NMR Study Directly Determining Drug Delivery Sites in Phospholipid Bilayer Membranes

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The drug delivery (DD) process of benzene derivatives, *n*-propylbenzene (PrBe) and benzyl alcohol (BzOH), from water to phospholipid vesicles is first monitored by noninvasive NMR technique. The bilayer interface and interior as delivery sites are unambiguously specified by taking advantage of the site selectivity of NMR. Chemical shift differences of the ring proton signals provide direct evidence for the penetration of the "drugs" into the bilayer within a few minutes. PrBe is deeply penetrated into the hydrophobic chain region of the bilayer core. In contrast, BzOH is preferentially trapped in the interfacial region near the carbonyl group of the phospholipid, with the methylene group oriented toward the inside of the bilayer. The delivery site of BzOH is characterized by the doublet of the ring proton NMR signal, which is ascribed to BzOH delivered into the outer and inner layers of the vesicle. This is also confirmed by ^{13}C NMR, for the first time applied to specify the delivery site. UV absorption spectra of PrBe and BzOH in vesicles are consistent with the delivery sites determined by NMR. Application of the molecular level study of the DD processes to recent severe problems of endocrine disruptors (EDs) is finally proposed as a basis for the comprehensive understanding of the molecular mechanism of the membrane disrupting action and for the purpose of detoxication and the prevention of ED accumulation.

Introduction

An urgent problem of endocrine disruptors (EDs) is nowadays coming up. EDs are said to be exogenous chemicals that interfere with the endocrine system of intact organisms by disrupting the synthesis, secretion, transport, receptor binding action, and elimination of natural hormones in the body. As a primary step of these "disrupting" actions, delivery of EDs into biomembranes is crucial. In this sense, a molecular level study of drug delivery (DD) into phospholipid bilayer membranes becomes of great importance. However, most of the physicochemical studies have discussed the mechanism of membrane−drug interaction in terms of induced structural changes of the membranes.[1,2] The equilibrium concentration of drugs in the membrane interior is, in general, too low to be detected experimentally. Much of the valuable information on the membrane transporting process of drugs has been due to molecular dynamics (MD) simulations (see the review, ref 3). On the other hand, a sensitive invasive probing method such as fluorescence can be utilized to overcome the difficulty of detection. Such probes are, however, a kind of drug. The membrane may be perturbed by their injection. No noninvasive spectroscopic procedures have been established yet. There are no systematic studies providing direct experimental evidence for the structure and dynamics of drugs trapped in membranes. Most recently, we have, for the first time, succeeded in determining the site and orientation of the most suspicious ED, bisphenol A (Bis A), solubilized in lipid bilayer membranes from the truly noninvasive NMR signal of Bis A itself.[4] Here, we concentrate on the direct detection of the DD process without alien probes, utilizing site-selective NMR. Our purpose is to directly determine DD sites and the orientation of drugs in bilayer membranes to develop basic concepts of the molecular mechanism of the DD from water to the lipid bilayer phase.

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We can consider three categories as molecular mechanisms of the membrane−drug interaction. Category I is that drugs are adsorbed on the hydrophilic surfaces of bilayer membranes by Coulombic interaction or hydrogen-bonding. In this case, drugs are generally polar and water-soluble. No significant effect of drugs is induced in the membrane interior. Category II is the case where drugs are slightly penetrated and trapped at the interfacial site of the membranes. These drugs are less hydrophilic than those of category I. Membrane structure is perturbed mainly at the interfacial glycerol and carbonyl sites of phospholipids. These drugs, however, do not significantly perturb the inner hydrophobic chains. Category III is that drugs are deeply penetrated into the hydrophobic chain region of the membranes. These drugs are almost insoluble in water, nonpolar, and highly lipophilic. In this case, the membrane interior can be most remarkably perturbed.

To strictly determine the trapped site of drugs at the atomic-site level from their direct NMR signals, we can rely upon the empirical rule that the NMR signals largely shift to a higher field when molecules are in a nonpolar environment.[5,6] According to this fact, the NMR signals of the trapped drugs should shift to a higher field in the order category I < category II < category III.

So far, several NMR studies have reported the binding of charged aromatic anions to cationic surfactant micelles.[5−7] Micellar interfaces as binding sites for the charged species have been specified, and the orientation of the anions has also been discussed. The aromatic anions used in the previous studies are, however, highly water-soluble. Our research, in contrast, first targets the delivery of neutral aromatic molecules that are practically insoluble in water. A small amount of the molecules can be delivered from water to the lipid phase because of the extremely low solubility in water of these molecules compared to the charged anions. It is, therefore, truly difficult to detect the solubilized molecules directly. The uncharged molecules are

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expected to have a much stronger affinity with the hydrophobic lipid phase than the charged species. Unlike the anions, the molecules can deeply penetrate into the lipid aggregates.

We first apply the above-mentioned empirical rule to the DD process to lipid bilayers. A similar approach has not been reported yet in the bilayer membranes, since the anisotropic structure of the bilayers with restricted motion of the lipid molecules makes the direct NMR observation difficult, compared to the micellar system.

We pay attention to the difference in the curvature between inner and outer layers of the membranes for the purpose of distinguishing between these two layers. This can be powerful for specifying the two membrane-trapped states of categories II and III. Remember that the NMR signal splitting demonstrates the membrane curvature difference most remarkably at the interfacial carbonyl site when phospholipid vesicles are used as simple models for the bilayer membranes.[8,9] A similar curvature difference should be recognized when drugs are trapped at the interfacial site (category II) whereas no effect occurs when incorporated deeply into the hydrophobic membrane interior (category III).

To obtain firm evidence for the above concepts, two benzene derivatives, *n*-propylbenzene (PrBe) and benzyl alcohol (BzOH), are added to small unilamellar vesicles (SUVs) of egg yolk phosphatidylcholine (EPC). The former drug is nonpolar and highly lipophilic. In contrast, the latter is relatively polar and considerably water-soluble. We use the low-field ring proton NMR signals in which no signals of EPC are overlapped. The difference of the delivery sites is also verified by UV absorption.

Experimental Section

Materials. EPC (>98% pure) was purchased from Nichiyu Liposome Co. and used without further purification. Reagent-grade PrBe and BzOH were from Wako Pure Chemical Industries and Nakalai Tesque Inc., respectively. Heavy water (D_2O, 99.9%) was used as received from CEA. Paramagnetic reagents $YbCl_3$ and $PrCl_3$ were from Nakalai Tesque Inc.

Sample Preparation. SUVs of EPC, with number-averaged diameter of 50 nm, were prepared as described elsewhere[10] according to a widely used ultrasonic irradiation method.

Injection of drugs into vesicles was performed by adding a desired amount of aqueous drug solution (in D_2O) to the vesicle dispersion. All procedures were carried out at room temperature (~22 °C). Since PrBe is sparingly soluble in water, saturated aqueous solutions were prepared by continuous stirring for at least 3 days. Final concentration of aqueous PrBe solution was at sub-mM level.[11] The solution was mixed together with the EPC vesicles, whose final concentration was 25−60 mM. BzOH was injected into 60−70 mM EPC with a mixing molar ratio of 2:1. The samples were subjected to NMR measurements within 1 min after the injection of the drugs into the EPC SUV.

NMR Spectroscopy. ^1H and ^{13}C NMR spectra were recorded with a high-resolution, multinuclear, and multipurpose NMR (JEOL JNM-EX270 wide-bore type) spectrometer equipped with an Oxford superconductor magnet (6.35 T). High-quality NMR tubes (Wilmad) with 10 mm o.d. were used. All measurements were performed at 30.0 ± 0.1 °C. Free induction decays (FID) were accumulated 8−128 and 4096 times for ^1H and ^{13}C NMR, respectively. The chemical shift of the HOD proton signal was used as the reference and was set to 4.70 ppm. In ^{13}C NMR, benzene (128.5 ppm) was used as an external reference. Experimental errors of the chemical shifts were ±0.001 ppm for ^1H NMR and ±0.01 ppm for ^{13}C NMR spectra.

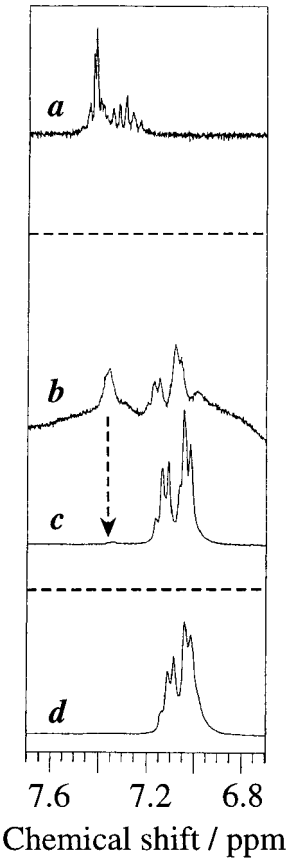

Figure 1. Ring proton region in the ^1H NMR spectra of PrBe: (a) in D_2O; (b) immediately; (c) 3 days after injection of EPC SUV. The ^1H NMR spectra of PrBe in *n*-hexane solution is also shown in (d).

UV Absorption Spectroscopy. UV absorption spectra of the benzene derivatives in water and in EPC vesicles were measured by a Hitachi UV-3400 spectrophotometer. The NMR sample solutions were diluted in the concentration range 0.6−1 mM. All measurements were performed at room temperature.

Results

Delivery of PrBe into EPC SUV. First, we describe the NMR specta of aqueous PrBe solution before going to the PrBe/EPC system. Figure 1a represents the ring proton region of the ^1H NMR spectra of saturated PrBe solution in D_2O. The ring proton NMR signal of PrBe is the strongest at 7.41 ppm in the aqueous environment.

Typical changes in NMR spectra are induced by the delivery of PrBe from water to EPC SUVs, as shown in parts b and c of Figure 1. Within a few minutes after mixing, we can observe a broad signal at 7.36 ppm and relatively sharp ones at 7.2−7.0 ppm (Figure 1b). Both signals are shifted to a higher field; the ring proton signal of PrBe dissolved in water appears at 7.41 ppm (Figure 1a). The difference in the chemical shift (δ) between the broad signal and that in water is, however, not large. On the basis of the empirical rule stated above, this is due to the PrBe molecules located near the polar environment. We can therefore ascribe this signal to the PrBe adsorbed on the SUV surface. The broad line width, which indicates the restricted molecular motion, also supports the adsorption of PrBe at the aqueous surface. On the other hand, the relatively sharp signals, observed at the higher field, are due to the PrBe molecules in considerably hydrophobic surroundings. We thus ascribe this to the PrBe deeply penetrated into the hydrophobic core of the membrane interior. The assignment is also supported by the

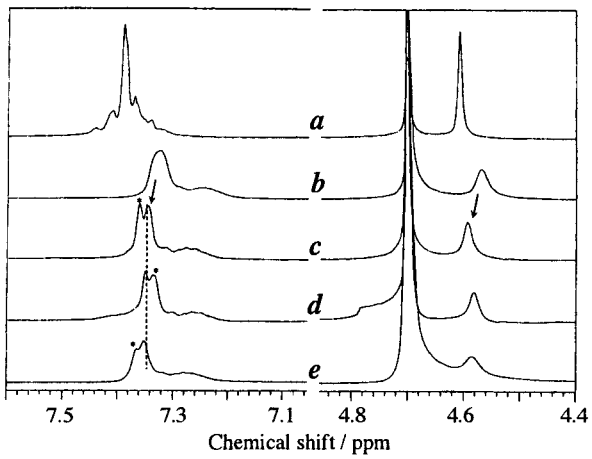

Figure 2. Ring proton and methylene signals in the ^{1}H NMR spectra of BzOH: (a) in D_2O; (b) immediately; (c) 15 min after injection of EPC SUV. Asterisks * indicate the ring proton signal of BzOH delivered to the outer layer of the SUV. The dashed line denotes the position of the BzOH signal trapped in the inner layer of the SUV. The ^{1}H NMR spectra of the BzOH/SUV system in the presence of 12 mM Yb^{3+} and 70 mM Pr^{3+} are also given in (d) and (e), respectively.

agreement of the signal with that of PrBe in *n*-hexane shown in Figure 1d; the doublet at the lower field (around 7.1 ppm) is due to the meta protons against the propyl group while the other doublet around 7.0 ppm to the protons at the ortho and para sites. There is no particular restriction to the motion of PrBe in the membrane core because the signal is sharp due to the motional narrowing effect. It is noteworthy that the present NMR study, for the first time, enables us to simultaneously distinguish the two different delivery sites for the DD process.

The PrBe, adsorbed at the SUV surfaces, is penetrated into the membrane and finally trapped in the hydrophobic core. This is clearly demonstrated by the ring proton signal change in Figure 1 (from part b to part c); that is, the disappearance of the broad signal at 7.36 ppm (shown by the dashed arrow) and the simultaneous increase of the sharp signals at 7.2−7.0 ppm.

Delivery of BzOH into EPC SUV. All BzOH molecules dissolved in water are also immediately transported and trapped in the lipid bilayer phase when it is mixed with EPC SUVs. However, the ^{1}H NMR spectra show that the trapped site of BzOH differs from that of PrBe. The ring proton signal of BzOH in water observed at 7.39 ppm (Figure 2a) is at first broadened and shifted to a high field (7.32 ppm) within a few minutes after mixing (Figure 2b). The upfield shift is then reduced within 15 min (Figure 2c). At the same time, the signal is split into two at 7.36 (shown by the asterisk) and 7.35 ppm.

The signal splitting is usually observed for the ^{13}C NMR signal of the interfacial carbonyl site of EPC SUVs. In this case, the doublet is ascribed to the carbonyls in the inner and outer layers of the SUVs.[8,9] We thus explain that, unlike the case of PrBe, BzOH is immediately trapped at the interfacial site of the SUVs. We assign the doublet of the ring proton signal to BzOH trapped in the outer (7.36 ppm) and the inner (7.35 ppm) layer of the SUVs, respectively; the reason is shown below.

To confirm the assignment described above, two kinds of paramagnetic lanthanide reagents ($YbCl_3$ and $PrCl_3$) were added to the sample from the outer aqueous phase. NMR signals are shifted to a high field in the presence of Yb^{3+}, whereas they are shifted to a low field by Pr^{3+} (the lanthanide-induced shift, LIS). Since neither the polar site in the inner layer nor the hydrophobic site can make contact with the lanthanide ions in the SUV system, the NMR signal shifted by LIS can be assigned to the hydrophilic site in the outer layer. In fact, the ring proton signal of the trapped BzOH at 7.36 ppm (shown by the asterisk) is shifted to 7.33 and to 7.37 ppm in the presence of Yb^{3+} and Pr^{3+}, respectively (see parts d and e of Figure 2). In sharp contrast, the proton signal of BzOH at 7.35 ppm essentially remains constant (see the dashed line). These results strongly support the above assignment.[12]

The δ values and the signal splitting are preserved even after 3 days. BzOH is, therefore, preferentially trapped at the interfacial site of the SUV. Finally, the ^{1}H NMR spectra shown in Figure 2b (a higher field and no splitting signal of BzOH) also give direct experimental evidence for the intermediate state in the BzOH delivery process; BzOH, first transported in the hydrophobic core of the SUV, is truly partitioned between the outer and inner layer at the interfacial site of the SUV.

TABLE 1: Change in the δ Value of the ^{1}H NMR Signals of the EPC SUV Associated with the Drug Delivery[a]

			5 min after	3 days after
choline CH_3	EPC	3.24		
	EPC + PrBe		3.22	3.22
	EPC + BzOH		3.15	3.15
chain $(CH_2)_n$	EPC	1.25		
	EPC + PrBe		1.23	1.20
	EPC + BzOH		1.26	1.26
chain CH_3	EPC	0.87		
	EPC + PrBe		0.85	0.84
	EPC + BzOH		0.87	0.87

[a] δ values in ppm.

TABLE 2: Carbon Atom Site of the EPC SUV Affected by the Delivery of BzOH, Together with Its Chemical Shift Difference Observed in the NMR Signal

	$\Delta\delta$ (ppm)[a]
choline CH_3	−0.07
glycerol CH	−0.08
carbonyl	−0.09 (out), +0.03 (in)[b]
chain α-CH_2	−0.07
chain β-CH_2	−0.09

[a] Chemical shift difference with an uncertainty of ±0.01 ppm. Negative values designate upfield shifts. [b] For the two components of the carbonyl signal, see text.

Structural Change of the EPC SUV during the Delivery Process. The delivery sites can be also demonstrated by analyzing δ values of the NMR signals of the EPC SUVs during the delivery process. The NMR signal of the EPC should be shifted because of the structural change induced by DD. The ^{1}H NMR result is summarized in Table 1. In the case of PrBe, the chemical shift change is small for the polar choline methyl proton. The δ values are, in contrast, gradually shifted to high field with time in the chain methylene and methyl signals in the bilayer core. On the other hand, the signal of the hydrophilic choline methyl proton is remarkably shifted upfield (by 0.09 ppm) immediately after the addition of BzOH; recall that the δ values are accurate within ±0.001 ppm. The changes in the δ values of the hydrophobic chains are, in contrast, relatively small.

Similar trends of the chemical shift change are recognized in the ^{13}C NMR spectra. In Table 2, we specify carbon atom sites of EPC, where the most prominent change is induced by the delivery of BzOH.[13] Here are given the chemical shift differences ($\Delta\delta$'s) of the EPC signals during the delivery process. Evidently, large $\Delta\delta$'s are observed at the choline methyl, glycerol C2, and carbonyl sites at the hydrophilic interface; significant change is recognized also in the chain α- and β-methylenes, two sites most adjacent to the headgroup. The $\Delta\delta$ values at the other sites are within experimental error (±0.01 ppm).

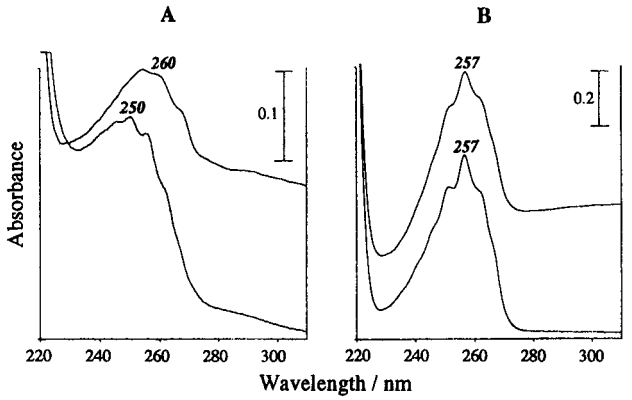

Figure 3. UV absorption spectra of (A) PrBe and (B) BzOH in EPC SUV (upper traces) and in D_2O (lower traces). The λ_{max} values of the major absorption are given. The absorption of the EPC SUV itself is subtracted in advance.

UV Absorption Spectra. UV absorption spectra of PrBe and BzOH in the EPC SUV further confirm the delivery sites. The 10 nm red shift is observed when the SUV is mixed into the aqueous PrBe solution (Figure 3A). In contrast, no substantial change in the UV absorption energy is recognized for the delivery of BzOH (Figure 3B). These results evidently indicate that PrBe is trapped at the hydrophobic site, whereas the trapped site of BzOH is essentially hydrophilic. The energy difference due to the 10 nm shift clearly reflects the difference in the environmental polarity.

Discussion

Characterization of the Different Delivery Sites by NMR. The present NMR study provides direct evidence for the different delivery sites of PrBe and BzOH. The results give convincing proof that most of the PrBe, mixed with EPC SUVs, deeply penetrate into the hydrophobic core of the SUV; no PrBe remain in water, or trapped at the bilayer interface, on the coexistence of the lipid membranes. All PrBe, trapped in the bilayer core, are in a solitary state with high motion and fluctuation. Changes in the signal fine structure (from Figure 1a to Figure 1c), probably due to the conformational change of PrBe, also provide evidence for PrBe far from the aqueous environment within the bilayer.

In contrast, BzOH is preferentially trapped at the interfacial site of the SUV. This is characterized by the doublet of the ring proton NMR signal, each of which is ascribed to BzOH delivered into the outer and inner layers of the vesicle. The signal splitting can be due to the difference in the magnetic susceptibility between the inner and outer layers originating from the difference in the molecular density and the hydration. Remember that the surface curvature of the SUVs induces the density difference between the two layers especially at the interfacial sites.

Additional evidence for the partitioning of the BzOH delivery between the outer and inner layers of the bilayers is provided in the EPC carbonyl ^{13}C NMR. It is known that the ^{13}C NMR signals are split into two at the carbonyl site in the EPC SUV; the low-field strong peak is assigned to the cabonyl of the outer layer with low packing density, while the high-field weak one is assigned to the inner layer.[8,9] This is also due to the curvature difference between the two layers. After the delivery of BzOH, both compoments of the carbonyl signal are affected; as demonstrated in Table 2, a significant upfield shift (0.09 ppm) is observed in the former peak, while the slightly downfield shift (0.03 ppm) is observed in the latter. These shifts consequently reduce the signal splitting. The most plausible interpretation is that the low-density carbonyl site of the outer layer is much highly packed as a result of the BzOH incorporation at the bilayer interface. Simultaneous dehydration at the same site is also probable. In contrast, the penetration of BzOH decreases the inherent packing density of the carbonyls at the inner surface. Consequently, the carbonyl sites of the two layers are less inequivalent. This is quite consistent with the ambiguous signal splitting observed for large unilamellar vesicles where both layers are almost equivalent at a molecular level.

TABLE 3: Chemical Shift Difference of the Ring Proton Signals of PrBe and BzOH Delivered to the EPC SUV from the Water Phase

	δ (ppm)[a]		
	in D_2O	in EPC SUV[b]	$\Delta\delta$[c]
PrBe	7.41	7.05	−0.36
BzOH	7.39	7.36 (out)	−0.03
		7.34 (in)	−0.05

[a] Chemical shift for the strongest signal. [b] Chemical shift 3 days after the drug injection. [c] Chemical shift difference in ppm. Negative values mean upfield shifts.

The differences in the delivery sites are also demonstrated by the structural change of the EPC SUV during the DD process shown in Tables 1 and 2. Here, the upfield shift of the ^1H and ^{13}C NMR signals of EPC is due to the perturbation of the structure of the EPC bilayers at the delivery site. Thus, the results strongly support the delivery sites of PrBe and BzOH mentioned above.

Determination of the Drug Delivery Sites by NMR. We further attempt to strictly determine the final delivery sites of the drugs. To do this, we analyze $\Delta\delta$ of the ring proton signals on the delivery from water to the bilayer phase. As clearly shown in Table 3, $\Delta\delta$ of BzOH is small (−0.03 and −0.05 ppm). In contrast, the absolute value of the $\Delta\delta$ of PrBe is relatively large (−0.36 ppm). This fact means the drugs deeply penetrate into the bilayer interior in the order PrBe > BzOH, which is in accord with the sequence of the insolubility in water.

The delivery sites of PrBe and BzOH can be finally determined, as illustrated in Figure 4. PrBe penetrates into the hydrocarbon tail region of the SUV. This is demonstrated by the significant high-field shift in the δ values of the EPC chain methylene and methyl groups most deeply buried in the bilayer core (see Table 3). The alkyl chain of the PrBe is expected to have a strong affinity with this site. These facts indicate that the bilayer interior is most perturbed by PrBe. In addition, there is no substantial shift in δ of the headgroup (choline methyl in Table 3) in a strong support for the delivery site determined. All kinds of NMR evidence demonstrate that PrBe belongs to the category III mentioned previously.

The delivery site of BzOH is the interfacial region, probably near the carbonyl group of EPC. This is confirmed by the splitting of the ring proton NMR signal assigned to the outer and inner layers of the SUV caused by the membrane curvature difference (Figure 2). ^{13}C NMR evidence strongly supports this site. No substantial shift in the δ values of the hydrocarbon chain (Table 1) also verifies the interfacial delivery site of BzOH.

Here, we can also discuss the orientation of BzOH trapped at the interfacial site of the SUV. The methylene group of BzOH is oriented toward the inside of the bilayer, not the outside, compared to the aromatic ring (see Figure 4). This can be explained by the absence of the signal splitting, which is ascribed to the curvature difference (Figure 2). The aromatic ring of BzOH cannot be penetrated deeply because of the bulkiness.

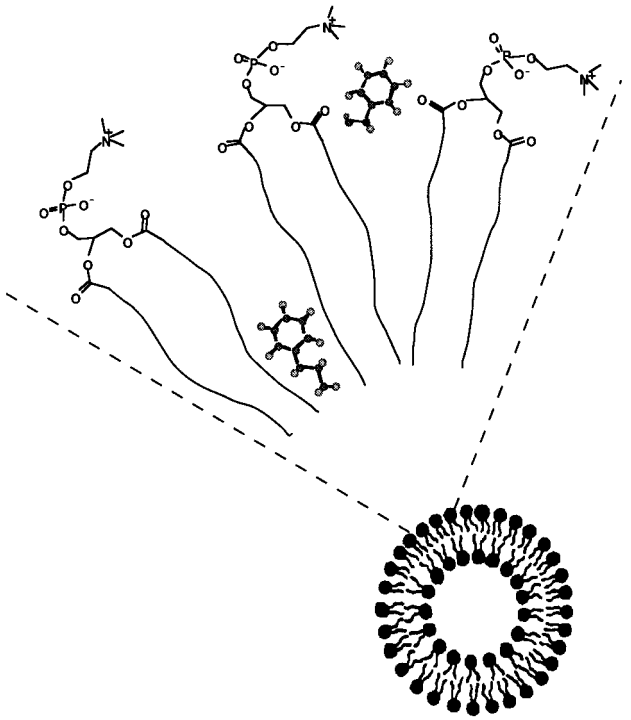

Figure 4. Schematic representation of the delivery sites of PrBe (left) and BzOH (right) in the EPC SUV determined by the NMR chemical shift difference.

The delivery site clearly demonstrates that BzOH belongs to category II.

It should be finally noted that the delivery site determination of BzOH by the NMR signal splitting is novel and unique. Delivery sites of aromatic anions are already investigated in surfactant micelles from the direct NMR signal of the ring protons.[5,6] The delivery into the water/micelle interfaces is also discussed. This is, however, only based on the δ values of the ring proton signals. No ^{13}C NMR methods have been combined with this phenomenon. The confirming evidence such as the signal splitting both in ^{1}H and in ^{13}C NMR spectra is inherent for the bilayers.

Conclusions

For the first time, we shed light on the molecular mechanism of the delivery process of benzene derivatives from water to the lipid bilayer phase, utilizing a site-selective NMR technique. We can also unambiguously specify the delivery site by truly noninvasive monitoring of signals of the drug itself. Our study provides direct evidence that the benzene derivatives injected penetrate into the bilayer within a few minutes and that they are delivered deeply into the bilayer interior in the order PrBe > BzOH. UV absorption spectra are quite consistent with the delivery sites determined by NMR.

The more deeply drugs are penetrated into the membrane, the more the bilayer interior is disrupted. The hydrophobicity of drugs increases the membrane damage. We are now verifying this by distinguishing delivery sites of alkylbenzenes with different hydrophobicity.

Our NMR study can also give comprehensive information on the prediction of the side effect and/or the toxicity of various drugs or EDs, especially lipophilic compounds. Most of the EDs have ring protons, similar to the benzene derivatives in this study. The NMR method developed here should make a significant contribution to the clarification of the molecular mechanism of the ED delivery into the lipid bilayer as a primary step of the membrane-disrupting action. Studies of ED concentration dependence, as well as that of the membrane structure (the size, the composition of vesicles, emulsions, etc.), are hoped to provide more detailed information about the mechanism. Finally, our method is also useful for monitoring the decomposition and the release of EDs accumulated in the membrane interior. NMR research is now in progress for the purpose of detoxification and the prevention of ED accumulation.

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(12) In parts b−e of Figure 2, there is a small but broad peak around 7.24−7.3 ppm, which is a high field compared to the strong sharp peak. The chemical shift of this peak is also changed by LIS. It could be assigned to the ring proton of BzOH trapped in the outer layer of some smaller vesicles.
(13) Here, we show the results of the BzOH/EPC system. A remarkable change is successfully detected, since a high drug/lipid ratio can be attained by the high solubility of BzOH in water.