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Biochimica et Biophysica Acta 1514 (2001) 206–216

BIOCHIMICA ET BIOPHYSICA ACTA
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Nuclear magnetic resonance study of doxorubicin binding to cardiolipin containing magnetically oriented phospholipid bilayers

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Received 4 April 2001; received in revised form 12 June 2001; accepted 15 June 2001

Abstract

Doxorubicin (DOX) is a potent anthracycline cancer drug whose interaction with anionic membrane phospholipids, such as cardiolipin (CL), is thought to contribute to its cytotoxicity as well as induce cardiotoxic side effects. We have studied the interaction of DOX with a CL containing model membrane system using high resolution, oriented sample ^{31}P and ^{2}H NMR. The model membrane system is composed of a bilayer forming phospholipid and a detergent that breaks the extended bilayers into disc-shaped micelles (bicelles) that can orient in a magnetic field. The effects of DOX on the phospholipid bilayer were monitored using samples containing dimyristoylphosphatidylcholine (DMPC), selectively deuterated in either headgroup or acyl chain positions, and measuring the changes in ^{2}H quadrupolar splittings as DOX was added. Changes in quadrupolar splittings upon DOX addition provide evidence for interaction with both surface and buried sites within the membrane bilayer.

Keywords: Drug–lipid interaction; Bicelle; Magnetic orientation

1. Introduction

The plasma membrane of a cell can serve either as a target for activity of a drug or as a barrier it must overcome on its journey to an intended internal cellular target. In the cytoplasm of eukaryotic cells, a large membrane surface area is available with which a drug can interact due to the presence of many membrane-encased organelles and vesicles. Clearly, understanding how drugs interact with membranes at an atomic level is important in understanding how drugs are transported to sites of action within cells [1]. Furthermore, these interactions are important in understanding the role membranes play in reducing the efficiency of drugs, either by involvement in multi-drug resistance mechanisms [2] or the development of unfavorable drug side effects. Unfortunately, the standard tools of structural biology (such as X-ray crystallography and solution nuclear magnetic resonance (NMR) spectroscopy) are notoriously difficult for high-resolution structural studies of lipid bilayer associated molecules. In this paper, we present results on the interaction of a potent antitumor drug with lipid membrane bilayers using oriented sample NMR methods that are effective in providing high-resolution, atomic-level structural information on liquid crystalline membrane bilayers.

Doxorubicin, also known as adriamycin, is a widely used, potent antitumor drug. Doxorubicin (DOX) consists of an amino sugar linked to an an-

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0005-2736/01/$ – see front matter
PII: S0005-2736(01)00371-6

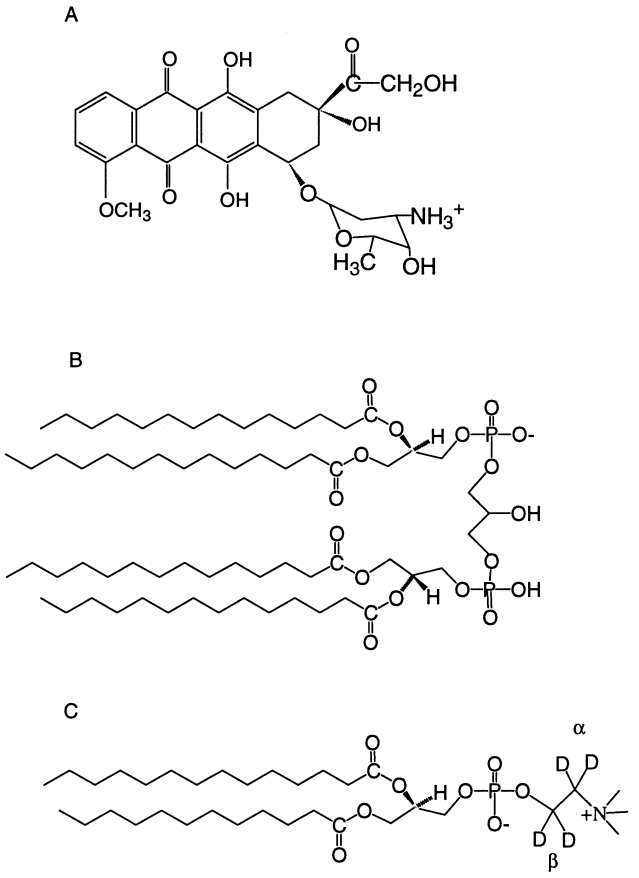

Fig. 1. Chemical structure of (A) doxorubicin (the amine group has a pK_a of 8.3); (B) cardiolipin (the pK_as of the two phosphates are 2.8 and 7.5 [21]); (C) dimyristoylphosphatidylcholine. Headgroup labeling sites for d4-DMPC choline tilt studies are labeled α and β.

thracycline ring (see Fig. 1A). Despite DOX's widespread clinical use, there are still questions left unanswered about its mode of action [3]. DOX has been primarily classified as a DNA intercalator. However, as an amphipathic molecule it is also able to interact with cellular membranes. There have been extensive studies on the interaction of DOX with membranes and DOX has been proposed to alter membrane fluidity, cause lipid peroxidation and interfere with membrane associated enzyme activity [4]. In fact, the interaction of DOX with membranes has been proposed to be the cause of many of the side effects of the drug, including cardiotoxicity, which ultimately sets the limit on the therapeutic dose [5].

Although DOX has been found to interact with membranes composed of a wide range of lipids, the binding capacity to zwitterionic lipids is low compared to acidic lipids such as cardiolipin (CL) [6]. The tetraacylphospholipid cardiolipin (see Fig. 1B) is found in several types of cellular membranes, but is particularly concentrated in mitochondrial membranes where it comprises ~15% of the total phospholipid [7]. Binding of DOX to mitochondrial CL is thought to be involved in the cardiotoxicity of the drug [8]. Interactions that have been proposed for the stabilization of the DOX–CL complex include electrostatic interaction between the protonated sugar amino group of DOX and an anionic phosphate of CL, as well as hydrophobic interaction of the drug with the acyl chains of the lipids. Two different binding sites in acidic phospholipids have been postulated: a superficial site and a site involving penetration of the drug into the hydrophobic region. However, the exact nature of the DOX–CL complex is not well understood and several views have been proposed based on a variety of different model membrane systems using a range of different biophysical methods [9]. Unanswered questions about the atomic-level details of the topology of the DOX–CL interaction is a problem that the oriented NMR methods presented here are well-suited to address.

Oriented sample NMR differs from normal solution NMR in that many of the interactions normally averaged to zero by motion in solution remain finite and can be measured and analyzed to determine high-resolution conformation and dynamics. The model membrane system used in this paper spontaneously orients in an applied magnetic field. It is composed of a mixture of bilayer-forming phospholipid (dimyristoyl phosphatidylcholine, DMPC) and detergent (dihexanoyl phosphatidylcholine, DHPC) that breaks up the extended bilayers into disc-shaped micelles (bilayered micelles, or bicelles) that are highly hydrated (~70% aqueous) [10]. The driving force for the macroscopic ordering is the anisotropy of the diamagnetic susceptibility of the acyl chains in the bilayer forming lipids. In this report, structural consequences of DOX binding to CL enriched DMPC bicelles are investigated using ^{31}P and ^{2}H NMR spectroscopy. In particular, ^{2}H NMR data for DMPC that has been deuterated either in the

headgroup or acyl chain regions provide evidence for interaction of DOX with both surface and buried sites within the membrane bilayer.

2. Materials and methods

2.1. Sample preparation

1,2-Dimyristol-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dimyristol-*sn*-glycero-3-phosphocholine-1,1,2,2-d (d4-DMPC), 1,2-dimyristol-d54-*sn*-glycero-3-phosphocholine (d54-DMPC), 1,2-dimyristol-*sn*-glycero-3-phosphoethanolamine-*N*-(poly(ethylene glycol)-2000) (PEG2000-PE), and 1,1′,2,2′-tetramyristol cardiolipin (CL) were purchased in powder form from Avanti Polar Lipids (Alabaster, AL) and stored at −20°C. 1,2-Dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC) was purchased from Avanti as a chloroform solution (20 mg/ml) and stored at −20°C. Doxorubicin hydrochloride was donated by Bristol-Meyers-Squibb (Princeton, NJ) as a 1:5 powder mixture with lactose. Additional DOX was purchased from Sigma (St. Louis, MO). The DOX from Bristol-Meyers-Squibb was purified away from lactose using Waters Sep-Pak 3 cc, C18 (reversed phase) cartridges. The C18 columns were conditioned with washes of methanol and equilibrated with washes of water. Lactose/DOX mixtures were loaded in water, the column washed with water to remove lactose and the DOX eluted in methanol. Methanol was then removed by rotary evaporation, and the remaining water was lyophilized. Purified DOX was compared by TLC (on silica gel plates; solvent system 100:50:14:6 v/v chloroform/methanol/acetic acid/water) [8] to DOX purchased from Sigma. Both compounds had a single spot at an R_f of 0.67. Concentration was determined from diluting aqueous stock solutions made just prior to use using an extinction coefficient at 480 nm of 10 600 $M^{-1} cm^{-1}$ [6]. The buffer 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was purchased from Fisher Biotech (enzyme grade). Deuterium-depleted water was purchased from Aldrich.

2.2. Preparation of NMR samples

Bicelle samples had a long-chain lipid (DMPC and CL) to DHPC ratio of 3.3:1 and were either 15% or 11.5% weight/volume (as specified in the figure legends). To conserve DOX, all samples with DOX were prepared with the lowest weight/volume that oriented consistently (11.5%). A typical sample volume was 350 μl. Bicelles were prepared by drying down DHPC from chloroform in a thin film using rotary evaporation followed by high vacuum (<100 millitorr) for at least 3 h. The DHPC was solubilized in one half of the total volume of deuterium-depleted 50 mM HEPES (pH 6.5) buffer, and this solution mixed directly in the NMR tube with the DMPC, CL, and PEG2000-PE powders. A small amount (0.5% molar to DMPC) of PEG2000-PE was included in all samples to improve sample stability [11]. For the collection of ^2H spectra, 4 mg of either d4-DMPC or d54 DMPC replaced 4 mg of DMPC in each sample. The NMR sample tube was then subjected to a series of heating to 50–60°C, cooling on ice, vortexing, and sonicating, until the mixture was homogeneous and clear below room temperature. Samples were then left for at least 3 h, and usually overnight, to fully homogenize. The DOX was then dissolved in the remaining volume of buffer and added in small aliquots to the sample with continuous vortexing. During DOX addition, the bicelles were kept on ice to maintain their fluidity and facilitate mixing of the drug. The samples were then immediately equilibrated in the NMR spectrometer at 40°C for 30 min prior to collecting spectra.

Unoriented dispersion samples used to collect the data for Fig. 5 were 50% w/v DMPC/CL, 90:10 in the same buffer used for all bicelle samples. Samples were prepared by cosolubilizing DMPC and CL in 1:1 chloroform/methanol in NMR tubes. The solvent was then blown off under a stream of nitrogen and the samples place under high vacuum overnight. One half of the total volume of buffer needed for the final samples was added to each of the NMR sample tubes which were then subjected to heating, cooling, vortexing, and bath sonicating, until well mixed. Samples were then left overnight, to fully homogenize. DOX was then dissolved in the remaining volume of buffer and added to the sample with continuous vortexing. The samples were then equilibrated in the NMR spectrometer at 40°C for 30 min prior to collecting spectra.

2.3. Collection of NMR data

NMR spectra were collected at 40°C on a Bruker Avance DRX 400 MHz spectrometer with a 5 mm tunable broadband probe. All samples containing deuterium labeled compounds were prepared in deuterium-depleted buffer so it was not possible to lock to a deuterated solvent. However, a similar control sample with D_2O present was used to create a shim file which was read before bicelle spectra were collected. All spectra were acquired with both the deuterium field frequency lock and sample spinning turned off.

^{31}P (161.98 MHz) spectra were collected using a 90° ^{31}P pulse (8.60 μs), WALTZ ^{1}H decoupling (^{1}H 90° pulse 17 μs), a 32 ms acquisition time and a relaxation delay of 3.0 s between scans. Spectra were collected with 2048 points, 32 scans, and a sweep width of 200.00 ppm centered at 0.00 ppm.

^{2}H spectra (61.4 MHz) were collected with a 90° pulse (15 μs) and a relaxation delay of 0.5 s between pulses. Headgroup deuterated spectra were collected with 512 points, 512 scans, and a sweep width of 25 kHz. Acyl chain-deuterated bicelles were collected with 2048 points, 1024 scans, and a sweep width of 61 kHz.

3. Results

3.1. Spectral properties of bicelles with CL

Fig. 2 demonstrates that CL can be incorporated into the magnetically oriented bilayers (bicelles) and that the system maintains homogeneous orientation.

Fig. 2A shows ^{31}P–^{1}H decoupled spectra for both neutral (top) and CL containing (bottom) bicelles. Since phosphorus does not require specific labeling to enable acquisition of ^{31}P spectra, it is often used as a simple and effective probe of bicelle formation and alignment. Both samples show sharp, well-resolved peaks, indicating that both were well-oriented and that the lipids are homogeneously distributed. There is one phosphorus peak present for each type of lipid in the bicelles and one can unambiguously assign the observed resonances. For the sample with no CL, the downfield lower intensity resonance belongs to the phosphorus of DHPC, while the upfield,

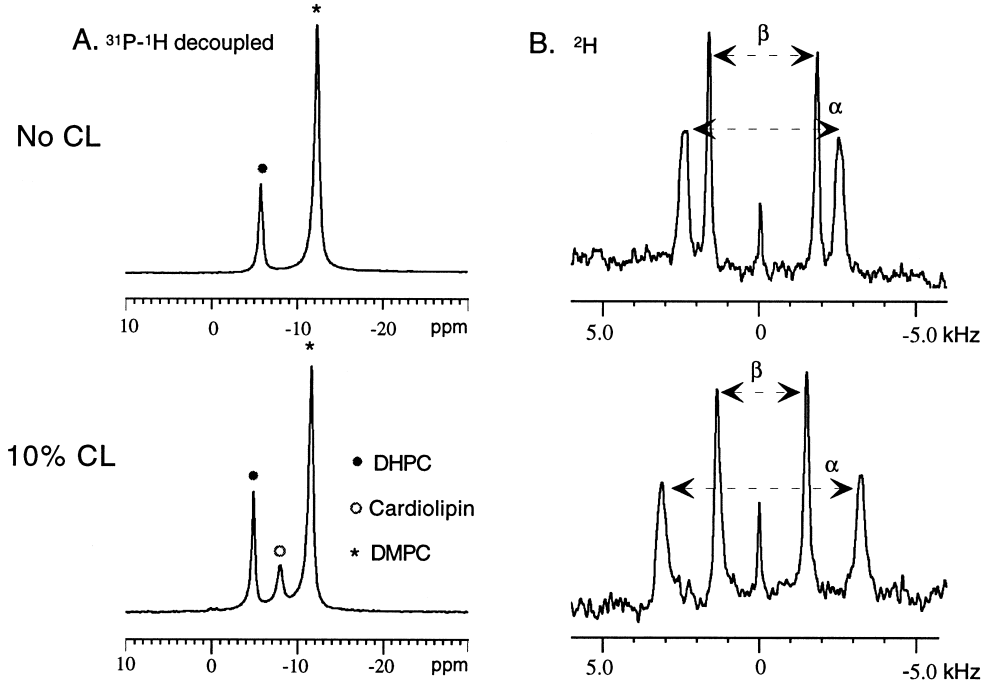

Fig. 2. ^{31}P–^{1}H decoupled (A) and ^{2}H (B) spectra of 15% w/v bicelles; neutral (top) and containing 0.10 mole fraction cardiolipin (bottom). The ^{31}P peaks corresponding to DHPC, cardiolipin, and DMPC are labeled in A. The quadrupolar splittings for α and β deuterons are labeled in B.

higher intensity resonance belongs to the phosphorus of DMPC. For the sample with 0.10 mole fraction CL, the new peak of intermediate chemical shift is assigned to CL. The chemical shifts of the ^{31}P peaks are determined largely by chemical shift anisotropy (CSA) and are sensitive to the overall order of the system [12]. Since the chemical shifts of the DMPC and CL resonances fall in the region that one expects for the 90° shoulder of the corresponding CSA powder patterns in nonoriented samples, the spectra observed are consistent with bicelles oriented with their bilayer normals perpendicular with respect to the director of the magnetic field as expected.

Fig. 2B are ^{2}H spectra for neutral (top) and CL containing (bottom) bicelles with incorporated headgroup-deuterated DMPC (labeling pattern indicated in Fig. 1C). The ^{2}H spectra show well-resolved doublets; one for the α deuterons and one for the β deuterons of the phosphocholine headgroup of DMPC. In the bicelles containing CL the α splittings are bigger and the β splittings smaller with respect to the neutral bicelles. Previous ^{2}H NMR studies have established that the headgroup of phosphatidylcholine changes conformation in response to surface charge due to the electric dipole moment of the choline headgroup [13]. The counterdirectional change in ^{2}H NMR quadrupolar splitting from DMPC-α-d2 versus DMPC-β-d2 is characteristic and results from a conformational change in the PC headgroup. The addition of negative charge to a PC membrane surface (as through the addition of CL) moves the positively charged choline end of the dipole towards the membrane surface, changing the orientation of the labeled PC headgroup and changing the observed orientational dependent ^{2}H quadrupolar splittings. (In the same way, the addition of positive charges at a membrane surface can have the opposite effect by forcing the positively charged quaternary nitrogen towards the water phase.) The sensitivity of ^{2}H spectra to changes in electrostatic surface charge has been called the 'choline-tilt' effect or molecular voltmeter and has proved very useful in studying the binding of charged ligands to membrane surfaces [14]. The majority of choline head tilt work has been done on unoriented lipid dispersions, although there is a recent report of the choline tilt effect being observed in bicelles [15]. In the work described in this paper the choline-tilt effect is very useful in investigating the binding of the positively charged DOX to bicelles containing negatively charged CL.

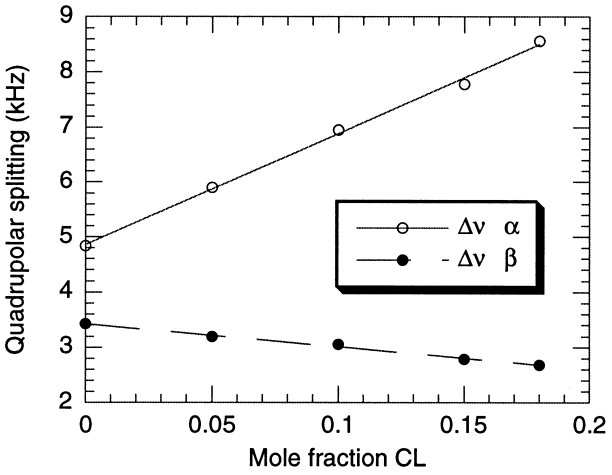

Fig. 3. Quadrupolar splittings of the α (○) and β (●) deuterons in 11.5% w/v bicelles with varying mole fractions of CL (relative to DMPC).

Fig. 3 shows the change in quadrupolar splitting for bicelles with varying amounts of CL (0–0.18 mole fraction to DMPC). The α splittings increase and the β splittings decrease with increasing amount of CL consistent with the choline head-tilt model which predicts the DMPC headgroup should rotate towards the bilayer surface as the amount of negative charge increases [13]. The choline tilt effect using ^{2}H NMR was observed in previous studies on unoriented multilamellar vesicles of diacylphosphatidycholines/CL mixtures [16]. Both those studies and our results with bicelles demonstrate a counterdirectional change in α and β splittings upon the addition of CL.

The bicelles with 0.10 mole fraction CL were chosen for all the DOX binding experiments because they were well-behaved and could be made reproducibly. (Bicelle samples above 0.18 mole fraction CL were unstable.) Finally, 0.10 mole fraction CL is within the range of concentrations found in naturally occurring (most often mitochondrial) membranes [7].

3.2. *Titration of DOX into oriented bicelles containing CL*

DOX was added to bicelles containing 0.10 mole fraction CL up to 0.10 mole fraction DOX, which corresponds to a ratio of 1:1 DOX:CL (Fig. 4) and the samples monitored using both ^{31}P and ^{2}H NMR.

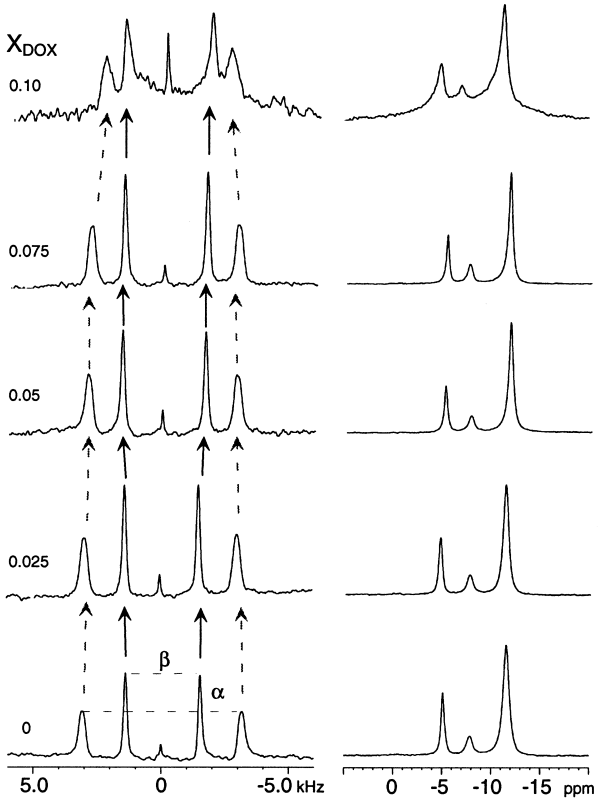

Fig. 4. ^{2}H (left) and ^{31}P (right) NMR spectra of 11.5% w/v bicelles with 0.10 mole fraction CL containing increasing amounts of DOX. Mole fractions DOX (X_{DOX}) are expressed relative to DMPC. $X_{DOX} = 0.10$ corresponds to a DOX/CL ratio of 1:1.

Up to 0.075 mole fraction DOX (3:4 DOX:Cl), each ^{31}P spectrum has three peaks at approximately the same positions indicating the samples maintain orientation over the titration. In the ^{2}H spectra, the quadrupolar splittings of the α deuterons decrease with increasing mole fraction of DOX as expected for a decrease in the net negative surface charge and a tilting of the head group away from the bilayer surface. There is also an overall increase in quadrupolar splitting over the DOX titration of the β deuterons, although the trend is not as consistent (see Table 1). Both ^{31}P and ^{2}H spectra have broadened peaks for the 0.10 mole fraction DOX sample which began to show evidence of partial phase separation and precipitation within a few hours of preparation. At high DOX concentration it is possible that precipitation could be due to drug-mediated inter-bicelle crosslinking. As suggested in a previous DOX membrane study [6], inter-bilayer crosslinking would be possible if DOX molecules located on one bilayer surface stack with DOX molecules of opposite orientation from a neighboring bilayer surface. In addition to possible mechanical interference resulting from crosslinking, it is possible that at high concentrations of DOX the magnetic susceptibility of the bicelle arrays changes enough to affect orientation. Highly aromatic molecules are known to have large diamagnetic susceptibilities [17] and it is possible that the highly aromatic DOX (possibly stacked in cooperative arrays when bound to membranes) could change the effective magnetic susceptibility of the bicelles in such a way as to change orientation properties. In fact, in a recent study using ^{31}P NMR [18], DOX is shown to induce partial magnetic orientation of 2:1 mixtures of CL/DOX where the bilayer normal of the lipids are oriented parallel to the applied magnetic field. However, their system had a significantly higher proportion of DOX to membrane

Table 1
Quadrupolar splittings (kHz) for α, β sites of d4-DMPC upon addition of DOX

X^{a}_{DOX}	$\Delta\nu_{\beta}$ (kHz)		$\Delta\nu_{\alpha}$ (kHz)		$\Delta\nu_{\alpha}$ (kHz)
	Unoriented[b]	Oriented[c]	Unoriented[b]	Oriented[c]	(calculated)[d]
0	3.69	2.93	8.13	6.26	6.86
0.025	4.08	2.90	7.56	5.99	6.48
0.05	4.26	3.26	7.34	5.82	6.10
0.075	4.36	3.25	7.13	5.71	5.73
0.1	4.42	3.41	6.97	4.91	5.35

[a]Mole fraction DOX to DMPC.
[b]Measured from unoriented dispersions of DMPC/CL, 90:10 from spectral maxima.
[c]Measured from oriented DMPC–DHPC bicelles with 0.10 mole fraction CL.
[d]Calculated for oriented bicelle system using Eq. 2.

forming lipid than our system and we suspect that the broadening seen in our spectra is caused by drug-mediated bicelle–bicelle interactions.

3.3. Titration of DOX into unoriented bicelles containing CL

To accurately interpret changes in quadrupolar splittings during the DOX titration in terms of our measurable of interest (local conformational changes of lipids due to drug binding), it is necessary to separate out other possible changes in the sample that could affect the measured quadrupolar splittings and complicate analysis. The other possible changes involve perturbation to the overall orientation and motion of the bicelles. Quadrupolar splitting, $\Delta\nu$, may be written as follows

$$\Delta\nu = \frac{3}{2}\frac{e^2qQ}{h}S_{\text{system}}\left\langle\frac{3\cos^2\theta-1}{2}\right\rangle \quad (1)$$

where the factor (e^2qQ/h) is the nuclear quadrupolar constant, θ is the angle between the deuterium-X nucleus bond axis and the bilayer normal, and the brackets surrounding the θ term indicate a time average of the enclosed function. Net overall orientation and motion of the bicelles are included in a single-order parameter (S_{system}) that scales all spectral parameters [12]. S_{system} is defined as $S_{\text{system}} = (-1/2)S_{\text{bilayer}}$, where the factor of $-1/2$ arises because the DMPC bilayer discs used in this study orient with their bilayer normals at $\sim$90° relative to the field and rotate rapidly about this axis. The bicelles also wobble about the average orthogonal direction and S_{bilayer} describes the residual order of the bilayer normal axis with respect to a fully extended bilayer membrane.

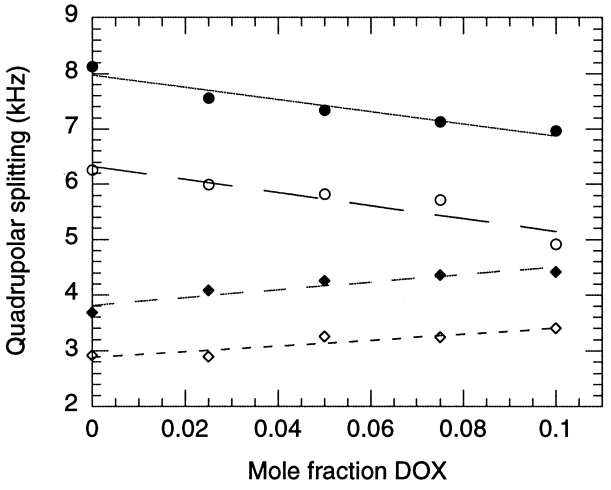

Fig. 5. Quadrupolar splittings of the α (circles) and β (diamonds) sites in d4-DMPC with various mole fractions of DOX. Open symbols were measured for oriented 11.5% w/v bicelles and filled symbols were measured using an unoriented dispersion sample.

Comparing analogous oriented and unoriented samples can allow the separation of global from local effects. The titration of DOX into DMPC bilayers with 0.10 mole fraction CL was repeated with unoriented lipid dispersions. Fig. 5 shows the observed α and β splittings upon DOX titration for both bicelle (open symbols) and unoriented samples (filled symbols). Quadrupolar splittings for the unoriented spectra are measured in kHz between the 90° maxima edges of the spectra. For both oriented and unoriented samples, similar trends are seen upon DOX titration: changes for the deuterons on the α carbon (circles) are opposite in sense to those for deuterons on the β carbon (diamonds). Furthermore, all the observed α and β splittings for bicelles are scaled down equally (within experimental error) from those seen in unoriented samples (scaled by 0.75 ± 0.04 for β sites and 0.77 ± 0.07 for α sites). This scaling by $\sim$0.75 between the unoriented dispersions and bicelles could be due either to a change in local headgroup conformation of lipids in unoriented dispersions with respect to bicelles, or more simply just a S_{bilayer} scaling due to fast axially symmetric motion of the bicelle arrays about the average bilayer normal. A value of 0.75 is consistent with other reported values for S_{bilayer} for bicelle systems [19] and is a likely reason for the difference seen between the oriented and unoriented data.

3.4. Effect of DOX on bilayer acyl chains

In order to investigate the effect of DOX binding on the acyl chain portion of the lipid bilayers, we examined ^{2}H spectra of bicelles containing a small amount of DMPC with perdeuterated acyl chains. If DOX binds with its anthracycline moiety imbedded in the membrane, the neighboring lipids could experience a change in the motional freedom of their acyl chains which in turn could change the local order and therefore $\Delta\nu$ of the acyl chain peaks. Deuterium

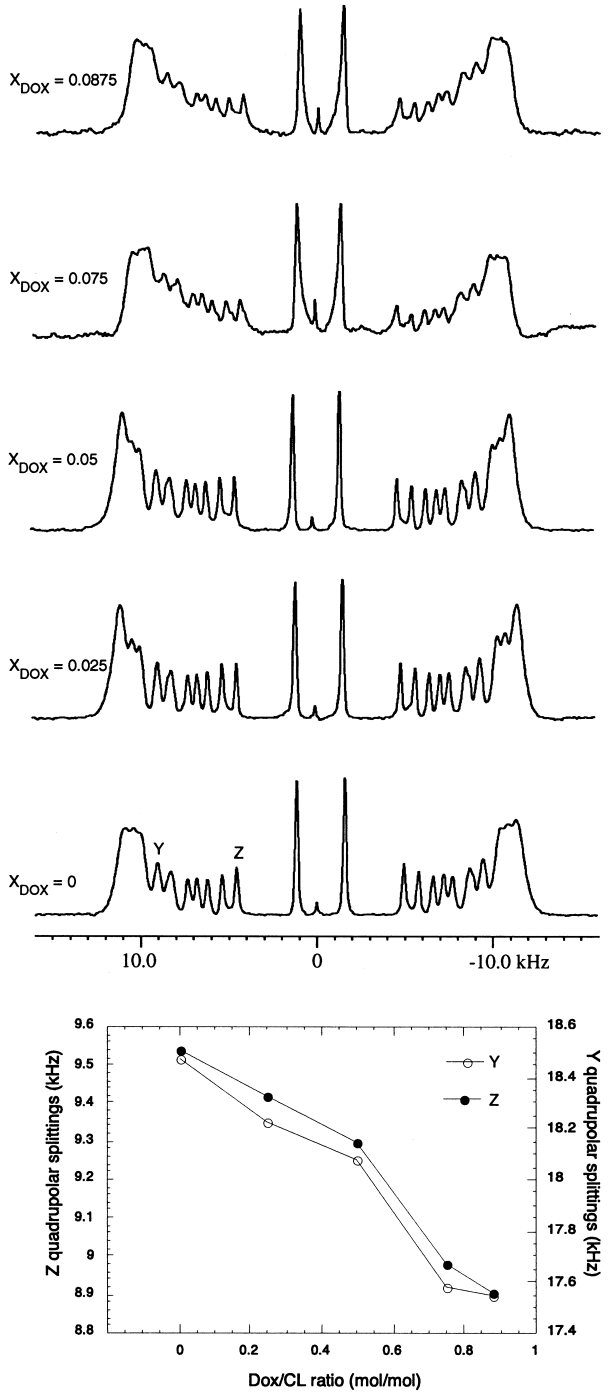

Fig. 6. ^{2}H spectra for 11.5% w/v bicelles containing d54-DMPC with increasing (from bottom to top) amounts of DOX. Graph at bottom shows change in quadrupolar splitting for two representative sites along the acyl chain (indicated Y and Z) as DOX is titrated in.

spectra of a series of d54-deuterated, CL-containing bicelles with varying amounts of DOX were collected (see Fig. 6). The appearance of each of the spectra are similar to spectra of ^{2}H-labeled bicelles found in the literature [10]. The biggest splittings in each represent deuterons from the methylenes in the high-order parameter plateau region of the acyl chains near the ester linkages, the smallest splittings in each are from the deuterons attached to the terminal methyl carbons that have the greatest degree of motional freedom, and the intermediate peaks are from the intermediate sites along the acyl chain.

Two observations can be made concerning the effect of the DOX titration on the acyl chain region of the bicelles. First, addition of DOX does not induce additional spectral components which indicates that the exchange of drug between the lipid molecules was rapid on the NMR time scale such that all acyl chains were similarly affected. Second, as mole fraction of DOX increased, quadrupolar splittings from all sites along the acyl chain decreased (percent decrease ranged from 5–10% depending on the site). Quadrupolar splittings for two representative sites along DMPC at different drug concentrations are shown in Fig. 6.

4. Discussion

In light of the proposed role of DOX–CL binding in the transport and cytotoxic properties of DOX, we have studied the interaction of this important antitumor drug using a magnetically oriented CL-containing model membrane system. Our results indicate that bicelles with 0.10 mole fraction CL can be successfully oriented and used to carry out NMR studies which provide high-resolution insight into the drug–membrane interaction. Changes in ^{2}H quadrupolar splittings for DMPC selectively deuterated in either headgroup or acyl chain positions provide evidence for the interaction of DOX with both surface and buried sites within the membrane bilayer. Below, we compare our ^{2}H data for both headgroup and acyl chain sites with related systems in the literature and discuss them in terms of the various models for the topology of interaction of DOX with CL membranes. Finally, we discuss experimental issues rele-

vant to the general use of magnetically oriented bicelles for the study of drug–membrane interactions.

One of the main applications of ^{2}H NMR choline tilt data has been the study of ligand binding to membrane surfaces and a variety of species ranging from small anions to peptides have been studied this way [20]. While it is thought that the main driving force for the observed changes in headgroup quadrupolar splittings is the reorientation of the PC headgroup dipole in response to changes in membrane surface charge, it has become clear that other effects (such as sterics) can come into play. Variations in the extent of choline tilt effects for different charged ligands has been attributed to the fact that different membrane-associated charged species can occupy different spatial positions with respect to the choline dipole [20].

If the addition of DOX to CL containing bicelles is simply reducing the bilayer surface charge, then the quadrupolar splittings of two bicelles with the same total surface charge should be identical regardless of the net mole fractions of the components. Assuming that at pH 6.5 each CL molecule has a −1 charge [21] and that DOX has a +1 charge, the quadrupolar splittings may be plotted against the 'surface charge' represented by $X_{DOX}-X_{CL}$. A comparison of the α splittings of CL bicelles to those of CL–DOX bicelles shows that quadrupolar splitting is not the same for bicelles with nominally identical surface charges (Fig. 7). Marassi and Macdonald showed that for a ternary lipid mixture of DMPC with cationic and anionic phospholipids, the quadrupolar splitting is not directly dependent on the macroscopic bilayer charge [22]. According to their theory, the degree of choline head tilt is not due to the global bilayer surface charge, but is a result of local, pairwise interactions between DMPC and neighboring charged species. The experimental quadrupolar splitting for the ternary mixture ($\Delta\nu_t$) can be approximated by the sum of the perturbations of the neutral quadrupolar splitting ($\Delta\nu_0$) by individual binary mixtures of membrane-bound cations and membrane-bound anions with DMPC

$$\Delta\nu_t(X_+, X_-) = \Delta\nu_0 + m_+X_+ + m_-X_- \quad (2)$$

where $\Delta\nu_t$ is the quadrupolar splitting of bicelles with X_- mole fraction of anionic molecules (here CL) and X_+ mole fraction of cationic molecules (here DOX),

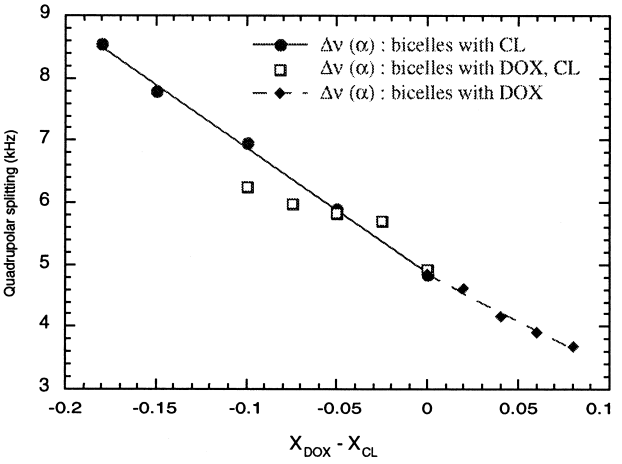

Fig. 7. Quadrupolar splittings of the α deuterons of d4-DMPC in 11.5% w/v bicelles, plotted against macroscopic bilayer charge expressed as mole fraction of positively charged molecules (DOX) minus mole fraction of negatively charged molecules (CL). Mole fractions are relative to DMPC. (●) Bicelles with varying CL content; (□) 0.10 mole fraction CL containing bicelles with varying DOX content; (♦) bicelles with varying DOX content.

$\Delta\nu_0$ is the quadrupolar splitting of neutral bicelles, m_- is the perturbation in $\Delta\nu$ per mole fraction of CL, and m_+ is the perturbation in $\Delta\nu$ per mole fraction of DOX. The slopes m_+ (−15.075) and m_- (20.208) were determined from Fig. 7 by fitting the data for the α splittings during the DOX titration and CL titration respectively. $\Delta\nu_t$ was calculated using Eq. 2 and compared to the values measured experimentally (Table 1). The experimental and calculated $\Delta\nu_t$ are within 10% for all bicelle samples, indicating that this pairwise interaction model may be appropriate for the analysis of choline head tilt in CL–DOX bicelles. There is, however, a potential problem with this method of analysis. Presumably, DOX binds preferentially to CL because of electrostatic interactions, and it may have a different binding mode to neutral bicelles, so m_+ might not be a reliable way to incorporate the effects of DOX on the PC headgroup into the model.

Our finding (demonstrated in Fig. 6) that increasing amounts of DOX decreases acyl chain order is consistent with previous ^{2}H NMR studies of DOX binding to pure anionic phospholipid dispersions [6], mixed zwitterionic/anionic lipid dispersions [23] and bacterial plasma membrane vesicles with varying ranges of anionic phospholipid content [4]. In each

of these studies, DOX induced a decrease in acyl chain order in the unoriented dispersions as manifested by a decrease in quadrupolar splitting. The percent decrease in quadrupolar splitting depended on anionic lipid content and conditions, but an observed average 10% decrease in mixtures of synthetic lipids containing 33% anionic lipids [23] is in line with the 5–10% decrease we observed here with our 10% CL containing bilayers.

The observed effect on acyl chain order suggests that binding is not purely electrostatic and localized to the membrane surface, but involves insertion of drug molecules into the lipid matrix. Another possible explanation for the DOX-induced acyl chain disorder is that DOX binds electrostatically to the bilayer surface without penetration, but increases the spacing between lipid headgroups upon binding, creating more room for acyl chain motion. De Wolf et al., however, found that binding of DOX to anionic phospholipid bilayers in their model membrane system did not cause the bilayer to expand significantly [6].

Many groups have studied the interaction of DOX with membranes using a wide variety of biophysical techniques on a range of different model membrane systems [24–26]. For example, surface potential data on monolayers showed no penetration of DOX into the hydrocarbon region of bilayers [25]. Another study used iodide fluorescence quenching to study DOX binding to CL-containing PC liposomes [9]. Since the quenching of bound DOX was biphasic they concluded that there were two types of binding environments for the drug, one relatively exposed and one more deeply buried in the membrane. The wide range of previous DOX–CL studies present a variety of different pictures for the binding of DOX to anionic phospholipid bilayers. Some of these differences may be due to the different methods and model membrane systems used to achieve these results. Most of the experiments previously carried out on DOX–CL binding, such as binding constants obtained from fluorescence experiments and measurements of enzyme activity, contain only indirect structural information from which the description of specific atomic-level details of binding modes is inferred. Also, several of the studies used highly curved SUVs that might not be a good model for physiologically relevant, near planar bilayered membranes. There have been a series of detailed NMR studies on the interaction of DOX with bilayered membranes, but most have used unoriented lipid dispersions where getting atomic level resolution data relies either on de-Paking powder patterns or the use of samples with single-site isotopic labels.

NMR on magnetically oriented bilayers provides atomic level structural information on the interaction of DOX with bilayered membranes. Information about the structure of the DOX–CL complex at the atomic level is essential for understanding DOX effects on membranes as well as potentially allowing modulation of binding by subtle changes of the molecular structure that could be of pharmacological interest. The results presented here demonstrate the ability to study the interaction of DOX with lipid bilayers by looking at isotopically labeled DMPC which makes up the bilayers. Furthermore, this report demonstrates methodology that could provide important insight into a wide range of drug–membrane interactions. The spectral perturbations seen upon drug titration to both the headgroup and acyl chain region of the DMPC bilayer indicate a binding mode (or modes) that involves both surface and buried interactions. Now that it is established that DOX does bind to CL bicelles, we are pursuing further studies where the key players in the interaction, the DOX and CL, are each in turn isotopically labeled.

Acknowledgements

This research was supported by funds from Swarthmore College and Grant GM57627-1 to K.P.H. from the National Institutes of Health. V.K. was supported with funds from the Howard Hughes Medical Institute.

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