug in both lipid head group and lipid chain region; Model 4: drug in lipid chain region.

Table SI - 5.3 Fitted parameters obtained for four different models of propranolol hydrochloride (20 mM) location in the lipid monolayers (see Table SI – 5.2)

	Model	Thick chain (Å)	Thick head (Å)	Thick extra layer (Å)	Solvation head (%)	Solvation extra layer (%)	Drug in head (%)	Drug in chain (%)	Surface excess lipid ($\mu\text{mol}/\text{m}^2$)	Surface excess drug ($\mu\text{mol}/\text{m}^2$)	Area / lipid molecule ($\text{\AA}^2$)
POPC/ drug 10 mN/m	1	9.4	11.5	/	0	/	70	0	1.67	3.02	99.6
	2	9.4	10.1	3.0	22	0	44	0	1.67	2.77	99.4
	3	9.8	11.4	/	2	/	68	5	1.66	3.02	100.1
	4	17.7	11.5	/	70	/	0	46	1.69	3.05	98.5
POPC/ drug 25 mN/m	1	9.8	10.9	/	3	/	64	0	1.73	2.63	95.9
	2	10.2	10.5	0.25	5	96	60	0	1.81	2.36	91.9
	3	9.9	10.9	/	4	/	63	1	1.73	2.60	95.9
	4	16.7	10.9	/	67	/	0	42	1.73	2.58	95.9
POPC/ POPG/ drug 10 mN/m	1	9.8	12	/	12	/	59	0	1.74	2.63	95.3
	2	10.2	10.7	1.9	8	80	59	0	1.80	2.50	92.1
	3	10.9	11.1	/	15	/	53	7	1.81	2.47	91.9
	4	16.9	10.9	/	69	/	0	49	1.72	2.69	96.8
POPC/ POPG/ drug 25 mN/m	1	10.3	10.9	/	2	/	65	0	1.82	2.65	90.9
	2	10.3	12.0	0.076	15	64	55	0	1.82	2.46	91.2
	3	10.7	10.9	/	7	/	59	2	1.86	2.47	89.1
	4	17.2	7.3	/	52	/	0	41	1.79	2.63	92.5

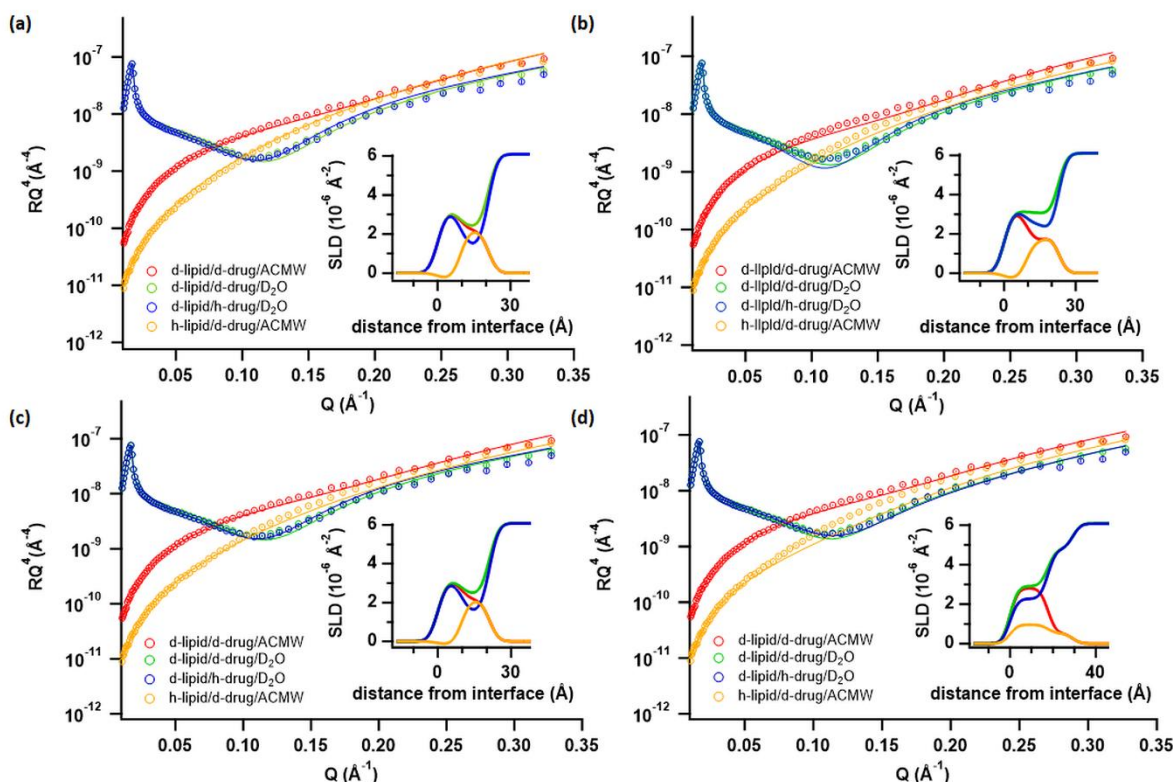


Figure SI - 5.2 NR data and SLD profile of POPC interacting with propranolol hydrochloride mimicking a starting surface pressure of 10 mN/m, fitted with (a) Model 1: drug in lipid head group region; (b) Model 2: drug in lipid head group region and additional sub-layer; (c) Model 3: drug in both lipid head group and lipid chain region; (d) Model 4: drug in lipid chain

region.

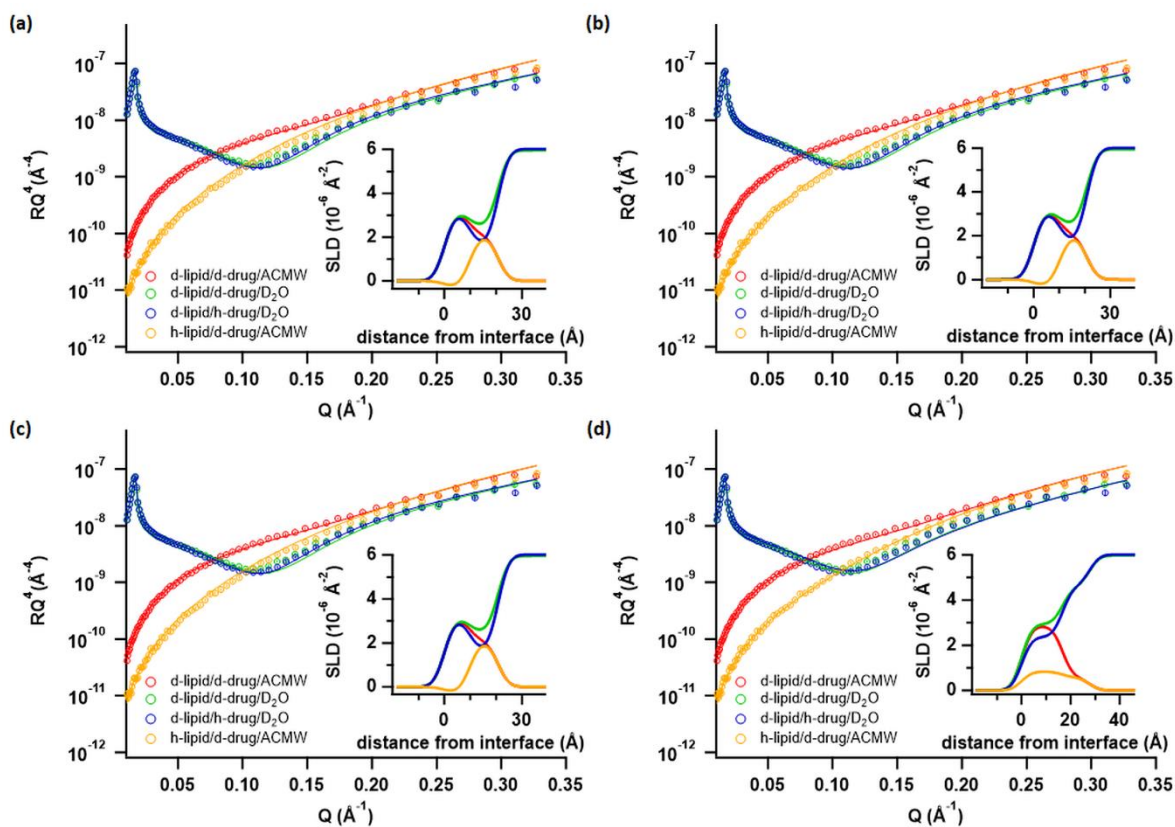


Figure SI - 5.3 NR data and SLD profile of POPC interacting with propranolol hydrochloride mimicking a starting surface pressure of 25 mN/m, fitted with (a) Model 1: drug in lipid head group region; (b) Model 2: drug in lipid head group region and additional sub-layer; (c) Model 3: drug in both lipid head group and lipid chain region and (d) Model 4: drug in lipid chain region.

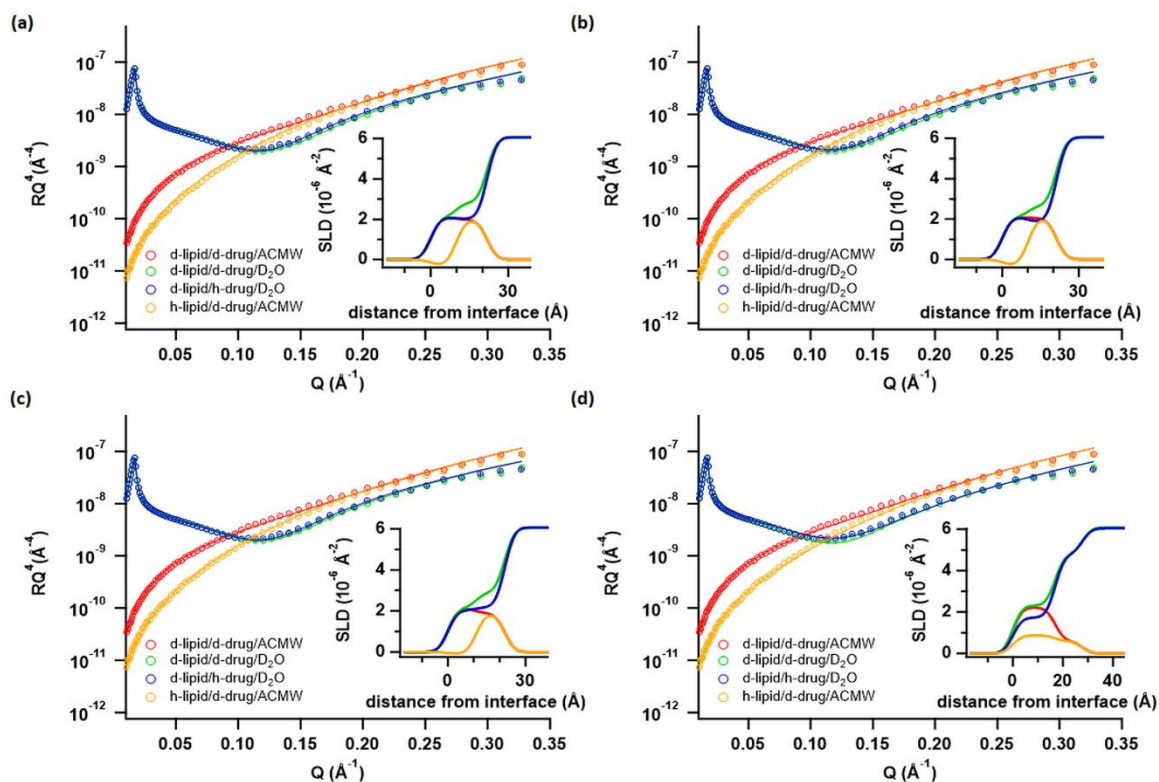


Figure SI - 5.4 NR data and SLD profile of POPC/POPG (7:3 molar ratio) interacting with propranolol hydrochloride mimicking a starting surface pressure of 10 mN/m, fitted with (a) Model 1: drug in lipid head group region; (b) Model 2: drug in lipid head group region and additional sub-layer; (c) Model 3: drug in both lipid head group and lipid chain region and (d) Model 4: drug in lipid chain region.

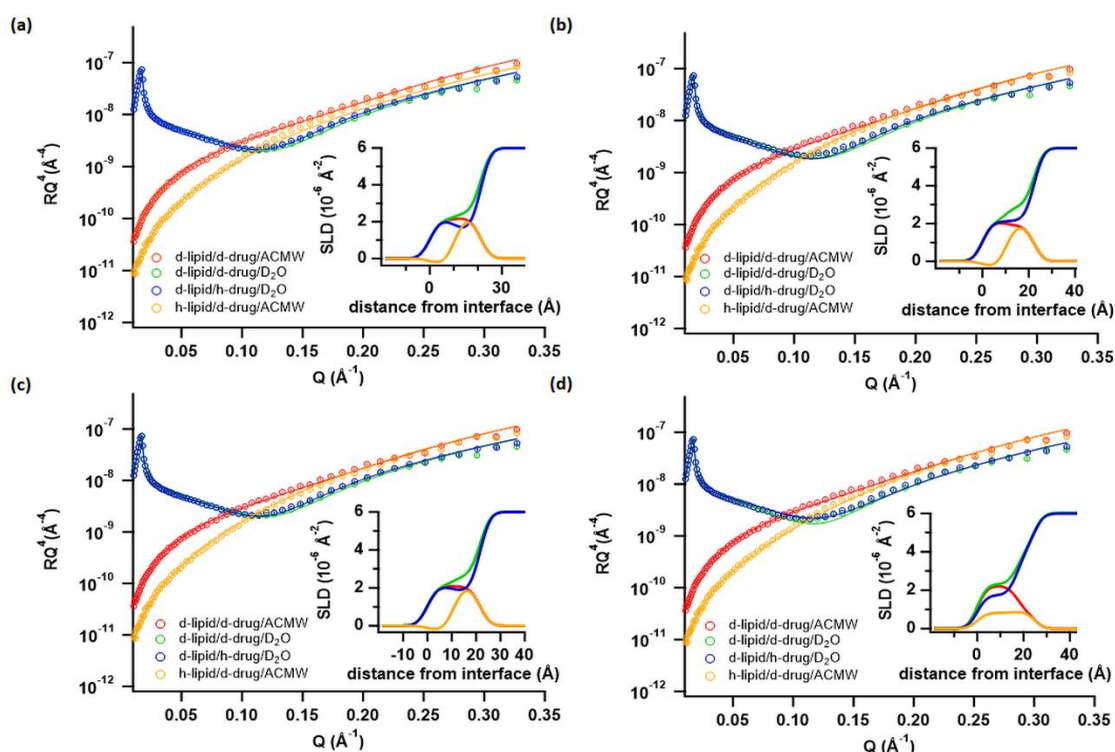


Figure SI - 5.5 NR data and SLD profile of POPC/POPG (7:3 molar ratio) interacting with propranolol hydrochloride mimicking a starting surface pressure of 25 mN/m, fitted with (a) Model 1: drug in lipid head group region; (b) Model 2: drug in lipid head group region and additional sub-layer; (c) Model 3: drug in both lipid head group and lipid chain region and (d) Model 4: drug in lipid chain region.

SI-3 Partition model

In consideration of the amphiphilic nature of propranolol hydrochloride, it was decided that the hydrophobic naphthalene ring may preferentially adopt a location with zero solvation and therefore reside in the lipid chain layer, whereas the side chains may adopt a location in either the chains or head groups layers. As a result, a novel partition model was developed that considers the propranolol molecule as being composed of two parts, one hydrophobic region containing the naphthalene rings, and one hydrophilic region comprising of the side chain with two hydrophilic centres where it was assumed that the naphthalene ring (termed ‘drug-ring’) exists only in the lipid chains, the distribution of the side chain (termed ‘drug-side chain’) between the hydrophobic lipid chain region and the hydrophilic solvated head group layer was examined. The surface excess of the drug-ring and drug-side chain was kept constant in the model to ensure physical reality of the same number of these components to equal a given number of drug molecules present in the membrane.

The partition model contains three variables:

- (1) volume fraction of ‘drug-ring’ in the lipid chain layer;
- (2) volume fraction of ‘drug-side chain’ in the lipid chain layer;
- (3) volume fraction of ‘drug-side chain’ in the lipid head group layer.

The first stage of the fitting procedure was to determine the correct amount of drug in the model membrane. To achieve this one of the variables was fixed, *e.g.* variable (2), and the other two variables were changed accordingly to obtain the surface excess of drug that gives the minimum χ^2 that was then used for further analysis.

The second stage was to vary the fixed variable while keeping the surface excess constant at the value selected in the first stage, while the values of the other two variables were changed accordingly, hence varying the ratio of drug-side-chain in the two layers.

The optimum fit is therefore determined from the combination of three variable that gives the minimum χ^2 , as shown as an example in Figure SI - 5.6.

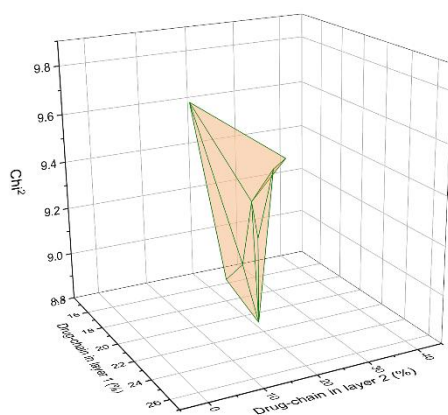


Figure SI - 5.6 Distribution of χ^2 with change in volume fraction of 'drug-side chain' in each layer (data from the POPC/drug system at 10 mN/m). The minimum point is referred to as the optimum fit.

6 A mechanistic study of the interaction of model lipid membranes containing cholesterol with the amphiphilic drug propranolol hydrochloride at the air/water interface

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6.1 Abstract

Propranolol hydrochloride is reported to interact with cell membranes due to its amphiphilic structure and exhibits membrane stabilising side effects. For human cell membranes, cholesterol is essential for stabilising their structure while maintaining the fluidity. In this work, the interaction between the drug propranolol hydrochloride and cholesterol containing model lipids membrane at the air/water interface has been studied, using a combination of optical interfacial techniques to resolve the mechanism of the drug-membrane interaction over a range of time and length scales. Effects of physiological ionic strength, content of cholesterol (physiological level and high level), starting surface pressure of the membrane as well as the drug concentration were studied. The results suggested that physiological content of cholesterol (30 mol%) improves the penetration of the drug in the membrane significantly. The effect of a physiological concentration of sodium chloride results in a combination of different factors that promote the interaction. On the contrary, a higher level of cholesterol (46 mol%) prohibits the drug penetration possibly through selective interaction with POPC over phase separated cholesterol domains. This study has provided insight into the drug-membrane interactions that may occur in human body, which may inspire an approach to improve the bioavailability of propranolol hydrochloride in clinical use.

6.2 Introduction

Propranolol hydrochloride is an amphiphilic drug that is reported to interact with cell membranes and to exhibit local anaesthetic and membrane stabilising side effects.^[1, 2] The interactions between

propranolol hydrochloride and membranes have been studied using various membrane models as platforms, such as erythrocyte membranes,^[3] vesicles,^[4] bilayers,^[5] monolayer.^[6] Most of these studies have focused on membranes comprising phospholipids or the overall cell membrane, with little attention given to the specific role of cholesterol in the drug-membrane interaction. Cholesterol is an essential component in mammalian cell membranes that stabilises the structure of the membrane, increase lipid packing density yet maintains the fluidity of the membrane.^[7, 8] Additionally, cholesterol assists the formation of lipid rafts, structures which are known to consist of cholesterol-rich highly ordered lipid domains, which are involved in many physiological processes.^[9, 10] Therefore, the role of cholesterol in the interaction between lipid membranes with propranolol hydrochloride strongly merits further study.

It has been reported that the presence of sodium chloride can change the membrane rigidity as well as its surface charge, processes which play an important role in membrane interactions with biochemical molecules, for example the drug-membrane interaction that is related to the drug delivery and bioavailability.^[11, 12] Magarkar and co-workers have proposed that the cholesterol level reduces the binding of Na⁺ to the lipid head groups.^[11] Under physiological conditions, the concentration of sodium chloride is 0.154 M, hence the effect of the presence of sodium chloride to the drug-membrane interaction can be significant. In this work, we have studied the interaction of POPC membranes containing 30 mol% cholesterol with propranolol hydrochloride in the presence of 0.154 M sodium chloride, to mimic the physiological environment, thus obtaining information about the drug-membrane interactions in the human body.

The percentage of cholesterol in mammalian cell membrane ranges from 30% up to 50%.^[13, 14] A study on the cholesterol-poor and cholesterol-rich DPPC monolayers has proposed that the cholesterol level affects the state of the cholesterol-containing phases of the membrane, from a mixture of liquid expanded phase and liquid condensed phase at cholesterol less than 10%, to the coexistence of several phases at cholesterol levels that exceed 17%, and then a homogeneous liquid condensed phase at cholesterol above 33%.^[15] Furthermore, the level of cholesterol in a cell membrane is not constant. Its level can be affected by factors such as biosynthesis, efflux and influx of external cholesterol and disease.^[9] Indeed, the content of cholesterol in membranes can play a significant role in the membrane interaction of propranolol hydrochloride. POPC is a zwitterionic unsaturated phospholipid, which is the major component of human cell membranes,^[16] for which the interaction

with propranolol hydrochloride has been studied in our previous work.^[17] Therefore in this study, two levels of cholesterol in a POPC monolayer have been studied as models of different biological membranes, namely either a commonly found level of cholesterol (*i.e.* 30 mol%) or a relatively high level of cholesterol (*i.e.* 46 mol%), in the text we simplify refer to these monolayers by their POPC:cholesterol molar ratio of 7:3 and 5:5, respectively. The goal of using these two membranes was to probe the effect of the content of cholesterol on the interaction of POPC with propranolol hydrochloride.

The overall aim of this work is to understand the mechanism of the interaction of model lipid membranes containing cholesterol with the amphiphilic drug propranolol hydrochloride on a molecular length scale. The effects of additional electrolytes, starting surface pressure, drug concentration as well as the content of cholesterol in membrane were studied using a series of interfacial techniques to resolve the interaction by the use of probes that are sensitive to different aspects of the interaction. A Langmuir trough was used to produce the model lipid monolayer at air/water interface, which provided a platform where other optical reflectometry techniques such as ellipsometry, Brewster angle microscopy and neutron reflectometry were in combination to resolve real-time changes of the drug-membrane interaction. Ellipsometry at air/water interface was applied to gain information on the heterogeneity of the membrane that is related to the membrane thickness and refractive index, by measuring the phase shift of the laser beam through refraction and reflection by the interfacial layer. Brewster angle microscopy was applied to visualise the lateral heterogeneity of the membrane on the micrometre scale. Neutron reflectometry was applied to reveal kinetic changes of the amount of the components and the structure of the components in the direction perpendicular to the interface both during kinetic changes and once the system had reached steady state.

6.3 Materials

1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC, $\geq 99.0\%$ TLC) and cholesterol ($\geq 99\%$) were provided by Sigma Aldrich. 1-palmitoyl-*d*₃₁-2-oleoyl-sn-glycero-3-phosphocholine (*d*₃₁-POPC, $> 99\%$) was supplied by Avanti Polar Lipids. ($\pm$)-Propranolol hydrochloride (99.9 %) was purchased from Alfa Aesar. ($\pm$)-Propranolol hydrochloride (*d*₇, 84%) was synthesised by the ISIS Deuteration Facility,

Rutherford-Appleton Laboratory, STFC, Didcot, Oxford. Deuterium oxide (D_2O , 99.9 atom % D) and sodium chloride (NaCl, 99.9 %) were purchased from Sigma Aldrich. Chloroform (≥ 99.8 %) were obtained from Fisher Scientific. Ultra-pure water of resistivity $18.2 \text{ m}\Omega \text{ cm}^{-1}$, supplied by an Integra L, Suez Pure Water System, was used throughout.

6.4 Methods

6.4.1 Surface pressure measurements

The variation in surface pressure (π) of a lipid sample at the air/water interface in the absence and presence of differing amounts of propranolol hydrochloride was measured using the Wilhelmy plate method and a Langmuir trough composed of Teflon fitted with two movable Delrin barriers of 16 mm width (Kibron, Finland). The trough was filled with 160 ml of either pure water or an aqueous sodium chloride (0.154 M) solution as sub-phase (total surface area = 28000 mm^2). The lipid dissolved in chloroform was added dropwise onto the surface of the aqueous sub-phase using a Hamilton syringe. After waiting 10 min for the evaporation of the chloroform, the area over which the lipid monolayer was spread was compressed by moving the two Delrin barriers at a rate of 10 mm/min until the π reached a value of either 10 mN/m or 25 mN/m whereupon the surface area of the trough was kept constant. The π of the resulting monolayer was monitored over time to ensure its stability, once this was established, concentrated drug stock solution was injected into the sub-phase and any subsequent variation in π monitored. Prior to the injection of the aqueous drug stock solution, 10 ml of the sub-phase was carefully removed to allow injection of 10 ml of the stock propranolol hydrochloride solution (either 150 or 300 mM), using a long needle (120 mm $\times$ 0.8 mm, B Braun). The π was monitored for at least 90 minutes after injection of the stock drug solution. All measurements were performed in room temperature (22 ± 1 °C) and repeated at least twice to ensure reproducibility.

Knowledge of the amount of lipid added to the air/water surface, coupled with the area over which it was spread, allowed determination of the area per lipid molecule, or in the case of a mixed lipid film, the average area per lipid molecule, both denoted as A and expressed in $\text{\AA}^2$. Generally, π is expressed as a function of A in what is known as a π - A isotherm. The surface data can also be expressed in the form of the compressibility modulus, C_s^{-1} , calculated by:

$$C_s^{-1} = -A \frac{d\pi}{dA} \quad (6.1)$$

The C_s^{-1} allows the phase of the lipid monolayer to be assessed, C_s^{-1} values between 12.5 and 50 mN/m indicates that the lipid(s) comprising the monolayer are in a liquid expanded phase while values of between 100 and 250 mN/m correspond to lipid(s) in a liquid condensed phase. Moreover, the C_s^{-1} limit values for the solid state (LS) are 1000–2000 mN/m.^[18]

6.4.2 Ellipsometry and Brewster angle microscopy

Any variation in the amount of lipid (and drug) at the air/water interface, along with the presence of any lateral inhomogeneities in the film at the surface, was assessed using ellipsometry. Ellipsometry is an optical reflection technique that uses polarised light to measure the phase shift (Δ) and amplitude ratio (ψ) that occur due to refraction and reflection as a result of its interaction with a film at an interface. By measuring the variation in phase shift, changes in film heterogeneity due to a phase separation of the film, which result in a difference of the refractive index and/or film thickness recorded with time can be resolved. In the present study, the quantity $\Delta\delta$ is used for analysis, which is the value of Δ measured for the sample minus the value measured for pure water. The $\Delta\delta$ measured in the present study was used to assess the amount of lipid (and drug) present at the air/water interface while the presence of any lateral heterogeneities in the film were inferred from fluctuations in the value of $\Delta\delta$ over time and are thought to be related to either lipid phase and/or regions where the binding of drug perturbing the ellipsometric response by passing in and out of the $\sim 1 \text{ mm}^2$ interface probed.

In the present study the ellipsometric data were obtained using an Accurion Nanofilm EP4 instrument (Goettingen, Germany) equipped with a blue diode laser of operating wavelength 489 nm at an incident angle of 50° in combination with the Langmuir trough set-up described earlier. The standard deviation and frequency of $\Delta\delta$ over 3.5 min before the injection and 3.5 min prior to the end of 100 min after the interaction (of the concentrated propranolol hydrochloride solution into the sub-phase are reported. The peak value of the distribution of the phase shift and its width are related to changes in film properties due the interaction of drug with the lipid monolayer. All measurements were performed in room temperature ($22 \pm 1^\circ \text{C}$) and repeated at least twice to ensure reproducibility.

Brewster angle microscopy (BAM) was used to visualise the lateral morphology of the lipid monolayers at the air/water interface on a micrometre length scale and changes in the presence of differing amounts of propranolol hydrochloride. In common with an ellipsometer, a Brewster angle microscope uses polarised light, but specifically p-polarised light at the Brewster angle for water (53.1°), so that no reflection occurs from the (bare) water surface (neglecting surface roughness), and reflection (and visualisation) will occur only if materials of a different refractive index than water are present at the air/water surface. The brightness and contrast of the BAM images are related to the thickness and anisotropy of material (here lipid (and drug)) at the interface.

An Accurion Nanofilm EP4 instrument was used to obtain BAM images using the same Langmuir trough set-up as described above. The BAM images were optimised by constant gamma correction using a software Irfanview (Germany). Due to the incident angle 53.1°, the illumination of the laser was elliptical and therefore the BAM images needed to be rescaled in length by a ratio of 1.66 to restore them to their original shape. All measurements were performed in room temperature (22 ± 1 °C) and repeated at least twice to ensure reproducibility.

6.4.3 Neutron reflectometry

Neutron reflectometry (NR) was used to determine the structure, thickness and composition of the lipid monolayers alone and in the presence of differing amounts of propranolol hydrochloride on approach to steady-state at the Å-scale normal to the interface. NR is a surface reflection technique which allows determination of the neutron reflectivity (R) from the momentum transfer of neutrons (Q) after the reflection and the neutron scattering length density (SLD) of the material, which is related to the refractive index of neutrons to the material.

$$R \approx \frac{16\pi^2 \rho^2}{Q^4} \quad (6.2)$$

where ρ is the symbol that represents the SLD. The SLD is calculated from the sum of the scattering lengths of all atoms of the material in combination with its molecular volume:

$$\rho = \frac{\sum_i^n b_i}{V_m} \quad (6.3)$$

where b_i is the scattering length of the i atom in a molecule of n atoms, and V_m is the molecular

volume of the molecule. The scattering lengths, molecular volumes and the SLDs calculated for the various materials used in the present study are listed in Table 6.1.

Table 6.1 Scattering lengths, molecular volumes and scattering length densities of different materials used in the present study.

Molecule	Moiety	Scattering length (fm)		Molecular volume ($\text{\AA}^3$)	Scattering length densities ($\times 10^{-6} \text{\AA}^{-2}$)	
POPC	Head ($\text{C}_{10}\text{H}_{18}\text{NO}_8$)	60.07		344 ⁽¹⁾	1.75	
	Tail ($\text{C}_{32}\text{H}_{64}$)	-26.62		937 ⁽¹⁾	-0.28	
d_{31} -POPC	Tail ($\text{C}_{32}\text{H}_{33}\text{D}_{31}$)	296.09		937 ⁽¹⁾	3.16	
Cholesterol	Head (OH)	2.06		5 ⁽²⁾	4.13	
	Tail ($\text{C}_{27}\text{H}_{45}$)	11.19		624 ⁽²⁾	0.18	
Propranolol	h-Ring (C_{10}H_7)	40.29		183 ⁽³⁾	2.20	
	d_7 -Ring (C_{10}D_7 , 84%D)	101.50		183 ⁽³⁾	5.55	
	Chain ($\text{C}_6\text{H}_{15}\text{NO}_2$)	5.59 (in ACMW)	15.17 (in D_2O)	264 ⁽⁴⁾	0.21 (in ACMW)	0.57 (in D_2O)

⁽¹⁾ Data sourced from reference [19]. ⁽²⁾ Total volume taken an average from reference [19, 20]. ⁽³⁾ Sourced from PubChem database. ⁽⁴⁾ Calculated from a physical density prediction using the Percepta Platform from ACD Labs in ref [21].

Significantly for the present study, replacement of a protium atom with a deuterium atom is exploited in isotopic substitution due to the fact that the neutron scattering length of deuterium is 6.671 fm, much larger than and of opposite phase to that of protium, -3.739 fm^[22]. As a result, deuterated/partially deuterated materials can be used as isotopic contrast agents to resolve the detailed structure of the systems of interest. The use of materials of differing SLDs can be used to highlight or even make ‘invisible’ to neutrons part of the system under study. For example, when studying films at the air/water surface, it is possible to highlight the film, by using air contrast matched water (ACMW), composed of 8.1% v/v D_2O in H_2O , and which makes the sub-phase ‘invisible’ to neutrons.

In the present work, the structural NR studies on POPC/cholesterol (7:3 molar ratio) and (5:5 molar ratio) were performed on the INTER reflectometer (ISIS Pulsed Neutron and Muon Source, Rutherford Appleton Laboratory, Harwell, UK) and the kinetic and structural NR studies on POPC/cholesterol (7:3 molar ratio) in the presence of 0.154 M sodium chloride were performed using the FIGARO reflectometer (Institut Laue Langevin (ILL), Grenoble, France). For the experiments on the INTER reflectometer, the lipid stock solution was spread directly on an aqueous solution containing 20 mM propranolol in a trough of 11900 mm² surface area. The amount of lipid solution to be spread onto

the surface to obtain the required π had been previously calibrated (Supplementary information SI-1). At least three and sometimes four isotopic contrasts were used on the INTER reflectometer namely *d*-lipid/*d*-drug/ACMW; *d*-lipid/*d*-drug/D₂O; *d*-lipid/*h*-drug/D₂O and *h*-lipid/*d*-drug/ACMW. Two incident angles of 0.8° and 2.3° were measured using neutrons of wavelength range of 1.5-17 Å. The experiments performed on the FIGARO reflectometer were performed using a 50 mL Nima Langmuir trough (area = 11900 mm²) where the protocol for preparation of the films was exactly the same as that described under the surface pressure measurements. Six isotropic contrasts were used on the FIGARO reflectometer namely *d*-lipid/*h*-drug/D₂O; *d*-lipid/*h*-drug/ACMW; *h*-lipid/*h*-drug/ACMW; *h*-lipid/*d*-drug/ACMW; *d*-lipid/*d*-drug/ACMW and *d*-lipid/*d*-drug/D₂O. The resulting data were analysed using the Motofit package in Igor 8, with the isotopic contrasts of the same system being co-refined.

By modelling the reflectivity profiles, information on the thickness, roughness, solvation degree and the volume fraction of each component in each layer can be resolved. In this work, the lipid membrane is regarded as a two-layer structure: a lipid chains layer and a solvated lipid head groups layer. The lipid head groups layer contains the PC head of POPC and the hydroxyl group of cholesterol. In the drug-membrane mixed system, since propranolol hydrochloride has an amphiphilic structure, the hydrophobic naphthalene ring (called 'drug-ring') is assumed to exist only in the lipid chains layer as a result of hydrophobic effects. By adjusting the volume fraction of drug-ring and the side chains of the drug (called 'drug-chains') in the two layers, it was possible to calculate the average SLD and solvation of the layers, thus obtain a corresponding optimum fit of the layer thickness. The optimum fits were determined based on the minimum χ^2 obtained that makes physical sense.

6.4.4 Low-Q measurements

For neutron reflectivity measurements at the air/water interface, structural information can be extracted from reflectivity profiles recorded at the relatively high Q range, while at very low Q (≤ 0.05 Å⁻¹), the reflectivity is related to the product of the thickness (*d*) and SLD (ρ) of the film and is therefore proportional to the surface excess (Γ) of every component, where:

$$\rho d = \Gamma b N_A \quad (6.4)$$

and where *b* is the neutron scattering length of the component, and *N_A* is the Avogadro's number.

For systems comprising two components, as is the case here with lipid and drug, the product ρd is simply the sum of the scattering from two components. By the use of judiciously selected deuterated/partially deuterated materials, it is possible to resolve the surface excess of the two components. A measurement of the low Q region was therefore an easy and useful approach to determine any time-dependent changes in the surface excesses of the two components. In the current work, low Q measurements were used to study the interaction of 20 mM propranolol hydrochloride with monolayers of POPC/cholesterol (7:3 molar ratio) in the presence of an aqueous solution of 0.154 M sodium chloride at a starting surface pressure of 25 mN/m. This system was chosen because non-monotonic changes were observed in the surface pressure kinetics that merited the application of this additional technique.

6.5 Results

6.5.1 POPC/cholesterol model membranes

π -A isotherms measured at room temperature for POPC/cholesterol monolayers at molar ratios of 7:3 and 5:5 spread on pure water, a POPC/cholesterol monolayer at a molar ratio of 7:3 spread on 0.154 M sodium chloride aqueous solution, all up to 30 mN m⁻¹, and for reference pure POPC and cholesterol monolayers spread on pure water up to 35 mN m⁻¹, are shown (Figure 6.1(a)); the reciprocal of the compressibility modulus is shown in the inset. With the exception of POPC and cholesterol, the isotherms were measured during a cycle of film compression (solid line) and expansion (dashed line), and while some hysteresis is observed for the POPC/cholesterol monolayers, it was of a low level. For the cholesterol monolayer, the value of C_s^{-1} increases steeply to 100 mN/m as the surface pressure increased from 0 to 3 mN/m. In this range the cholesterol monolayer is in a liquid expanded state. However, as the π increases further, C_s^{-1} increases to very high values of greater than 600 mN/m at π of about 30 mN/m, which suggests that the cholesterol molecules are in a highly condensed state (LC).^[23] By way of contrast, whilst the π -A isotherms show that no obvious phase transitions occur for the POPC or POPC/cholesterol monolayers, the compressibility data suggest that the monolayers of POPC and POPC containing 30 mol% cholesterol are in a liquid expanded phase only at the lower of the two surface pressures that will be studied below using other techniques (*i.e.* 10 mN/m) while they are in a liquid condensed phase at the higher value (*i.e.* 25

mN/m), yet the POPC monolayer containing 50 mol% cholesterol is present in the liquid condensed phase at both surface pressures. As can be seen, the more cholesterol the POPC monolayer contains, the more similar its surface behaviour to the of the cholesterol monolayer (Figure 6.1(a)).

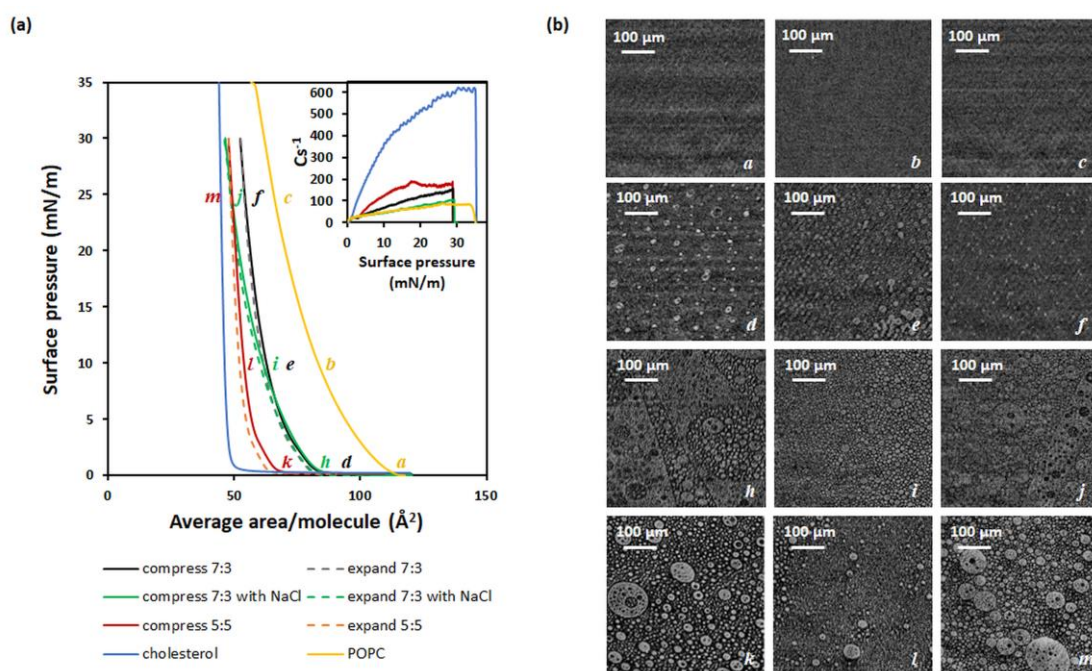


Figure 6.1 (a) Lipid isotherm of POPC, cholesterol, and mixed POPC/cholesterol membrane of 7:3 (in the absence or presence of 0.154 M sodium chloride) and 5:5 molar ratio upon compression and expansion, respectively. (b) BAM images taken at 0, 10 and 25 mN/m upon compression, *a-c*: POPC, *d-f*: POPC/cholesterol (7:3), *h-j*: POPC/cholesterol (5:5), *k-m*: cholesterol, corresponding to the letters labelled in (a).

BAM images of the two cholesterol containing monolayers spread on pure water (Figure 6.1(b)) show increasing the ratio of cholesterol present influences the formation of lipid domains, in that at the same π , the size of the lipid domains formed in the presence of 46 mol% cholesterol (Figure 6.1(b)*k-m*) are larger than those formed in the presence of 30 mol% cholesterol (Figure 6.1(b)*d-f*). In the monolayer containing 46 mol% cholesterol, the higher π of 25 mN/m (Figure 6.1(b)*m*) is dominated by the presence condensed lipid domains when compared to a π of 10 mN/m, considering the turning point of C_s^{-1} at around 17 mN/m, it indicates a change of phase upon compression.

The NR data obtained for the POPC/cholesterol monolayers containing 30 and 50 mol% cholesterol at π of 10 mN/m and 25 mN/m, and the POPC/cholesterol monolayer with 30 mol% cholesterol in the presence of 0.154 M sodium chloride is shown in Figure 6.2 and Figure 6.3, respectively. The optimum

fits modelled assuming two layers, one comprising of lipid tails and the other solvated lipid head groups, yielded information on the thickness of the two layers, solvation, and the amount of lipid present in the monolayers (Table 6.2). For purposes of comparison the corresponding NR data obtained for POPC monolayers is reported in Table 6.2.^[17] For the POPC monolayer and both cholesterol containing membranes, the increase of π contributes to the increase of the thickness of lipid chain layer, because of more closely packed lipid molecules at the higher π . Furthermore, the addition of cholesterol increases the thickness of the tail layer by about 3-4 Å, irrespective of how much cholesterol is present in the monolayer. Interestingly, however, regardless of π there was no difference in the thickness of the head group layer between the POPC and the monolayer containing 30 mol% cholesterol, 9.4 ± 2.7 Å for POPC and 9.5 ± 3.0 Å for POPC containing 30 mol% cholesterol at 10 mN/m. Yet when the cholesterol content is increased to 50 mol% the thickness of the head group layer decreased from ~ 9.5 to 7.4 Å, probably a consequence of the very small volume of the hydroxyl head group present in cholesterol resulting in an increase in space available to the PC head groups and their rearrangement. It should be cautioned that the uncertainty in the global fit means that this difference falls within the stated error, but the key isotopic contrast that reveals the total interface thickness (*i.e.* *d*-lipid in D₂O) shows a clear shift in fringe position for the system with 46 mol% cholesterol. Despite the difference in head group volumes, the total amount of lipid in the monolayer at the same π is comparable for both cholesterol containing membranes (Table 6.2).

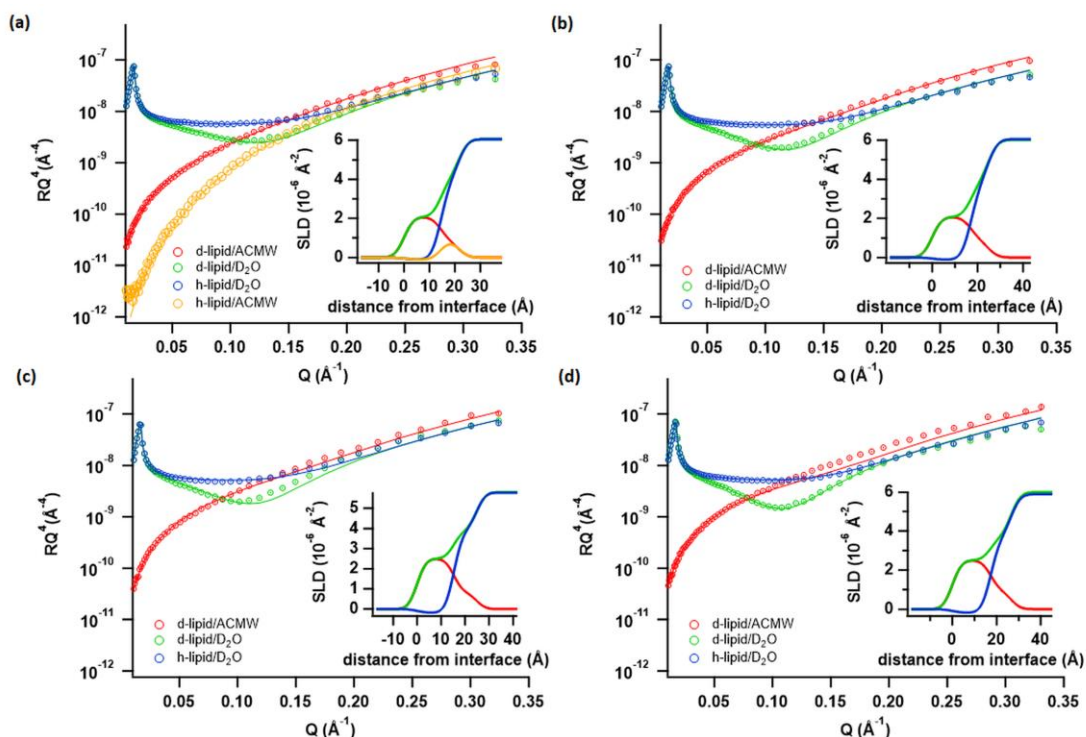


Figure 6.2 Reflectivity data of POPC/cholesterol membrane of 5:5 and 7:3 molar ratio at 10 and 25 mN/m, respectively. (a) POPC/cholesterol (5:5 molar ratio) at 10 mN/m, (b) POPC/cholesterol (5:5 molar ratio) at 25 mN/m, (c) POPC/cholesterol (7:3 molar ratio) at 10 mN/m, (d) POPC/cholesterol (7:3 molar ratio) at 25 mN/m. The insets are the SLD plotted against the distance from interface. The insets are the SLDs of each component plotted against the distance from the interface.

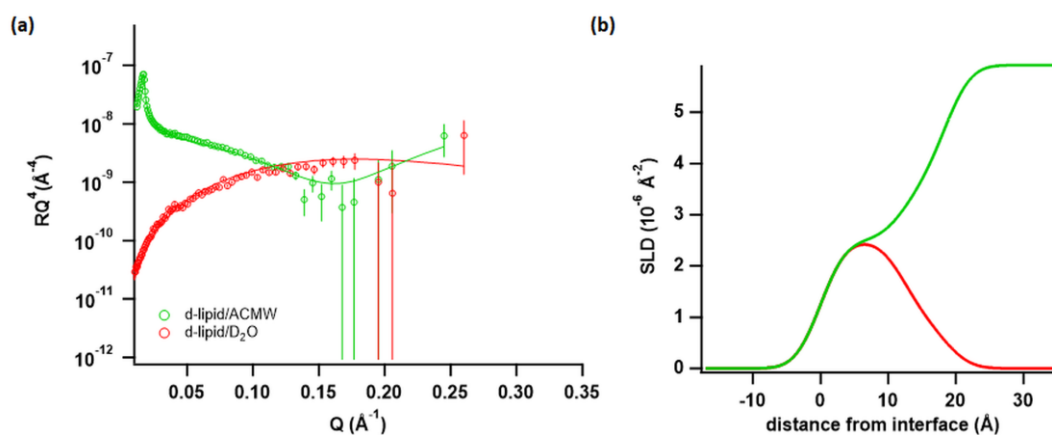


Figure 6.3 Reflectivity data (a) and the SLD profile (b) of the POPC/cholesterol (7:3 molar ratio) monolayer with the presence of 0.154 M NaCl.

Table 6.2 Fitted structural parameters of POPC monolayers neutron reflectivity data recorded in either 3 or 4 isotopic contrasts.

System and starting surface pressure	Lipid chain thickness (Å)	Lipid head thickness (Å)	Head group solvation (vol%)	Surface excess of lipid ($\mu\text{mol}\cdot\text{m}^{-2}$)	Average area per lipid molecule ($\text{\AA}^2$)
POPC at 10 mN/m*	11.4 ± 0.5	9.4 ± 2.7	55 ± 13	2.02 ± 0.14	82.2 ± 6.2
POPC at 25 mN/m*	14.1 ± 0.2	8.9 ± 1.3	42 ± 9	2.49 ± 0.04	66.6 ± 1.1
POPC/cholesterol 7:3 at 10 mN/m	15.3 ± 0.6	9.5 ± 3.0	53 ± 10	3.02 ± 0.12	54.9 ± 2.1
POPC/cholesterol 7:3 at 25 mN/m	17.3 ± 0.5	9.3 ± 1.7	46 ± 7	3.42 ± 0.10	48.6 ± 1.4
POPC/cholesterol 7:3 at 25 mN/m with 0.154 M NaCl	12.2 ± 0.4	6.4 ± 1.9	45 ± 11	2.41 ± 0.08	68.9 ± 2.3
POPC/cholesterol 5:5 at 10 mN/m	14.5 ± 0.4	7.1 ± 2.1	53 ± 19	3.04 ± 0.08	54.7 ± 1.1
POPC/cholesterol 5:5 at 25 mN/m	17.0 ± 0.4	7.8 ± 1.6	49 ± 12	3.57 ± 0.09	46.6 ± 1.2

*data from reference [17].

In the present study, the addition of 0.154 M of NaCl to the aqueous sub-phase was used as a simple surrogate of the physiological environment as it is well known that the presence of ions can influence the structural properties of phospholipid monolayers.^[24] In contrast, however, it was considered unlikely that the presence of sodium and chloride ions would alter the behaviour of the hydroxyl head group present in non-ionic cholesterol. The π -A isotherm of POPC monolayers containing an equimolar amount of cholesterol in the presence of a relatively low amount of 0.154 M NaCl is comparable in behaviour to the POPC monolayer as evidenced by the variation in its compressibility modulus, which are virtually superimposable, suggesting that the presence of a relatively low amount of sodium and chloride ions has fluidized the monolayer which is in a more expanded state that compared to the one prepared in its absence. The BAM images in the presence of sodium and chloride ions appear less intense (or bright) than the images obtained in the absence of added salt, suggesting that the monolayer is thinner or less tightly packed than the monolayer presence without salt, which would be consistent with the observation from the π -A isotherm of a more expanded monolayer formed in the presence of NaCl.

Analysis of the NR data in the presence of NaCl at a π of 25 mN/m (Table 6.2) provides information on the effect of sodium and chloride ions on the structure of the monolayer. It is noticeable that there is a significant decrease of the thickness of both the tail and head group region monolayer from 17.3

± 0.5 to 12.2 ± 0.4 Å and 9.3 ± 1.7 to 6.4 ± 1.9 Å, respectively, a result which is consistent with the presence of less intense regions from thinner domains observed with BAM. Furthermore, the presence of NaCl results in a severe decrease in the amount of lipid at the air/water interface, from $3.02 \mu\text{mol}\cdot\text{m}^{-2}$ in the absence of 0.154 M NaCl to $2.41 \mu\text{mol}\cdot\text{m}^{-2}$ in its presence. The observation correlates with the more expanded nature of the cholesterol containing monolayer in the presence of NaCl.

6.5.2 The interaction of propranolol hydrochloride with POPC/cholesterol (7:3 molar ratio) model membranes

The interactions of propranolol hydrochloride with a model membrane composed of POPC/cholesterol (7:3 molar ratio) were studied using the same techniques as above as well as ellipsometry following injection of drug solution into the subphase beneath lipid monolayers prepared at 10 or 25 mN/m. For comparative purposes, the results of the interaction of propranolol hydrochloride with POPC model membranes published previously^[17] are first summarised in brief (Figure 6.4): propranolol hydrochloride induce immediate lipid loss upon exposure, increasing membrane heterogeneity and forming ordered lipid domains, accompanied with the penetration of drug into the membrane. The starting surface pressure, which determines the initial state of the membrane, plays an important role in the interaction. Also, the interaction is dependent on the concentration of propranolol hydrochloride that the increase of drug concentration leads to higher degree of drug penetration.

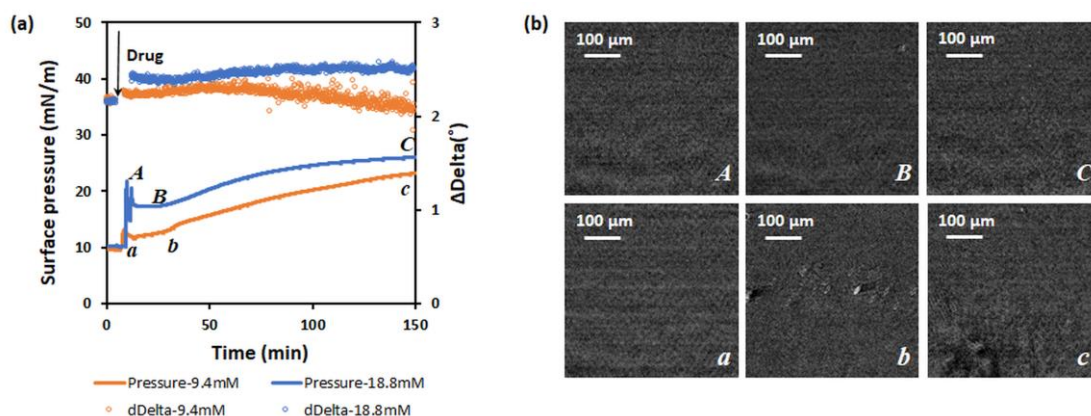


Figure 6.4 (a) Surface pressure and $\Delta\delta$ of POPC monolayers upon interaction with 9.4 mM and 18.8 mM propranolol hydrochloride at initial surfaces pressures of 10 mN/m. (b) BAM images of the interaction of POPC with 9.4 mM and 18.8 mM propranolol hydrochloride at 10 mN/m. Figure modified from reference [17].

For the POPC/cholesterol (7:3 molar ratio) monolayers, a similar sharp rise then a fall in π soon after injection of drug, attributed to loss of lipid from the membrane possibly through formation of mixed propranolol hydrochloride/phospholipid micelles lost to the bulk. Thereafter, the slow increase in π is recorded for the POPC/cholesterol (7:3 molar ratio) membrane over the 100 minutes, considered to be a consequence of drug molecules penetrating the model membrane. Also, the extent of the interaction with propranolol hydrochloride with the model membrane is dependent upon the starting π . For example, the recorded increase in π upon approach to steady state is greatest at a starting π of 10 mN/m with increases of ~ 11 mN/m and 12 mN/m being observed upon the addition of drug concentrations of 9.4 mM and 18.8 mM, respectively. At a starting π of 25 mN/m, the increase in π upon approaching steady state is smaller at ~ 8 mN/m, regardless of the propranolol hydrochloride concentration. Clearly the effect of drug concentration is less pronounced at the higher of the two starting π , an effect which we attribute to the more ordered packing of lipid molecules at 25 mN/m, coupled with the slight increase in the number of condensed lipid domains.

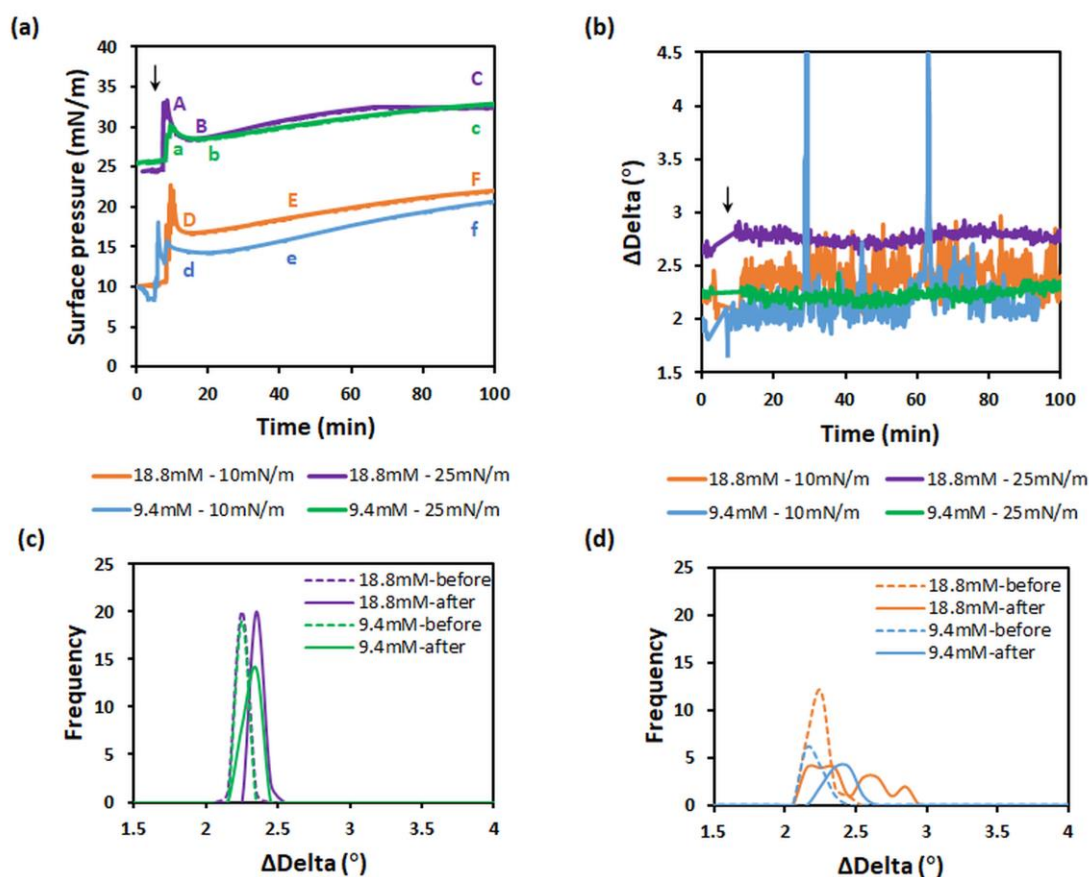


Figure 6.5 (a) Surface pressure and (b) $\Delta\delta$ of POPC/cholesterol (7:3 molar ratio) monolayers upon interaction with 9.4 mM and 18.8 mM propranolol hydrochloride at initial surfaces pressures of 10 and 25 mN/m. The arrows point indicate the time at which injection of drug solution occurred. (c) and (d) show the frequency of $\Delta\delta$ of 3.5 min before the injection of propranolol hydrochloride and 3.5 prior to the end of the 100 min interaction period, at 25 mN/m and 10 mN/m, respectively.

Table 6.3 The mean value and standard deviation of $\Delta\delta$ obtained from the POPC/cholesterol (7:3 molar ratio) membranes before* and after^ injection of propranolol hydrochloride into the aqueous sub-phase at room temperature.

Starting surface pressure (mN/m)	Propranolol hydrochloride concentration (mM)	Before		After	
		Mean (°)	Standard deviation (°)	Mean (°)	Standard deviation (°)
10	9.4	2.21	0.069	2.40	0.071
	18.8	2.23	0.066	2.43	0.22
25	9.4	2.25	0.027	2.31	0.028
	18.8	2.24	0.032	2.36	0.028

*3.5 min prior to injection of propranolol hydrochloride into the sub-phase; ^ 3.5 min prior to the end of the 100 min measurement period.

The variation in $\Delta\delta$ before and after addition of propranolol hydrochloride, measured using ellipsometry at a starting π of 10 and 25 mN/m is shown in Figure 6.5(b). The data show a difference between the two starting π , in that, at a starting π of 10 mN/m, the value of $\Delta\delta$ varies to a greater

extent than the values obtained at the higher π of 25 mN/m. At the lower π , the frequency distribution does not follow a normal distribution, exhibiting a standard deviation of 0.22° in the presence of 18.8 mM propranolol hydrochloride, compared to standard deviation of only 0.028° for the same drug concentration at the higher π of 25 mN/m. The origin of the fluctuation of $\Delta\delta$ is thought to be the heterogeneity of the membrane resulting from lipid orientation and anisotropy as domains move in and out of the region of the interface probed by the laser. It is not surprising, therefore, that the extent of drug-induced membrane heterogeneity is greater at the lower π , where the more disordered packing of lipid is thought to occur. It is noticeable that the peak of the frequency distribution of $\Delta\delta$ shifts to slightly higher values after interaction of the membrane with drug. Furthermore, the increase of $\sim 0.2^\circ$ seen at the lower starting π of 10 mN/m, is greater than the value of $\sim 0.1^\circ$ seen at 25 mN/m, suggesting that the starting π plays an important role in the interaction of propranolol hydrochloride with the POPC/cholesterol (7:3 molar ratio) membrane.

In contrast, the effect of drug concentration on its interaction with the lipid model membrane is less pronounced, especially at the higher starting π of 25 mN/m, where drug concentration appears to have little effect on the interaction, in respect to both the final π and $\Delta\delta$ value which are relatively similar at both concentrations of drug. The reason for the lack of difference is thought to be the consequence of the fact that the interaction between the drug and membrane is effectively saturated at the lower of the two drug concentrations because of the close packed nature of the lipids in the membrane coupled with the phase separation of lipid. In contrast at the lower π of 10 mN/m, the less dense packing of the lipid molecules in the model membrane allows more of the drug to interact with it resulting in slight concentration dependent changes.

The images obtained from BAM showed that, at a starting π of 25 mN/m (Figure 6.6, *a-c* and *A-C*), branched-like domains are formed in the presence of both concentrations of drug, while in contrast similar domains were observed only at the higher starting π of 10 mN/m using the higher concentration of drug of 18.8 mM (Figure 6.6, *d-f*). It is thought the reason for the system prepared at the lower drug concentration and lower π not forming these structures is the less dense packing of the lipid monolayer at the lower π . In the case of the higher drug concentration it was observed that the introduction of 18.8 mM of propranolol hydrochloride into the sub-phase resulted in the immediate formation of the worm-like domains, which are thought to result in a higher π surface pressure, explaining the greater jump in π immediately after the injection. In the case of the lower

drug concentration, the formation of lipid domains was observed to take a longer time, although at the end of the 100 min incubation period, similar domains were seen in the systems containing both drug concentrations.

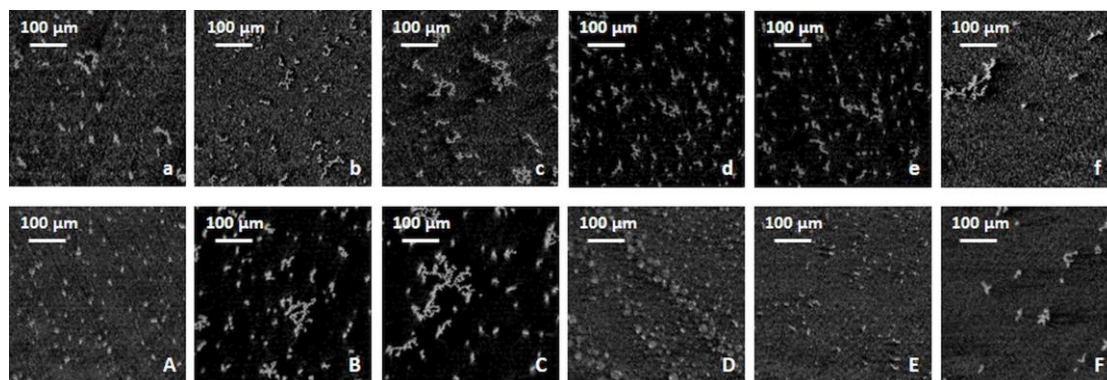


Figure 6.6 BAM images of the interaction of POPC/cholesterol (7:3 molar ratio) with 9.4 mM and 18.8 mM propranolol hydrochloride at 10 and 25 mN/m, respectively, corresponding to the letters in Figure 6.5 (a).

The NR data of POPC/cholesterol (7:3 molar ratio) model membranes with 20 mM propranolol hydrochloride at starting π of 10 and 25 mN/m are shown in Figure 6.7 along with the volume fraction profiles of each component as a function of distance across the interface, calculated from the parameters used to obtain the optimised fits to the data (Table 6.4). Also included in Table 6.4 are the equivalent data for POPC membranes published previously.^[17] It is very noticeable that the difference between the sets of parameters obtained at the two starting π for the POPC/cholesterol (7:3 molar ratio) model membranes are very comparable with the greatest variation being seen in the amount of drug and lipid present at the interface. However, compared to a monolayer of POPC/cholesterol (7:3 molar ratio), the surface excess of lipid has decreased significantly, from 3.4 to 2.0 $\mu\text{mol}\cdot\text{m}^{-2}$ at 25 mN/m and 3.0 to 1.8 $\mu\text{mol}\cdot\text{m}^{-2}$ at 10 mN/m, a result which confirms the proposed loss of lipid from the interface during its interaction with propranolol hydrochloride. Furthermore, the NR results confirm that propranolol hydrochloride molecules can penetrate and orientate themselves in the lipid membrane, because that the hydrophobic rings reside in the lipid tail region while about half of the hydrophilic side chains are located in the solvated lipid head group region. The observation from the NR studies that the increase in starting π leads to a decrease in the amount of drug penetrating the model lipid membrane is consistent results from with the π -A experiments, which suggest that the interaction between drug and membrane tends to be weaker at the higher of the two starting π , most

likely the result of the close lipid packing.

To summarise, the interaction between propranolol hydrochloride with POPC model membrane containing 30 mol% of cholesterol results in immediate lipid loss from the interface, the penetration and orientation of drug within the lipid layer and resulted in the phase separation of the membrane. The extent of the interaction of drug with the membrane largely depends on the starting structure of the lipid model membrane, and in particular the surface density of the lipid molecules, the orientation of the of lipid molecules and the heterogeneity of the membrane as well as the concentration of propranolol hydrochloride.

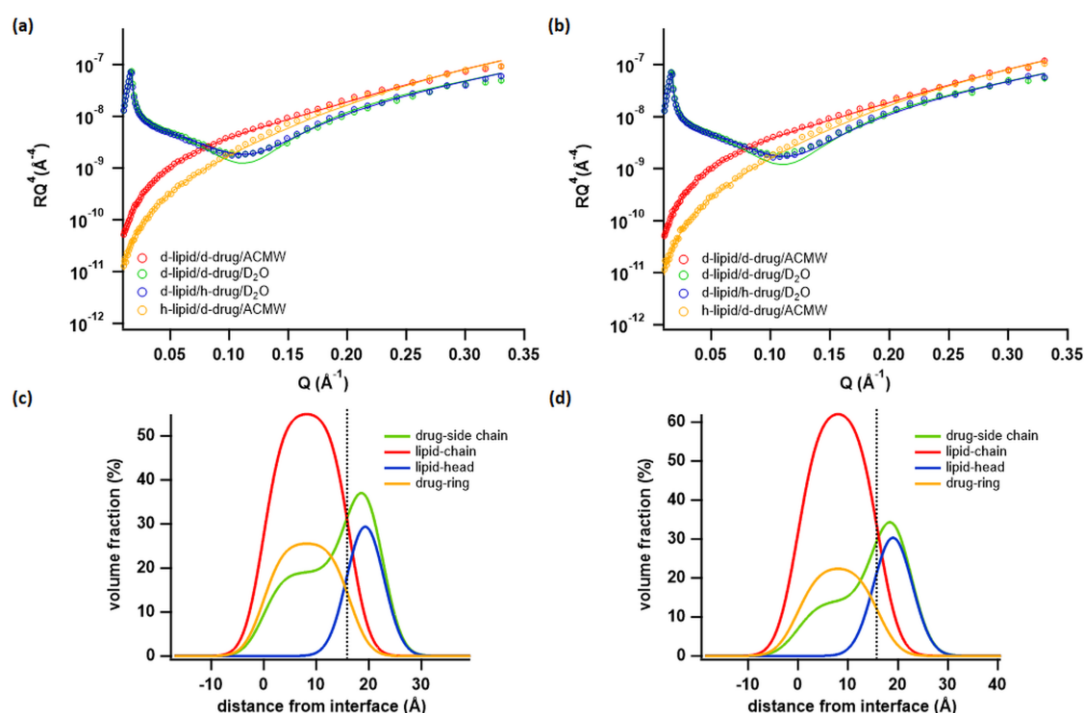


Figure 6.7 Neutron reflectivity data of POPC/cholesterol (7:3 molar ratio) model membranes with 20 mM propranolol hydrochloride at starting surface pressures of (a) 10 and (b) 25 mN/m. The volume fraction profiles of each component as a function of distance across the interface, calculated from the optimised fits to the data are shown at starting surface pressures of (c) 10 and (d) 25 mN/m. Experiments performed at ambient temperatures.

Table 6.4 Fitted structural parameters of POPC and POPC/cholesterol (7:3 molar ratio) monolayers following their interaction with propranolol hydrochloride because of the optimum model to fit a two-layer model to neutron reflectivity data recorded using 4 isotopic contrasts.

Starting surface pressure (mN/m)	Lipid chain thickness (Å)	Lipid head thickness (Å)	Solvation in head (%)	Drug naphthalene ring in layer 1 (%)	Drug hydrophilic chain in layer 1 (%)	Drug hydrophilic chain in layer 2 (%)	Surface excess of lipid ($\mu\text{mol}\cdot\text{m}^{-2}$)	Surface excess of drug ($\mu\text{mol}\cdot\text{m}^{-2}$)	Area per lipid molecule ($\text{\AA}^2$)
10	16.3 ± 0.5	6.0 ± 0.6	8 ± 3	25.7 ± 2.4	19 ± 1.7	49 ± 4.5	1.78 ± 0.06	3.80 ± 0.35	93.3 ± 3.2
25	16.0 ± 0.7	6.0 ± 0.7	1 ± 4	22.8 ± 2.0	14 ± 1.2	50.3 ± 4.5	1.99 ± 0.09	3.30 ± 0.29	83.5 ± 3.8

The upper lipid chain region is noted here as 'layer 1' and the solvated lipid head group region is denoted here as 'layer 2'.

6.5.3 The interaction of propranolol hydrochloride with POPC/cholesterol (7:3 molar ratio) model membranes in the presence of 0.154 M sodium chloride

The effect of 0.154 M sodium and chloride ions on the interaction of POPC/cholesterol (7:3 molar ratio) and propranolol hydrochloride was examined using the same experimental techniques as above. Significantly, although the π data shows a similar trend in behaviour to that seen as for the interactions in pure water, there is a significant increase in the extent of the drug-membrane interaction in the presence of sodium chloride (Figure 6.8(a)) with an increase in π in the presence of NaCl upon approaching steady state at 90 min of ~ 20 mN/m at starting π of 10 mN/m and ~ 10 mN/m at the higher starting π of 25 mN/m. One of the reasons for this enhanced effect is thought to be the state of the membrane in the presence of NaCl, namely its increased fluidity and phase separation of the membrane, which makes it easier for the propranolol hydrochloride molecules to interact with and penetrate the membrane. In addition, there is a possibility that the drug exhibits a preferential interaction with POPC over cholesterol. Assuming the condensed lipid domains are composed mainly of cholesterol, as cholesterol is well known to be a key component of lipid rafts,^[25] the POPC lipids surrounded the domains will 'occupy' more space and be in a more fluid state, thereby improving the interaction between propranolol hydrochloride and POPC lipids, which would result in the more significant effects on π observed. Moreover, the decrease in surface pressure after the injection of drug tends to be greater in the presence of NaCl, which is probably related to an increased lipid loss.

The values of $\Delta\delta$ in the presence of NaCl show increased fluctuations (as evidenced by the standard deviation) compared with those seen in the absence of NaCl (Figure 6.8(b)). Furthermore, the shift of the frequency distribution peak of $\Delta\delta$ increases from $\sim 0.2^\circ$ at 10 mN/m in pure water

to $\sim 0.8^\circ$ at the same surface pressure in 0.154 M NaCl (Table 6.5). Therefore, the presence of NaCl acts synergically with propranolol hydrochloride.

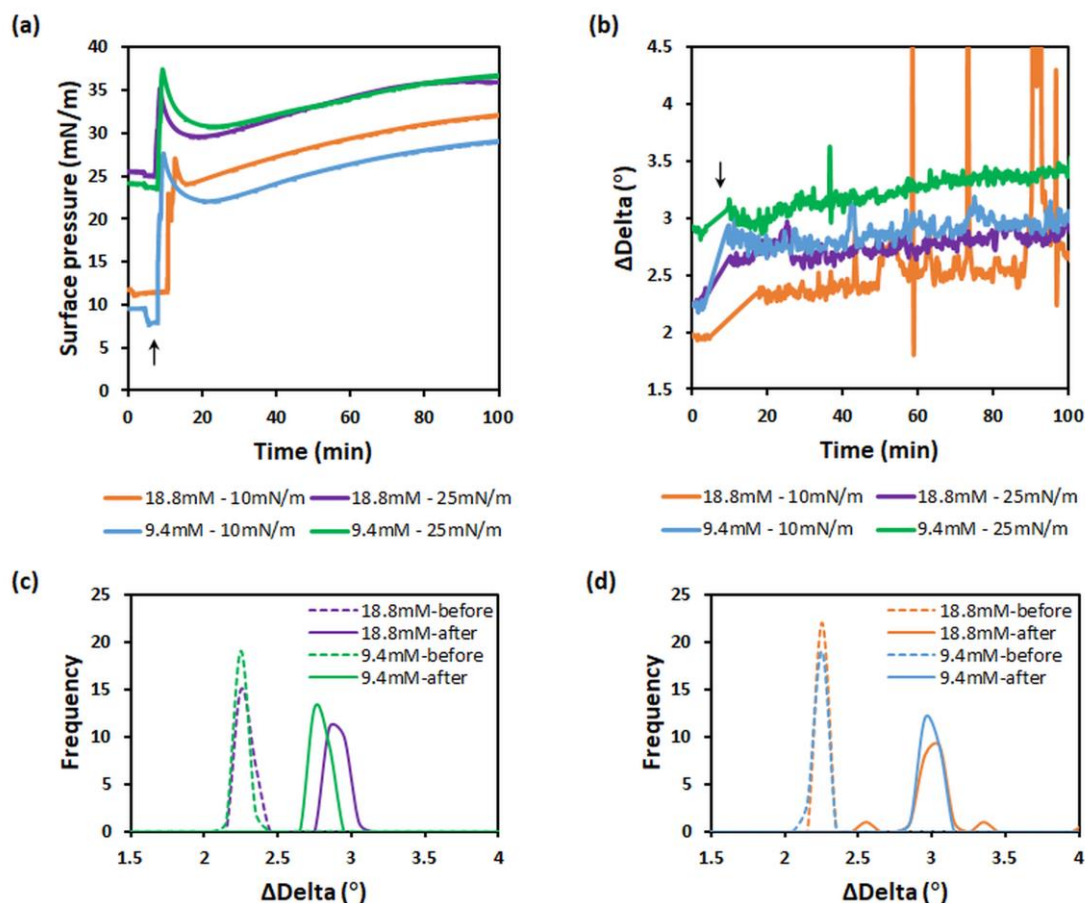


Figure 6.8 (a) Surface pressure and (b) $\Delta\Delta$ of POPC/cholesterol (7:3 molar ratio) interact with 9.4 mM and 18.8 mM propranolol hydrochloride with the presence of 0.154 M sodium chloride at 10 and 25 mN/m. The arrows show the injection of propranolol hydrochloride into the subphase. (c) and (d) shows the frequency of $\Delta\Delta$ of 3.5 min before the injection of propranolol hydrochloride and 3.5 min approaches 100 min, at 25 mN/m and 10 mN/m, respectively.

Table 6.5 The mean value and standard deviation of $\Delta\Delta$ obtained from the POPC/cholesterol (7:3 molar ratio) membranes in the presence of 0.154 M sodium chloride, before* and after^ injection of propranolol hydrochloride into the aqueous sub-phase at room temperature.

Starting surface pressure (mN/m)	Propranolol hydrochloride concentration (mM)	Before		After	
		Mean (°)	Standard deviation (°)	Mean (°)	Standard deviation (°)
10	9.4	2.24	0.029	2.99	0.054
	18.8	2.26	0.014	3.09	0.37
25	9.4	2.27	0.027	2.80	0.034
	18.8	2.28	0.044	2.89	0.056

*3.5 min prior to injection of propranolol hydrochloride into the sub-phase; ^ 3.5 min prior to the end of the 100 min measurement period.

Kinetic NR experiments at low- Q have been performed to obtain the real-time changes in the amount of drug and lipids during the interaction, as shown in Figure 6.9(a). The purpose was to correlate the changes in surface excess of the components to the strongly nonmonotonic changes in the surface pressure data (figure 6.9(a)); the injection of propranolol hydrochloride occurred at time 0–2 min. The surface excess of drug increases rapidly corresponding to the first increase in surface pressure, and then it undergoes a gradual decrease in the first 30 min after the injection. Noticeably, the surface excess of lipid had an immediate decrease as soon as the injection starts, then kept relatively stable for the rest of the time. The loss of both components from the membrane correlates to the second phase of the surface pressure data where the values decrease. The BAM images taken at this stage (Figure 6.9(b)a) showed the same effect that the amount of lipid domains observed has decreased significantly compared with the one before the injection of drug (Figure 6.1(b)f). The reduced lipid amount in the membrane confirms our speculation of lipid loss from surface pressure data, which probably involves the drug-induced solubilisation of lipids. However, the continuous decrease of drug with unchanged lipid in the following 30 min suggested a different process. One possible explanation is the uneven distribution of drug result from the injection, hence the local high drug concentration decreased to the average level. Figure 6.9(b)b that taken at this stage of the interaction suggested that the decrease of drug at interface is accompanied with the formation of small lipid domains. Additionally, due to the amphiphilic structure of propranolol hydrochloride, the drug molecules probably self-assembled to form aggregates due to the local high concentration, therefore have left the surface into the bulk. As a result of both processes, the last phase of the surface pressure changes where the values increase gradually is explained by reorganisation of the components in the membrane rather than changing surface excesses of either of the components, as their amounts in the membrane remain constant during this period. It may be noted that the steady state surface excesses of drug and lipid from this low- Q analysis approach match the values obtained from the full Q range NR data measured after the system had reaches steady state, as shown in Table 6.6.

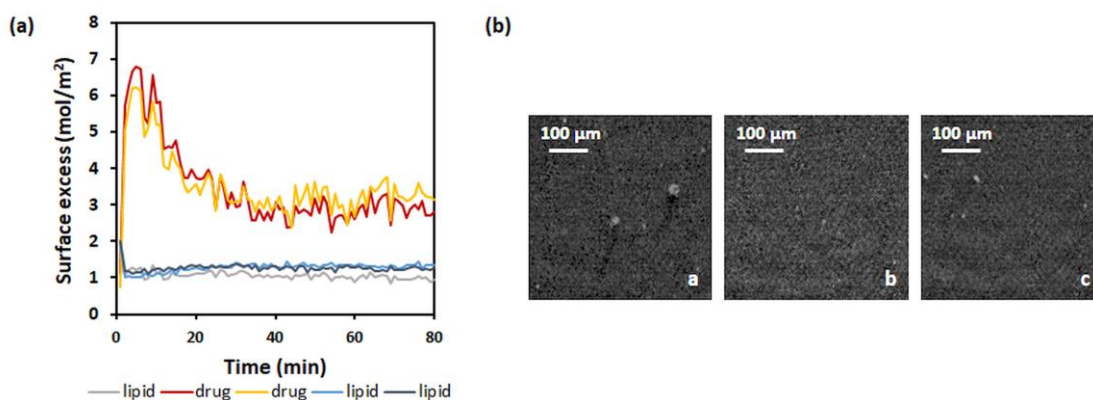


Figure 6.9 (a) Changes of surface excess of drug and lipid calculated from the low-Q reflectivity data (using different isotopic contrasts) of POPC/cholesterol (7:3 molar ratio) with 20 mM propranolol hydrochloride in the presence of 0.154 M sodium chloride. (b) BAM images of POPC/cholesterol (7:3 molar ratio) membrane: *a*. surface pressure decreases after inject drug, *b*. at the minimum surface pressure, *c*. at steady state.

The structure of the system containing 0.154 M NaCl after the interaction reached steady state was measured using full *Q* range NR. Surprisingly, the fitting of the data suggested that all drug molecules penetrated to the lipid chain region, which is very different from the results in the absence of NaCl, where most of the drug side chains reside in the solvated lipid head group region. A possibility is that the addition of Na⁺ decreased the ionization of propranolol hydrochloride, which exists as cationic molecule in pure water with a *pK_a* of 9.35.^[26] Meanwhile, the binding of Na⁺ to the phosphate group on the PC head in the presence of cholesterol makes the lipid more comparable to a neutral lipid.^[11] As a result, the electrostatic interaction between drug and PC heads will decrease, making the hydrophobic effect plays the predominant role of the drug-membrane interaction. Due to the severe lipid loss observed, the amount of lipid at interface has decreased significantly, which therefore enables more drug molecules penetrate the membrane.

To summarise, the presence of NaCl has modified the membrane lateral packing that the phase separation has increased, the condensed domains are considered mainly to be composed of cholesterol, while the surrounding POPC molecules exhibit higher fluidity. Propranolol hydrochloride is likely to show preference to interact with POPC rather than cholesterol, hence the interaction has been strengthened in the presence of NaCl, because of the loosely packed POPC molecules compared with that in water. In addition, the binding of Na⁺ to the phosphate group on PC heads and the decreased ionization of propranolol hydrochloride both contributes to the modelling result that drug molecules penetrate the lipid chain layer, most likely driven by the hydrophobic effects.

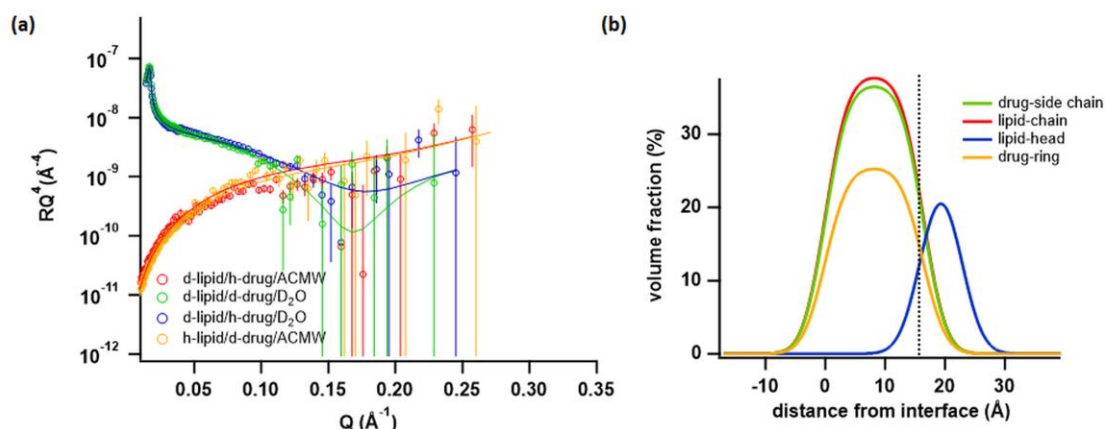


Figure 6.10 (a) Reflectivity data of POPC/cholesterol (7:3 molar ratio) with 20 mM propranolol hydrochloride at 25 mN/m in the presence of 0.154 M sodium chloride. (b) shows the volume fraction of each component at different layers calculated from the optimum fits of the data.

Table 6.6 Fitted structural parameters of POPC/cholesterol (7:3 molar ratio) monolayers in the presence of 0.154 M sodium chloride at 25 mN/m following their interaction with propranolol hydrochloride because of the optimum model to fit a two-layer model* to neutron reflectivity data recorded in 4 isotopic contrasts.

Lipid chain thickness (Å)	Lipid head thickness (Å)	Solvation in head (%)	Drug naphthalene ring in layer 1 (%)	Drug hydrophilic chain in layer 1 (%)	Drug hydrophilic chain in layer 2 (%)	Surface excess of lipid ($\mu\text{mol}\cdot\text{m}^{-2}$)	Surface excess of drug ($\mu\text{mol}\cdot\text{m}^{-2}$)	Area per lipid molecule ($\text{\AA}^2$)
16.3 ± 0.6	6.0 ± 9.5	70 ± 18	25.4 ± 2.6	36.7 ± 3.8	0 ± 4.9	1.22 ± 0.05	3.77 ± 0.51	136.2 ± 5.5

6.5.4 POPC/cholesterol (5:5 molar ratio) with propranolol hydrochloride

The content of cholesterol in human cell membranes varies in different organs. For example, the amount of cholesterol in brain cells is higher than that in stomach.^[27] The effects of the increased content of cholesterol from molar ratio of 30 to 46 mol% have been studied under the same conditions as above. Figure 6.11(a) shows the surface pressure data of the interaction of propranolol hydrochloride with the 5:5 POPC/cholesterol molar ratio. It can be noted that the total increase of surface pressure is very similar to that with 30 mol% cholesterol; however, the increase of surface pressure is more linearly correlated with 46 mol% cholesterol, indicating that the interaction takes a longer time to reach steady state. This difference is attributed to the higher extent of phase separation of the membrane with more cholesterol, or in other words, the significantly increased number, and sizes of the lipid domains.

The $\Delta\pi$ values at a starting π of both 25 mN/m and 10 mN/m became very fluctuated after the

interaction, with the corresponding standard deviation increased significantly from $\sim 0.03^\circ$ with 30 mol% cholesterol to $\sim 0.2^\circ$ with 46 mol% cholesterol at 25 mN/m. Additionally, the mean values of $\Delta\delta$ also increased from $\sim 2.2^\circ$ (before) $\sim 2.3^\circ$ (after) with 30 mol% cholesterol to $\sim 2.6^\circ$ (before) and $\sim 2.9^\circ$ (after) with 46% mol cholesterol. These effects suggested that the POPC/cholesterol (5:5 molar ratio) membrane is much more heterogeneous, *i.e.* higher extent of phase separation to form rigid lipid domains.

The BAM images of the drug-membrane interaction with the 5:5 POPC/cholesterol molar ratio membranes are shown in Figure 6.12. The BAM results exhibit similar trends of the interaction with the effects of drug concentrations and starting pressure to that of 30 mol% cholesterol systems. The high drug concentration promotes the formation of irregular shaped domains as soon as the drugs were injected. The higher starting surface pressure contributes to the formation of a larger population of the reticular domains. An interesting observation is the huge reticular shaped domain for 18.8 mM drug at the starting surface pressure of 10 mN/m, while there is no evidence of the existence of similar domains for the systems with less cholesterol (30 mol%). Therefore, it can be concluded that the increase of the content of cholesterol greatly promotes the phase separation and the formation of large lipid domains especially when interacting with propranolol hydrochloride.

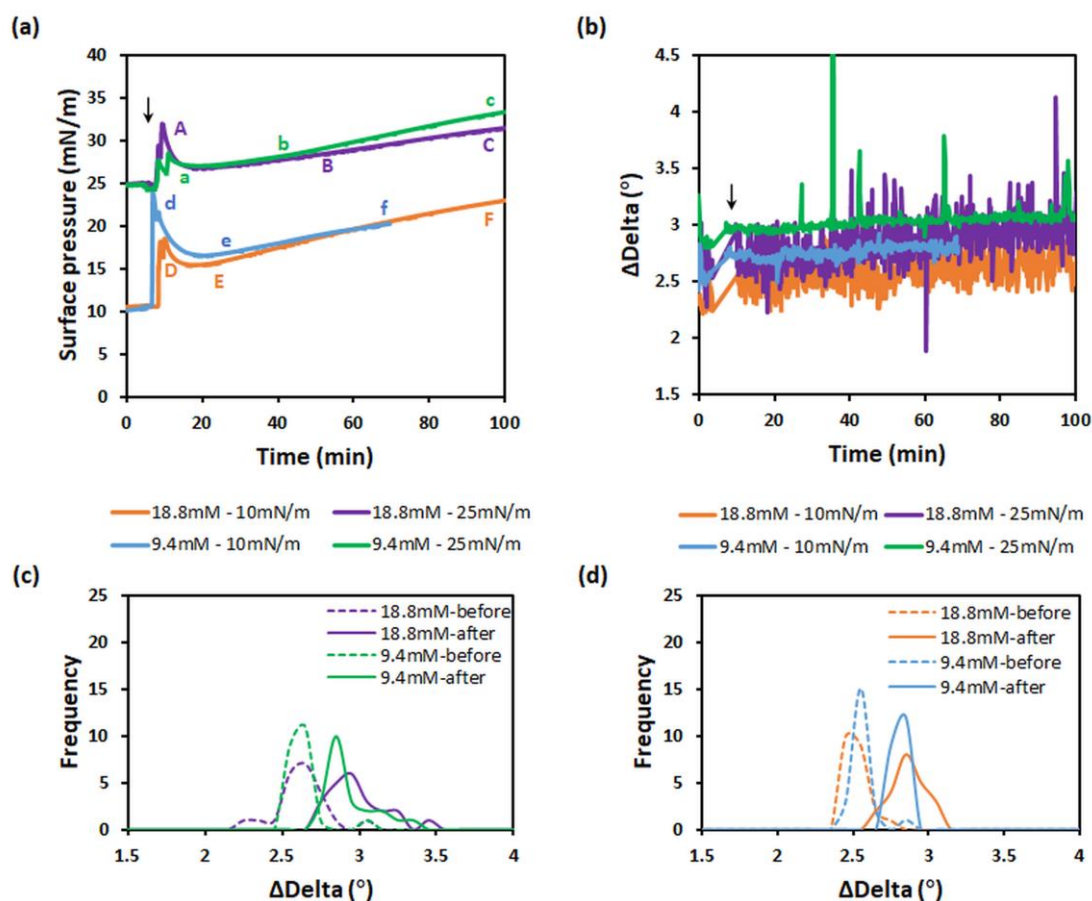


Figure 6.11 (a) Surface pressure and (b) $\Delta\Delta$ of POPC/cholesterol (5:5 molar ratio) interact with 9.4 mM and 18.8 mM propranolol hydrochloride at 10 and 25 mN/m. The arrows show the injection of drug. (c) and (d) shows the frequency of $\Delta\Delta$ of 4 min before the injection of propranolol hydrochloride and 3.5 min approaches 100 min (last 3.5 mins for 9.4 mM drug at 10 mN/m), at 25 mN/m and 10 mN/m, respectively.

Table 6.7 The mean value and standard deviation of $\Delta\Delta$ obtained from the POPC/cholesterol (7:3 molar ratio) membranes in the presence of 0.154 M sodium chloride, before* and after^ injection of propranolol hydrochloride into the aqueous sub-phase at room temperature.

Starting surface pressure (mN/m)	Propranolol hydrochloride concentration (mM)	Before		After	
		Mean (°)	Standard deviation (°)	Mean (°)	Standard deviation (°)
10	9.4	2.56	0.084	2.81	0.038
	18.8	2.52	0.073	2.87	0.12
25	9.4	2.62	0.10	2.93	0.16
	18.8	2.63	0.16	2.98	0.18

*3.5 min prior to injection of propranolol hydrochloride into the sub-phase; ^ 3.5 min prior to the end of the 100 min measurement period.

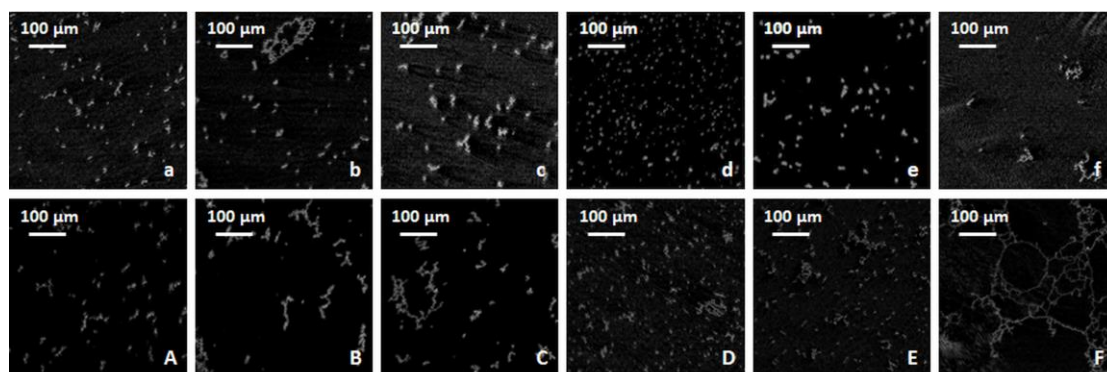


Figure 6.12 BAM images of the interaction of POPC/cholesterol (5:5) with 9.4 mM and 18.8 mM propranolol hydrochloride at 10 and 25 mN/m, respectively, corresponding to the letters in Figure 6.11 (a).

The neutron reflectivity data of the POPC/cholesterol 5:5 molar ratio membranes are shown in Figure 6.13, while the parameters obtained from the optimum fits are listed in Table 6.8. Compared with the 7:3 molar ratio systems, the amount of drug penetrated in the membrane has decreased by approximately 50%, due to the more rigid packing of lipid molecules with higher content of cholesterol. The increased thickness for both the lipid chain and lipid head group layer confirmed the estimation that the lipid molecules are arranged in a highly ordered manner. However, unlike the 7:3 molar ratio membrane systems where the orientation of propranolol hydrochloride in membrane remains unchanged with the increase of the starting surface pressure, the drug side chains in the 5:5 molar ratio membrane systems reorient to locate in the lipid head group layer from 10 mN/m to 25 mN/m. This effect is a result of the highly ordered packing of lipid chains that blocks the penetration of drugs. In conclusion, the increase of cholesterol content in the membrane has weakened the interaction between propranolol hydrochloride and lipids, most likely the POPC, by increasing membrane phase separation to form highly ordered lipid domains, which therefore suppresses the penetration of drug molecules into the membrane.

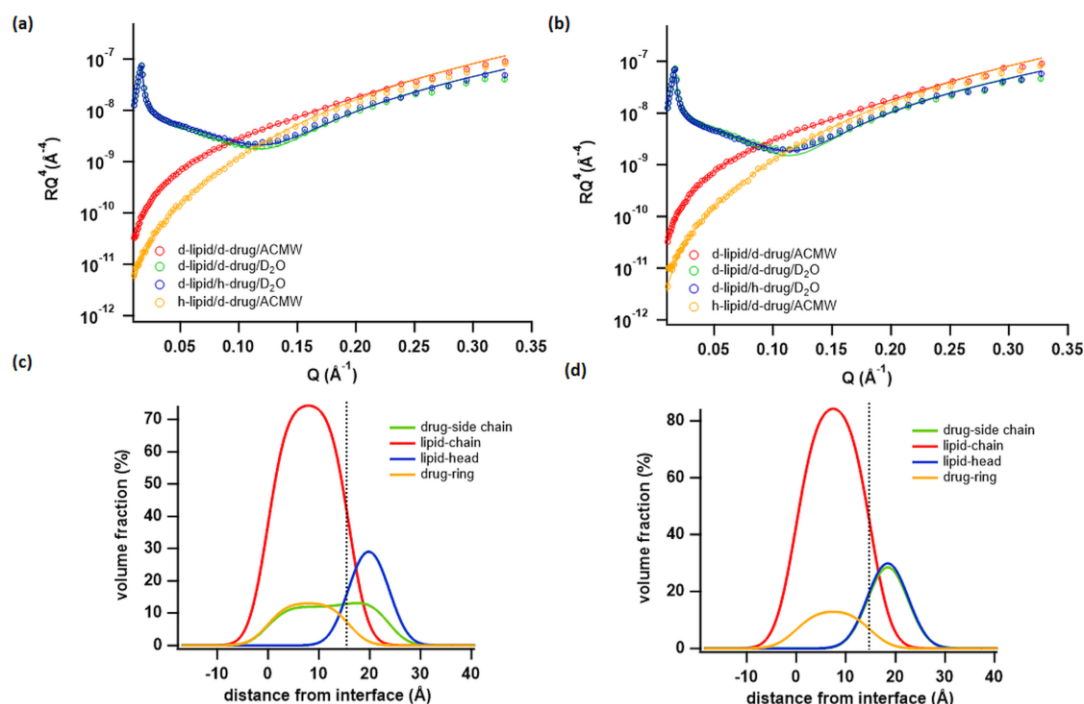


Figure 6.13 Reflectivity data of POPC/cholesterol (5:5 molar ratio) with 20 mM propranolol hydrochloride at 10 and 25 mN/m. (c) and (d) show the volume fraction of each component at different layers calculated from the optimum fits of the data.

Table 6.8 Fitted structural parameters of POPC/cholesterol (5:5 molar ratio) monolayers following their interaction with propranolol hydrochloride because of the optimum model to fit a two-layer model* to neutron reflectivity data recorded in 4 isotopic contrasts.

Starting surface pressure (mN/m)	Lipid chain thickness (Å)	Lipid head thickness (Å)	Solvation in head (%)	Drug naphthalene ring in layer 1 (%)	Drug hydrophilic chain in layer 1 (%)	Drug hydrophilic chain in layer 2 (%)	Surface excess of lipid ($\mu\text{mol}\cdot\text{m}^{-2}$)	Surface excess of drug ($\mu\text{mol}\cdot\text{m}^{-2}$)	Area per lipid molecule ($\text{\AA}^2$)
10	15.9 ± 0.4	7.8 ± 1.4	50 ± 5	13.1 ± 1.7	12 ± 1.6	14 ± 1.8	2.93 ± 0.07	1.89 ± 0.24	56.6 ± 1.3
25	14.9 ± 0.4	7.1 ± 1.1	17 ± 7	13.3 ± 1.4	0 ± 2.7	40.5 ± 4.3	3.13 ± 0.09	1.80 ± 0.19	53.0 ± 1.5

* The upper lipid chain region is noted here as 'layer 1' and the solvated lipid head group region is denoted here as 'layer 2'.

6.6 Conclusions

Cholesterol containing POPC membranes have very different properties to pure POPC membranes in that cholesterol promotes the rearrangement of lipid acyl chains to form highly ordered lipid domains. The addition of a physiological concentration of sodium chloride (0.154 M) has changed the structure of the membrane slightly, the membrane exhibits higher fluidity and heterogeneity, indicating a slightly looser packing of lipids. On the contrary, an increase of cholesterol content in membrane to 46 mol% has greatly decreased the membrane fluidity to form more rigid membrane structure.

Our previous study on drug-membrane interactions involving the beta blocker propranolol hydrochloride concluded that the starting state of the membrane plays an important role in the interaction.^[17] Compared to the interaction with pure POPC membranes, the addition of 30 mol% of cholesterol induces increased drug penetration but, less pronounced changes on surface pressure. This observation can be attributed to the more heterogeneous morphology of the membrane containing 30 mol% cholesterol. NR data suggests that the amount of drug that penetrates the lipid head group greatly increases, with volume fractions rising from 19% in pure POPC membranes to 49% in POPC/cholesterol (7:3 molar ratio) membranes at 10 mN/m. It is due to the fact that cholesterol has only a hydroxyl group in solvated lipid head group region,^[20] the density in lipid head group layer is less than that of the pure POPC membrane at the same surface pressure. Therefore, this difference has enabled the possibility of the increased amount of drug penetration in the head group layer. Furthermore, it is likely that propranolol hydrochloride prefers to interact with POPC over cholesterol, which is considered to be a reason for the increased phase separation occurring in the membrane subsequent to its interaction with propranolol hydrochloride. In the mixed POPC/cholesterol membrane, cholesterol is assumed to be the major component of the highly ordered domains,^[28] leaving the surrounding disordered fluid phases to be dominated by the remaining POPC molecules. As a result, the extent of the interaction between drug and POPC is high. In addition, the effect of drug concentration on the nature of the interaction is less pronounced in the cholesterol containing membrane, an observation which is attributed to the more closely packed lipid chains that limit the amount of drug in the lipid chain region.

Using a physiological strength of sodium chloride, significant effects on the drug-membrane interaction have been observed, namely: 1) more pronounced effects on surface pressure and $\Delta\pi$; 2) enhanced drug penetration and severe lipid loss; 3) relocation of drug side chains penetrated in membrane from lipid head group region to lipid chain region. The non-monotonic changes induced by the higher electrolyte concentration can be regarded as a combination of several different effects. The electrostatic interactions of Na^+ with a phosphate group and the Cl^- with a choline group in the PC head group regions, are likely to influence the membrane charge and the solvation of the lipid head group regions,^[29, 30] hence contributing to the increased heterogeneity of the membrane. Propranolol hydrochloride is 90% ionized in aqueous solution at physiological pH.^[31] The presence of electrolytes in solution acts to decrease the ionization of propranolol hydrochloride, making the drug more hydrophobic. Therefore, the strengthened hydrophobic effects of the drug will lead to a

rearrangement of drug in the lipid chain layer. Additionally, it has been elucidated in the previous work that the presence of NaCl significantly promotes the self-association of propranolol hydrochloride in aqueous solution,^[32] as pre-micellization was observed at 30 mM (the lowest concentration considered in the study). Therefore, it can be deduced that some drug molecules probably form mixed micelles with the lipids, which results in the loss of lipid at the interface as a result of the interaction between drug and membrane. Such observations have been made in related studies,^[33, 34] although further work is required to elucidate the mechanism of lipid extraction.

For the POPC/cholesterol membranes containing a higher level of cholesterol, 46 mol%, the interaction of propranolol hydrochloride is weaker, with less drug penetrating the membrane and taking a longer time to reach a steady state. The higher amount of cholesterol in the membrane alters its structure resulting in a highly ordered, rigid state with reduced fluidity, and a suppressed penetration of drug. Moreover, unlike the interaction with the membrane containing the lower amount of cholesterol (30 mol%) where the starting surface pressure makes little difference to the orientation of drug, the increase of starting surface pressure involves a reorientation process of the drugs when interacting with the membrane containing 46 mol% of cholesterol, *i.e.* nearly no drug side chains penetrated the highly ordered lipid chain region. Therefore, it is concluded that 30 mol% cholesterol in POPC membrane improves the membrane penetration of propranolol hydrochloride, while a further increase of cholesterol to 46 mol% antagonizes the drug-membrane interaction, a result of the reduced fluid state of the membrane.

This study has provided insight into the mechanism of interaction of propranolol hydrochloride with a simple model of the human cell membrane, under physiological conditions of the cholesterol content and ionic strength. The study demonstrated that the interaction is a complicated process that is influenced by many different factors, including the initial state of the membrane, bulk ionic strength, as well as the cholesterol content. However, the process of drug-membrane interaction involves even more lipid species in the body, which may lead to even more significant effects resulting from the drug-membrane interactions. Therefore, it is of importance to further understand the mechanism of the interaction of propranolol hydrochloride with model membrane to improve the bioavailability of the drug in clinical use.

Statements:

NR experiments at FIGARO (ILL, France) were performed by Samuel Winnal, and the experiments at INTER (ISIS, UK) were performed by Dr. Maximilian Skoda. All the data analysis and other experiments were done by Yixuan Yan. The deuterated drug was synthesised by Jasmine Hind and Dr. James Tellam. The project was conceived and supervised by Professor Jayne Lawrence and Dr. Richard Campbell.

6.7 Acknowledgement

Neutron experiments at the ISIS Neutron and Muon Source were supported by beamtime allocation from STFC, and the NR data from INTER are available at DOIs: 10.5286/ISIS.E.RB2010646 and 10.5286/ISIS.E.RB2010570. Data from FIGARO, ILL are available at DOI: doi.ill.fr/10.5291/ILL-DATA.9-13-930.

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Supplementary information

SI-1 Calibration of POPC/cholesterol (7:3 molar ratio) on 20 mM drug solution for NR experiments.

As the neutron experiments performed on INTER at the ISIS laboratories in Oxford were performed using an adsorption (and not a Langmuir) trough by spreading lipid on the surface of an aqueous drug solution, pre-calibration of the amount of lipid required to reach 10 mN/m and 25 mN/m surface pressure was required using the same trough in advance of the neutron experiments. The amount of a 0.5 mg/mL chloroform solution of *h*-POPC/cholesterol (7:3 molar ratio) required to reach a surface pressure of 10 mN/m was established to be 42 μL , while an extra 6 μL (total 48 μL) was needed for the surface pressure to reach 25 mN/m. For the corresponding *d*₃₁-POPC/cholesterol (7:3 molar ratio) chloroform solution, a volume of 48 μL was required to reach a surface pressure of 10 mN/m, while an extra 8 μL (total 56 μL) was necessary for a surface pressure of 25 mN/m. The corresponding area per lipid molecule at surface pressures of 10 and 25 mN/m was 61.0 $\text{\AA}^2$ and 53.4 $\text{\AA}^2$, respectively – a value in good agreement with the measured lipid isotherms (Figure 6.1(a)).

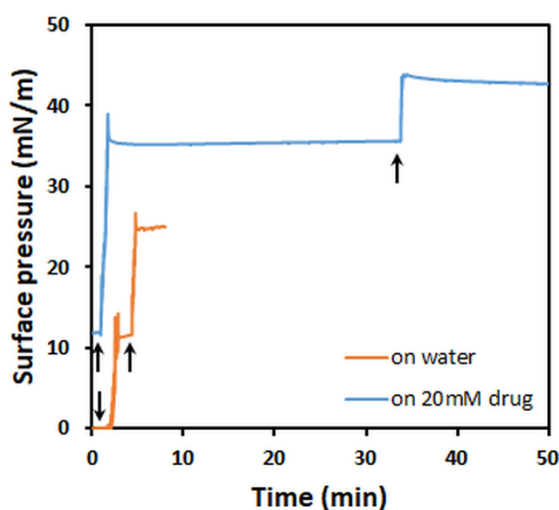


Figure SI - 6.1 Surface pressure of lipid stock solution of POPC/cholesterol (7:3 molar ratio) spread dropwise on pure water and on 20 mM propranolol hydrochloride solution. The arrows indicate the start of the spreading process.

SI-2 Interaction of POPC/cholesterol (7:3 molar ratio) with propranolol hydrochloride in the presence of 0.5 M sodium chloride.

To further understand the effect of added electrolyte on the drug-membrane interaction, surface pressure and ellipsometry measurements were performed by the addition of 0.5 M sodium chloride to the aqueous sub-phase containing 18.8 mM propranolol hydrochloride. Although the effect of the addition of 0.5 M NaCl was similar to that seen with 0.154 M NaCl, its effect was more exaggerated. Figure SI-6.2(a) shows the surface pressure-time data for both surface pressures measured at the higher salt concentration, both data sets showed a significant variation in the shape of the surface pressure time curves subsequent to the decrease in surface pressure seen after the injection, suggesting the period of immediate lipid loss had been extended to 10 – 15 minutes. One of the reasons for this observation was thought to be the increase of sodium ions in the bulk which results in an increased association of the lipid molecules via electrostatic interactions of the phosphate groups. After the initial decrease in pressure lasting $\sim 10 - 15$ minutes, a continuous increase in surface pressure was observed which approached equilibrium quicker than was seen when the sub-phase was pure water or contained 0.154 M NaCl. On the basis of these observations, it was considered that the increase in the ionic strength of sub-phase resulted in a strengthened drug-membrane interaction.

The changes of $\Delta\delta$ at 25 mN/m before and after the interaction of the membrane with propranolol hydrochloride (Table SI-6.1) are very similar to those seen in the presence of in 0.154 M NaCl. In contrast the changes observed at 10 mN/m show a greatly increased mean value of $\Delta\delta$, as a result of which it was deduced that the effect of increasing the ionic strength of the bulk was more significant at the lower surface pressure of 10 mN/m due to the looser packing of the lipid molecules.

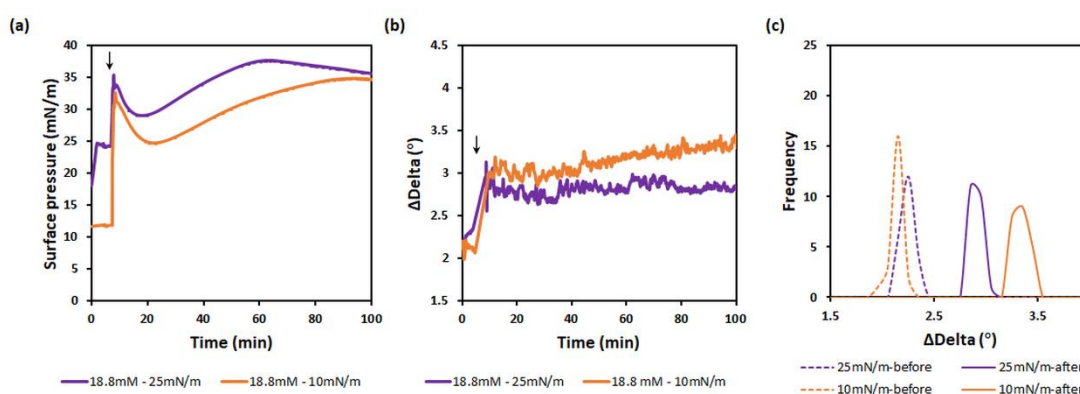


Figure SI - 6.2 (a) Surface pressure and (b) $\Delta\delta$ changes due to the interaction of POPC/cholesterol (7:3 molar ratio) with 18.8 mM propranolol hydrochloride at 10 and 25 mN/m in the presence of 0.5 M NaCl. The arrows indicated the

time of injection of the drug solution. (c) frequency distribution of $\Delta\delta$ determined over 3.5 minutes prior to injection and the 3.5 minutes prior to the 90 min after interaction.

Table SI - 6.1 The mean value and standard deviation of $\Delta\delta$ obtained from the POPC/cholesterol (7:3 molar ratio) membranes in the presence of 0.5 M NaCl, before* and after^ injection of propranolol hydrochloride into the aqueous sub-phase at room temperature.

Starting surface pressure (mN/m)	Propranolol hydrochloride concentration (mM)	Before		After	
		Mean (°)	Standard deviation (°)	Mean (°)	Standard deviation (°)
10	18.8	2.12	0.055	3.34	0.059
25	18.8	2.25	0.066	2.81	0.021

*3.5 min prior to injection of propranolol hydrochloride into the sub-phase; ^ 3.5 min prior to the end of the 100 min measurement period.

7 Summary and Conclusion

Amphiphilic behaviour is a relatively common feature of many commercial drugs, with a wide range of drug molecules exhibiting an amphiphilic nature, ranging from small, low molecular weight molecules such as the beta blocker propranolol hydrochloride,^[1, 2] the calcium channel blocker verapamil,^[3] the local anesthetic lidocaine,^[4] non-steroidal anti-inflammatory ibuprofen^[5] through to larger more complex structures such as cannabinomimetics^[6] and amphotericin B.^[7] The amphiphilic nature of a drug is thought to play a major role in its therapeutic effect(s), which can be exploited in the delivery of the drug and in its clinical applications. It is acknowledged that the pharmacological activity of a drug is normally assigned to the monomer, whereas the drug aggregates may not only alter the apparent solubility but also the toxicity and activity, as well as the interaction of drug with other physiological ingredients *in vitro* and *in vivo*.^[8]

The current study focuses on a typical amphiphilic drug, propranolol hydrochloride, and uses several different techniques, including experimental and computational methodologies, covering different length and time scales to resolve the self-association of the drug in the bulk solution, as well as the interaction of the drug with model lipid membranes at the air-water interface. For example, contrast variation SANS and NR techniques covered the molecular length scale, focussing on the bulk and surface behaviour of the drug, respectively. BAM visualized the lateral properties of the model membranes before and after interaction with the drug on the micrometre length scale. Contrast variation SANS provided the time averaged behaviour, while MD simulations obtained nanosecond time shots of the of the drug aggregates in solution. Ellipsometry, in combination with measurements made using a Langmuir trough, recorded the changes over the whole process of the drug-membrane interaction, while NR yielded structural information at the steady state.

7.1 Self-association of propranolol hydrochloride

The amphiphilic structure enabled propranolol hydrochloride to self-associate in aqueous solution, driven by the hydrophobic nature of the naphthalene rings present in its structure. There have been some studies reporting the self-assembly of propranolol hydrochloride in aqueous solution, and detailing its critical micelle concentration.^[9, 10] However, there have been no studies detailing the self-assembly of propranolol hydrochloride using contrast variation SANS in combination with ¹H

NMR and atomistic MD simulation. In the present study, pre-micellar aggregates consisting of only a few molecules of propranolol hydrochloride have been resolved in detail at concentrations much lower than the CMC determined from both contrast variation SANS and ^1H NMR as well as surface tension measurements. Atomistic MD simulations suggested the presence of very polydisperse sizes drug aggregates that appeared to self-assemble in a stepwise manner. The concentration dependent nature of aggregates' growth observed with the MD simulations was also seen with the contrast variation SANS and ^1H NMR.

As regard the shape of the propranolol hydrochloride aggregates in aqueous solution, a combination of both experimental and computational techniques has helped to resolve the morphology of the aggregates. The partial ring-deuterated propranolol hydrochloride used in the SANS experiments in combination with contrast variation offered the opportunity to model the SANS data by separating the propranolol hydrochloride molecules into a (hydrophobic)-ring and a (hydrophilic)-side chain, which suggested a core-shell aggregate morphology comprising of a dry core composed of naphthalene rings, surrounded by a solvated shell of drug side chains. The overall geometry of the aggregates obtained from SANS were well-modelled as prolate ellipsoid, while the geometry of the aggregates obtained from atomistic MD studies were much more varied. However, reassuringly the equivalent dimensions of the aggregates obtained from the MD simulations are consistent with those obtained from the SANS analysis, analysed constrainedly using an equivalent ellipsoid model.^[11] Both the MD simulations and the ^1H NMR studies indicate that the interaction of the naphthalene rings occurs via CH- π interactions rather than the originally anticipated π - π stacking.

The effect on the self-assembly of propranolol hydrochloride under a series of physiological relevant conditions, namely temperature, the presence of a physiological concentration of salt, as well as the presence of individual optical isomers, was studied using ^1H SANS. The present study showed an increase in experimental temperature was antagonistic to the process of self-association in that the average size of the aggregates decreases at higher temperature. This is a result of the increased Brownian motion observed at higher temperatures, which leads to a breaking of the structured water molecules surrounding the hydrophobic, naphthalene groups, resulting in an increase in the free energy required for self-association. Similar effects of temperature have been recorded for ionic surfactants.^[9]

Furthermore, it was demonstrated that the addition of 0.154 M sodium chloride significantly increases the size and aggregation numbers recorded for the aggregates, most likely a consequence of the decreased ionization of propranolol hydrochloride seen in the presence of salt, resulting in a reduced electrostatic repulsion between adjacent side-chains in the aggregates. Furthermore, SANS measurements at drug concentrations above 50 mM, in the presence of 0.154 M NaCl, indicated the presence of wide angle scattering at high Q values which was fitted assuming a distance of 4 – 6 Å, potential indicative of interactions between aromatic rings. It was concluded that the intermolecular CH- π stacking indicated from the ^1H NMR and MD simulations has been altered with the presence of salt.

Due to the fact that propranolol hydrochloride has a chiral carbon in its structure, the effect of optical isomers on the drugs aggregation behaviour was studied using SANS. It has been widely reported that although the clinically used form of propranolol hydrochloride is the racemate, the R- and S-isomers exhibit different therapeutic effects.^[12] The result of these investigations suggested that the S-isomer tended to form very slightly larger aggregates than the R-isomer as well as the racemate, which is probably related to differential packing in the crystals.^[12, 13]

7.2 Interaction of propranolol hydrochloride with model membrane

The interaction of amphiphilic drugs, such as propranolol hydrochloride, with cell membranes play an important role in the drug's therapeutic behaviour. In the present work, the drug-membrane interaction of propranolol hydrochloride was studied using a lipid monolayer prepared at the air/water interface as a model of a single leaflet of a fluid cell bilayer membrane. The application of several reflectometry techniques, namely ellipsometry, Brewster angle microscopy and neutron reflectometry, enabled the resolution of the interaction of the drug with the cell membrane in detail. From these studies, it is suggested that the interaction between propranolol hydrochloride and POPC in the form of a monolayer (with and without the presence of POPG) is a complex process. Specifically, the presence of propranolol hydrochloride results in the immediate loss of lipid from the monolayer, followed by a modification of monolayer heterogeneity and the penetration of drug into the lipid monolayer, resulting in the presence of more drug than lipid at equilibrium. This part of the study focused on varying several conditions, namely starting surface pressure, drug concentration

and the presence of negatively charged lipid, to probe their effect on the interaction of drug and model cell membrane.

The interaction of drug with the model membrane depends upon the initial physical state of the membrane, *i.e.* the higher packing of the membrane at the higher starting surface pressure led to less pronounced effects on surface pressure upon approaching steady state. Also, the ordered lipid domains formed in the presence of 30 mol% POPG results in similar effects on the membrane as those seen in its absence, albeit that the changes in surface pressure are of a smaller extent. Significantly, the NR results provided information on the location of drug within the membrane. Specifically, the naphthalene rings and some of the drug's side chains penetrate lipid chain region of the monolayer, while the rest of the drug's side chains reside in the lipid head group region. The NR studies were best modelled in the present study using a partition model which separates the drug-ring and drug-side chain to determine location. Such a separation of the drug into ring and side-chain regions was only possible because of the availability of partially deuterated drug. The partition model was important in helping to understand the changes in the orientation of drug with a variation in starting surface pressure, in that the orientation of drug molecules in the POPC membrane probably varied from a side-chain "laying" to "standing" conformation since the amount of the drug's side-chain in the lipid head and lipid chain regions has changed significantly.

The interaction of different concentrations of propranolol hydrochloride with the model cell membrane was studied. And the result suggested a concentration dependent manner of the drug's interaction with the POPC monolayer. The higher concentration of drug led to a greater extent of penetration, and the more significant the variation in membrane lateral heterogeneity. However, the effect of drug concentration (in the range of 9.8 mM to 18.4 mM) on the POPC/POPG (7:3 molar ratio) membrane was not as significant as that seen with the POPC membrane. Originally, it had been anticipated that the negatively charged POPG would strengthen the interaction with the positively charged drug via electrostatic attractions. Surprisingly, the interaction of propranolol hydrochloride with the POPC/POPG (7:3 molar ratio) membrane exhibited less lipid loss but a very similar amount of drug penetration as seen with the POPC membrane, highlighting the complexity of the system under study. The presence of POPG in the model membrane makes a difference to the membrane's lateral properties by resulting in a phase separation forming many evenly distributed condensed phase lipid domains, contributing to the less pronounced changes in surface pressure observed at

steady state in the POPG-containing membrane. It can therefore be concluded that the drug-membrane interaction involves multiple parallel processes.

The immediate loss of lipid observed in the surface pressure and NR measurements may be attributed to the drug-induced solubilisation of lipid. Indeed, a similar effect has been seen with the amphiphilic drug, amitriptyline where is solubilised part of a supported lipid bilayer.^[14] Until recently few/if any studies have recorded loss of lipid upon addition of drugs and similar molecules. However, recently studies exploiting neutron reflection have reported lipid loss effects, for example after addition of peptide to a DPPG monolayer.^[15] It has been discussed above (chapter 3) that propranolol hydrochloride starts to self-associate at concentrations much below its CMC observed from surface tension, SANS, ¹H NMR and atomistic MD simulations. It should be noted that for a mixed surfactant system, the CMC of the mixture is normally below the CMC of either of the single component.^[16] Therefore, it is considered that the presence of lipid probably led to the formation of drug-lipid mixed micelles, resulting in the solubilisation of lipid from the monolayer.

As regard to the interaction of propranolol hydrochloride with POPC/cholesterol membranes containing 30 mol% cholesterol, similar effects were observed yet with enhanced drug penetration. The reason is attributed to the increased phase separation of the membrane due to the ordering effects of cholesterol on the lipid acyl chains.^[17] As cholesterol is assumed to have a only hydroxyl group in the solvated lipid head group layer, the density of this layer will be lower when there is more cholesterol in the membrane, making it easier for drugs to interact, which explains the increased amount of penetrated drug. This point can also explain the resulting change in drug orientation in POPC/cholesterol membrane containing 30 mol% cholesterol from that in POPC membrane, as most drug chains stay in the lipid head group layer when there is cholesterol present in the membrane.

Under a physiological concentration of sodium chloride (0.154 M), the interaction of propranolol hydrochloride with POPC/cholesterol (7:3 molar ratio) exhibits stronger effects on both the loss of lipid from the membrane and the amount of drug penetration. In addition, the location of the drug molecules within the membrane moved to the lipid chain region. This difference is considered to be the result of a combination of the electrostatic interaction between Na⁺ and the phosphate group of the lipid head groups and the reduced ionization of the drug that increased its hydrophobicity. Moreover, the presence of sodium chloride ions may promote the drug-induced solubilisation of

lipids, since propranolol hydrochloride has been shown to exhibit stronger self-assembly with the addition of sodium chloride.

On the contrary, the interaction of drug with POPC/cholesterol membrane of a higher level of cholesterol (46 mol%) has been suppressed, which is attributed to the highly condensed structure of the membrane formed with 46 mol% cholesterol, *i.e.* a higher area coverage of condensed cholesterol or cholesterol-rich domains. In summary, 30 mol% cholesterol in POPC/cholesterol membrane promotes the penetration of propranolol hydrochloride, while further increase of the cholesterol content in membrane (46 mol%) prohibits the penetration of drug in membrane, which may relate to the application in cholesterol-rich cell membranes caused by diseases.

7.3 Prospects and future works

The current study focuses on the β -blocker propranolol hydrochloride, however as discussed in the introduction section, the structure of most β -blockers has a common base structure that make the drug ideal for the study of structure-aggregation relationships. For example, the β -blocker acebutolol hydrochloride has a smaller hydrophobic group and much higher aqueous solubility compared with propranolol hydrochloride. As a consequence of its smaller hydrophobic region, it is expected that acebutolol hydrochloride will exhibit weaker capacity for self-association. Indeed, it is reported to exhibit a CMC of 170 mM.^[2] Therefore, it is considered as the next interested drug to study to gain an understanding of the structure-aggregation relationship of the β -blockers.

Additionally, as discussed above, the SANS data of the self-assembly of propranolol hydrochloride in the presence of salt showed a wide-angle scattering peak at high Q region, which is just on the upper Q limit of the SANS instrument used in the present study. It would be interesting to establish if propranolol hydrochloride in the absence of salt showed a wide-angle scattering peak at higher Q . Further studies using different techniques could therefore be considered, for example wide angle X-ray scattering or liquid neutron diffraction in order to resolve further details of the intermolecular interactions occurring within the aggregates.

For the interaction of propranolol hydrochloride with the mixed POPC/POPG (7:3 molar ratio) membrane, the addition of PG to slightly increase the complexity of the membrane in that its

interactions with drug appear to be less pronounced, despite a similar extent of drug penetration into the monolayer being recorded. The introduction of other components such as sterols, membrane proteins, lipids of different chain lengths and saturation degree may lead to interesting effects on the interaction. Such variables are worth studying since the human cell membrane is a system of high complexity. Additionally, it is not possible to determine the composition of any separated phase domains in the monolayer using the current methodologies, thereby making it difficult to understand in detail some of the observations. Therefore, to resolve the process of phase separation in the model lipid membranes, other complementary techniques, such as MD simulations, need to be applied.

The processes of drug self-assembly and drug-membrane interaction occurring in the human body involve many more variables, such as physiological ionic strength, temperature, pH and the presence of different lipid types. The effects of ionic strength and temperature on the drug self-assembly were studied, as well as the variables of lipid components and ionic strength for drug-membrane interaction, while the interactions were only measured at room temperature as result of a study limitation. However, the effect of pH is of importance due to the fact that propranolol hydrochloride can be administrated orally, hence the behaviour of the drug may be affected by the acidic environment in stomach. In summary, further investigation is needed to understand the behaviours of amphiphilic drug in human body.

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Appendix

Equivalent ellipsoid modelling of the atomic structures of micelles allows for more detailed comparison of the findings from molecular dynamics and small angle scattering experiments

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Abstract The derivation and application of simple methodologies are reported that afford an improved modelling of the geometry of micellar aggregates using atomic co-ordinates furnished through molecular dynamics simulations. Use of these methodologies – based around an equivalent ellipsoid modelling of micelle geometries – is demonstrated for the case of micelles formed by the surfactants, sodium dodecyl sulfate and Triton X-100, and those formed by the anti-hypertensive drug, propranolol hydrochloride. The structures of these micelles, as provided through full atom molecular dynamics simulations, are compared against the structures derived through model-fitting of the corresponding small angle neutron scattering data, and the equivalent ellipsoid modelling is shown to provide for a more complete/more satisfying comparison to be made between theory and experiment.

Introduction The manner in which amphiphilic molecules self-associate in aqueous dispersion is pertinent across a wide range of disciplines [1-4], and recent years have borne witness to an increasing number of studies of these systems wherein the process of self-association, and the supramolecular architecture of the aggregates formed, have been probed through the conjoint application of small angle X-ray and/or neutron scattering and full atom molecular dynamics (MD) simulations (*cf.*, [5-10]). In some such studies, where the experimental data have been obtained through small angle X-ray scattering (SAXS), the findings from the MD simulations have been used to calculate theoretical scattering profiles, and these then compared (in a semi-quantitative manner) against those measured by experiment (*cf.*, [11-12]). With this approach, the MD trajectories are first analysed to derive time-averaged radial distribution functions $g_{ij}(r)$ for all pairs of atom types i and j , and these are each then spatially Fourier transformed to give the corresponding pairwise structure factor, $S_{ij}(q)$, as a function of the scattering vector, q . All $S_{ij}(q)$ are then summed to yield the total structure factor, $S(q)$, which can then be compared against the experimental small angle scattering profile. More commonly, however, the correlation between the scattering experiments and MD simulations is made simply by comparing various derived geometric descriptions of the aggregates.

The sizes of the MD-modelled aggregates are typically characterised in terms of their radius of gyration (R_g), calculated as:

$$R_g = \sqrt{\frac{1}{N} \sum_{k=1}^N (r_k - r_{com})^2}$$

where R_g is the root mean square distance of the atoms composing the aggregate (r_k) measured from its

centre of mass (r_{com}) (cf, [6, 7, 10, 13]). This description of the aggregate's size is readily obtained (using tools often provided within the MD program suites) and the R_g so calculated can be compared against the value computed (given certain approximations) through a (shape independent) Guinier analysis of the experimentally determined low- q scattering data. Such a description of aggregate size is perfectly adequate if the particles are relatively monodisperse and spherical but is much less useful for particles that are polydisperse and show significant asymmetry. Moreover, if a comparison is to be made between the sizes of the MD-modelled aggregates and those determined *via* modelling of the small angle scattering recorded over the full measured q -range, it is preferable to approximate the aggregates as some form of spheroid/ellipsoid, and then to quantify their size in terms of the particle volumes.

The ellipsoidal descriptions of the molecular aggregates seen in MD simulations are most often provided through calculation of their principal moments of inertia. The moment of inertia tensors are calculated directly from the atomic co-ordinates of the modelled aggregate(s), with the three principal moments (I_1 , I_2 , and I_3) obtained as the inverse square roots of the eigenvalues (λ_1 , λ_2 , and λ_3), that are obtained *via* diagonalisation of the aggregate's momental matrix (the matrix of its moments and products of inertia), $\mathbf{M}$. When expressed in matrix notation, the diagonalisation of $\mathbf{M}$ simply involves finding a solution to the eigen problem:

$$\mathbf{M}\mathbf{v} = \lambda\mathbf{I}\mathbf{v}$$

where $\mathbf{I}$ is the identity matrix, $\mathbf{v}$ is the matrix of eigenvectors that transforms the old arbitrary reference frame to the new momental ellipsoid-centred reference frame, and $\mathbf{M}$ is calculated (considering only non-hydrogen atoms, and treating each as having equal mass) as:

$$\mathbf{M} = \begin{bmatrix} \sum y^2 + z^2 & \sum -xy & \sum -xz \\ \sum -yx & \sum x^2 + z^2 & \sum -yz \\ \sum -zx & \sum -zy & \sum x^2 + y^2 \end{bmatrix}$$

The principal moments of the aggregate are ordered so that $I_1 < I_2 < I_3$, and if $I_1 \approx I_2 < I_3$, the aggregate has the form of an oblate ellipsoid, and if $I_1 < I_2 \approx I_3$, it is a prolate ellipsoid. In the case of an MD-modelled population of particles, the asymmetry is quantified in terms of their mean eccentricity, ε , calculated as:

$$\varepsilon = 1 - \frac{3 \langle I_1 \rangle}{\langle I_1 + I_2 + I_3 \rangle}$$

where $\langle I_1 \rangle$ is the mean value of I_1 . The eccentricity, ε , is zero if the aggregates are completely spherical, and otherwise indicates the extent of their deviation from sphericity.

As with the size of an MD-modelled aggregate being described by means of its radius of gyration, so too there is a deficiency in describing its shape by means of its momental ellipsoid. While the momental ellipsoid calculated for a particle models its form quite closely, there is an alternative description – provided by the so-called equivalent (equi-momental or Legendre's) ellipsoid – that furnishes a more faithful modelling of the particle's shape (see Fig. 1). Indeed, for a solid ellipsoidal particle, the equivalent ellipsoid superposes the original exactly (whereas the momental ellipsoid does not). The equivalent ellipsoid modelling was previously employed in describing the shapes of globular proteins [14] and was subsequently used as the basis of a method for predicting a protein's continuous antigenic determinants [15].

In MD simulations where a population of aggregates showing a distribution of shapes and sizes is produced, the calculation of their equivalent ellipsoidal geometries also allows that a representative mean

geometry can be computed, and this can then be compared against the geometry modelled by fitting to the small angle X-ray and/or neutron data, where the time- and space-averaged scattering of the particles is weighted by particle volume fraction.

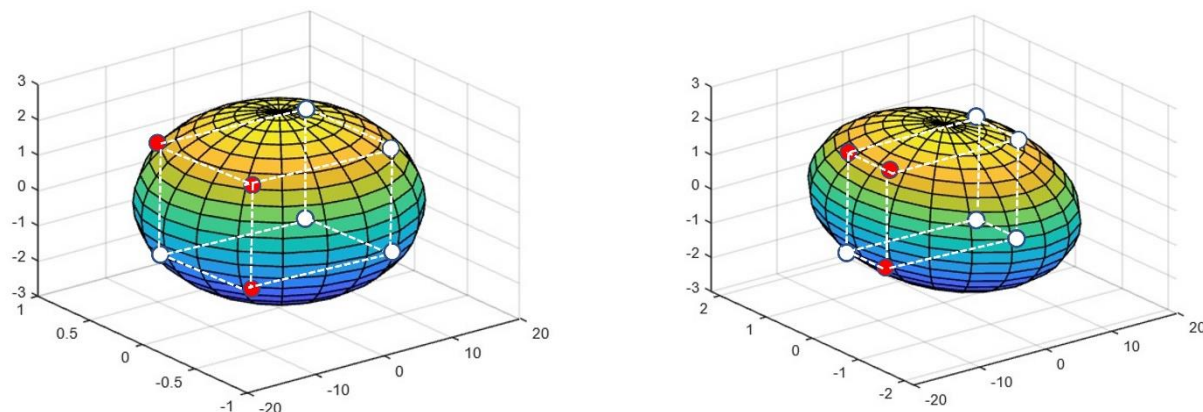


Fig. 1 The equivalent ellipsoid (left; with scaled semi-axes: $17 \times 0.85 \times 2.55$) and the corresponding momental ellipsoid (right, with scaled semi-axes: $14 \times 2.2 \times 2.23$) calculated for an array of points placed at the corners of a rectangular box measuring $20 \times 1 \times 3$. The semi-axes dimensions for both ellipsoids are scaled so that all points lie on the bounding surface. As the figures make clear, the equivalent ellipsoid models the shape of the rectangular box rather better than the momental ellipsoid.

In the following work, we show how the equivalent ellipsoid description of MD-modelled aggregates thus affords a better comparator than the momental ellipsoid when correlating the MD results with the findings obtained from small angle scattering experiments, and how, by an extension of this modelling, with account taken of the loci of explicit solvent molecules in and around the aggregates, it is possible also to determine the shape and size of their hydrophobic core, and the thickness of their hydrophilic shell – and thereby to provide for a more complete/more satisfying comparison to be made between theory and experiment.

Materials and Methods

The methodology for comparing micelle geometries was tested here with reference to the micelles formed by the anionic surfactant, sodium dodecyl sulfate [9], the non-ionic surfactant, Triton X-100 (TX-100; [16]), and those formed by the hydrochloride salt of the anti-hypertensive drug, propranolol [17] (Fig. 2).

Simulation of the (pre-assembled) sodium dodecyl sulfate micelle was performed with LAMMPS [18], using the CHARMM force field [19, 20], with the final 80 ns production run (performed at 1 atm pressure and 300 K) involving a periodic box of dimensions, $126 \text{ \AA} \times 126 \text{ \AA} \times 126 \text{ \AA}$, containing 80 surfactant molecules, along with 64,083 water molecules. Simulation of the (pre-assembled) Triton X-100 micelle was performed with GROMACS [21], using the CHARMM36 force field [22] with the final 200 ns production run (performed at 1 atm pressure and 303 K) yielding a periodic box with dimensions, $111.94 \text{ \AA} \times 119.4 \text{ \AA} \times 119.4 \text{ \AA}$, containing 150 surfactant molecules, along with 52,266 water molecules. Simulation of the (spontaneously assembled) propranolol hydrochloride system was performed with GROMACS [18], using the GROMOS 53A6 force field [23], with the final 60 ns production run (performed

at 1 atm pressure and 300 K) yielding a periodic box of dimensions, 149 Å x 149 Å x 149 Å, containing 406 drug molecules, along with 103,035 water molecules.

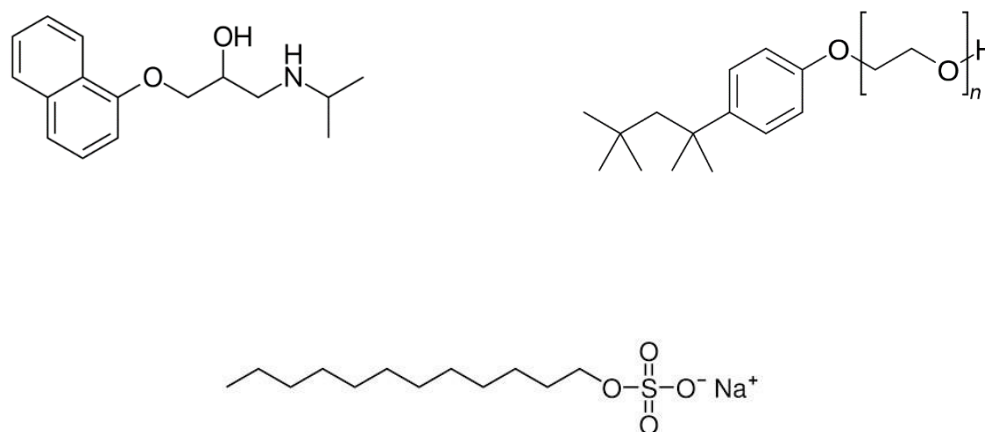


Fig. 2 Chemical structures of propranolol (top left), Triton X-100 (where $n = 9 - 10$; top right), and sodium dodecyl sulfate (bottom).

For each of the modelled systems, the Cartesian co-ordinates of the non-hydrogen atoms making up each aggregate (excluding those of any entrained solvent molecules) were transformed so that their centroid was placed at the reference frame origin, and the second moment matrix, $\mathbf{S}$, obtained, calculated as:

$$\mathbf{S} = \begin{bmatrix} \sum x^2 & \sum xy & \sum xz \\ \sum yx & \sum y^2 & \sum yz \\ \sum zx & \sum zy & \sum z^2 \end{bmatrix}$$

A solution was then sought for the eigen problem:

$$\mathbf{S}\mathbf{v} = \lambda\mathbf{I}\mathbf{v}$$

yielding (*via* matrix diagonalisation) the 3 x 3 matrix of eigenvectors, $\mathbf{v}$, that rotates the aggregate's atomic co-ordinate set into the new (equivalent ellipsoid-centred) reference frame, and the corresponding column matrix of eigenvalues, λ , from which the lengths of the semi-axes of the equivalent ellipsoid (a , b , and c) are obtained as:

$$a = \sqrt{\lambda_1}, \quad b = \sqrt{\lambda_2}, \quad c = \sqrt{\lambda_3}$$

Given that the size of the equivalent ellipsoid is not generally the same as that of the modelled molecular aggregate, however, the semi-axes lengths so derived must be scaled (but with preservation of the axial ratios), such that the bounding ellipsoid encloses a given fraction of its constituent atoms. For the studies reported here, this scaling was performed stepwise, incrementally increasing the lengths of the semi-axes until the bounding ellipsoid enclosed a given fraction of the atoms. With the atoms of the aggregate appropriately transformed (such that their centroid is set at the origin and the reference frame axes are

made co-incident with the axes of the equivalent ellipsoid), the atoms falling within the scaled equivalent ellipsoid (with semi-axes lengths, a' , b' and c') are determined simply by checking which of them has co-ordinates (x , y , z) that satisfy the condition:

$$\left(\frac{x}{a'}\right)^2 + \left(\frac{y}{b'}\right)^2 + \left(\frac{z}{c'}\right)^2 < 1$$

The co-ordinates of waters entrained within the MD-modelled aggregate are then considered, and the size of the equivalent ellipsoid is again incrementally scaled, but in this case with the termination criterion that any further increase in the semi-axes dimensions leads to the inclusion of solvent molecules within the enclosed volume. If the semi-axes dimensions are scaled here so as to preserve the same axial ratios as the outer bounding ellipsoid, the resulting hydrophilic shell has a thickness that varies pro-rata with the ellipsoid radii. However, if the semi-axes lengths are scaled by fixed increments, so that the axial ratios of the (solvent-free) hydrophobic core are then different from those of the outer bounding ellipsoid, the hydrophilic shell that results has a fixed thickness.

Small angle neutron scattering (SANS) data for the three modelled micellar systems were performed at the STFC ISIS facility in Didcot, UK, with the data in each case recorded for the protiated surfactant/drug samples dispersed in D2O and maintained at 298 K. The data for a 3 wt% dispersion of sodium dodecyl sulfate were measured on LOQ, and those for the 10 wt% dispersion of Triton X-100 and the 200 mM dispersion of propranolol hydrochloride on Sans-2d. Model-fits of the SANS data were performed using SasView 5.0 [24], employing either a uniform scattering length density triaxial ellipsoid or a core-shell prolate/oblate ellipsoid geometry [25]. Aggregation numbers for the micelles were calculated from the micelle core and surfactant/drug hydrophobe volumes.

Results

In the (ten) snapshots sampled from the MD production run for the 3 wt% sodium dodecyl sulfate system, the micelle structures were each modelled by an equivalent ellipsoid enclosing 85% - rather than 100% - of the atoms in the associated molecules, making due allowance, therefore, for molecules that (as judged by inspection) are (by virtue of the dynamic nature of micellar aggregates) caught in the act of dissociation (Fig. 3). The mean whole particle dimensions were then obtained as 18.6 Å x 22.5 Å x 26.6 Å, and the dimensions of the water-free core averaged as 9.1 Å x 13.0 Å x 17.1 Å (Fig. 3). The maximum axial ratio/ellipticity of the micelle varied over the range 1.3 – 3.1, with a mean of 2.0. The mean volumes of the micelle core and whole particle were obtained as 9 nm³ and 46 nm³, and the uniform shell modelled with a mean thickness of 9.6 Å (varying over the different snapshots with a range of 7.3 Å – 13.6 Å). As can be seen in Fig. 3, the core of the micelle contains only atoms of the surfactant alkyl chains, whereas the shell comprises primarily the (hydrated) sulfate head groups but also (because of the radial staggering of the head groups) some alkyl chain atoms. The mean (neutron) scattering length densities computed for the modelled core and shell were $-0.26 \times 10^{-6} \text{ Å}^{-2}$ and $2.8 \times 10^{-6} \text{ Å}^{-2}$, respectively.

The corresponding SANS data for the 3 wt% sodium dodecyl sulfate dispersion were found to be rather poorly modelled assuming a triaxial ellipsoid model (data not shown) but were well-modelled assuming a core-shell prolate ellipsoid model (convoluted with a Hayter Penfold structure factor; Table 1, Fig. 4). The outer fitted dimensions of the micelles in the dispersion were obtained as 18.7 Å x 18.7 Å x 27.2 Å, which are gratifyingly similar to the corresponding dimensions of the MD-modelled micelles – albeit that they describe a prolate rather than a triaxial ellipsoid – and the SANS-derived volume of the micelle corresponds closely with that of the fitted equivalent ellipsoid (*viz.*, 40 nm³ *vs.* 46 nm³). The SANS-model-fitted shell of the micelle, on the other hand, is much thinner than that for the MD-derived model – 2.0 Å *vs.* an average of 9.6 Å – but has a fitted scattering length density (of $3.0 \times 10^{-6} \text{ Å}^{-2}$) that is nevertheless close to that calculated from the MD models ($2.8 \times 10^{-6} \text{ Å}^{-2}$).

The difference in the modelled dimensions of the micelle core (and the resulting difference in thickness of the micelle shell) are not unexpected, given that the bounding *triaxial* ellipsoid of the core in the MD-

derived models was defined only on the criterion of no enclosed solvent, whereas the model-fit to the SANS data was performed with the equatorial semi-axis dimension of the *prolate* ellipsoidal core fixed to the extended length of a C12 chain, 16.7 Å, and the scattering length density fixed to that for anhydrous C₁₂ chains ($-0.39 \times 10^{-6} \text{ Å}^{-2}$). The use of such constraints on the core dimensions and composition is common practice in the model-fitting of micelle small angle scattering data (*cf.* [26, 27]) but it necessarily proscribes the inclusion of methylene groups within the micelle shell and assumes that all methyl groups are located within the core.

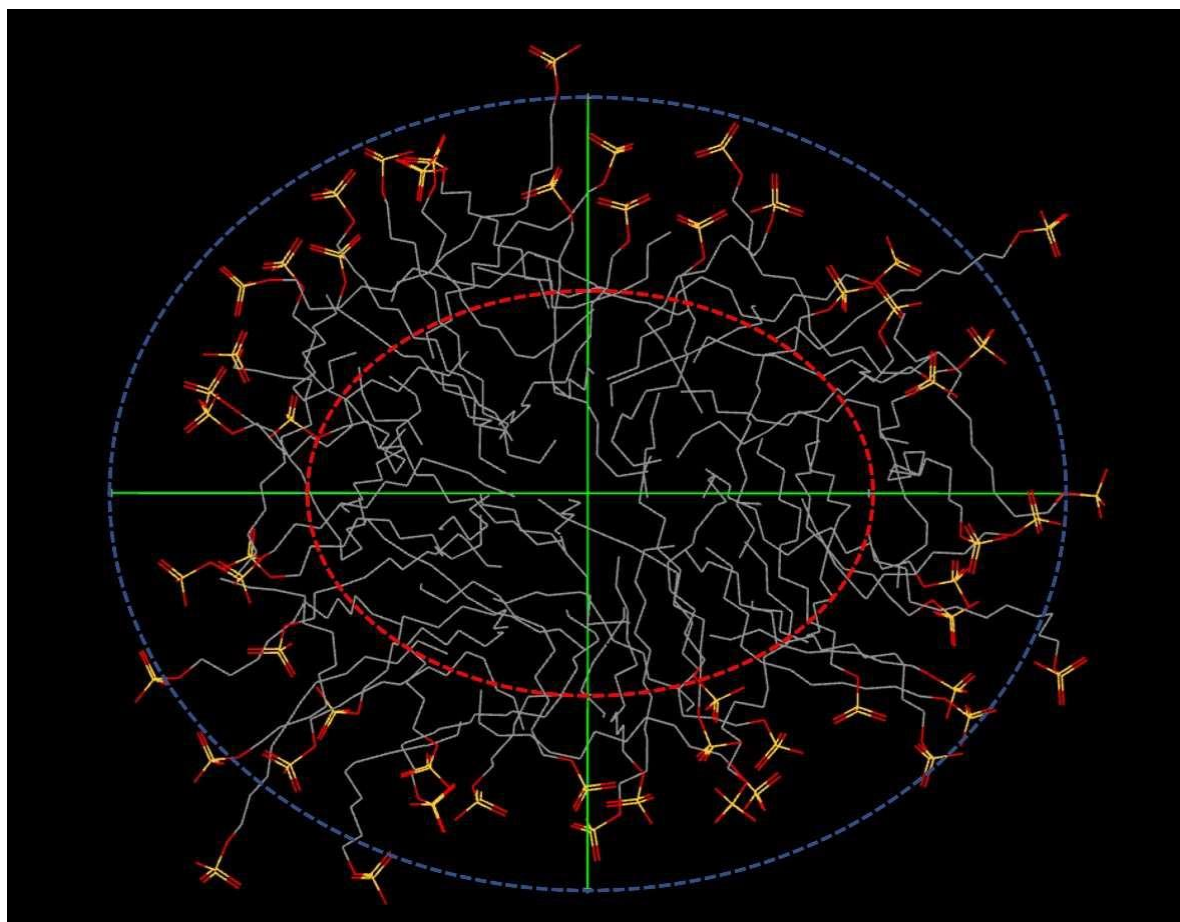


Fig. 3 Cross-sectional view of the atomic structure of a sodium dodecyl sulfate micelle, as seen in one of the ten analysed MD snapshots (colour coded by atom type; with C, grey, O, red, S, yellow), showing the semi-axes and perimeters of the scaled equivalent ellipsoids delimiting the water-free core and the outer surface of the particle (respectively shown by the dashed red and blue ellipses). The semi-axes dimensions of the ellipsoids fitted to the whole particle and the micelle core are, in this instance, 18.6 Å x 22.2 Å x 29.0 Å, and 9.5 Å x 13.1 Å x 19.9 Å, respectively, and the micelle has a uniform shell thickness of 9.2 Å.

Table 1 Fitted parameters for a core-shell prolate ellipsoid model fitted to the SANS profile for 3 wt% protiated sodium dodecyl sulfate dispersed in D₂O.*

Whole particle ellipsoid semi-axes dimensions / Å			Core semi-axes dimensions / Å			Scattering length densities / 10^{-6} Å^{-2}		Shell thickness / Å
a	b	c	a	b	c	Core	Shell	
18.7	18.7	27.2	16.7 [†]	16.7 [†]	25.2	-0.39 [†]	2.8	2.0

* The model fit was performed assuming a Hayter-Penfold structure factor with the particle radius taken as the radius of the equivalent volume sphere. The fitted value of the charge *per* surfactant molecule was 0.28 e, and the calculated aggregation number, 83.

† The equatorial semi-axis of the core was fixed at the extended length of a C₁₂ alkyl chain, and the scattering length density of the core fixed as that of the surfactant/SDS C₁₂ chain.

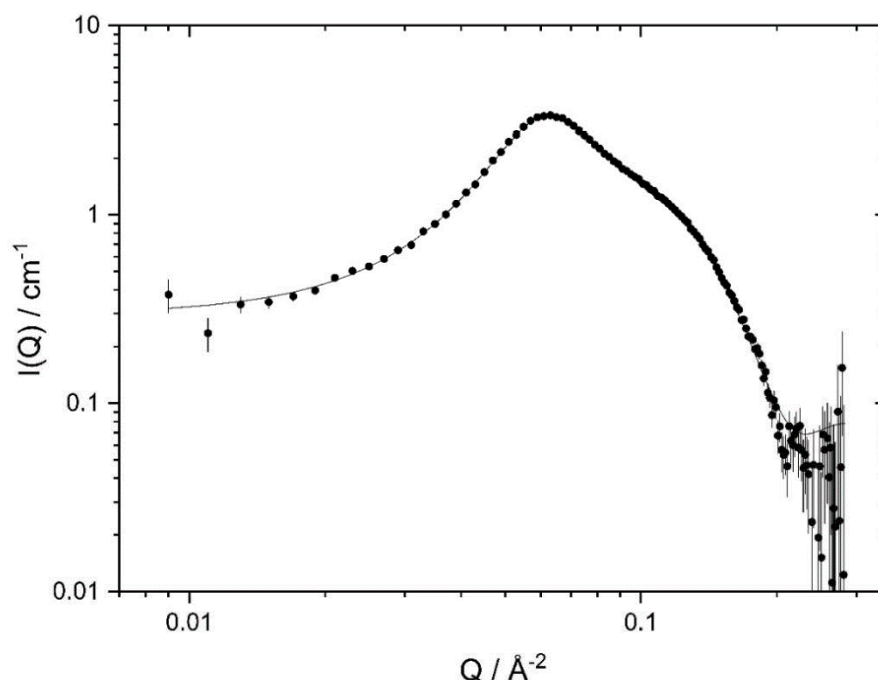


Fig.4 Small angle neutron scattering profile (showing scattering intensity, $I(Q)$, as a function of momentum transfer, Q) measured for a 3 wt% dispersion of protiated SDS in D₂O. Plotted points show the measured data and the solid line shows the fit to the data calculated assuming a core-shell prolate ellipsoid model convoluted with a Hayter Penfold structure factor. The corresponding values of the fitted parameters are shown in Table 1. Error bars in the plot represent the standard errors on the measured data, and in most cases are subsumed within the plotted points.

In the MD simulation of the 10 wt% aqueous dispersion of Triton X-100, the micelle was previously reported [13] as having roughly prolate ellipsoidal geometry, with the particle long dimension averaging 108 ± 1 Å, and with the ellipticities varying over time through the production run and spanning the range 1.5 to 2.0. In the (10) snap-shots from this simulation that were analysed here, the micelle has an aggregation number of 150, and the equivalent ellipsoids scaled to enclose 75% of the atoms had semi-axes dimensions averaging 42.4 Å $\times$ 32.9 Å $\times$ 28.1 Å, with the maximum axial ratios of the particles thus averaging as 1.51, and the end-to-end length of their longest axis around 85 Å (Fig. 5). Note here, that the criterion to enclose 75% of the atoms within the scaled equivalent ellipsoid, as opposed to the 85% used in modelling the sodium dodecyl sulfate aggregates, was chosen so as to avoid having the particle size unreasonably biased by the small number of poly-oxyethylene head groups that straggle from the main body of the particle, snaking out into the surrounding solvent (Fig. 5). Calculation of the water-free core of the TX-100 micelles was initially modelled assuming a uniform shell thickness but this gave a micelle core that appeared highly asymmetric and unrealistically small. In this case, therefore, the micelle core was considered to be better modelled assuming that the shell thickness varied pro-rata with the particle radius. With this assumption, the mean dimensions of the core were obtained as 20.4 Å $\times$ 15.8 Å $\times$ 13.5 Å,

and the shell thickness varied between 15 Å (along the shortest axis) and 22 Å (along the longest axis). The mean scattering length densities for the TX-100 micelle core and shell were calculated as $0.1 \times 10^{-6} \text{ Å}^{-2}$, and $2.2 \times 10^{-6} \text{ Å}^{-2}$, respectively, and the whole particle density obtained as $2.3 \times 10^{-6} \text{ Å}^{-2}$.

Table 2 Fitted parameters for triaxial and core-shell prolate ellipsoid models fitted to the SANS profile for 10 wt% TX-100 dispersed in D₂O.*

Model	Whole particle ellipsoid semi-axes dimensions / Å			Scattering length densities / 10^{-6} Å^{-2}			Hydrophilic shell thickness / Å
	a	b	c	Whole particle	Core	Shell	
Triaxial ellipsoid	20.4	32.5	53.7	1.67	-	-	-
Core-shell ellipsoid	43.1	43.1	19.9	-	0.46 [†]	2.13	17.1 – 7.9

* Both model fits were performed assuming a hard sphere structure factor with the hard sphere radius taken as the radius of the equivalent volume sphere.

[†] The scattering length density of the micelle core was fixed as that of the TX-100's 1,1,3,3,-tetramethylbutylbenzene moiety.

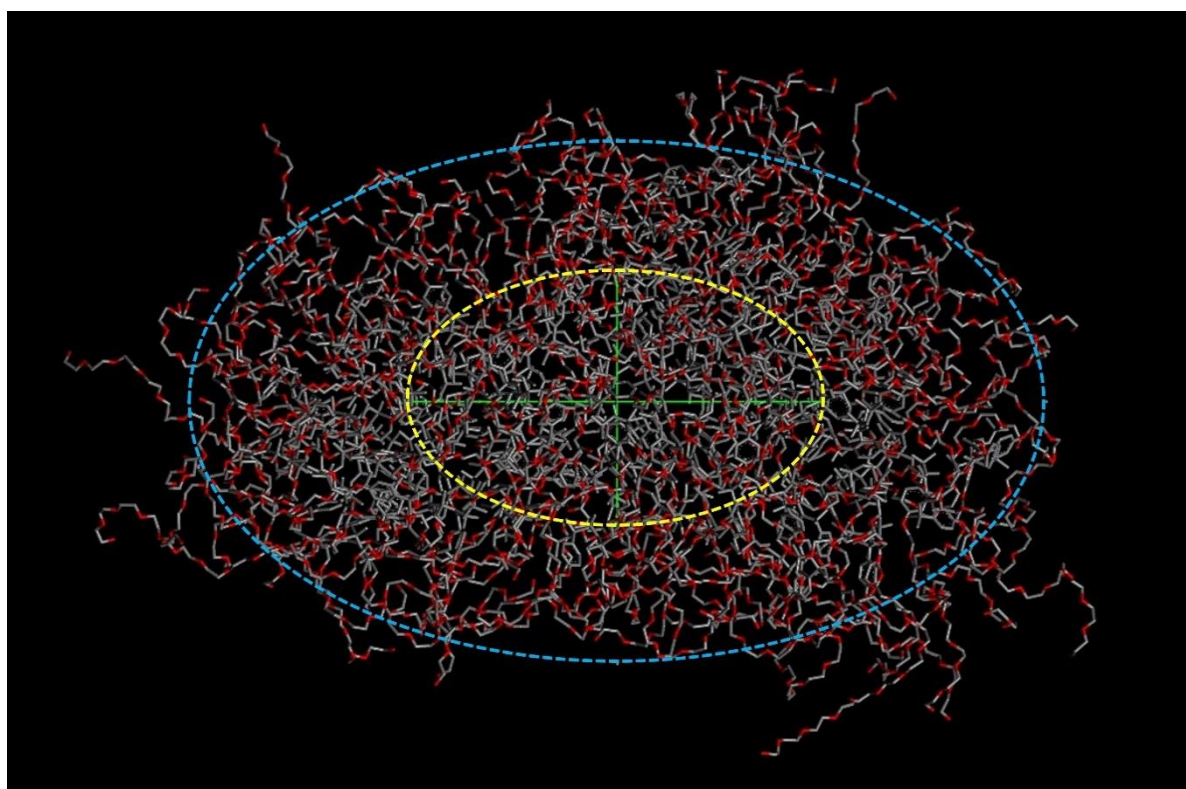


Fig. 5 Atomic structure of an MD-modelled Triton X-100 micelle overlaid with a semi-transparent rendering of its solvent accessible surface (colour coded by atom type; with C, grey, O, red), and showing the semi-axes and perimeters of the scaled equivalent ellipsoids delimiting the water-free core and the outer surface of the particle (respectively shown by the dashed blue and yellow ellipses). The semi-axes dimensions of the ellipsoids fitted to the whole particle and the micelle core are $46 \text{ Å} \times 28 \text{ Å} \times 29 \text{ Å}$, and $22 \text{ Å} \times 14 \text{ Å} \times 14 \text{ Å}$, respectively, and the micelle has a shell thickness that varies pro-rata with the radius of the particle, varying between 24 Å at the equator, and

15 Å at the poles.

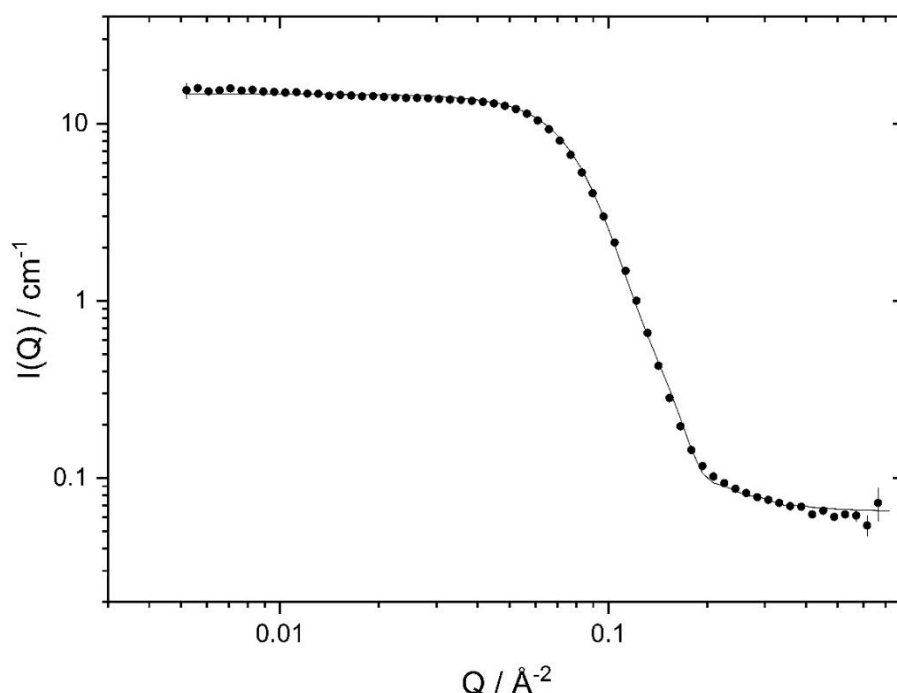


Fig. 6 Small angle neutron scattering profiles (showing scattering intensity, $I(Q)$, as a function of momentum transfer, Q) measured for a 10wt% dispersion of TX-100 in D_2O . Plotted points show the measured data and the solid lines show the fits to the data calculated (A) assuming a uniform scattering length density tri-axial ellipsoid model, and in (B) assuming a core-shell oblate ellipsoid with the shell thickness varying pro-rata with the particle radius, in each case convoluted with a hard sphere structure factor. The corresponding values of the fitted parameters are shown in Table 2. Error bars in the plots represent the standard errors on the measured data, and in most cases are subsumed within the plotted points.

Model fitting of the corresponding SANS data for the 10 wt% TX-100 system [17], assuming uniform scattering length density triaxial ellipsoids convoluted with a hard sphere structure factor (data not shown), gave fitted semi-axes dimensions of 20.4 Å x 32.5 Å x 53.7 Å, with the major semi-axis thus slightly longer, and with one of the minor semi-axes roughly the same and the other slightly larger than the corresponding MD-modelled micelle dimensions. The more detailed modelling of the SANS data gave a best-fit solution using a hard sphere structure factor coupled with a core-shell oblate ellipsoid micelle geometry, and with the shell thickness varying pro-rata with the particle radius, as per the modelling of the aggregate seen in the MD simulation (Table 2; Fig. 6). The fitted semi-axes dimensions for the whole particle were then obtained as 43.1 Å x 43.1 Å x 19.9 Å (Table 2) with the major semi-axis length approximately the same as the longest dimension of the MD-modelled micelle (42.4 Å), and the minor semi-axis dimension shorter than the two minor MD-modelled semi-axes (28.1 Å, 32.9 Å). The shell thickness of the micelle in this case varied between 17.1 Å along the equatorial axes, and 7.9 Å along the polar axis, such that the water-free core had semi-axes dimensions of averaging 26.0 Å x 26.0 Å x 12.0 Å – and leading to a core volume almost twice that calculated for the MD models (*viz.*, 34 nm³ *vs.* 18 nm³).

Inspection of the water-free core of the MD-modelled micelle reveals that its contents, in addition to the expected 1,1,3,3-tetramethylbutylbenzene moieties, also include a significant proportion of polyoxyethylene head groups, particularly towards the outer perimeter (Fig. 7). In consequence of this, the calculated scattering length densities of the MD-modelled micelle core average at $0.1 \times 10^{-6} \text{ Å}^{-2}$, somewhat lower, therefore, than the value of $0.46 \times 10^{-6} \text{ Å}^{-2}$ that was assumed in modelling the SANS data.

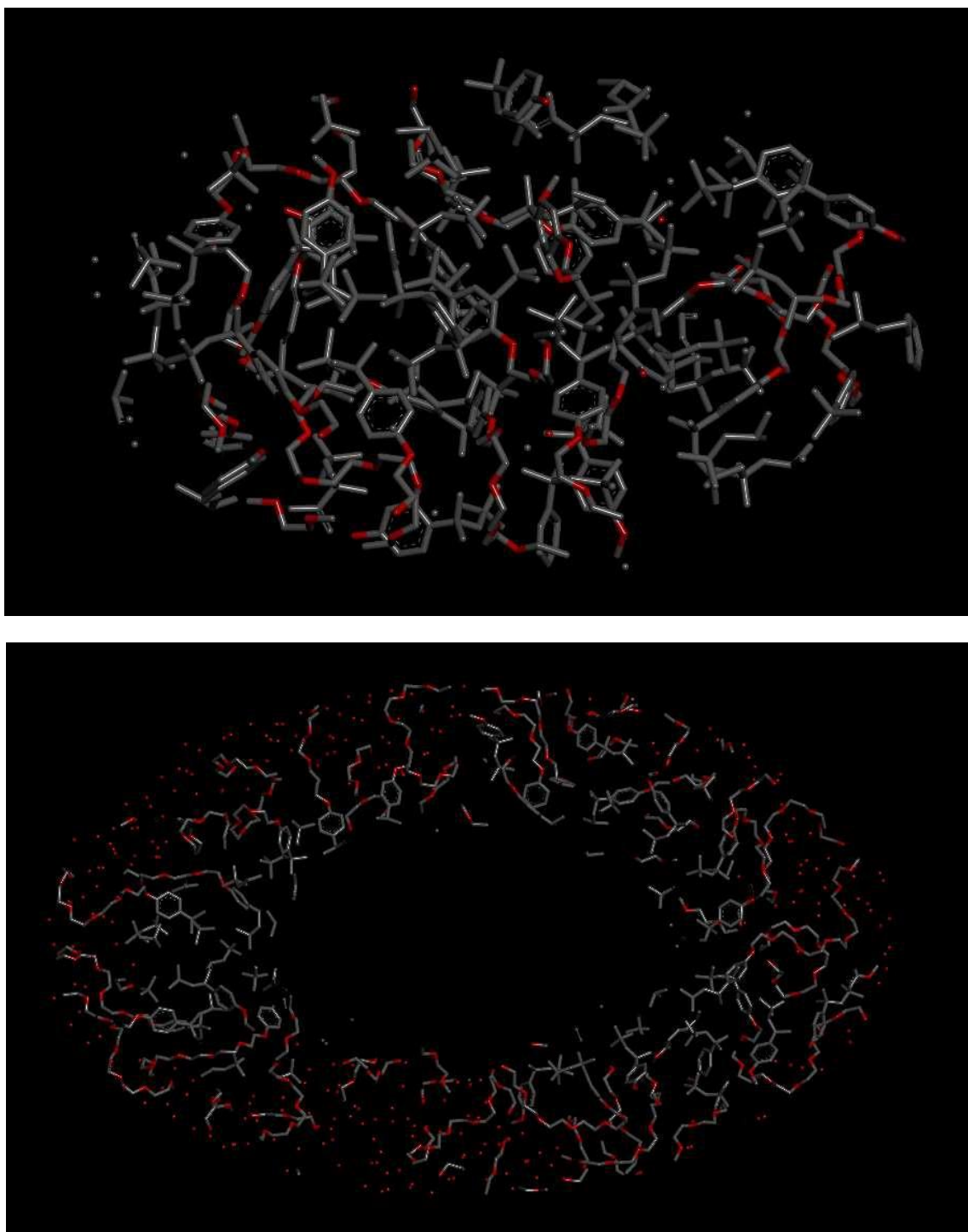
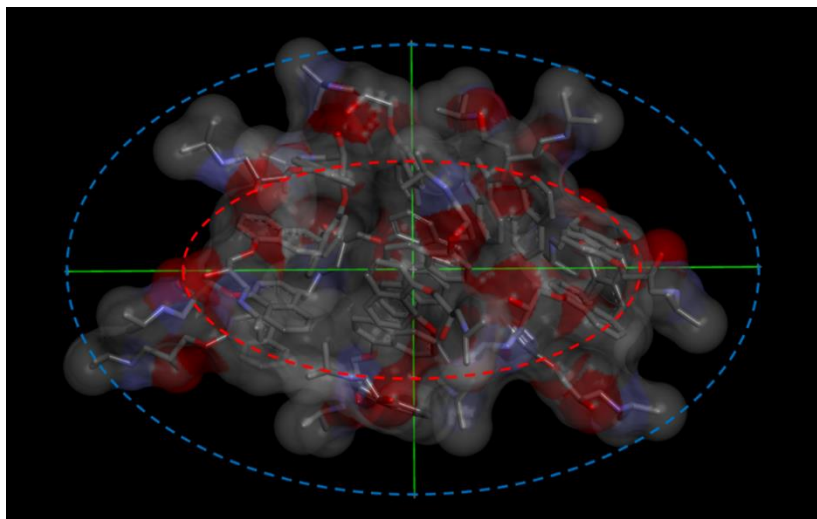


Fig. 7 Molecular models showing the contents of the TX-100 micelle core (upper panel) and shell (lower panel). Only heteroatoms are shown (carbon, grey; oxygen, red), with waters (present only in the shell) represented simply as small red spheres. Dimensions are as given in Fig. 5.

The SANS- and MD-derived scattering length densities of the micelle shell compare rather more favourably, with the former, $2.1 \times 10^{-6} \text{ \AA}^{-2}$ and the latter, $2.2 \times 10^{-6} \text{ \AA}^{-2}$. The thickness of the MD-modelled micelle shell, however, is almost twice as thick along the ellipsoid short axis as the thickness determined on the basis of the SANS data, *viz.*, $15 \text{ \AA}$ vs. $8 \text{ \AA}$, and somewhat thicker too along the major axis, *viz.*, $22 \text{ \AA}$ vs. $17 \text{ \AA}$.

A



B

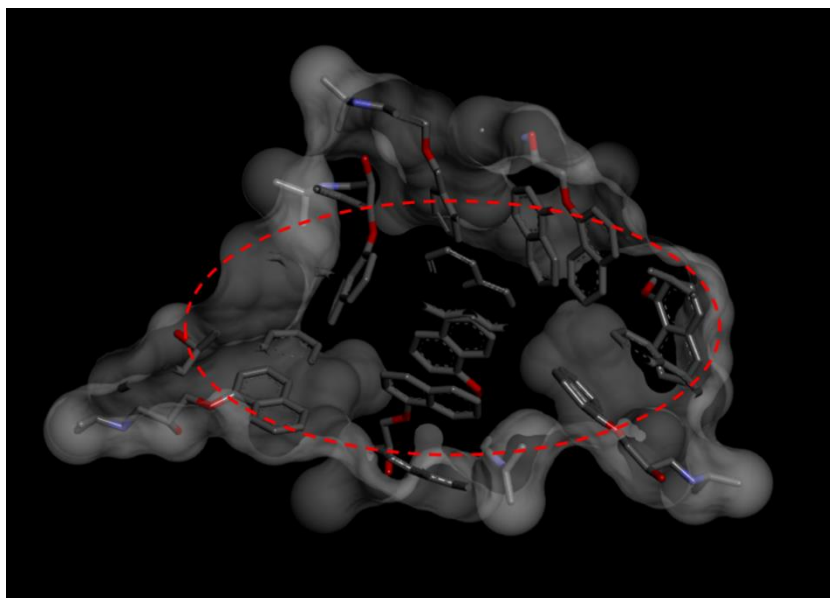


Fig. 8 (a) Atomic structure of an MD-modelled propranolol hydrochloride micelle overlaid with a semi-transparent rendering of its solvent accessible surface (colour coded by atom type; with C, grey, O, red, N, blue), and showing the semi-axes and perimeters of the scaled equivalent ellipsoids delimiting the water-free core and the outer surface of the particle (respectively shown by the dashed blue and red ellipses). The semi-axes dimensions of the ellipsoids fitted to the whole particle and the micelle core are $12.9 \text{ \AA} \times 14.3 \text{ \AA} \times 19.7 \text{ \AA}$, and $6.2 \text{ \AA} \times 7.6 \text{ \AA} \times 13.1 \text{ \AA}$, respectively, and the micelle has a uniform shell thickness of $6.8 \text{ \AA}$. (b) The same micelle as shown in (a) but here with the solvent accessible surface cut away to reveal the naphthyl groups comprising the core of the particle.

In the snapshot taken from the MD simulation of the 200 mM propranolol hydrochloride system, the periodic box contained two singlet drug molecules, together with 38 aggregates of varying shapes and sizes, with aggregation numbers (N_{agg}) ranging from 5 to 19. When each of the modelled aggregates was approximated by an equivalent ellipsoid scaled to enclose 95% of its constituent atoms (this cut-off once again chosen to ensure that individually protrusive molecules exert no undue bias on the modelled micelle size; Fig. 8), the computed volumes of the particles were found to vary between 6 nm³ and 16 nm³, with a mean of ca. 11 nm³, and the volume-weighted mean N_{agg} calculated as 14. The geometries of the aggregates varied between near spherical, triaxial, oblate, and prolate ellipsoids, with ellipticities ranging from 1.2 to 13.1, and with a mean of 2.8.

The volumes of the water-free (apolar) cores of the modelled aggregates ranged from 1.0 nm³ to 3.2 nm³, with a mean of 1.6 nm³. The (uniform) thickness of the shell layer varied between 4.4 Å and 9.7 Å, with a mean of 6.6 Å. The scattering length densities of the core and shell varied between $1.7 \times 10^{-6} \text{ Å}^{-2}$ and $2.3 \times 10^{-6} \text{ Å}^{-2}$, and $3.0 \times 10^{-6} \text{ Å}^{-2}$ and $4.4 \times 10^{-6} \text{ Å}^{-2}$, respectively, with corresponding means of $2.1 \times 10^{-6} \text{ Å}^{-2}$ and $3.9 \times 10^{-6} \text{ Å}^{-2}$. The whole particle scattering length density varied between $3.2 \times 10^{-6} \text{ Å}^{-2}$ and $3.9 \times 10^{-6} \text{ Å}^{-2}$, with a mean of $3.6 \times 10^{-6} \text{ Å}^{-2}$.

When the SANS data for the 200 mM propranolol hydrochloride system [18] were modelled with a Hayter-Penfold structure factor and the drug aggregates treated simply as uniform scattering length density triaxial ellipsoids, the fitted dimensions obtained (Table 3) yielded a whole particle volume of 11.9 nm³ – which is close to the MD-modelled mean of 10.6 nm³. The fitted scattering length density of the particles is also comparable to the MD-derived value at $3.36 \times 10^{-6} \text{ Å}^{-2}$, as too is the ellipticity at 3.3. In the more detailed modelling of the SANS data [18], the best fit was achieved assuming a core-shell prolate ellipsoid geometry convoluted with a Hayter-Penfold structure factor (Table 3; Fig. 9), and this gave whole particle and core volumes of 16.6 nm³ and 5.0 nm³ – both of which values are a little higher than the maxima calculated for the MD models. Likewise, the SANS-derived N_{agg} of 27, is higher than the value (of 14) calculated from the MD models, whereas the ellipticity calculated from the SANS- fitted dimensions is almost the same as the MD-derived value, at 2.9. The scattering length density fixed for the core of the aggregates in the SANS model-fitting is gratifyingly close to the mean value calculated from the MD-models ($2.2 \times 10^{-6} \text{ Å}^{-2}$ vs. $2.1 \times 10^{-6} \text{ Å}^{-2}$), and the bounding ellipsoid of the core seen in the MD-modelled aggregates clearly surrounds the drugs' naphthyl moieties (*cf.* Fig. 8) – which was the assumption made when fitting the SANS data. The SANS- and MD-derived shell thicknesses are also quite close (*viz.*, 4.8 Å vs. 6.6 Å), as too are the scattering length densities of the shell layer ($4.7 \times 10^{-6} \text{ Å}^{-2}$ vs. $3.9 \times 10^{-6} \text{ Å}^{-2}$).

Table 3 Fitted parameters for triaxial and core-shell prolate ellipsoid models fitted to the SANS profile for 200 mM propranolol hydrochloride dispersed in D₂O.*

Model	Whole particle ellipsoid semi-axes dimensions / Å			Scattering length densities / 10^{-6} Å^{-2}			Hydrophilic shell thickness / Å
	a	b	c	Whole particle	Core	Shell	
Triaxial ellipsoid	7.4	15.9	24.3	3.36	-	-	-
Core-shell ellipsoid	12.2	12.2	24.4	-	2.2 [†]	4.7	4.9

* Both model fits were performed assuming a Hayter-Penfold structure factor with the particle radius taken as the radius of the equivalent volume sphere. Fitted values of the salt concentration and charge per drug molecule were 0.006 and 0.17 e, and 0.006 and 0.22 e, for the triaxial and core-shell ellipsoid modelling,

respectively, and the calculated aggregation numbers were 19 and 27, respectively.

† The scattering length density of the micelle core was fixed as that of the propranolol's naphthyl moiety.

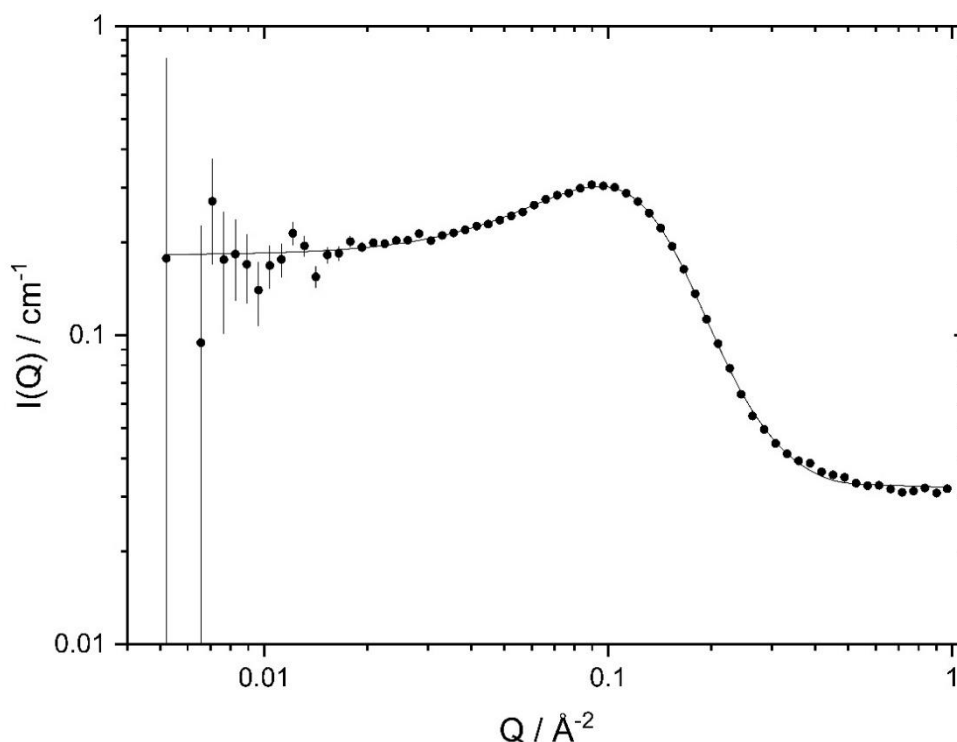


Fig. 9 Small angle neutron scattering profiles (showing scattering intensity, $I(Q)$, as a function of momentum transfer, Q) measured for a 200 mM dispersion of propranolol hydrochloride in D_2O . Plotted points show the measured data and the solid line shows the fit to the data calculated assuming a core-shell ellipsoid model convoluted with a Hayter-Penfold structure factor. Error bars represent the standard errors on the measured data, and in most cases are subsumed within the plotted points. The values of the model-fitted parameters are shown in Table 3.

Discussion & Conclusions

In previous work, an alternative methodology was presented to define the location of the interface and geometry of micelles, wherein a continuous intrinsic surface is calculated for the interface without being confined to classical geometric forms [10, 28, 29]. In the case of SDS micelles, this methodology served to demonstrate the protrusion of molecules from the micelle into the surrounding solvent [28]; for Triton X-114 micelles, it revealed the penetration of ethylene oxide headgroups into the micelle core [29], and in both of these systems it proved of value in showing the degree of solvent penetration into the micelle interior. This methodology is thus demonstrated as a valuable tool for analysing the structures of micelles that can be reasonably approximated as spheres, but it is less insightful for micelles that are poorly approximated as spheres, and the descriptions of the particle core and shell that are furnished cannot in any case be compared *directly* against the architectures determined through model-fitting of small angle scattering data.

The equivalent ellipsoid approximation of micelle geometry that is described here is shown to

provide for a more complete comparison of the findings afforded through small angle scattering experiments and molecular dynamics simulations. Not only does the equivalent ellipsoid modelling allow better comparison of the overall sizes and shapes of the particles, it is also readily extended to allow direct comparison between the MD- and SANS/SAXS-derived dimensions and compositions of their hydrophobic core and hydrophilic shell.

For the test case of propranolol hydrochloride micelles, there is good agreement between theory and experiment (Table 4): the shapes and dimensions of the full-atom models generated in MD simulation quite closely match those derived through model-fitting of the corresponding SANS data, and the calculated core and shell of the MD-modelled micelles have dimensions and compositions that match those determined through model-fitting of the corresponding SANS data. There is a good general agreement seen too for the sodium dodecyl sulfate micelles (Table 4), with the MD- and SANS-derived models showing the same particle sizes and ellipticities, and closely similar core and shell scattering length densities, but with the dimensions of the core and shell differing as a consequence of assumptions applied in the analyses. For the micelles formed by the non-ionic surfactant, Triton X-100, however, the agreement between theory and experiment is a little less satisfying: the shape and size of the whole micelle and the thickness and scattering length density of its shell are quite well matched between the two, but the composition of the core differs significantly from the SANS-derived value (Table 4).

In part, the discrepancies that are seen between the SANS-derived and MD-modelled micelle architectures arise because of the difference in the time-scales of the simulations and experimental measurements (nanoseconds *vs.* minutes or hours), but also because the computer models are never perfectly oblate or perfectly prolate, whereas the models that are available to fit the SANS data and can provide detail on the core and shell of the particles are currently limited to those geometries. For the purposes of comparing the overall shape of the micelles, the SANS data can, as shown here, be modelled assuming triaxial ellipsoid geometry, but one then has to assume a particle with uniform scattering length density and, as a consequence, gains no knowledge of its internal structure. In the ideal, one would use a triaxial core-shell ellipsoid model to fit the SANS data, but such a model is not presently available.

It is clear from the studies reported here too that there are particular problems posed in modelling the (single contrast) SANS data for aggregates formed from surfactants like TX-100, where dehydrated polar head group moieties are likely to intrude into the particle interior, and such problems can also limit the agreement between theory and experiment when – as in the case of sodium dodecyl sulfate micelles – the micelle shell contains a proportion of hydrocarbon moieties. Since it is not obvious *a priori* to know the extent of mixing of an amphiphile's apolar and polar moieties, and since optimisation of both the dimensions and scattering length densities of the core and shell without the use of constraints will generally prove highly problematic, and frequently lead to physically implausible solutions, it is essential in such cases to make measurements using multiple H/D contrasts, and then to perform simultaneous constrained model fitting.

Accepting these various caveats, we maintain that our equivalent ellipsoid approximation of micelle geometry, and the extrapolation from this to determine the micelle core and shell, will be useful to others concerned with correlating the findings got from small angle scattering experiments and MD simulations. It can be expected to work well for any spheroidal micelle that has a clearly defined core and shell (that is, for micelles formed from amphiphiles that have hydrophobe and head group moieties that exhibit little mixing). The same methodology might also find use in comparisons made for rod-shaped aggregates, given that capped cylinders and long prolate ellipsoids are geometrically very similar.

Table 4 Comparison of MD- and SANS-derived micelle structures

Sodium dodecyl sulfate		MD-models	SANS model
Particle	Ellipticity [†]	1.3 – 3.1 (2.0)	1.51
	Volume / nm ³	42 – 53 (46)	40
	Aggregation number	79	83
Shell	Thickness [†] / Å	7.3 – 13.6 (9.6)	2.0
	Scattering length density / 10 ⁻⁶ Å ⁻²	2.6 – 3.2 (2.8)	3.0
Core	Volume [†] / nm ³	3.3 – 12.2 (9)	29
	Scattering length density / 10 ⁻⁶ Å ⁻²	-0.24 – -0.27 (-0.26)	-0.39
Triton X-100		MD-models	SANS model
Particle	Ellipticity [†]	1.33 – 1.64 (1.51)	2.17
	Volume / nm ³	152 – 175 (163)	155
	Aggregation number	150	98
Shell	Thickness [†] / Å	15 – 22	8 – 17
	Scattering length density [†] / 10 ⁻⁶ Å ⁻²	2.0 – 2.3 (2.2)	2.1
Core	Volume / nm ³	17.4 – 18.9 (18)	34
	Scattering length density [†] / 10 ⁻⁶ Å ⁻²	-0.05 – 0.14 (0.1)	0.46
Propranolol HCl		MD-models	SANS model
Particle	Ellipticity [†]	1.2 – 13.1 (2.8)	2.9
	Volume [†] / nm ³	6 – 16 (10.6)	16.6
	Aggregation number [†]	5 – 19 (14)	27
Shell	Thickness [†] / Å	4.4 – 9.7 (6.6)	4.8
	Scattering length density [†] / 10 ⁻⁶ Å ⁻²	3.0 – 4.4 (3.9)	4.7
Core	Volume [†] / nm ³	1.0 – 3.2 (1.6)	5
	Scattering length density [†] / 10 ⁻⁶ Å ⁻²	1.7 – 2.3 (2.1)	2.2

[†] mean values of parameters given in parentheses

Author contributions

DJB – conceptualisation, formal analysis, methodology, resources, writing–original draft, review & editing. MJL – supervision, funding acquisition, resources, formal analysis, writing–review & editing. CDL– supervision, funding acquisition, formal analysis, resources, writing–review & editing. YY – formal analysis, writing–review & editing. AL – formal analysis, writing–review & editing. DA – formal analysis, writing–review & editing. HI – formal analysis, writing–review & editing.

Conflicts of Interest

There are no conflicts of interest to declare.

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