Detection of Odorous Substances by Using a Lipid-Coated Quartz-Crystal Microbalance in the Gas Phase^{1,2)}

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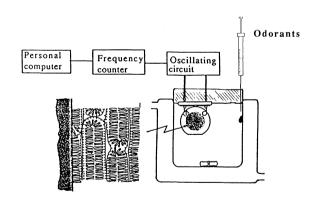
The adsorption behaviors and partition coefficients of various odorants and perfumes in a lipid matrix were measured in the gas phase using a dimethyldioctadecylammonium poly(styrene-4-sulfonate) multibilayer film-coated quartz-crystal microbalance (QCM), the resonance frequency of which changes linearly with increasing the absorption amount on the lipid matrix on the QCM. A good correlation was observed between the partition coefficients of various odorous substances or perfumes in the lipid matrix and the odor intensity in humans obtained by smelling tests for the same compounds. A similar relation was observed regarding the absorption behavior to an olfactory epithelium-coated QCM in the gas phase. Odorant having a strong intensity showed higher adsorption in the lipid matrix on the QCM. The partition coefficients increased in the solid state of the lipid matrix, compared with the fluid-liquid crystalline state. As compared with absorption experiments in the aqueous phase, the high partition coefficients of odorants in the lipid matrix and the good correlation between the partition coefficients and the odor intensity in humans were observed in the gas phase.

The olfactory reception of various odorants and perfumes is thought to occur by receiving at the olfactory cell membrane and producing electric signals in our bodies.3) However, the molecular mechanism of the reception of chemical substances and the transaction process in olfactory cells are not well understood at present. The chemical structures of odorants are extremely diverse and it is difficult to find any one chemical structure that is common to those substances. Odorant molecules are relatively hydrophobic and the threshold concentration of general odorants is roughly determined by the partition coefficients between water and oil.4) Kurihara and coworkers reported that the surface pressure of the lipid monolayer at the air-water interface from bovine olfactory epithelium⁵⁾ and the membrane potential of liposomes^{6,7)} or planner bilayer membranes⁷⁾ from synthetic phospholipids are selectively changed by the addition of various odorants in the aqueous phase. Their magnitude of the response has good correlation with olfactory reception in humans.^{5–7)} Responses to odorants are found not only in olfactory cells, but also in nonolfactory cells.4,5,8) These results suggest that the first step of olfactory reception takes place upon the adsorption of odorous substances at a lipid bilayer matrix without any specific receptor proteins in biological cells.32) Many approaches to elucidate olfactory reception have been undertaken using biological systems in the aqueous phase, although odorant molecules actually interact with olfactory epithelium in the gas phase.

These biological results prompted us to study the partition process of various odorants and perfumes in a lipid matrix by using a quartz-crystal microbalance (QCM) coated with a synthetic multibilayer-immobilized film in the gas phase. QCMs are known to provide very sensitive mass measuring devices at nanogram levels, since the resonance frequency changes sharply upon deposition of a given mass on the

electrode of the quartz plate. The experimental setup is shown in Fig. 1. A synthetic multibilayer film [dimethyldioctadecylammonium poly(styrene-4-sulfonate), 2C₁₈N+2C₁/PSS⁻], a naturally-occurring phospholipid (DPPC) cast film, and human olfactory epithelium were used as a membrane on the QCM. The adsorption experiments were carried out in the gas phase.

We have reported that lipid-coated QCM can detect the adsorption behaviors and partition coefficients of bitter substances,⁹⁾ general and local anesthetics,^{1,10)} and antibiotics¹¹⁾ into lipid bilayer membranes *in an* aqueous solution. Their bioactive magnitude can be



Multibilayer-immobilized film-coated Quartz-Crystal Microbalance

Fig. 1. Experimental setup for frequency measurements of the 2C₁₈N+2C₁/PSS⁻ multibilayer film-coated quartz-crystal microbalance (QCM) in the gas phase. DPPC cast film or olfactory epithelium was also employed as a membrane on the QCM.

explained in terms of the adsorption behaviors of these molecules into a lipid matrix. 12,13)

Experimental

Materials. Preparations of a polyion-complex-type synthetic bilayer-forming amphiphile, dimethyldioctadecylammonium poly(styrene-4-sulfonate) (2C₁₈N+2C₁/PSS⁻), have been reported elsewhere. 14,15) 1,2-Dipalmitoyl-sn-glycero-3-phospho-choline (DPPC), odorants, and perfumes were commercially available from Sigma Co. and Tokyo Kasei Co. Some odorants and perfumes were received from Takasago Fragrance Co., Tokyo.

Lipid-Coated QCM. A quartz-crystal microbalance (QCM, 8 mm diameter, AT cut, 9 MHz) was connected to a homemade oscillator designed to drive quartz at its resonance frequency and was driven at 5-V dc.^{9–13)} The frequency of the vibrating quartz was followed by a frequency counter (Iwatsu Elec. Co., Model SC7201) attached to a microcomputer system (NEC Co., Model PC9801). The following equation was obtained for the AT-cut shear mode OCM:^{16,17)}

$$\Delta F = \frac{-2F_{\rm o}^2}{A\sqrt{\rho_{\rm q}\mu_{\rm q}}} \,\Delta m,\tag{1}$$

where ΔF is the measured frequency shift (Hz), F_0 the parent frequency of QCM (9×10⁶ Hz), Δm the mass change (g), A the electrode area (0.20 cm²), ρ_q the density of quartz (2.65 g cm⁻³), and μ_q the shear modulus (2.95×10¹¹ dyn cm⁻², 1 dyn=10⁻⁵ N). Calibration of the QCM used in our experiments showed that a frequency decrease of 1 Hz corresponds to a mass increase of 1.05±0.01 ng on the electrode of QCM,^{9–13)}

$$\Delta m = -(1.05 \pm 0.01) \times 10^{-9} \,\Delta F. \tag{2}$$

A chloroform solution of 2C₁₈N+2C₁/PPS- or DPPC was cast on electrodes on both sides of OCM dried in air and aged in a hot water at 60 °C for 1 h in order to prepare welloriented multilamellar structures in the film. diffraction analyses showed that 2C₁₈N+2C₁ amphiphiles form extended lamellar structures of lipid bilayers (3.8 nm thick) parallel to the film plane (the QCM plate) in a polyion complex with poly(styrene-4-sulfonate) anions (PSS-).14,15) The multibilayer film showed a sharp endothermic peak at 45 °C with differential scanning calorimetry (DSC) in an aqueous solution, corresponding to the phase transition from the solid-to-liquid crystalline state. $^{14,15,18)}$ When the $2C_{18}N+2C_1/PSS$ film was cast, 20±2 μg on electrodes (20 mm²×2) on both sides of the QCM, the vibration frequency decreased by 2100±200 Hz in the air, which was consistent with the mass deposited on the eletrode in line with Eq. 2. Polymer (20±2 μg)-coated QCMs were prepared by casting a chloroform solution on the plate. Biological olfactory cells were cast from an aqueous dispersion of human olfactory epithelium. The membranecoated QCM was set in a 60-ml closed vessel which had been saturated with the vapor of odorous substances by the injection of 2 µl of odorants; the frequency change of the OCM due to the adsorption of odorants in a lipid matrix was followed with time in the gas phase under stirring.

Results and Discussion

Absorption Behaviors of Odorants. Figure 2 shows the frequency changes of a 2C₁₈N+2C₁/PPS- multibilayer film (20 µg, 1.0 µm thick)-coated QCM when the QCM was set in saturated vapor of β -ionone in a 60-ml closed vessel. The concentration of β -ionone was calculated to be 6.12 µg/60 ml of air from the saturated vapor pressure (9.9×10⁻³ mmHg at 25 °C, 1 mmHg= 133.322 Pa). The frequency of the OCM decreased immediately and reached to the equilibrium ($\Delta F = 720$ ± 5 Hz) within 5 min., which corresponding to an adsorption of 760±5 ng in a lipid matrix on the QCM from Eq. 2. The frequency reverted to the original value after the QCM was removed from the vessel to the atmosphere (arrow b in Fig. 2), indicating a leaving of adsorbed β -ionone from the lipid matrix. These reversible adsorption and desorption phenomena could be repeated many times without damaging the membrane and were observed for other odorants and perfumes. The adsorbed amount in the 2C₁₈- $N+2C_1/PSS$ - film on the QCM increased linearly with increasing concentration (1.0-30 µg/60 ml in air) of odorants. Partition coefficients (P) of the odorants in the lipid matrix were obtained when the concentration (g dm⁻³) of the adsorbed substance in the lipid matrix was divided by the concentration (g dm⁻³) of substances in the vessel calculated from the saturated vapor pressure.

The time courses of the frequency decrease in Fig. 2 show penetration or diffusion process of substances into the lipid matrix. The apparent diffusion rate constants (D) can be calculated from the slope of a plot of $\Delta m_t/\Delta m_\infty$ versus $t^{1/2}$ according to the approximations of the Hill and MacBain equation,¹⁹⁾

$$\frac{\Delta m_t}{\Delta m_{\infty}} = 2\sqrt{\frac{D}{\pi L^2}} t^{\frac{1}{2}} \qquad (\frac{\Delta m_t}{\Delta m_{\infty}} < 0.335), \qquad (3)$$

where Δm_t and Δm_{∞} are the adsorption amount at the time t and at the equilibrium, respectively. L is the

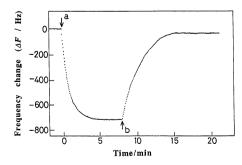


Fig. 2. Typical frequency changes of the $2C_{18}N^+2C_1/PSS^-$ film (20 µg)-coated QCM responding to setting in saturated vapor of β -ionone (6.12 µg/60 ml of air) at arrow a and brought in the atmosphere at arrow b.

Table 1. Absorption Amounts (Δm) and Partition Coefficients (P) of β -Ionone in Membrane-Coated QCMs in tha Gas Phase at 25 °Ca)

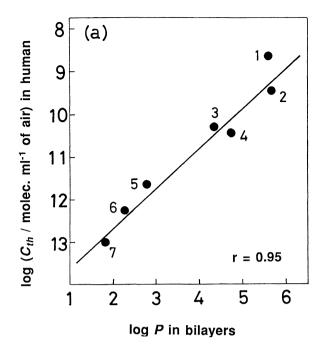
Membranes on QCN	$\Delta m/{ m ng}$	$P^{ m b)}/10^3$
Uncoated	8±2	4.1
2C ₁₈ N+2C ₁ /PSS- cast film	760 ± 10	390
DPPC cast film ^{c)}	540 ± 10	280
Olfactory cell membrane	640 ± 10	330
$C_{16}N^{+3}C_1/PSS^{-}$ film ^{d)}	155 ± 10	80
Polystyrene	74±4	38
Poly(vinyl alcohol)	26 ± 3	13
Poly(methyl L-glutamate)	42 ± 5	22
Bovine plasma albumine	32 ± 5	16
Kelatine	28±4	14

a) Obtained in the saturated vapor of β -ionone (6.12 µg) in 60 ml of air. The cast amount of membranes was 20±2 µg on the QCM. b) Containing ±5% of experimental errors. c) Dipalmitoylphosphatidylcholine. d) Hexadecyltrimethylammonium poly(styrene-4-sulfonate).

membrane thickness.

The absorption amounts and partition coefficients of β -ionone to various membrane-coated QCMs are summarized in Table 1. β -Ionone hardly adsorbed on to the uncoated QCM and hydrophilic or hydrophobic polymer-coated QCMs. The partition coefficients in proteins, such as albumine and kelatine, were also very small. In contrast, β -ionone absorbed specifically onto the lipid bilayer matrix of synthetic 2C₁₈N+2C₁/PSS⁻ and naturally-occurring DPPC cast films. A similar large adsorption was observed on the olfactory cell membranes in humans on the QCM. However, the partition to the cast film prepared from the single-chain amphiphile [hexadecyltrimethylammonium poly(styrene-4-sulfonate), C₁₆N+3C₁/PSS-] on the QCM was relatively small, in which single-chain amphiphiles cannot form oriented multibilayer structures in the cast film. These results indicate that odorants tend to adsorb into the lipid bilayer matrices, but not proteins, and that the partition behaviors hardly depend on the detailed structure of the hydrophilic head groups of lipid matrixes; further, the well-packed dialkyl bilayer structure is important for absorbing odorous substances. Although it is difficult to compare the absorption behaviors onto simple proteins, such as albumine and kelatine, with that onto membrane proteins in a cell membrane, it seems that the adsorption amount of odorants onto the proteins in the cell membrane is very small compared with that of a lipid matrix; the odor intensity is mainly determined by the adsorption behavior to the lipid bilayers.

Absorption experiments were carried out on seven odorous substances which are commonly employed in biochemical and biological experiments^{6,7,20} (β-ionone, coumarin, citral, 1-octanol, isopentyl acetate, methyl acetate, and diethyl ether) by using the 2C₁₈N+2C₁/PPS⁻ film-coated QCM in the gas phase at 25 °C.



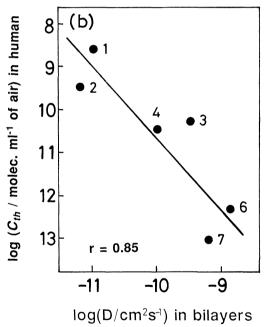


Fig. 3. Relations between (a): partition coefficients, *P*, or (b): diffusion rate constants, *D*, of odorants to the 2C₁₈N+2C₁/PSS⁻ multibilayer film on the QCM and their threshold concentration, *C_{th}*, for olfactory reception in humans. 1: β-ionone, 2: coumarin, 3: citral, 4: 1-octanol, 5: isopentyl acetate, 6: methyl acetate, 7: diethyl ether.

Partition coefficients (P) were calculated by dividing the absorption amount in the lipid matrix by the concentration of odorants in the vessel. Diffusion coefficients (D) were also calculated from the initial time course of absorptions according to Eq. 3.

Figure 3 shows a correlation between the $\log P$ or

log D values of various odorants in the $2C_{18}N^+2C_1/PSS^-$ film on the QCM and the logarithm of the threshold concentration (C_{th} , the number of odorant molecules in 1 ml of air) for the same odorants to cause olfactory reception in humans.⁵⁾ There was a good correlation between log P and the biological threshold values for the odorants employed (Fig. 3a). A plot of the log D values versus the biological log C_{th} values for odorants also gave a linear correlation. Odorous substances having a lower C_{th} value (stronger smell intensity) showed a higher partition and a slower diffusion rate in a lipid matrix. However, a better correlation (r=0.95) was observed in a plot of log P vs.

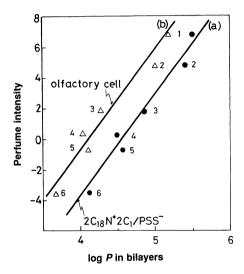


Fig. 4. Relations between partition coefficients of perfumes in (a): the synthetic 2C₁₈N+2C₁/PSS⁻ film or (b): the olfactory epithelium on the QCM and their perfume intensities in humans. 1: 1-undecanol, 2: *p*-anisaldehyde, 3: anethol, 4: citral, 5: phenethyl acetate, 6: benzyl acetate.

log C_{th} than that (r=0.85) of log D vs. log C_{th} . Thus, the intensity of olfactory reception seems to be mainly determined by the adsorption amounts (partition coefficients) to the lipid matrix on the QCM. These substances hardly adsorbed onto the protein matrix on the QCM (see Table 1).

Absorption Behaviors of Perfumes. QCM absorption experiments were also applied to detect various perfumes and fragrances in the gas phase at 25 °C in the same manner. The partition coefficients of six typical perfumes (1-undecanol, p-anisaldehyde, anethol, citral, phenethyl acetate, and benzyl acetate) in the synthetic 2C₁₈N+2C₁/PSS- film-coated QCM and the human olfactory epithelium-coated QCM were obtained and plotted against the perfume intensity proposed originally by Appell in Fig. 4. determined the perfume intensity by smelling vapors from solutions of standard concentration and normalized by setting the intensity of citral to unity.²¹⁾ Since Appell's intensities were not considered to be the true concentration of perfume in air, we corrected Appell's values by dividing them by the partial vapor pressure of each perfume. We found a good correlation between the log P values of perfumes in the lipid matrix, or the log P values in the olfactory epithelium, and the modified perfume intensity, as well as the correlation for various odorants. The perfume having the stronger intensity showed the higher partition to the lipid matrix or the olfactory cell membrane on the QCM.

These results for odorants and perfumes agree with the proposal^{3a,5,7)} that the first step of olfactory reception takes place upon the adsorption of odorous substances at a lipid bilayer matrix, without any specific receptor proteins in the biological cells. The intensity of olfactory reception is mainly determined

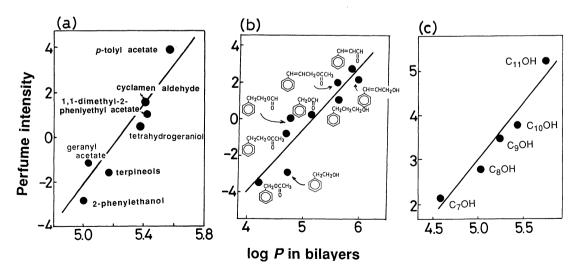


Fig. 5. Relations between partition coefficients of (a): floral perfumes, (b): aryl perfumes, and (c): long-chain alcohols in the 2C₁₈N+2C₁/PSS⁻ film on the QCM and intensities of those perfumes in humans.

by the partition amounts in the lipid matrix of biological olfactory cells. The partition coefficients in the olfactory epithelium is smaller than those in the synthetic $2C_{18}N^+2C_1/PSS^-$ membranes, probably because of the smaller amount of lipid matrix in the cell membrane, compared with a synthetic multibilayer film.

Absorption experiments were also carried out for other specific perfumes such as floral-smelling perfumes (p-tolyl acetate, cyclamen aldehyde, 1,1-dimethyl-2-phenylethyl acetate, tetrahydrogeraniol, geranyl acetate, terpineols, and 2-phenylethanol), aryl compounds and linear long-chain aliphatic alcohols $(C_7OH-C_{11}OH)$ by using the $2C_{18}N+2C_1/PSS$ - filmcoated QCM in gas phases at 25 °C. The obtained $\log P$ values are plotted against the each perfume intensity in Fig. 5a—c. Good linear correlations were obtained for floral perfumes, aryl compounds, and long-chain alcohols. The proposal that the smell intensity can be determined mainly by the partition amount of odorants in the lipid matrix can be extended widely to various kinds of odorous substances and fragrances.

Absorption Mechanisms. Figure 6 shows the effect of the membrane thickness of the $2C_{18}N+2C_1/PSS^-$ multibilayer film on the QCM on the absorption amount of β -ionone in the gas phase at 25 °C. The absorption amount increased with increasing the membrane thickness. Similar dependences on the membrane thickness were observed for Langmuir-Blodgett (LB) lipid films on the QCM. When monolayers of dialkyl amphiphiles, β -(N,N-dioctadecylcarbamoyl)propionic acid), were deposited on a QCM by the LB method, the adsorption amount of odorants, such as β -ionone, increased linearly with increasing the number (n=2-30) of layers of the lipid LB films.

The phase transition from solid-to-liquid crystalline states of the dialkyl chain is one of the fundamental

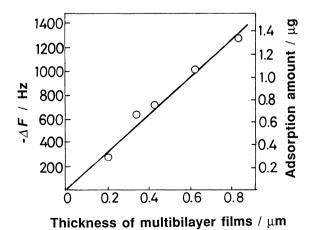


Fig. 6. Effect of the membrane thickness of the $2C_{18}N^+2C_1/PSS^-$ multibilayer film on the QCM on the absorption amount of β -ionone at 25 °C.

properties of the lipid bilayer matrix.^{14,15,18)} Partition coefficients (P) and diffusion coefficients (D) were obtained for the adsorption of β -ionone at various temperatures both below and above the phase-transition temperature (T_c =45 °C) of the 2C₁₈N+2C₁/PSS- multibilayer film on the QCM; results are shown in Fig. 7 in a form of Arrhenius plots. The T_c of the 2C₁₈N+2C₁/PSS- bilayer film was observed separately by differential scanning calorimetry (DSC). The diffusion constants increased drastically at temperatures above T_c =45 °C, compared with those below T_c . On the contrary, the partition coefficients decreased unexpectedly at temperatures above T_c relative to those

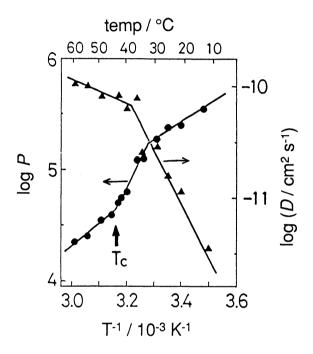


Fig. 7. Temperature dependences of partition coefficients (P) and diffusion rate constants (D) of β-ionone in the 2C₁₈N+2C₁/PSS⁻ film on the QCM. T_c value was obtained separately from DSC measurements.

Table 2. Effect of the Alkyl Chain Length of Dialkylammonium Multibilayer Film (2C_nN+2C₁/PSS⁻) on Partition Coefficients of β-Ionone in the Gas Phase at 25°C

Bilayer membranes	$T_{\rm c}/{}^{\circ}{ m C}^{ m a)}$	P/10 ³
2C ₁₈ N+2C ₁ /PSS-	45	390
$2C_{18:1}N^{+}2C_{1}^{b)}/PSS^{-}$	<0	160
$2C_{16}N+2C_{1}/PSS-$	37	340
$2C_{14}N+2C_{1}/PSS-$	26	250
$2C_{12}N^{+}2C_{1}/PSS^{-}$	15	100

a) Obtained by DSC measurements.

below T_c . Thus, in the fluid and disordered liquid crystalline state above T_c , the adsorption amount of odorant molecules decreased, although their penetration rate into the lipid matrix increased.

Table 2 shows the effect of the alkyl-chain length of bilayer-forming amphiphiles on the QCM on the partition coefficients of β -ionone in the gas phase at 25 °C. The phase-transition temperatures of these 2C_nN+2C₁/PSS- films decreased with decreasing the alkyl-chain length of the lipid. Dimethylbis(2oleyloxyethyl)ammonium salts (2C_{18:1}N+2C₁/PSS-) having two unsaturated alkyl chains showed T_c below The $2C_{18}N+2C_1/PSS^-$ and $2C_{16}N+2C_1/PSS^$ films have their T_c at 45 and 37 °C, respectively, and exist in the solid state at 25 °C. On the contrary, the $2C_{12}N+2C_1/PSS$ - film ($T_c=15$ °C) having the shortest dialkyl chains and the 2C_{18:1}N+2C₁/PSS- film having the unsaturated dioleyl chains exist as the fluid-liquid crystalline state at 25 °C. The partition coefficients in the fluid membrane of the 2C_nN+2C₁/PSS- film decreased compared with those in the solid state membrane at 25 °C. These results are consistent with the temperature dependence shown in Fig. 7; odorant molecules tend to adsorb in the solid state of the lipid matrix compared with the fluid state.

Comparison with Aqueous Systems. When absorption experiments of odorants were carried out in distilled water by using the 2C₁₈N+2C₁/PSS- filmcoated OCM at various temperatures in the same manner,9) the results were different from those in the gas phase: the partition coefficients of β -ionone increased in the fluid-liquid crystalline state above T_c relative to the solid state lipid matrix (not shown). These opposite temperature dependences both in gas and the aqueous phases can be explained as follows. In an aqueous system, odorous molecules may aggregate around each other because of their hydrophobic properties; these aggregate molecules can adsorb and penetrate largely into the disordered fluid lipid matrix above T_c . On the contrary, in the gas phase odorous molecules can disperse molecularly and tend to penetrate into small defects in a solid lipid matrix, compared with the largely disturbed lipid multibilayer film.

The partition coefficients of various perfumes in the 2C₁₈N+2C₁/PSS⁻ film on the QCM were also obtained in an aqueous solution at 25 °C. The correlation between log *P* obtained in an aqueous solution vs. the human perfume intensity is shown in Fig. 8, together with that in the gas phase. The correlation in the aqueous phase was incorrectly compared with that in the gas phase, and partition coefficients in the aqueous solution was 100-times smaller than those in the gas phase. These results can again be explained by suggesting that odorous molecules aggregate around each other in the aqueous phase and that the partition coefficients of the aggregated molecules obtained in an

aqueous system do not show a good correlation with the perfume intensity obtained by smelling in the gas phase.

In biological olfactory cell membranes, it is known that the membrane surface is covered with a wet mucous membrane, which is believed to play an important role in olfactory reception. We studied the effect of this wet layer of the lipid matrix on the absorption of odorants by using the QCM system in the gas phase. We prepared three kinds of multibilayer cast films on the QCM: i) the usual $2C_{18}N+2C_1/PSS$ -film (0.5 µm thick) whose contact angle toward water

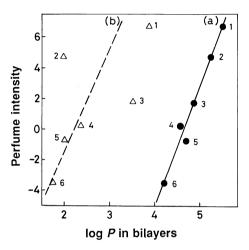


Fig. 8. Relations between perfume intensities in humans and partition coefficients of the same compounds in the 2C₁₈N+2C₁/PSS⁻ film (a): in a gas phase or (b): in an aqueous phase. 1: 1-undecanol, 2: *p*-anisaldehyde, 3: anethol, 4: citral, 5: phenethyl acetate, 6: benzyl acetate.

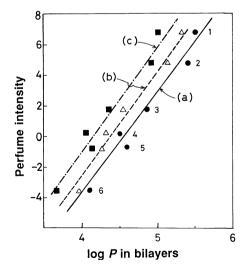


Fig. 9. Relations between perfume intensities in humans and partition coefficients of the same compounds in (a): the 2C₁₈N+2C₁/PSS⁻ film, (b): the PVA-overcoated 2C₁₈N+2C₁/PSS⁻ film, and (c): the blend film of 2C₁₈N+2C₁/PSS⁻ and PVA. Numbers show the same as those in Fig. 8.

is 76°, showing the hydrophobic surface similar to that of polystyrene; ii) the swelled poly(vinyl alcohol) film (PVA, 0.1 µm thick) was cast from an aqueous solution on the $2C_{18}N+2C_1/PSS$ - film (0.5 µm thick) whose contact angle toward water is 34°, showing the hydrophilic surface similar to that of PVA; and iii) the 2C₁₈N+2C₁/PSS- was blended with swelled PVA (5:1) and cast on the QCM whose contact angle toward water is 50°. The partition coefficients of various perfumes in these membranes were obtained and are plotted against the perfume intensity in Fig. 9. Good correlations were found between $\log P$ values and the perfume intensity for those membranes; however, the partition coefficients for the PVA-overcoated membrane and the blend membrane with PVA decreased compared with those in the 2C₁₈N+2C₁/PSS- film having a hydrophobic surface. Thus, the partition of relatively hydrophobic odorous molecules seems to decrease in the presence of the hydrophilic layer on the lipid matrix, and the mucous membrane on the olfactory epithelium may be not absolutely required for the detection of odorants.

Conclusion

The partition coefficients of various odorants and perfumes into the lipid matrix can be easily and quantitatively obtained by using the synthetic multibilayer film-coated QCM in the gas phase. The intensity of various odorants and perfumes can be explained by the absorption amount of a lipid matrix. The lipid-coated QCM is both physically stable and reusable, and will provide a new sensor system to determine the intensity of odorants and perfumes in the gas phase.

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