Partition Property of Pyrene onto Synthetic Membrane Vesicles and the Effects of Natural Organic Matters

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Abstract:

In this study, we investigated the partition property of pyrene onto cell membrane using synthetic membrane vesicles consisted of several phospholipids and the effect of natural organic matters (NOM) on the partition. Membrane fluidity and membrane phase affected partition coefficient (P_{mw}) of pyrene onto the membrane. P_{mw} was larger for the liposome in liquid crystalline phase > in ripple phase > in gel phase. Pyrene did not undergo the steric interfere in partition onto the membrane used in this study. Negative charge of the membrane did not affect P_{mw} of pyrene.

Suwannee River Natural Organic Matter (NOM) and Nordic fulvic acid affected the partition of pyrene onto the TRANSIL® membrane. The reason was confirmed that NOM associated pyrene did not partition onto the membrane. Low molecular weigh fraction of SRNOM had strong effect on the partition as much as high molecular weigh fraction. From calorimetrical analysis, it was found that SRNOM did not affect the fluidity of the dimyristoylphosphatidylcholine (DMPC) membrane.

Keywords:

Pyrene, Polyaromatic Hydrocarbons (PAHs), Synthetic Membrane Vesicles, Fluidity, Partition Coefficient between Membrane and Water (P_{mw}), Natural Organic Matter (NOM), K_{oc}

1. Introduction

Pyrene is categorized into polyaromatic hydrocarbons (PAHs) and one of the typical contaminants in the natural water systems and water treatment plants. It is suspected to have adverse effects on the aquatic organisms, especially strongly with sunlight (International Programme on Chemical Safety, 1998). Therefore, great attention should be paid to maintain the sound ecosystems.

Pyrene is a hydrophobic organic compound, and it is considered to penetrate into the cell membrane by passive diffusion. Pyrene partition onto the cell membrane, which is quantified with partition coefficient (P_{mw}), is very important with the reasons below.

One is that cell membrane is the target site for the baseline toxicity of hydrophobic organic compounds to the aquatic organisms (Escher and Hermens, 2002). Therefore P_{mw} evaluates that toxicity of those pollutants.

Another is that P_{mw} is a kinetic (toxicokinetic) parameter to determine the uptake rate of hydrophobic organic compounds into the body from water (Parsons and Opperhuizen, 1987). When bioconcentration or toxicity is estimated, P_{mw} is critically important to simulate the distributions of hydrophobic organic compounds in the body. P_{mw} is also significant in the field of biological treatment process of wastewater to access the sorptive removal onto the sludge or degradation capability of hydrophobic organic compounds.

P_{mw} is often substituted by *n*-octanol /water partition coefficient (K_{ow}). Cell membrane is, however, inherently different from octanol in the physicochemical property (Gobas *et al*., 1988). Cell membrane has steric structure called lipid bilayer with surface charge from twitter ions of lipids which consist of cell membrane. Therefore, hydrophobic organic compounds may interact electrostatically with cell membrane and/or undergo the interference by rigid structure when they partition into the lipid phase in cell membrane, although they do not in the case of octanol which is a bulk solvent.

Natural organic matter (NOM) can take a large role when aquatic organism or activated sludge uptakes pyrene. Hydrophobic organic compounds easily sorb to the NOM and aquatic organism has

been believed to uptake only freely dissolved fraction, because the fraction associated with NOM can not pass the cell membrane. Several researchers (Haitzer *et al*., 1999; Mccarthy *et al*., 1994; Kukkonen and Oikari, 1991) reported NOM decreased the bioconcentration or toxicity of hydrophobic organic compounds, but they have some questions due to the uncertainty of bioassay (Haitzer *et al*., 2001). Then we need to prove the assumption that NOM associates can not be taken into the cell membrane.

In this study, we investigated the partition property of pyrene onto cell membrane using synthetic membrane vesicles. The lipids which consist of actual organisms are different for the kind of organisms and organs and the physicochemical property of the membrane is varied. Therefore we focused the effects of the lipid component, which affects the fluidity and surface charge of membrane, on the partition of pyrene. This may contribute to the estimation of pyrene uptake by the variety of aquatic organisms for the future. We also demonstrated the effects of NOM on pyrene partition onto cell membrane. NOMs are ubiquitous and have different structures and properties for their origins. We used several NOMs and investigated how the NOM property impacted their effects.

2. Materials and Methods

2.1 Chemicals and quantification

Pyrene is selected as a target chemical and other three PAHs are selected for comparison in the investigation of partition property of pyrene. Pyrene (98%) as 4-rings PAH, and fluorene (95%), phenantherene (95%) and anthracene (98%), as 3-rings PAHs, were purchased from Wako (Japan). PAHs concentrations were basically measured by fluorescence (pyrene; ex.335 nm, em.371 nm, fluorene; ex.264 nm, em.301 nm, phenantherene; ex.294 nm, em.363 nm, and anthracene; ex.250 nm, em.379 nm). Measured fluorescence was corrected for the inner filter effect according to the method developed by Shimizu (Shimizu and Liljestrand, 1991). Pure water (specific resistance >18 MΩcm)

was used by processing distilled water with epw-200 (Advantec, Japan) water purification system equipped with ion exchange column and filtration column. Other chemicals used were higher grade than reagent grade.

2.2 Effects of the lipid component (membrane fluidity) on the partition of pyrene onto cell membrane

2.2.1 Preparation of liposome solution

For this study, we made five kinds of liposome, as synthetic membrane vesicles, using five phospholipids purchased from Sigma (Japan). Dilauroylphosphatidylcholine (DLPC: C12), dimyristoylphosphatidylcholine (DMPC: C14), dipalmitoylphosphatidylcholine (DPPC: C16), distearoylphosphatidylcholine (DSPC: C18) and diarachidoylphosphatidylcholine (DAPC: C20), which are listed in descending order of acyl chain length, were selected and synthesized into five liposomes which had different fluidity of membranes. DLPC liposome has the highest fluidity and DAPC liposome has the lowest.

The liposomes were synthesized with evaporation method developed by Moscho (Moscho *et al*., 1996) with slight modification by us and refined with rapid extrusion to lower the polydispersity (Mayer *et al*., 1986). First, the lipid solution in chloroform and 10 mM phosphate buffer (pH 7) were added in round-bottom flask. Chloroform was then removed with rotary evaporator by two steps of decompression. The liposome solution was left in the phosphate buffer and flowed by rapid extrusion with 1.2 μm polycarbonate filter in water bath at higher than 10 ^{o}C of their main phase transition temperatures. The resulting liposome diameters were around 1 μm.

2.2.2 Measurement of partition coefficients of PAHs onto the liposomes

Batch sorption experiments were conducted for measurement of partition coefficient (P_{mw}) of

PAHs onto the varieties of liposomes. PAHs concentrations were set below their 50% solubility. PAHs and liposomes were incubated with rotary shaker in the 15 mL test tube for 24 hour at 25 ^{o}C in 10 mM phosphate buffer (pH7), followed by quantification of freely dissolved PAHs concentration. Liposomes as sobent were prepared in five different concentrations. P_{mw} was calculated by the next equation (Takahashi *et al*., 2003).

$$C_0/C = 1 + P_{mw}[lip] \quad (1)$$

In equation (1), C_0 is freely dissolved PAHs concentration without liposome, C is freely dissolved PAHs concentration with liposome and [*lip*] is liposome (synthetic membrane vesicle) concentration.

Freely dissolved concentration could not be measured directly, because the separation of liposome from the aqueous phase was practically impossible. Then we used fluorescence enhancement method we developed before (Takahashi *et al*., 2003). Briefly, PAHs associated with liposome emit a strong fluorescence. Aqueous phase fluorescence was the sum from freely dissolved PAH and liposome associated PAH. After sorption experiment reached equilibrium, we can adjust the aqueous liposome concentration by centrifugation. The solution fluorescence measured with Shimadzu (Japan) RF-5000 spectrofluorometer, was plotted to liposome concentrations measured by absorbance at 350 nm (UV2500PC, Shimadzu, Japan). The fluorescence correlates the aqueous liposome concentration and we can get the fluorescence from freely dissolved PAHs as the intercept of liner correlation. This method has the advantage that plastic devices were not used and lower the error from soptive loss to those, which is often problem for using hydrophobic organic compounds.

2.3 Effects of the lipid component (membrane surface charge) on the partition of pyrene onto cell membrane

2.3.1 Synthetic membrane vesicle selection

To evaluate the effects of membrane surface charge on the partition of pyrene onto cell membrane, we prepared both negative charged and neutral charged synthetic membrane vesicles. Manufacture synthesized membrane vesicles named as TRANSIL® (Nimubus biotechnology, Germany) were purchased for this study. TRANSIL® has silica beads (specific gravity>1) in its inner aqueous phase and is, therefore, easy to separate from the aqueous phase by centrifugation (Loidl-Stahlhofen *et al*., 2001) For sorption experiment, this is superior to liposome due to easy measurement of freely dissolved sorbate, although this is more expensive than liposome which can be synthesized in the laboratory. In this study, we used neutral charged TRANSIL® consisted of egg yolk phosphatidylcholine (EPC-TRANSIL®) and negative charged TRANSIL® consisted of 80% EPC and 20% palmitoyl-oleoylphosphatidylserine (POPS-TRANSIL®).

2.3.2 Measurement of partition coefficient (P_{mw}) of PAHs onto the TRANSIL®

Batch sorption experiments were conducted basically in same way as section 2.2.2. In this experiment, freely dissolved PAHs concentration was measured after perfectly removing TRANSIL® from the aqueous phase by centrifugation.

2.4 Effects of NOM on pyrene partition onto cell membrane.

2.4.1 NOMs

Suwannee River natural organic matter (SRNOM) obtained from International Humic Substances Society (IHSS) was used for this study. This NOM has been often used in the environmental researches as representative NOM of colored water river. Nordic fulvic acid (NFA) also was obtained from IHSS and used. SRNOM and NFA were extracted from different flesh waters and have different physicochemical properties (IHSS, 2007).

To use NOM of definitely different property, we fractionated SRNOM based on molecular weight using ultrafiltration membrane device (Pellicon2 equipped with regenerated cellulose membrane of molecular weight cut off 1,000 Da, Millipore, Japan). SRNOM solution (400 mgC/L) in 20 mM phosphate buffer (pH7.2) was processed in transmembrane pressure; 50 psi and retentate flow rate; 1.8 L/min. The resulted retentate was high molecular weight fraction named as HMNOM and the permeate was low molecular weight fraction named as LMNOM.

2.4.2 Measurement of partition coefficient (P_{mw}) of pyrene onto the TRANSIL® with NOM

In order to evaluate the effects of NOM on pyrene partition onto cell membrane, P_{mw} was measured with the presence of NOM in different concentrations. In this case, the aqueous phase and/or membrane phase have both of freely dissolved fraction and NOM associated fraction. Then, P_{mw} means distribution ratio of pyrene between the aqueous phase and membrane phase. Therefore, if NOM associate does not partition onto the membrane, P_{mw} should be lower with NOM than that without NOM.

In this study, EPC-TRANSIL® was used as synthetic membrane vesicle. Batch sorption experiments were conducted basically in same way as in section 2.3.2, although the concentration of phosphate buffer was changed to 20 mM and pH was changed to 7.2. This time P_{mw} was calculated by equation (1) but C_o means the aqueous PAHs concentration without liposome and C means the one with liposome.

Aqueous pyrene concentration including freely dissolved fraction and NOM associated fraction was quantified with Waters 600E HPLC (Japan) equipped with Waters 474 scanning fluorescence detector (Japan) using HS Discovery C18 column (4.6 x 150 mm 5 μm, SPELCO, Japan) in the isocratic condition (flow rate: 1.0 mL/min, elluent: 80% as volume of acetonitrile and 20% as volume of water). We previously confirmed that when pyrene concentrations were identical in the solutions, the analytical signal corresponding to pyrene were identical in regardless of the presence

of NOM under the NOM concentration in this study.

2.5 Measurement of sorption coefficient (K_{oc}) of pyrene to NOM

In order to mechanistically evaluate the effects of NOM on the partition of pyrene onto the synthetic membrane vesicle, sorption coefficient (K_{oc}) of pyrene to NOM was measured. Batch sorption experiments were conducted basically in same way as in section 2.4.2. K_{oc} was calculated by fluorescence quenching method (Gauthier *et al*., 1986). Solution fluorescence was quantified with Hitachi F-4000 spectrofluorometer (Japan) and absorbance with Shimadzu UV-2500PC (Japan).

2.6 Membrane fluidity measurement

The fluidity of the membrane dramatically increases above main phase transition temperature (T_m), where phase change occurs from gel phase to liquid crystalline phase. T_m is determined by the strength of lipids interaction within the membrane and high fluidity membrane has low T_m (Blume, 1983).

T_m of the concerned membrane is expected to be changed if some forces change its fluidity. We tried to test hypothesis that NOM interacts the membrane and changes the fluidity. SRNOM and the TRANSIL® which was consisted of dimyristoylphosphatidylcholine (DMPC-TRANSIL®) were mixed for 24 hours at 25°C in 20 mM phosphate buffer (pH7.2). Then the solution was calorimetrically analyzed with Shimazdu DSC-60 differential scanning calorimeter (Japan) in order to measure endothermic energy in the phase transition and determine T_m. At DSC analysis, heating rate was 2.0°C/min and reference pan was filled with phosphate buffer. The effect of NOM on the fluidity was evaluated by comparing the results with NOM and without NOM.

3. Results and Discussions

3.1 Partition property of pyrene onto synthetic membrane vesicles

Table 1 summarized P_{mw} of PAHs onto the varieties of the liposomes with different acyl chains. Correlation coefficients and intercepts in equation (1) for calculation of P_{mw} were listed together. Acceptable correlation coefficients (R^2>0.68) mean the determination of P_{mw} was acceptable. Fluorescence enhancement method had been applied to the liposome in gel phase before (Takahashi *et al*., 2003) for the first time. The applicability of this method to the liposome in the liquid crystalline and ripple phase was also confirmed in this research.

The effect of the acyl chain length of the phospholipids in the liposome on P_{mw} of pyrene was shown in Fig.1. P_{mw} increased with decrease of the acyl chain length, when the fluidity of the liposome membrane increased. DAPC liposome, DSPC liposome and DPPC liposome are in gel phase in 25 ^{o}C (the sorption experiment condition). On the other hand, DMPC liposome is in ripple phase and DLPC liposome is liquid crystalline phase. The fluidity of liposome membarene was increased dramatically with change of the phase from gel to ripple and ripple to liquid crystalline (Gennis, 1989) and P_{mw} was increased in the same way. It was found that fluidity and phase of the membrane have large effects on the partition of pyrene to the membrane.

The log P_{mw} of PAHs onto DPPC liposome (gel phase) and DLPC liposome (liquid crystalline phase) was plotted to their log K_{ow} in Fig.2. Log P_{mw} has good correlation to log K_{ow} for both liposomes (R^2=0.84 for DPPC liposome and R^2=0.94 for DLPC liposome). This means the driving forces of partition is same between P_{mw} and K_{ow}. K_{ow} is the index to evaluate the hydrophobicity. Then, these PAHs may partition onto the membrane basically due to the hydrophobic interaction. If there is the interference originated from the rigid structure of the membrane and the size of PAH molecule in the penetration of PAHs into the membranes, the relation of log P_{mw} and log K_{ow} must be

convex upward because bulk solvent octanol does not exert that interference for K_{ow} (Gobas *et al*., 1988). However, that relation was not found for both liposomes of gel phase and liquid crystalline phase. The steric interference does not exist in the partition of pyrene onto these membranes. From Fig.2, P_{mw} of 3-4 ring PAHs might be estimated from K_{ow}. However, precise estimation was not expected because it was found that P_{mw} of phenanthrene which has higher K_{ow} was lower than anthracene which has lower K_{ow}. P_{mw} should be measured directly.

Table 2 summarized P_{mw} of PAHs for EPC-TRANSIL® and POPS-TRANSIL®. log P_{mw} was also plotted to log K_{ow} in Fig.3. Good correlation was shown between log P_{mw} for negative charged EPC-TRANSIL® and for neutral charged EPC-TRANSIL®. This meant that electrostatic interaction was little important in partition of PAHs onto the membrane. P_{mw} of PAHs for EPC-TRANSIL® was not perfectly identical to P_{mw} for POPS-TRANSIL®. This is because not only surface charge but also acy chain composition was different between EPC-TRANSIL® and POPS-TRANSIL®, although both membranes are in the gel phase.

3.2 Effects of NOM on pyrene partition onto the synthetic membrane vesicles

Table 3 summarized K_{oc} of pyrene to SRNOM and NFA. Pyrene was found to sorb to SRNOM almost as much as to NFA. The ratio of freely dissolved fraction when NOM exists is calculated by the next equation (Mccarthy *et al*., 1994).

$$\text{(ratio of freely dissolved fraction)} = 1/\ (1+K_{oc}\ [NOM]) \qquad (2)$$

When SRNOM concentration is 40 mgC/L, ratio of freely dissolved fraction is 0.7, which means 30% of pyrene was associated to SRNOM. Alhough K_{oc} of pyrene may be varied with the varieties of NOMs (Gauthier *et al*., 1987) and this result may not directly be able to apply to the other water

system than Suwannee River, NOM associated pyrene should be took into account to control pyrene pollution in the natural water with high concentration of NOM like peatland water.

Fig.4 showed P_{mw} of pyrene onto EPC-TRANSIL® with the presence of SRNOM and NFA. For both NOM, P_{mw} dramatically decreased as the concentration of NOM increased. These NOMs decrease the partition of pyrene on the membrane, which meant that the uptake of pyrene by aquatic organisms might decreased significantly with NOM.

If it is assumed that NOM associated pyrene can not partition onto the membrane, P_{mw} was calculated by the next equation (Gauthier *et al*., 1987).

$$P_{mw}\ (x)\ /\ P_{mw}\ (0)\ =\ 1/\ (1+K_{oc}\ [NOM]) \tag{3}$$

In equation (3), $P_{mw}\ (x)$ is P_{mw} when the concentration of NOM is x. P_{mw}, which was estimated from this equation assigned by measured K_{oc}, was shown in Fig.4. Estimated P_{mw} satisfactorily fitted in the measured P_{mw}. This meant that only freely dissolved pyrene could partition onto the membrane.

It was considered that the NOM associated pyrene could not partition onto the membrane because the size of associated pyrene became bigger than pyrene molecule, so that the steric interfere was originated and/or associated pyrene had higher hydrophilicity, therefore it lost driving force, hydrophobic interaction, to partition onto the membrane.

Fig.5 showed P_{mw} of pyrene onto EPC-TRANSIL® with the presence of HMNOM and LMNOM, respectively, which are the fraction of SRNOM separated by molecular weight cut off 1,000 Da UF membrane. It was found that both NOMs decreased P_{mw} of pyrene to almost same extent. From this result, the relatively low molecular weight NOM may be also important role in the fate of pyrene in natural waters.

Fig.6 showed the NOM effect on the main phase transition temperature of DMPC-TRANSIL®. Endothermic peaks, indicating the phase transition, was found at the same temperature. It meant that

10 and 40 mg/L of SRNOM did not change T_m at all. There was no evidence that SRNOM changed the fluidity of the membrane in DMPC-TRANSIL®. We had previously conducted the sorption experiment of SRNON to EPC-TRANSIL® and had found that NOM did not sorb onto EPC-TRANSIL® (Data not shown). We did not confirm for DMPC-TRANSIL® used in this study, although SRNOM was not considered to sorb to DMPC-TRANSIL®.

4. Conclusions

In this study, we investigated the partition property of pyrene onto cell membrane using synthetic membrane vesicles. The membrane fluidity and the membrane phase affected P_{mw} of pyrene. P_{mw} for DLPC liposome in liquid crystalline phase was highest and P_{mw} for DMPC liposome in ripple phase was the next and P_{mw} for DPPC, DSPC and DAPC liposome in gel phase was low. Pyrene did not undergo the steric interfere in partition onto the membrane used in this study. Negative charge of the membrane did not affect P_{mw} of pyrene. These results will contribute the estimation of pyrene uptake by varieties of aqueous organisms which have many types of the membranes.

We also demonstrated the effects of NOM on pyrene partition onto cell membrane. SRNOM and NFA affected the partition of pyrene onto the TRANSIL® membrane. The reason was found that NOM associated pyrene did not partition onto the membrane. From calorimetrically analysis, it was found that SRNOM did not effect the fluidity of the DMPC membrane. These results suggest that the NOM associated pyrene was not toxic but not biodegradable and moves to the other environment like sediment from the natural water body. Therefore the degradability of the associates and the toxicity to the sediment organisms like soileater is critically considered and necessary to be investigated for the control of pyrene pollution.

In this study, we used pyrene, one of non-polar hydrophobic organic compounds. Hydroxylpyrene is one of the pyrene metabolites and its interaction to the membrane is considered to be different

from pyrene because electrostatic interaction is expected in partition onto the membrane and sorption to NOM. In the future we will take up hydrophobic organic compound with hydrophilic functional groups.

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Table 1 P_{mw} of PAHs onto the liposome of different acyl chain phospholipids

PAHs	$\log K_{ow}$	P_{mw} (L/kgC) [a]				
		lipid of liposome	DLPC	DMPC	DPPC	DSPC	DAPC
		number of acyl chain carbon	12	14	16	18	20
		T_m [b]	0	23	43	55	64
		membrane phase at 25°C	liquid crystalline	ripple	gel	gel	gel
pyrene 	4.88[c]		5.4×10^5 (1.8) (0.72)	1.4×10^5 (2.2) (0.68)	3.8×10^4 (0.95) (0.99)	2.0×10^4 (1.0) (0.98)	1.5×10^4 (0.92) (0.75)
phenanthrene 	4.52[d]		8.9×10^4 (0.98) (0.99)		7.8×10^3 (1.0) (0.97)		
anthracene 	4.34[d]		8.7×10^4 (1.0) (0.96)		1.1×10^4 (0.94) (0.94)		
fluorene 	4.18[e]		3.0×10^4 (1.0) (0.95)		3.1×10^3 (0.96) (0.80)		

(a) In the column, the first line is P_{mw}, the second and the third are the intercept and R^2, respectively, for fitted curve corresponding to the equation (1).

(b) Gennis (1989).

(c) Hansch (1979).

(d) Kenega and Goring (1980).

(e) Hansch and Fujita (1964).

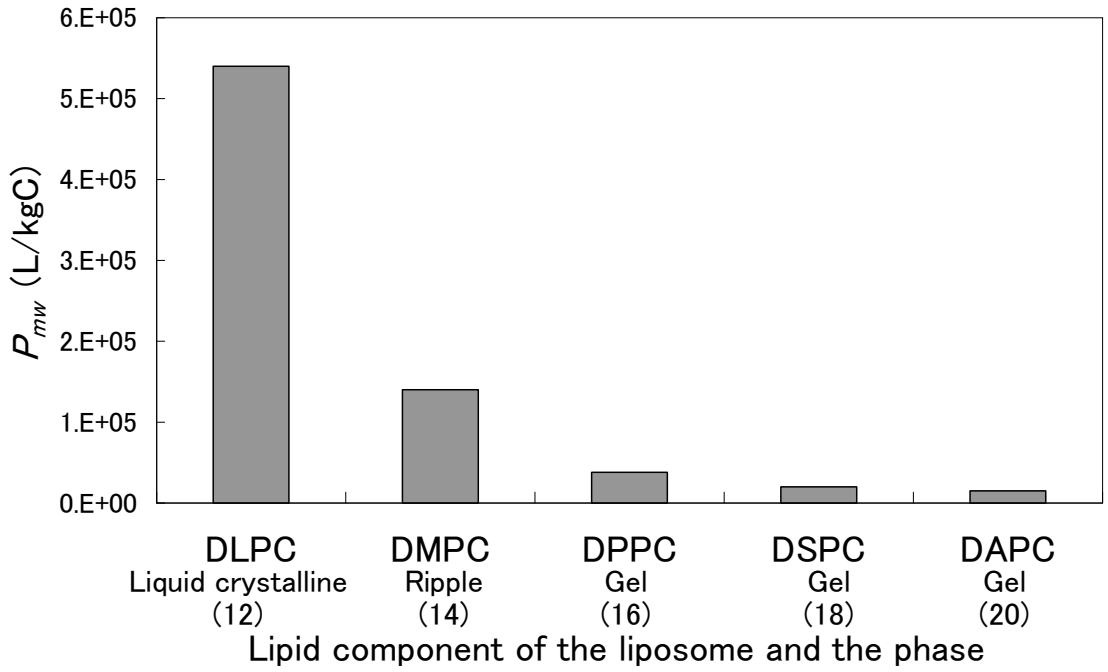


Fig.1 The effect of the fluidity of the membrane on P_{mw} of Pyrene

The values in parenthesis indicate the number of chain carbon.

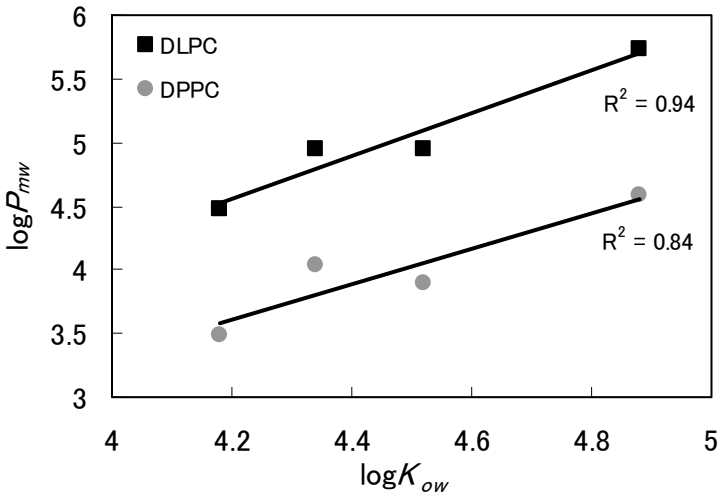


Fig.2 The correlation between logP_{mw} and logK_{ow}

Table 2 P_{mw} of PAHs onto EPC-TRANSIL® and POPS-TRANSIL®

PAHs	EPC-TRANSIL®	POPS-TRANSIL®
fluorene	2.8×10^4	2.2×10^4
phenanthrene	9.4×10^4	6.1×10^4
anthracene	1.2×10^5	9.1×10^5
pyrene	6.7×10^5	5.9×10^5

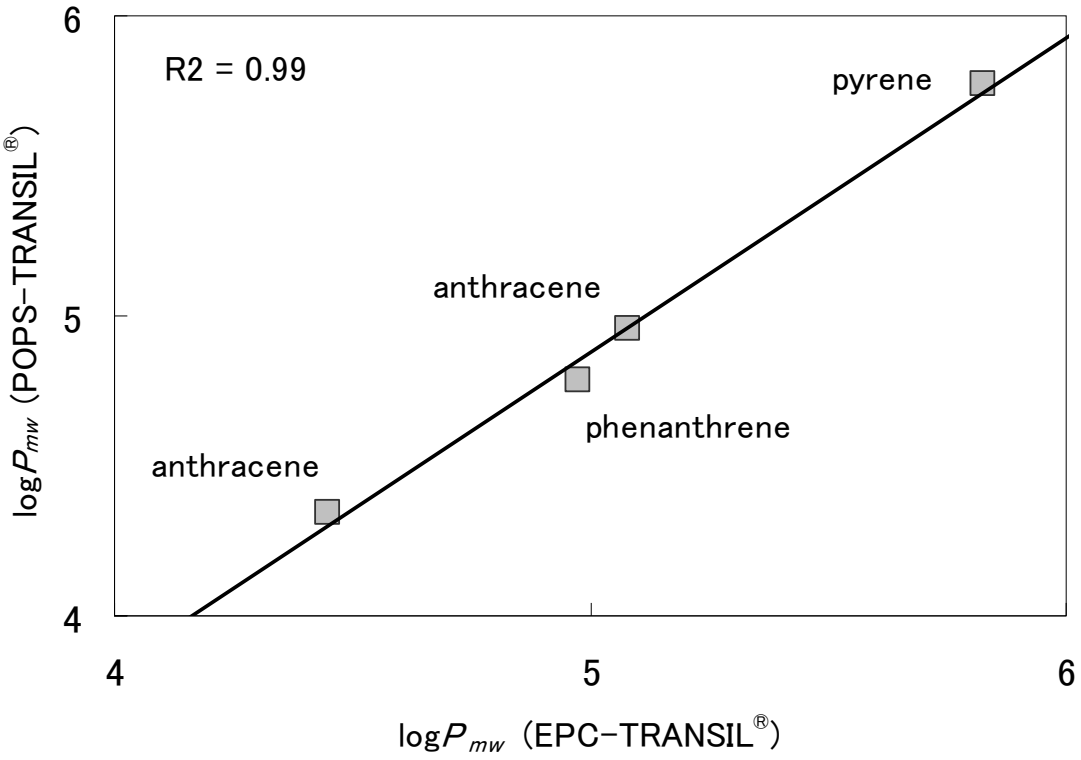
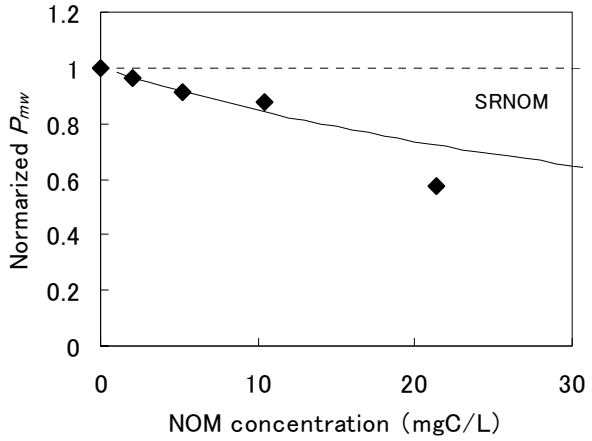


Fig.3 Correlation between P_{mw} for POPS-TRANSIL® and EPC-TRANSIL®

Table 3 K_{oc} of pyrene to NOMs

NOM	SRNOM	NFA
K_{oc}	1.7×10^4	1.8×10^4



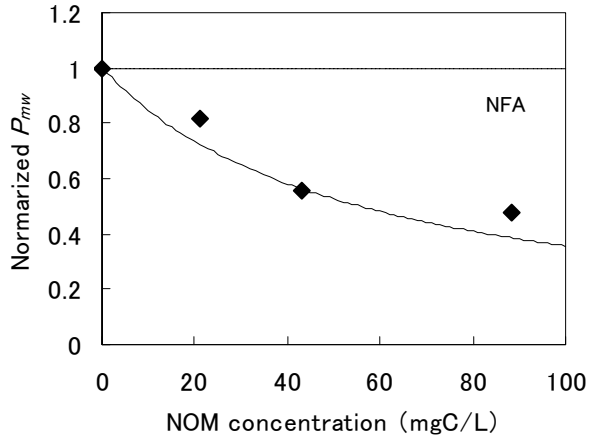


Fig.4 The effect of NOM on P_{mw} of pyrene onto EPC-TRANSIL®

Plots are measuered P_{mw}, the solid lines are estimated from equation (3) substituted by measured K_{oc}.

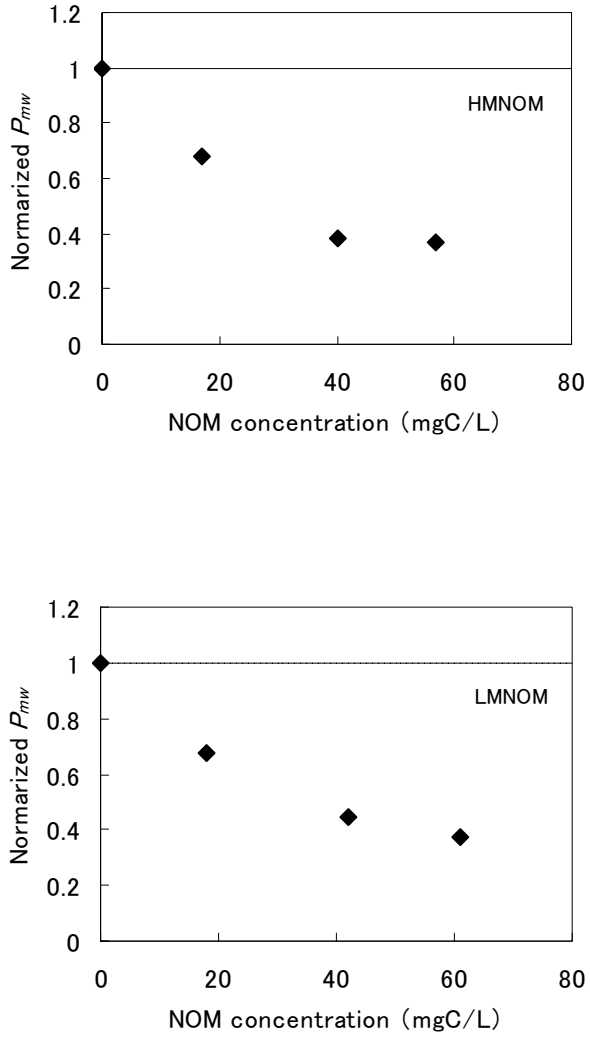


Fig.5 The effect of high molecular weight NOM and low molecular weight NOM on P_{mw} of pyrene onto EPC-TRANSIL®

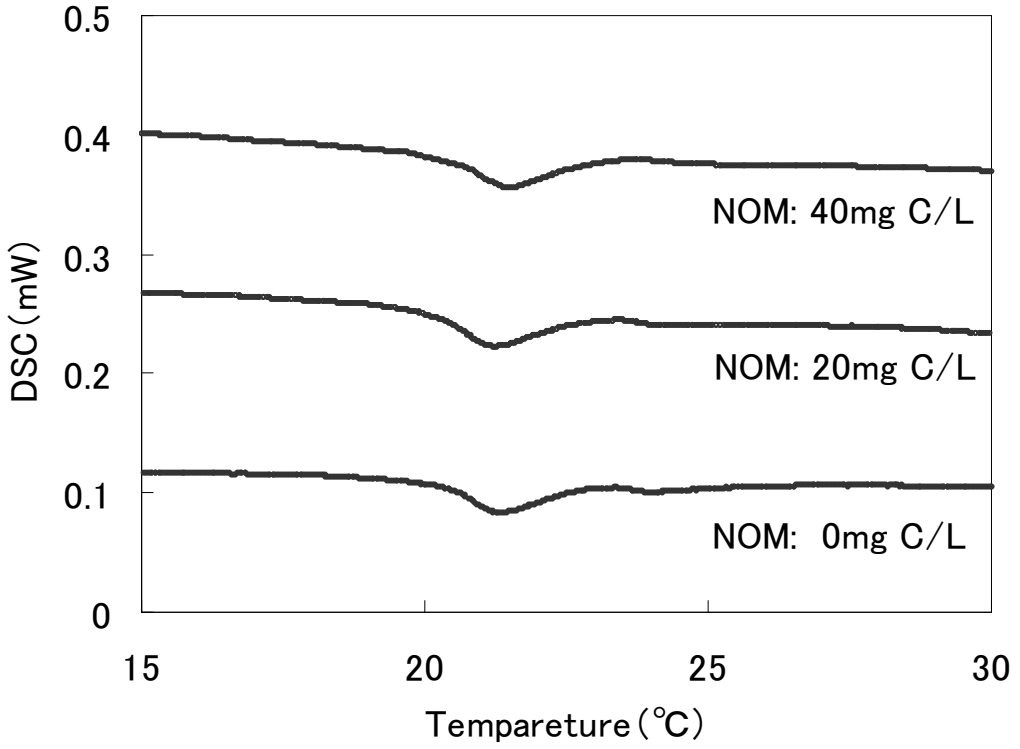


Fig.6 The effect of NOM on main phase transition temperature of DMPC-TRANSIL® membrane