



17 β -estradiol (E2) in membranes: Orientation and dynamic properties



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ABSTRACT

Non-genomic membrane effects of estrogens are of great interest because of the diverse biological activities they may elicit. To further our understanding of the molecular features of the interaction between estrogenic hormones and membrane bilayers, we have determined the preferred orientation, location, and dynamic properties of 17 β -estradiol (E2) in two different phospholipid membrane environments using ²H-NMR and 2D ¹H-¹³C HSQC in conjunction with molecular dynamics simulations. Unequivocal spectral assignments to specific ²H labels were made possible by synthesizing six selectively deuterated E2 molecules. The data allow us to conclude that the E2 molecule adopts a nearly "horizontal" orientation in the membrane bilayer with its long axis essentially perpendicular to the lipid acyl-chains. All four rings of the E2 molecule are located near the membrane interface, allowing both the E2 3-OH and the 17 β -OH groups to engage in hydrogen bonding and electrostatic interactions with polar phospholipid groups. The findings augment our knowledge of the molecular interactions between E2 and membrane bilayer and highlight the asymmetric nature of the dynamic motions of the rigid E2 molecule in a membrane environment.

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1. Introduction

17 β -Estradiol (estra-1,3,5(10)-triene-3,17 β -diol, estradiol) (E2) (Fig. 1), one of the most important estrogenic hormones, is responsible for growth and the development of sexual characteristics and reproductive capacity in the female. The biological activity of estrogens has traditionally been held to reflect its regulation of gene expression by interacting with two classical nuclear hormone receptors, ER α and ER β [1]. More recently, accumulating evidence supports the proposition that interactions between E2 and constituents of biological membranes act as determinants of E2 bioactivity. Fatty acyl esterification of the steroid backbone at its 17-OH position to generate E2 is catalyzed by a membrane-associated acyltransferase [2]. A growing body of literature [3–5] points to the presence of E2 receptors—particularly the G-protein coupled E2 receptor 1 (GPER1) and its ER α alternative splicing variant,

ER α 36—in the plasma membrane and intracellular membranous organelles including the endoplasmic reticulum and Golgi apparatus. As with other amphipathic molecules [6–8], E2 may partition into biomembranes and access intramembranous estrogen receptors through lateral diffusion within the membrane-phospholipid bilayer. In addition, neuroprotective properties of estrogens against cellular damage may reflect E2's ability to preserve mitochondrial function and structural integrity [9–12], help maintain the electrical activity of the excitable neuronal membrane [13], and stabilize cell membranes [14,15]. Notably, differential spatial partitioning of E2 molecules within lipid bilayer systems has been correlated with E2 neuroprotective potency against oxidative insult [14].

Associations between E2 biosynthesis/activity and various membrane phenomena have made it critical to delineate the orientation and dynamics of E2–membrane interaction at the (sub)molecular level. For this purpose, NMR has been used to probe the physicochemical parameters of E2 orientation and location in membrane systems [16–18]. In 2006, Cegelski et al. [16] studied E2 and its structural analogs in 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) bilayers and deduced from the distance distributions afforded through solid-state rotation echo double resonance (REDOR) NMR that E2 favors an orientation toward the bilayer center with the E2 phenol moiety near the phospholipid fatty acyl-chains. Scheidt et al. [17] subsequently applied a suite of solid-state NMR methods to study E2 in synthetic 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) membranes and concluded that E2 is stably inserted and broadly distributed within the membrane and may undergo dynamic rotations without condensing

Abbreviations: E2, 17 β -Estradiol; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DHPC, 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; PSPC, 1-palmitoyl-2-stearoyl-*sn*-glycero-3-phosphocholine; MD, molecular dynamics.

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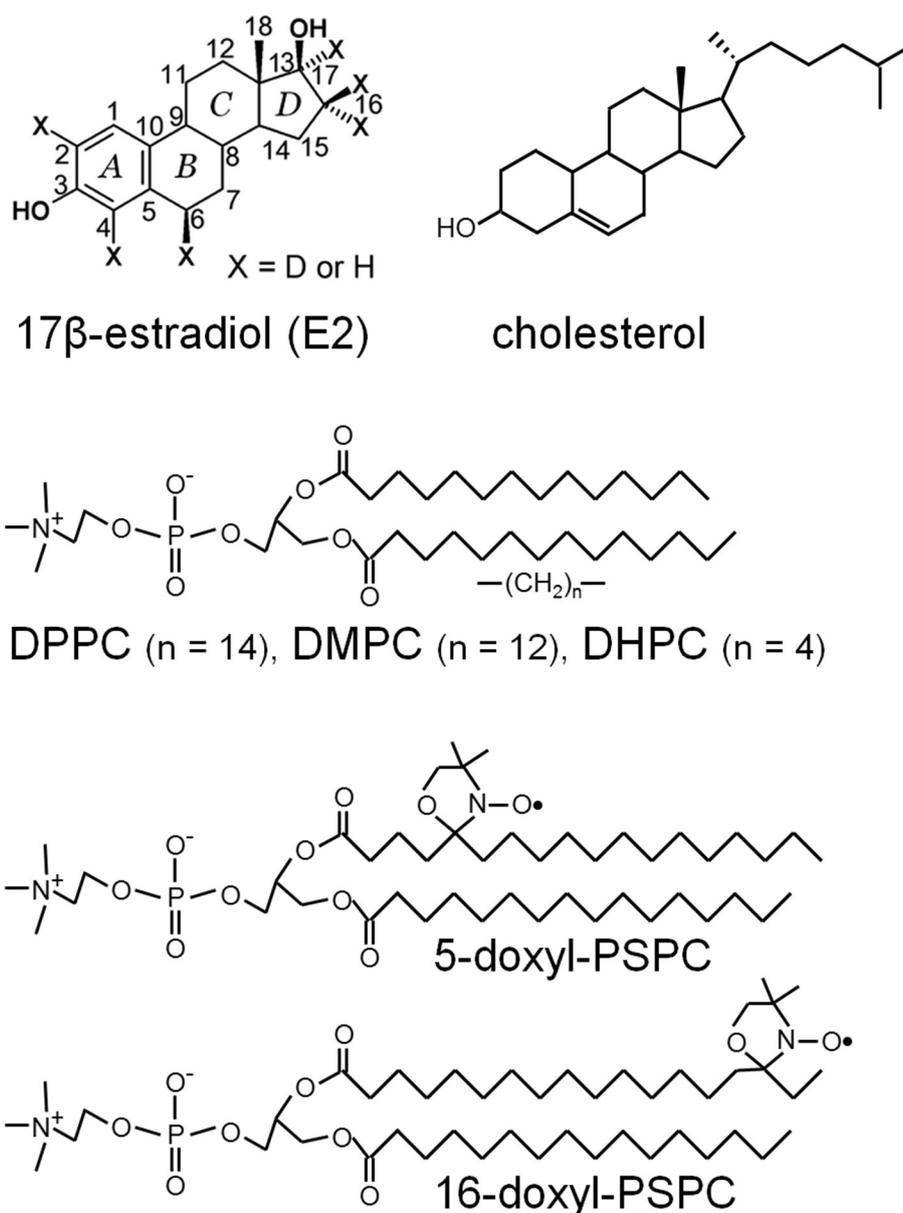


Fig. 1. Molecular structures of 17β-estradiol (E2), cholesterol, DPPC, DMPC, DHPC, and two PSPC molecules with 5-doxy and 16-doxy free radical spin labels.

the surrounding phospholipids. Using a combination of experimental NMR spectroscopy and *in silico* molecular dynamics (MD) simulation, Vogel et al. [18] recently concluded that E2 preferentially adopts a horizontal orientation in multilamellar vesicles with the E2 long axis perpendicular to the membrane normal and rarely embedded deeply among the membrane-lipid acyl-chains. Their experimental and modeling data suggested the probability of a more complex, dynamic distribution involving a superimposition of two orientations.

In light of the importance of E2 to mammalian physiology and the impact that E2–membrane interaction has on the synthesis and bioactivities of E2, we have conducted NMR and MD simulation investigations of E2 in both multilamellar vesicles and bicelles to determine the orientation, location, and dynamic properties of this important hormone in membrane-phospholipid bilayers. As distinct from prior investigations, we strategically synthesized six deuterated E2 molecules, each containing either one or two ^2H -labels at different positions (Fig. 1), and employed these to assign unambiguously the experimentally observed quadrupolar splittings ($\Delta\nu_Q$) to individual ^2H labels. This approach enabled us to use a straightforward mathematical method for determining the preferred orientation and dynamics properties of E2 in membrane.

Furthermore, since E2 in a lipid bilayer is relatively insensitive to the type of membrane environment [18], we also utilized paramagnetic relaxation agents as probes to determine the spin–spin relaxation rates of protons on different parts of the E2 molecule in phospholipid bicelles. Our data provide verification of and extend previous evidence that E2 prefers a nearly “horizontal” orientation in which the long axis of the E2 molecule is essentially perpendicular to the phospholipid fatty acyl-chains. In this orientation, both the 3-OH and 17β-OH groups of E2 are well positioned to engage in hydrogen bonding and electrostatic interactions with the membrane-phospholipid polar groups. More broadly, our method of analysis may also be useful in studies aimed at determining the orientation and dynamics of other hydrophobic or amphipathic molecules in membrane bilayers.

2. Materials and methods

2.1. ^2H -NMR experiments of E2 in DPPC multilamellar vesicles

17β-estradiol molecules individually labeled with one or two ^2H -labels at 2, 4, 6β, 16α, 16β, and 17 positions were synthesized in our

laboratory. Details of their chemical synthesis are beyond the scope of this paper and will be reported elsewhere. All ^2H -NMR samples were prepared by first dissolving DPPC (Avanti Polar Lipids, Alabaster, AL) and estradiol (molar ratio 10:1) in chloroform: methanol (3:1) (Sigma–Aldrich, St. Louis, MO). The solvent was then evaporated under a nitrogen stream, and the residue was placed under vacuum (0.1 mmHg) for 12 h. The resulting powder was then hydrated by adding an appropriate amount of deuterium-depleted water (Cambridge Isotope Laboratories, Andover, MA) followed by a combination of mechanical blending, heating, and cooling until a homogeneous preparation (lipid concentration 50% w/v) was obtained. Stationary ^2H -NMR experiments were carried out on a Bruker AVANCEII 400 MHz high-resolution NMR spectrometer using a 5-mm direct-observe probe with the broadband channel tuned at 61.402 MHz. ^2H -NMR spectra were recorded at 42 °C using a quadrupole echo pulse sequence that consisted of a pair of 90° pulses separated by an interval τ of 45 μs and $\pi/2$ out of phase.

2.2. 1D ^1H - and 2D ^1H - ^{13}C HSQC NMR experiments of E2 in DMPC/DHPC bicelles

Acyl-chain perdeuterated 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC- d_{54}), 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC- d_{22}), and 1-palmitoyl-2-stearoyl-(5-doxy)-*sn*-glycero-3-phosphocholine (5-doxy-PSPC) were purchased from Avanti Polar Lipids. 1-Palmitoyl-2-stearoyl-(16-doxy)-*sn*-glycero-3-phosphocholine (16-doxy-PSPC) was synthesized in our laboratory (see Fig. 1 for structures of these lipids). Unlabeled E2, cholesterol (Fig. 1), and manganese chloride (MnCl_2) were from Sigma–Aldrich. All samples were prepared by first mixing appropriate amounts of E2 or cholesterol, DMPC- d_{54} , DHPC- d_{22} , and/or 5-doxy-PSPC or 16-doxy-PSPC as described in Section 2.1. The powder mixture was hydrated by adding an appropriate amount of $\text{H}_2\text{O}/\text{D}_2\text{O}$ (90/10). The preparation then underwent a combination of mechanical blending, heating, and cooling until a clear and homogeneous system was obtained. All bicelle samples had a total lipid concentration of 10% (w/v), and the molar DMPC:DHPC ratio (q) was 0.5. The molar ratio of E2 relative to the long acyl-chain lipid DMPC was 1:10. For samples containing paramagnetic agents, the concentration of MnCl_2 was 0.2 mM, and that of 5 doxy-PSPC or 16-doxy-PSPC was 1 mol% with respect to total phospholipid. All 1D proton and 2D ^1H - ^{13}C HSQC experiments were performed at 38 °C on a Bruker AVANCEII 700 MHz NMR spectrometer. Water suppression was achieved by using excitation sculpting [19] for the 1D and WATERGATE [20,21] for the 2D experiments.

2.3. Molecular dynamics (MD) simulations

A 250 ns molecular dynamics (MD) simulation of E2 in DPPC membrane bilayer was performed using Desmond 3.6 molecular dynamics software package [22] with the optimized potentials for liquid simulations-all atom (OPLS-AA) parameter set [23]. The initial structure for E2 in bilayer membrane was constructed by placing one minimized E2 molecule into pre-equilibrated membrane bilayer containing 64 DPPC molecules and 1871 SPC (simple point charge) water molecules. The system was minimized first by steepest descent method until a gradient threshold of 25 kcal/mol/Å was reached followed by limited-memory Broyden–Fletcher–Goldfarb–Shanno (LBFGS) algorithms until a convergence on the gradient of 1.0 kcal/mol/Å. The molecular dynamics simulation was then performed using the so-called NPAT ensemble with fixed number of molecules (N), constant pressure ($P = 1$ atm), constant membrane surface area (A), and constant temperature ($T = 315$ K). Coordinates were retrieved every 2 ps from a total of 250 ns dynamics simulation for further analysis.

3. Results

3.1. Orientation of E2 determined from ^2H quadrupolar splittings

Selected ^2H -NMR spectra from E2 molecules with individual, positionally defined ^2H -labels are shown in Fig. 2 (left panel), where the corresponding quadrupolar splittings ($\Delta\nu_Q$) are indicated. Fig. 2 (middle panel) shows simulated Pake patterns matching the experimental spectra, an approach that enhances the accuracy of the $\Delta\nu_Q$ measurements. We observed that all measured $\Delta\nu_Q$ values were less than 25 kHz and appreciably below the expected “rigid-lattice” value of ~ 130 kHz [24]. This narrowing effect is evidence for the motion or wobbling of E2 within the membrane’s liquid crystalline bilayer. The unique $\Delta\nu_Q$ value (ranging from 1 to 23 kHz) given by each ^2H -label suggests that the wobbling motion of E2 in the membrane is not isotropic. This result is congruent with the nature of the membrane bilayer as an anisotropic medium in which E2 would tend to assume a preferred orientation rather than tumbling freely. The ^2H -NMR spectra of three ^2H -labeled E2 species (2,4- d_2 , and 6 β - d) yielded three $\Delta\nu_Q$ values, 23.10, 12.73, and 20.71 kHz, respectively. (Fig. 2(a and b), also see Supplementary Data for detailed spectral assignments). These $\Delta\nu_Q$ values are comparable to those reported by Vogel et al. [18] of 2, 4, 16 α , 16 β - d_4 -estradiol in POPC multilamellar vesicles (2.6, 17.3, 18.5, and 23.4 kHz, respectively). Their experiment, however, did not permit distinctive $\Delta\nu_Q$ assignments to individual deuterium labels.

To determine the preferred orientation of E2 from our experimental quadrupolar splitting values, we needed to first take into account the dynamical motions of E2 in a bilayer membrane. In a previous solid-state REDOR NMR study [16], the E2 molecule in model membranes was considered being disposed with its long axis essentially parallel to the lipid acyl-chains, similar to the orientation of cholesterol [25,26], with uniaxially symmetric motions. This construct prompted us to incorporate into our analysis the ratio method [27,28], which assumes a uniaxially symmetric wobbling motion. The ratio method is based on the theory that each $\Delta\nu_Q$ is related to the molecule’s geometry and dynamics by

$$\Delta\nu_Q = \frac{3}{4}A_Q \left(\frac{3 \cos^2\theta - 1}{2} \right) S_{\text{mol}}$$

where θ represents the angle between the particular C–D bond and the common direction of lipid acyl-chains of the bilayer and S_{mol} measures the dynamics (the “molecular order parameter,” common to all C–D bonds) [24,29]. The quadrupolar constant A_Q assumes the values of 185 kHz or 170 kHz for an aromatic or aliphatic C–D bond, respectively [30]. When the ratio between any two $\Delta\nu_Q$ values is taken, the order parameter S_{mol} cancels out, and the orientation of the molecule can be determined. In our case, we calculated three θ values, allowing us to “orient” the molecule in the bilayer through triangulation. (See Supplementary Data for the mathematical details.) Results from the ratio method using E2 with ^2H -labels at the 2-, 4-, and 6 β -positions are shown in Fig. 3(a). We found a nearly perfect fit between the calculated $\Delta\nu_Q$ values and the corresponding measured values by using $\theta_2 = 107^\circ$, $\theta_4 = 133^\circ$, $\theta_{6\beta} = 108^\circ$, and $S_{\text{mol}} = 0.445$.

In order to extend and refine these results, we introduced three other ^2H -labels into the E2 molecule (16- d_2 and 17- d). Their ^2H -NMR spectra are shown in Fig. 2(c and d), and the quadrupolar splitting values of 1.40, 19.60, and 22.53 kHz were assigned to 16 α , 16 β , and 17, respectively (see Supplementary Data for details). We then extrapolated from the above determined orientation to the three new C–D bonds and found that their θ angles to be $\theta_{16\alpha} = 49^\circ$, $\theta_{16\beta} = 83^\circ$, and $\theta_{17} = 67^\circ$. When combined with $S_{\text{mol}} = 0.445$, this particular orientation yields theoretical $\Delta\nu_Q$ values of 7.57, 27.10, and 15.64 kHz, for 16 α , 16 β , and 17, respectively [Fig. 3(b)]. The substantial differences between these two sets of values suggest that the ratio method and its

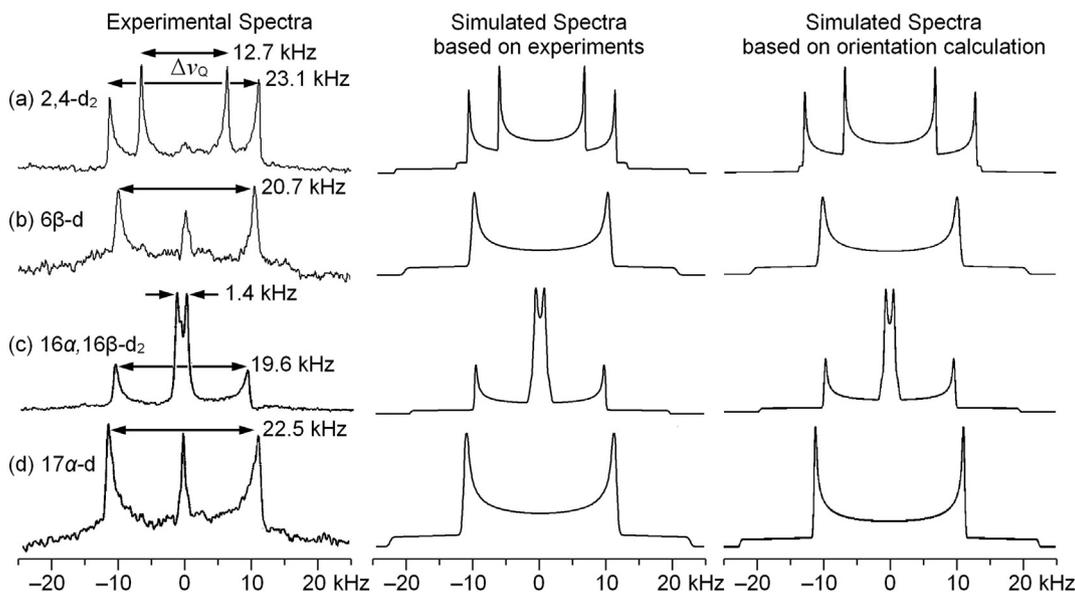


Fig. 2. Experimental ^2H -NMR spectra (left) of 17β -estradiol (E2) with six deuterium labels (at 2, 4, 6β , 16α , 16β , and 17 positions) in DPPC multilamellar membrane bilayers. The peak at 0.0 kHz in the experimental spectra is due to trace amounts of D_2O present in the hydrated samples. Simulated Pake patterns (middle) were produced to match the experimental spectra for more accurate measurements of the quadrupolar splitting values ($\Delta\nu_Q$). The simulated spectra on the right panel were based on the calculated orientation of E2 in the bilayer.

attendant assumption of uniaxial motions of E2 in the membrane are not applicable to E2's membrane disposition.

This discordancy led us to adopt a matrix approach. For this purpose, we incorporated the expanded set of all six ^2H quadrupolar splitting values into our calculations and removed the constraint of uniaxial symmetry [31,32]. The theory behind the matrix method rests on the concept that the order parameter S is actually a second-order tensor, represented by a 3×3 matrix. Each $\Delta\nu_Q$ value is a matrix "inner" product of the tensor and the vector V of each C–D bond comprising three "direction cosines" (see Supplementary Data for mathematical details):

$$\Delta\nu_Q = \frac{3}{4}A_Q(V \cdot S \cdot V^T)$$

The direction cosines of each C–D bond were derived from coordinates of heavy atoms in E2 based on our previous solution ^1H -NMR study [33]. Using our experimental quadrupolar splitting values ($\Delta\nu_Q$) from six individual deuterium labels, we were able to calculate the

order parameter tensor S and find its principal Z-axis which represents the lipid acyl-chain direction (the bilayer normal). This allowed us to determine the most probable orientation of E2 within DPPC membrane bilayers. In this orientation, a complete set of theoretical $\Delta\nu_Q$ values of the six deuterium labels was also computed, and as shown in Fig. 4, they are in good agreement with experimental values. For comparison with the experimental spectra, we used these theoretical $\Delta\nu_Q$ values and produced the corresponding Pake patterns, as shown in Fig. 2, right panel. Our calculations yielded the following geometric data: $\theta_2 = 44^\circ$, $\theta_4 = 134^\circ$, $\theta_{6\beta} = 78^\circ$, $\theta_{16\alpha} = 129^\circ$, $\theta_{16\beta} = 78^\circ$, and $\theta_{17} = 102^\circ$, where θ is the angle between each C–D bond and the membrane bilayer normal.

Fig. 5 shows the orientation of E2 in a DPPC bilayer by two perspectives of the molecule (left: viewed along the principal X-axis, right: viewed along the principal Y-axis) as determined by the matrix method. In this diagram, the vertical lines represent the direction of the lipid acyl-chains, and a θ angle is indicated between each C–D bond and the acyl-chain direction. In this nearly "horizontal" orientation, the long

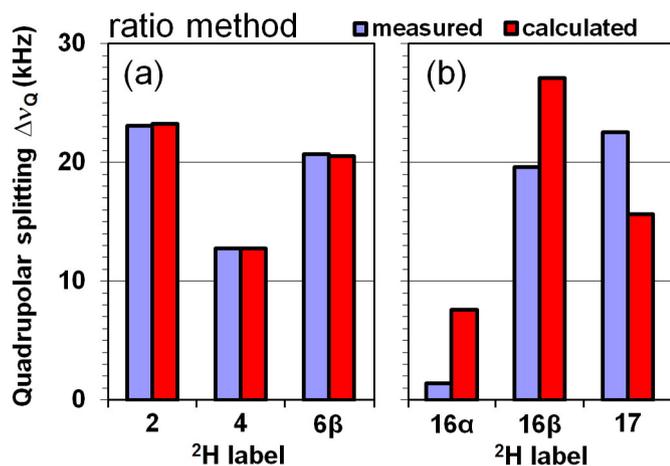


Fig. 3. (a) Using the ratio method, a good agreement was obtained between measured and calculated quadrupolar splittings $\Delta\nu_Q$ (absolute values) from three ^2H labels (2, 4, 6β) on 17β -estradiol (E2). (b) Extrapolating from the orientation obtained in (a), we calculated quadrupolar splittings $\Delta\nu_Q$ for 16α , 16β , and 17, but they deviate significantly from the measured $\Delta\nu_Q$ values.

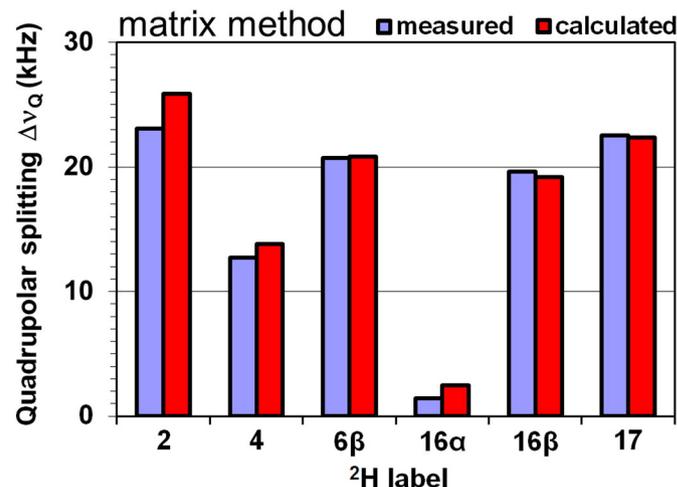


Fig. 4. The matrix method provides an overall good agreement between measured and calculated ^2H -quadrupolar splittings $\Delta\nu_Q$ (absolute values) from all six ^2H labels on 17β -estradiol (E2), which is wobbling within the DPPC multilamellar membrane in an asymmetric anisotropic manner with a preferred orientation.

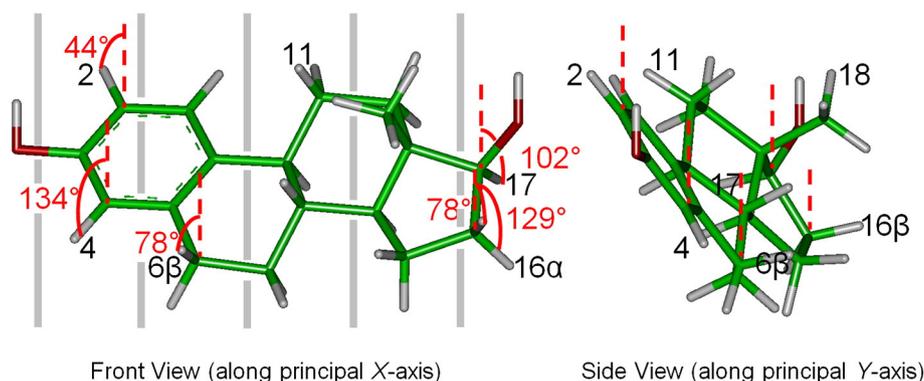


Fig. 5. Preferred orientation of 17 β -estradiol (E2) in the DPPC membrane bilayer as determined by solid-state ^2H -NMR. The vertical lines represent the direction of acyl-chains of the membrane lipid. The angles between the acyl-chain direction and the six C–D bonds are indicated in the diagram. The E2 molecule is shown by two perspectives: one front view (along the principal X-axis) and one side view (along the principal Y-axis). We can clearly see that the side of E2 molecule that contains C2, C11, and C18 faces toward the membrane surface.

axis of estradiol is essentially perpendicular to the lipid acyl-chains while the side of E2 molecule that contains C2, C11, and C18 facing toward the membrane surface. This allows both the 3-OH and 17 β -OH groups to engage in hydrogen bonding and electrostatic interactions with the polar phospholipid groups, thus minimizing the overall energy of the E2-phospholipid membrane supramolecular assembly.

3.2. Location and orientation of E2 determined from proton relaxation rates

To corroborate and extend our data on the membrane topology of E2, we conducted relaxation experiments using high-resolution 1D ^1H - and 2D ^1H - ^{13}C HSQC. The paramagnetic relaxation agents added were MnCl_2 , 5-doxyl-PPSPC, or 16-doxyl-PPSPC. MnCl_2 produces a magnetic Mn^{2+} ion in aqueous solution, and each of the doxyl groups contains a free radical. These paramagnetic agents are frequently used for semi-quantitative measurements of the depth of insertion of molecules in lipid vesicles [34,35], micelles [36], and bicelles [37,38].

Fig. 6(a) shows representative ^1H -NMR spectra of E2 in an isotropic bicelle preparation in the absence or presence of 5-doxyl-PPSPC. Although the DMPC and DHPG used for our bicelle preparation have perdeuterated acyl-chains, the resonances arising from their headgroups overwhelm those from E2. Only the aromatic protons of E2 display resonances far enough downfield to be discernible in the spectra (see the magnified inset in Fig. 6(a)). Since the remaining E2 resonances overlap with the lipid signals in the 1D spectrum, we used a 2D ^1H - ^{13}C HSQC experiment to perform quantitative relaxation measurements. As shown in Fig. 6(b), previously unresolved resonances of the non-aromatic protons of estradiol are now clearly distinguishable from one another and from the lipid resonances. Complete assignments of the ^1H and ^{13}C chemical shifts could thus be achieved straightforwardly based on our previous conformational study of E2 in DMSO [33], and ^1H -resonance linewidths could be measured accurately by extracting 1D proton “slices” from the corresponding 2D-HSQC spectrum.

The data in Fig. 7(a) demonstrate that the paramagnetic Mn^{2+} ion and the 5-doxyl and 16-doxyl free radicals are located at three different depths in the membrane. Thus, each would significantly influence the proton relaxation within its respective bilayer region. Identical 2D ^1H - ^{13}C HSQC NMR spectra were recorded from the above isotropic bicelle sample in the absence or presence of each of the three paramagnetic agents. We then extracted 1D proton “slices” from each of the corresponding HSQC spectrum. The linewidth ($\Delta\nu_{1/2}$) of each resonance was determined using an automatic simulation program in Bruker TopSpin 2.1. Representative peaks used for linewidth measurements are shown in Fig. 6(b) below the 2D spectrum. The increases in spin–spin

relaxation rate (ΔR_2) for each individual proton after the addition of paramagnetic relaxation agent is

$$\Delta R_2 = \pi \left(\Delta\nu_{1/2}^p - \Delta\nu_{1/2}^c \right)$$

where $\Delta\nu_{1/2}^c$ is the linewidth from the control and $\Delta\nu_{1/2}^p$ is that from the corresponding sample containing one of the three paramagnetic agents.

As expected, the three paramagnetic relaxation agents perturbed three specific regions of the bilayer. The relative increases of spin–spin relaxation rates (indicated by the height of vertical bars) of the α , g3, g1, C3, C13, and C14 protons of DMPC are shown in Fig. 7(b). The Mn^{2+} ions, located near the negatively charged phosphate groups, selectively enhanced the relaxation of the α , g3, and g1 protons in the lipid–water interface region. The free radical effect of 5-doxyl is limited mainly to the g1 and C3 protons of the upper acyl-chains, while that of the 16-doxyl is concentrated at the C13 and C14 protons located at the bilayer center. Fig. 7(c) shows that, for the 5-doxyl sample, the relaxation rate R_2 of all listed E2 protons increases much more significantly (red graph) when compared to any broadenings of E2 resonances caused by Mn^{2+} (blue) and 16-doxyl (green). The fact that the 5-doxyl group has an essentially equal effect on all protons of E2 clearly demonstrates that all four rings of the E2 molecule are located near the upper acyl-chain region. These experiments strongly support the solid-state ^2H -NMR results that E2 adopts a nearly “horizontal” orientation in the membrane with its long axis perpendicular to the bilayer lipid acyl-chains.

The robustness of this approach was further confirmed by applying this same method to cholesterol, which has been demonstrated across several consensus studies to assume a “vertical” orientation in membrane bilayers [25,26,39]. Fig. 7(d) shows the relaxation data (ΔR_2) from two groups of protons in cholesterol: 1, 3, 4, and 6 near the 3-hydroxyl group and 25, 26, and 27 at the end of the hydrocarbon chain. The data show that Mn^{2+} , 5-doxyl, or 16-doxyl affect differently the two groups of protons in cholesterol. Specifically, the Mn^{2+} (blue) and 5-doxyl (red) affect cholesterol’s A-ring, and the 16-doxyl (green) affects the resonances of the hydrocarbon tail. These results clearly demonstrate the vertical orientation of membrane-associated cholesterol, with the 3-OH group of the molecule oriented toward the polar lipid region and its hydrocarbon tail deeply imbedded within the central bilayer core.

3.3. Molecular dynamics simulations

In support of our experimental findings, we performed a 250 ns MD simulation of E2 in membrane bilayers with an E2:DPPC molar ratio of 1:64. Before the start of the 250 ns dynamics calculation, we

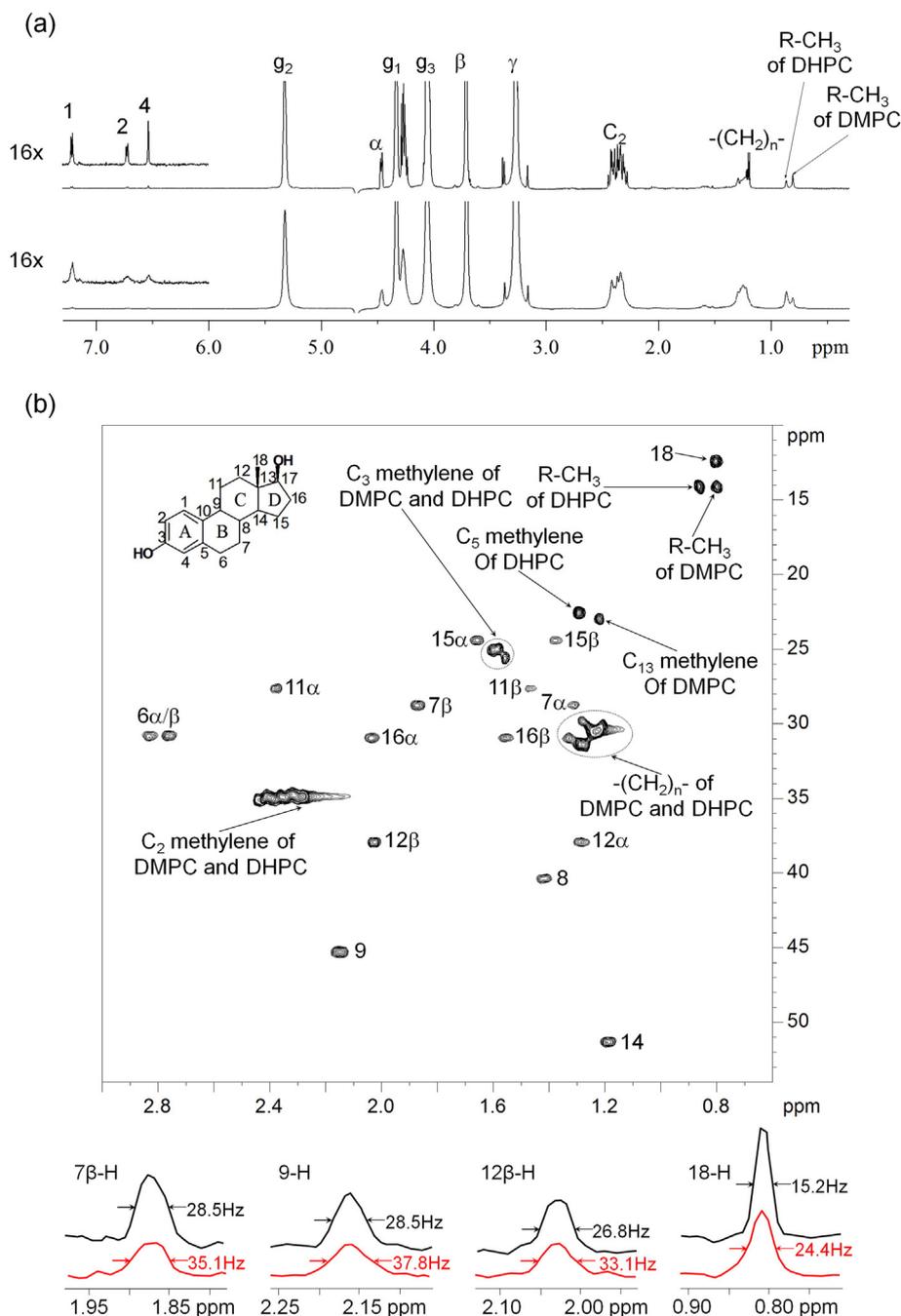


Fig. 6. (a) 1D ^1H -NMR spectrum from E2 in DMPC/DHPC isotropic bicelle solution as a control (top) and from an equivalent bicelle sample in the presence of 5-doxyl-PSPC at 1 mol% with respect to the total phospholipids (bottom). (b) Expansion of the ^1H - ^{13}C HSQC spectrum from the control sample highlighting the assignment of the aliphatic resonances of both E2 and the lipid acyl-chains. Representative peaks (black: control; red: with 5-doxyl-PSPC) used for linewidth measurements are shown below the 2D spectrum.

intentionally placed the E2 molecule in a “vertical” orientation with its long axis essentially parallel to the lipid acyl-chains and its A-ring close to the membrane interface. In order to sample fully all possible orientations, no distance constraints were imposed during the entire MD simulation process. After 0.25 ns, E2 has completed its transition to a “horizontal” orientation and remained in the vicinity of such an orientation. To quantitatively study the E2 orientation and its fluctuational motions in membrane, we first defined the long axis of the E2 molecule as the line crossing its C16 and C3 atoms and then measured the angle (β) between this E2 long axis and the bilayer normal. This long axis is the same as the “internal” Z_{int} -axis in an earlier work by Vogel et al. [18]. The insets on top of Fig. 8 show the three internal axes on the E2 molecule and the Euler angle α and β that are used to describe the orientation

of E2 relative to the bilayer normal direction. While the angle β indicates how the long axis of E2 orients with the lipid acyl-chain direction, the angle α measures the turning of E2 around its own long axis. The left panel in Fig. 8 depicts the time dependence of β from 0 to 0.25 ns for every 0.002 ns and from 0.25 ns to 250 ns for every 0.5 ns. Despite the fluctuations, the average value of β is 86° while approximately 80% of the time E2 is oriented with $60^\circ < \beta < 120^\circ$. In a few occasions (e.g., ~ 45 and 140 ns), E2 turned to an almost “vertical” orientation ($\beta \approx 0^\circ$ or 180°) for several nanoseconds and then flipped back to the “horizontal” orientation. This indicates that in a phospholipid membrane environment, E2 prefers a “horizontal” orientation with its long axis essentially perpendicular to the lipid acyl-chains. Because the angle α may vary from -180° to $+180^\circ$, its time dependence is not

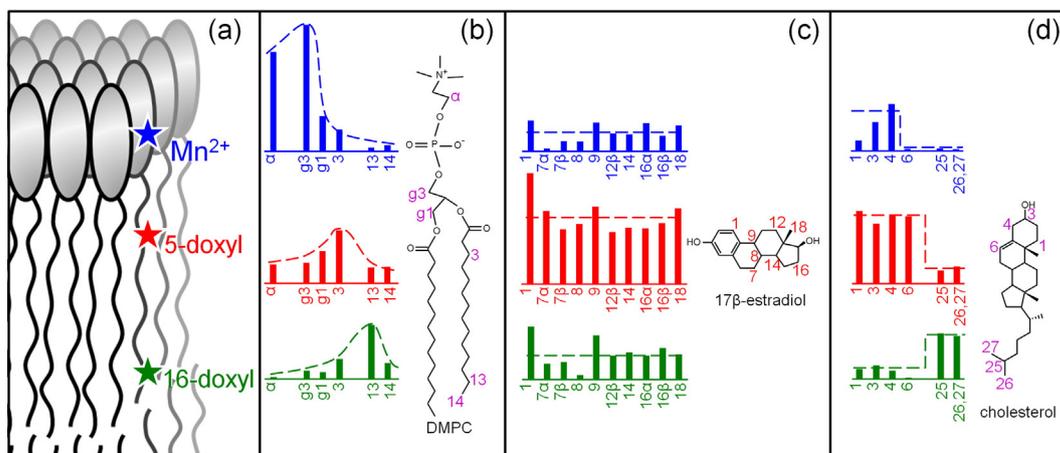


Fig. 7. Effects of paramagnetic agents on the proton resonances of DMPC, E2, and cholesterol in isotropic bicelles. (a) Schematic representation of Mn^{2+} , 5-doxyl, and 16-doxyl in different regions of the bilayer. (b) Bar graphs showing that Mn^{2+} affects the DMPC headgroup (blue), 5-doxyl affects the upper acyl-chain (red), and 16-doxyl affects the lower acyl-chain (green). The height of each vertical bar is proportional to ΔR_2 , an increase in the spin–spin relaxation rate of proton resonances. (c) 5-doxyl increases the relaxation rates of E2 protons from all four rings equally and more significantly than Mn^{2+} and 16-doxyl. (d) Mn^{2+} and 5-doxyl mostly affect cholesterol A-ring, whereas 16-doxyl affects the hydrocarbon tail.

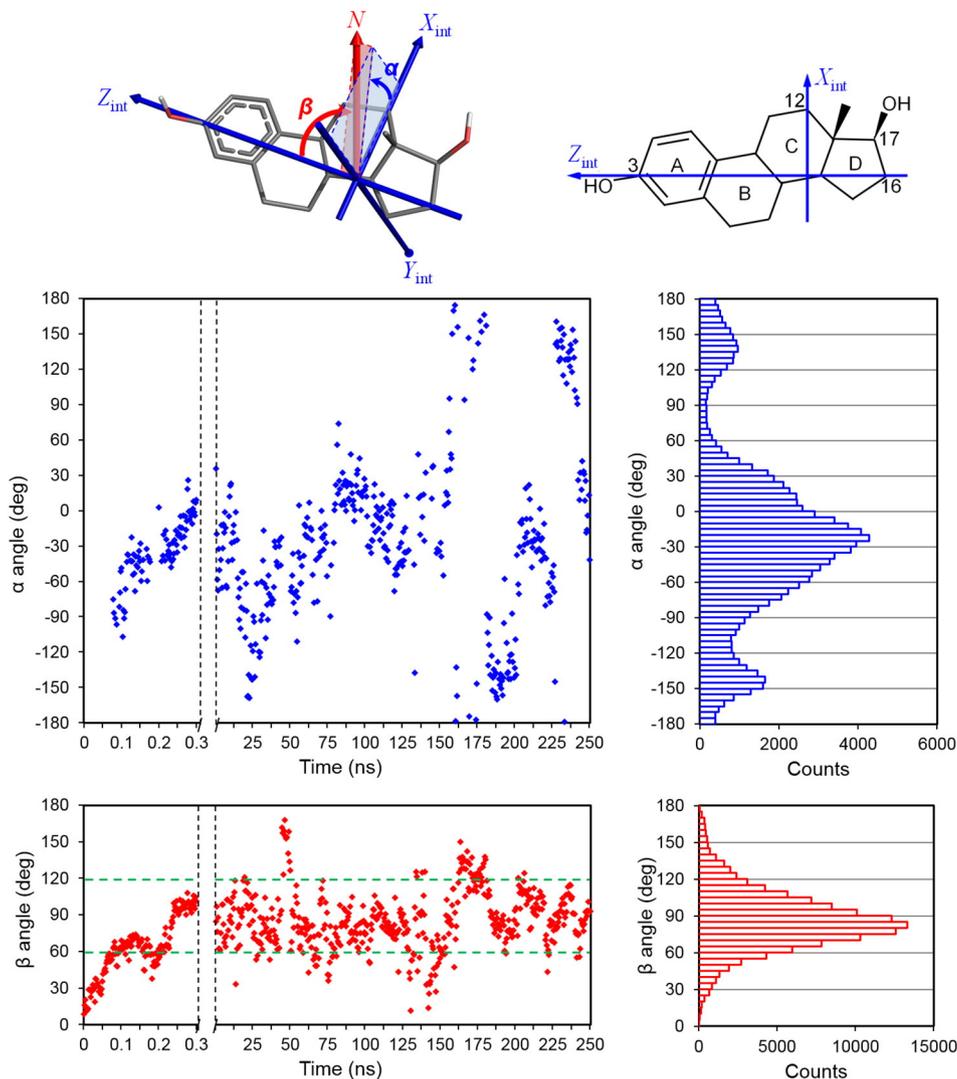


Fig. 8. Time dependence and histograms of the two Euler angles α and β , extracted from our MD simulation. The angles α and β are defined in the insets on top, with respect to the molecular-fixed internal axes X_{int} and Z_{int} , which are exactly the same as the α_M and β_M in the earlier work by Vogel et al., for easy comparison. The angle β indicates how the long axis of E2 orients with the lipid acyl-chain direction, and the angle α measures the turning of E2 around its own long axis. During the first 0.25 ns (note the data are shown with a 250 \times expanded horizontal scale), which is 1/1000 of the total simulation time, E2 transitioned to an orientation with its long axis nearly perpendicular to the acyl-chains and persisted in the vicinity of such an orientation for about 80% of the remaining simulation time. The overall average value of β is 86°. The histograms (right panels) show the distribution of angle β from all 125,000 frames extracted from MD simulation as well as the distribution of angle α from the 101,072 frames wherein $60^\circ < \beta < 120^\circ$.

straightforward to interpret visually. Therefore, we calculated the distributions of α and β in 5° intervals, shown in the right panels in Fig. 8. The histogram for β is well defined, with a prominent peak at 85° and about 80% probability of falling between 60° and 120° . We then chose to extract α angles from those cases where β falls in this range, and the histogram for α clearly shows a large peak at $\alpha = -25^\circ$, whereas the other peaks are considerably smaller.

In the context of these internal axes (X_{int} , Y_{int} , Z_{int}), the orientation we determined from our ^2H -NMR quadrupolar splittings by matrix method can be described by the following Euler angles: $\alpha = -32.3^\circ$ and $\beta = 79.5^\circ$. Therefore, our *in silico* MD simulation data are clearly congruent to our experimental ^2H -NMR results, and this orientation is also consistent with one set of Vogel's fitting parameters ($\alpha = 0^\circ$ with an offset of 24.8° and $\beta = 90^\circ$). Fig. 9 shows a representative snapshot that exhibits an E2 orientation similar to the one we obtained from the ^2H -NMR experiments. All four rings of the E2 molecule remain at the interface between the polar and hydrophobic regions of the membrane, allowing both the 3-OH and 17 β -OH groups to engage in hydrogen bonding and electrostatic interactions with the polar phospholipid head groups.

4. Discussion

Our ^2H -NMR and ^1H - ^{13}C HSQC experimental results provide a thorough description of the location, orientation, and dynamic properties of E2 in membrane bilayers. The study is complementary to, yet distinct from, earlier solid-state NMR reports on E2 membrane topology [16–18]. A REDOR NMR study designed to measure intermolecular distances between an estradiol 3- ^{13}C label and a phosphorus- or fluorine-labeled phospholipids localized the E2 A-ring in proximity to the membrane interface [16]. Other solid-state NMR data indicate that the most preferred membrane orientation of E2 has its long axis perpendicular to the bilayer lipid acyl-chains such that appreciable condensation of the surrounding phospholipids does not occur [17,18], in contrast to cholesterol's reported behavior [24,25,37]. The experimental approach adopted in this report is made distinct from prior related studies on E2 membrane localization and dynamics [16–18] through our use of a family of selectively deuterated E2 molecules, which enabled us to assign unambiguously all six quadrupolar splittings ($\Delta\nu_Q$) and apply a simplified mathematical method for determining the E2 orientation and dynamics in phospholipid membranes.

Another distinction of this work from prior reports is the incorporation of several paramagnetic relaxation agents that perturb spin–spin relaxations of proton resonances and, by this property, can serve as probes for characterizing the location and orientation of E2 in bicellar membranes. Our data indicate that all four rings of the E2 molecule are located near the upper acyl-chain region, about 4 Å below the polar headgroup-hydrocarbon boundary. Within the membrane, E2 is best accommodated by a near “horizontal” orientation that allows for a simultaneous interaction of its two hydroxyl groups with the bilayer polar surface, thus minimizing the overall energy of the E2-phospholipid membrane supramolecular assembly.

The asymmetric nature of the E2 wobbling motion in the multilamellar membrane based on the mathematical analysis of our solid-state ^2H -NMR results is noteworthy. The work by Vogel et al. [18] has provided a clear distinction and quantitative comparison between two experimentally determined order parameters, one from solid-state ^2H -NMR experiments and the other from ^1H - ^{13}C dipolar coupling and chemical shift (DIPSHIFT) measurements. From our analysis using the ratio method, we found that a uniaxial rotation for E2 around its long axis based on a molecular order parameter, as used in earlier studies, is not adequate to describe the dynamic motions of E2 within membrane bilayers. Compared with the ratio method, the matrix method is a more rigorous and self-consistent approach because it imposes no assumption of uniaxial symmetry and relies on a total of six deuterium labels. As a result, analyses using the matrix method provide us the order parameter tensor with the directions of three principal axes as well as the order parameter along each axis, which is much more accurate to characterize the dynamic motions of E2 in bilayer membranes. From our calculations, the order parameter of E2 along each of the three principal axis is $S_{xx} = -0.395$, $S_{yy} = -0.179$, and $S_{zz} = 0.573$. The asymmetry parameter η can be derived as follows:

$$\eta = \left| \frac{S_{xx} - S_{yy}}{S_{zz}} \right|$$

The η -value for E2, $\eta = (0.395 - 0.179)/0.573 = 0.377$, is significantly greater than that reported for cholesterol in lipid membranes [$\eta = 0.02$ [26]; $\eta = 0$ from assumed uniaxial symmetry [25]]. A uniaxial symmetry assumption has often been made in studying the structure and dynamics of lipids [40], transmembrane peptides [41], and other small molecules including local anesthetics [42] trifluoperazine [43], and serotonin receptor agonists [44] in membrane environments. Our data

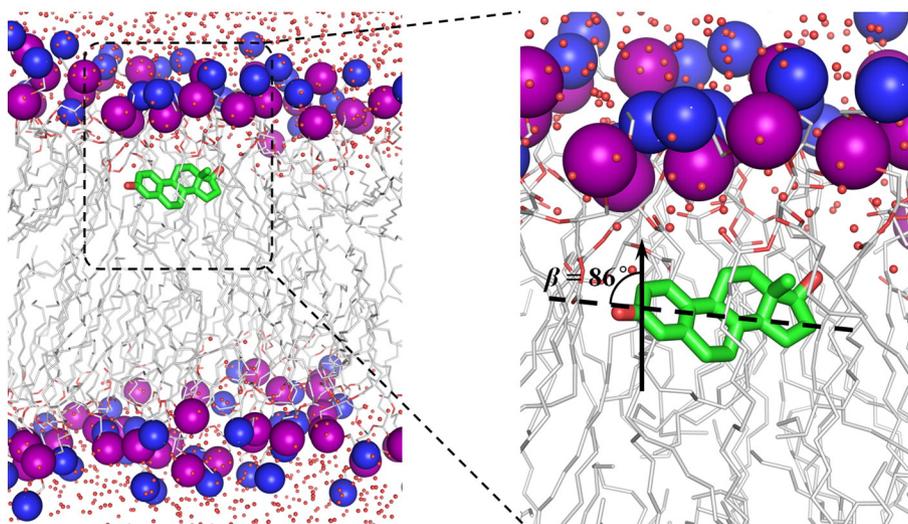


Fig. 9. One typical frame ($\beta = 86^\circ$) retrieved from 250 ns molecular dynamics simulation of 17 β -estradiol in DPPC membrane bilayers. The system for simulation was constructed by placing one E2 molecule into a pre-equilibrated membrane bilayer containing 64 DPPC molecules and 1871 H_2O molecules (purple ball: phosphorus; blue ball: nitrogen; small red ball: oxygen of water molecule).

now show that the dynamic motions of E2 within membranes are far from uniaxially symmetric and underscore the importance of the full tensor analysis (*i.e.*, the matrix method) as opposed to assuming $\eta = 0$ (*i.e.*, the ratio method). We suggest that when studying the motion of a rigid and/or bulky molecule in a membrane environment, uniaxial symmetric motion should not be invoked *a priori* in lieu of considering the possibility of asymmetric interactions.

5. Conclusions

We have demonstrated that the E2 molecule adopts a “horizontal” orientation in a membrane-phospholipid environment with its long axis essentially perpendicular to the lipid acyl-chains. All four rings of the estradiol molecule are located near the membrane interface, allowing both the E2 3-OH and the 17 β -OH groups to participate in hydrogen bonding and electrostatic interactions with polar groups on the phospholipid membrane. Our findings regarding the estradiol orientation and dynamic properties in the membrane bilayer contribute to a better understanding of its interactions with the cell membrane and membrane-associated estrogen receptors. Our matrix approach involving the unique use of six deuterium labels in E2 serves as an exemplar for determining unambiguously the orientation and dynamic properties of molecules in membrane bilayers. The results reported here also underline the highly asymmetric nature of dynamic motions for a rigid molecule in a membrane environment.

Transparency document

The Transparency document associated with this article can be found, in online version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbamem.2015.11.015>.

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