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Research article

Development of a membrane impregnated with a poly(dimethylsiloxane)/poly(ethylene glycol) copolymer for a high-throughput screening of the permeability of drugs, cosmetics, and other chemicals across the human skin

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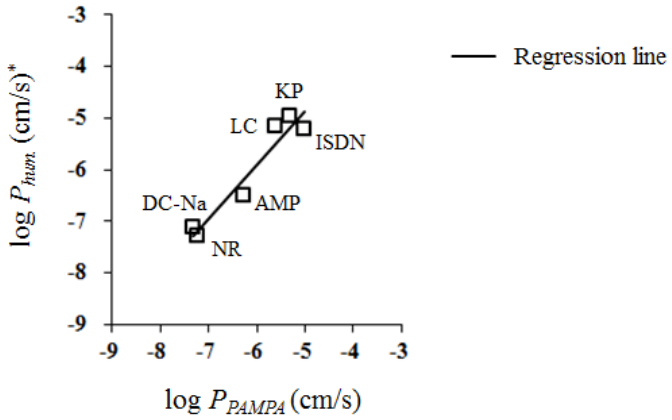
Graphical abstract

We obtained an ideal correlation between the permeability coefficients across the poly(dimethylsiloxane)/poly(ethylene glycol) 6000 (PDMS/PEG 6000) copolymer by using a 96-well plate and those obtained across the human skin.



ABSTRACT

We aimed to develop a high-throughput screening (HTS) system for preliminary predictions of human skin permeability by using an artificial membrane that can mimic the permeation behaviour of lipophilic and hydrophilic compounds across the human skin. In this study, we synthesized a copolymer containing poly(dimethylsiloxane) (PDMS) and poly(ethylene glycol) (PEG) 6000 and impregnated it onto a supportive membrane filter to prepare a PDMS/PEG 6000 copolymer-impregnated membrane. In addition, we synthesized another polymer without PEG units and used it to prepare an impregnated membrane for determining the role of PEG 6000 units in the PDMS/PEG 6000 copolymer-impregnated membrane. The permeation characteristics of the impregnated membranes were evaluated on the basis of the permeability coefficients of 12 model compounds with different lipophilicities, by using a 2-chamber diffusion cell, and these permeability coefficients were compared with those across the human skin. We obtained a good correlation between the permeability coefficients across the PDMS/PEG 6000 copolymer-impregnated membrane and human skin. Further, we evaluated the permeation characteristics of a 96-well plate model of the PDMS/PEG 6000 copolymer by using 6 model compounds. We obtained an ideal correlation between the permeability coefficients across the PDMS/PEG 6000 copolymer using a 96-well plate and those

across the human skin. Thus, the PDMS/PEG 6000 copolymer would be a good candidate for preliminary evaluation of the permeability of lipophilic and hydrophilic compounds across the human skin.

1. Introduction

Estimation of the skin permeability of various compounds is important for determining the potential of transdermal application of pharmaceutical and cosmetic products and for assessing the risk of toxic chemicals. Preliminary evaluations of the permeability of a large number of compounds and their formulations by *in vivo* and *in vitro* studies using human or animal skin are not feasible. Thus, a high-throughput screening (HTS) system is a desirable method for these studies.

The parallel artificial membrane permeation assay (PAMPA) was developed as a HTS system for estimation of passive membrane permeability of molecules, and the PAMPA model has been used mainly to predict the gastrointestinal absorption (Avdeef et al., 2007; Kansy et al., 1998; Sugano et al., 2003). However, few studies have reported the use of the PAMPA for determining the skin permeability (Ottaviani et al., 2006; Sinkó et al., 2012). The ability of several synthetic membranes to predict skin permeability has been studied by using a 2-chamber diffusion cell (Dias et al., 2007; Geinoz et al., 2002; Hatanaka et al., 1992; Wasdo et al., 2009). While these membranes could be used to predict the skin permeability of lipophilic compounds, they could not be used for hydrophilic compounds; moreover, fewer studies have been performed using these

membranes in hydrophilic compounds than in lipophilic compounds.

Typically, the permeability of the skin is lower for hydrophilic compounds than for lipophilic compounds because the stratum corneum (SC) is a highly lipophilic barrier. Evaluation of the permeability of the skin for both lipophilic and hydrophilic compounds is required, and an artificial membrane is required to perform these preliminary evaluations.

In a previous study, we synthesized copolymers using the lipophilic materials, methyl methacrylate (MMA) and glycidyl methacrylate (GMA), and the hydrophilic macro-azo-initiator poly(polyoxyethylene-azobiscyanopentanoate) (VPE-0601), which had poly(ethylene glycol) (PEG) 6000 units with an average molecular weight (MW) of approximately 6000 Da (Miki et al., 2010). We identified the possibility that the PEG 6000 units formed a hydrophilic microdomain in the copolymer membranes.

Silicone, poly(dimethylsiloxane) (PDMS), is a popular rubbery polymer component; it is stable, inert, and used for the dissolution of chemicals (De Coensel et al., 2007). The permeability of hydrophilic compounds across PDMS membranes has often been undetectable because of the highly lipophilic nature and non-aqueous pore structure of these membranes.

Our aim is to develop a HTS system for predicting the permeability of the human

skin using an artificial membrane that could mimic the permeation behaviour of lipophilic and hydrophilic compounds across the human skin. In this study, we synthesized a copolymer with PDMS and PEG 6000 units. To enable comparison with a polymer containing PEG 6000 units, we also synthesized a polymer without PEG 6000 units. We evaluated the average MW and composition of the synthetic polymers by using gel permeation chromatography (GPC) and proton nuclear magnetic resonance (^{1}H-NMR). The synthesized polymers were viscous liquids, and thus, membranes impregnated with these polymers could be prepared easily using a membrane filter and could be used in a PAMPA model with a 96-well plate. We evaluated the permeation behaviours of model compounds with different lipophilicities through the impregnated membranes containing the polymers with or without PEG units (Table 1) by using a 2-chamber diffusion cell, and we compared them with those through the human skin (Morimoto et al., 1992). Further, we evaluated the permeation behaviours using the PAMPA model with the synthesized polymer.

2. Materials and methods

2.1. Materials

VPE-0601, 2,2′-azobis(2-methylbutyronitrile) (AMBN), aminopyrine (AMP), antipyrine (ANP), ketoprofen (KP), and PEG 400 were obtained from Wako Pure Chemical Industrial Co. (Osaka, Japan). Cyclobarbital (CB), diclofenac sodium (DC-Na), isoproterenol hydrochloride (IPH), and lidocaine (LC) were purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Dopamine hydrochloride (DPH) was purchased from Yodogawa Pharmaceutical Industries Co. (Osaka, Japan). Isosorbide dinitrate (ISDN) and flurbiprofen (FP) were kindly supplied by Toko Pharmaceutical Industries Co. (Tokyo, Japan). L-dihydroxyphenylalanine (L-dopa; L-DP) was obtained from Sigma–Aldrich Japan (Tokyo, Japan). Nicorandil (NR) was obtained from Nisshin Flour Milling Co. (Tokyo, Japan). α-Butyl-ω-(3-methacryloxypropyl)polydimethylsiloxane (SILAPLANE; FM-0711), with PDMS units of an average MW of 724 Da, was generously provided by JNC Co. (Tokyo, Japan). All other chemicals and solvents were of reagent grade and were obtained commercially. VPE-0601 was refined by recrystallization in chloroform, as the good solvent, and hexane, as the poor solvent (Miki et al., 2010). All of the above chemicals, except VPE-0601, were used without further

purification. We used 12 compounds (Morimoto et al., 1992) as penetrants in the permeation study performed using a 2-chamber diffusion cell for each impregnated membrane (Tables 1-2). We used six compounds (KP, LC, ISDN, AMP, DC-Na and NR) as penetrants for the PAMPA experiments (Morimoto et al., 1992).

2.2. Synthesis of polymers

For the initiation of the radical polymerization reaction, specified amounts of purified VPE-0601 or AMBN and FM-0711, weighing a total of 9.0 g, were placed into a glass tube with 30 ml of chloroform to synthesize polymers A and B (Fig. 1 and Table 3). The tube was sealed using a 3-way cock and was freeze-thawed to remove the oxygen. The tube was kept at 65°C in a water bath for 24 h to allow the reaction to take place. After dismounting the 3-way cock, the tube was kept at 65°C for an additional 24 h to allow the solvent to evaporate. The reaction mixture and 20 ml of the poor solvent were blended and were stirred for 1 h to refine the hydrophilic low-MW polymer and the unreacted initiator from the mixture. A mixture of methanol and water (70:30) was used as the poor solvent for polymer A, and ethanol was used for polymer B. After stirring, the blended liquid was kept still at room temperature for 1 h for it to separate into two phases; the upper phase then was removed using a Pasteur pipette. In addition, for

polymer B, a mixture of ethanol and chloroform (80:20) was used as a second poor solvent to remove any unreacted FM-0711, and the same procedure was performed for refinement. Then, the lower phases in the reaction mixture were dried at 100°C for 48 h in an oven to remove any remaining solvent.

2.3. Evaluation of synthetic polymers

2.3.1. Estimation of the average MW

GPC was used to determine the number-average MW (Mn), the weight-average MW (Mw), and the polydispersity (Mw/Mn) of the polymers. GPC was performed using a column (K-804L; Showa Denko, Tokyo, Japan) at 40°C and a refractive index detector (RI-101; Showa Denko), using chloroform as the eluent at a flow rate of 0.5 ml/min (Miki et al., 2010). Narrowly distributed poly(styrenes) (Showa Denko) were used as the MW standards.

2.3.2. Measurement by using ^{1}H-NMR

^{1}H-NMR spectroscopy was used to determine the composition of the synthetic polymer A (JNM-ECA600; JEOL Ltd., Tokyo, Japan); deuterated chloroform ($CDCl_3$;

99.96% D; ISOTEC, Inc., Miamisburg, OH, USA) at room temperature was used as the solvent. The sample solution was prepared at a concentration of 4.0 mg/ml (Miki et al., 2010).

2.3.3. Calculation of polymer composition

The chemical shifts of the functional groups of the chemicals and of the synthetic polymers used in the present study are shown in Table 4. In addition, the values of the chemical shifts, except for those of AMBN, were obtained from the literature and were used as a reference (Hamurcu et al., 1996; Hesse. et al., 2000; Laverty et al., 1977); Mojsiewicz-Pieńkowska et al., 2003; Tamura, 1991). The values of the chemical shifts of FM-0711 and of VPE-0601 were consistent with those reported in the literature. The chemical shifts at δ -0.1~0.2 (symbol: a) and δ 3.60~3.65 (symbol: f) were selected for calculating the mole ratio of each unit in polymer A (Fig. 1 and Table 4). VPE-0601 and FM-0711 were converted to PEG 6000 and PDMS units in the calculation of the polymer composition (Miki et al., 2010). The mole ratio of each unit was calculated according to the signal intensity ratio. Finally, the mole ratios were converted to weight ratios by multiplying them by the MW of the components (PDMS units, 724 Da and PEG 6000 units, 6000 Da; Table 3).

2.4. Preparation of polymer-impregnated membranes for the 2-chamber diffusion cell experiment

The synthetic polymers were dissolved in chloroform to a concentration of 100 mg/ml. We dropped 200 μl of the polymer solution onto a polyethylene terephthalate (PET) film (NF SP-PET 751031; LINTEC Co., Tokyo, Japan) placed on a flat glass plate. Then, a sheet of hydrophilized polytetrafluoroethylene (PTFE) membrane filter (Omnipore JH; pore size, 0.45 μm; diameter, 25 mm; Nihon Millipore, Tokyo, Japan) was overlaid immediately on the polymer solution. The impregnated membrane was kept still at ambient temperature for 1 h and then under ordinary pressure at 100°C for 24 h in an oven to dry and to ensure complete removal of the solvent. The impregnated membranes containing polymer A and polymer B were designated as membrane A and membrane B, respectively.

2.5. *In vitro* permeation study using a 2-chamber diffusion cell

The physicochemical properties of the model compounds used for the permeation study are shown in Tables 1-2. Table 1 contains apparent octanol/water partition coefficient (log $K_{O/W}$). We obtained log $K_{O/W}$ from a previous report (Morimoto et al.,

1992). The values of $K_{O/W}$ is obtained by calculating the solubility ratio in octanol/water at 37°C on a set of experiments (Morimoto et al., 1992). Note that the values of $K_{O/W}$ depend on the data source, and often can be different according to the methods (other methods e.g. shake flask method) and experimental condition (solvent, their pH and temperature and so on). And, even though described as the same method and condition, the values of $K_{O/W}$ may not be necessarily the same. The range of MWs for these compounds was within a factor of 1.69 (MW, 188 to 318 Da), whereas the $K_{O/W}$ values varied over a factor of 10^8 (log $K_{O/W}$, -4.70 to 3.86). Thus, the permeability through the lipophilic membranes was expected to be influenced predominantly by the $K_{O/W}$ values but not by the MW. We defined the lipophilic compounds as those with a log $K_{O/W} \geq 0$ and the hydrophilic compounds as those with a log $K_{O/W} < 0$. In the case of membrane B, the apparent permeability coefficient (P) (cm/s) was determined only for compounds with a log $K_{O/W} \geq -2.0$. The cumulative amount of L-DP permeated up to 48 h was less than the detection limit (0.05 μg/ml); similarly, the concentration of the two other hydrophilic compounds, IPH and DPH, was also less than the detection limit, and thus, their concentrations could not be determined. The prepared membranes were mounted in the 2-chamber diffusion cell (effective diffusion area, 0.95 cm^2) (Okumura et al., 1989). The donor compartment was filled with 2.6 ml saturated compound solutions in purified water

containing an excess of the compounds to maintain the maximal thermodynamic activity. For ANP, IPH, and DPH, the donor concentrations of which were 100 mg/ml in purified water, the receiver compartment was filled with 2.6 ml purified water. In the case of FP and KP, maintaining sink condition was difficult because these compounds were highly lipophilic. Therefore, we used an aqueous solution of 40% PEG 400 as the receiver solution to increase the solubility of these two lipophilic compounds (Yamaguchi et al., 1997). The donor and receiver phases were stirred using magnetic stirrers and kept at 37°C by circulating warm water in the water jackets of the chambers. A suitable volume of the receiver solution was removed at predetermined times and then the same volume of fresh purified water or the aqueous solution of 40% PEG 400 was added to the receiver compartment to maintain constant volume. The concentrations of the compounds in the receiver solution were assayed to determine the cumulative amount of the compounds permeated at each sampling time point. The cumulative amounts were plotted against time, and the steady-state flux (*J*, rate of compound permeated per unit area) was derived from the linear part of the profiles. *P* values were calculated by dividing *J* by the solubility of each compound for the suspended solution or the specified concentration: 100 mg/ml for ANP, IPH, and DPH. The sink condition ensured that concentration of the compound in the receiver phase was less than 10% of the concentration of the compound

in the donor phase.

2.6. Preparation of the PAMPA model for polymer A

Polymer A was dissolved in chloroform to a concentration of 100 mg/ml. We dropped 17 μl of the polymer solution onto a hydrophobic polyvinylidene difluoride (PVDF) membrane place in a 96-well plate (MultiScreen® IP filter plate; pore size, 0.45 μm; Nihon Millipore). The upper plates were kept still at ambient temperature for at least 12 h to completely evaporate the solvent.

2.7. *In vitro* permeation study using the PAMPA model

Permeation experiments were performed using the polymer A-impregnated 96-well plate. The donor plate was filled with 280 μl of the compound in purified water, citrate-phosphate buffer, or phosphate buffer. For ISDN and NR, purified water was used as the donor solvent. Donor solvents for other compounds were citrate-phosphate buffer or phosphate buffer adjusted at a pH value corresponding to that of the aqueous suspension of the compound (Table 2) (Morimoto et al., 1992). The donor concentrations of tested compounds were set at 20% of the saturated solubility of each compound (Table 1). The receiver plate was filled with 280 μl purified water. For KP, an aqueous solution

of 40% PEG 400 was used as the receiver solution. A donor plate mounted on a receiver plate (MultiScreen® IP filter plate; pore size, 0.45 μm; Nihon Millipore) was incubated at 37°C for predetermined time. Permeation periods were for KP, LC, ISDN, AMP, DC-Na and NR were 2.5, 2.5, 0.5, 1.0, 5.0 and 5.0 h, respectively. After the specified permeation periods, the donor and receiver plate were separated, and then, a suitable volume of receiver solution was immediately removed and assayed. For experiments performed using the 96-well plate, apparent *J* (J_{PAMPA}) was obtained by dividing the amount of compounds permeated from the start time to that at the end of the permeation period by the permeation period; the assumed permeation profiles were almost linear. Apparent *P* (P_{PAMPA}) values were calculated by dividing the apparent *J* by the initial donor concentration. The sink condition ensured that the concentration of the compound in the receiver phase was less than 10% of the concentration of the compound in the donor phase.

2.8. Analytical methods

The concentrations of the compound in the receiver phase were determined using an ultraviolet (UV) spectrophotometer with a microplate reader (SpectraMax M2^e; Molecular Devices, Sunnyvale, CA, USA). The sample solution or reference solution was

placed in each well of the 96-well UV-transparent microplate (BD Falcon, NJ, USA) or UV-star Half Area microplate (Greiner Bio-one, Tokyo, Japan). The UV detection wavelengths are shown in Table 1 (Hatanaka et al., 1990). An HPLC system was used to determine the concentration of L-DP obtained using the 2-chamber diffusion cell (Miki et al., 2010), and the HPLC conditions were similar to those reported previously (Hatanaka et al., 1990) for performing detection with a high sensitivity.

2.9. Statistical analysis

Correlation analysis was used to examine the relationships between log $K_{O/W}$ values of the compounds and the logarithm of the *P* through the membranes A and B. In addition, correlation analysis was used to examine the relationships between the logarithm of the *P* through each impregnated membrane and through human skin obtained from both permeation studies using a 2-chamber diffusion cell and a 96-well plate. A level of $p < 0.05$ was considered significant.

3. Results and Discussion

In this study, we synthesized two polymers (polymers A and B), one containing

PDMS and PEG 6000 units and the other without PEG 6000 units. The polymers were used to prepare membrane A with PEG units and membrane B without PEG units.

3.1. Evaluation of synthetic polymers

The unit composition of PDMS and PEG 6000 might be a crucial factor for determining the properties of the polymer. Thus, we evaluated the properties of the polymers using GPC and ^{1}H-NMR. The feed ratio, estimated composition of PDMS and PEG units, and average MW of the polymers are shown in Table 3. The Mn of polymers A and B was 157 and 52 kDa, and the Mw was 209 and 106 kDa, respectively. The estimated unit content of polymer A was 96.5 wt% for PDMS units and 3.5 wt% for PEG 6000 units, which reflected the feed ratio of FM-0711 and VPE-0601. This trend was consistent with that observed in our previous study performed using VPE-0601 (Miki et al., 2010).

3.2. An *in vitro* permeation study using the 2-chamber diffusion cell

3.2.1. Permeation behaviours of the compounds through the impregnated membranes

We performed permeation studies using a 2-chamber diffusion cell and evaluated the permeation behaviours of the compounds through membranes A and B. The cumulative

amounts of ISDN and NR that permeated across membranes A and B are shown in Fig. 2 as typical permeation profiles. The values of *J*, *P*, and lag time of the compounds through the impregnated membranes are shown in Table 5. We used L-DP, a highly hydrophilic compound, and confirmed that membrane B cannot be used for appropriate evaluation of the permeation behaviour of hydrophilic compounds. No permeation of L-DP through membrane B was detected until 48 h (the concentration of L-DP in the receiver phase was below the detection limit of 0.05 μg/ml, and the *P* value of L-DP through membrane B was below 1.58×10^{-10} cm/s). This finding confirmed that membrane B was a highly lipophilic barrier without any hydrophilic region or pores. The value of log *P* through the human skin (log $P_{hum.}$) (Morimoto et al., 1992) and through the impregnated membranes A and B (log $P_{mem.\ A}$ and log $P_{mem.\ B}$, respectively) were used for the various analyses detailed below (Table 6). The relationship between log $K_{O/W}$ and log $P_{mem.}$ is shown in Fig. 3. The values of log $P_{mem.\ B}$ and log $P_{mem.\ A}$ were dependent on the $K_{O/W}$ for the compounds with a log $K_{O/W} \geq -2.0$ (Fig. 3). In contrast, the values of log $P_{mem.\ A}$ for the compounds with a log $K_{O/W} < -2.0$ were constant at -6.717 to -6.438 (Fig. 3b). The regression lines obtained for the compounds with log $K_{O/W} \geq -2.0$ (solid line in each panel) are shown in Eq. 1 and 2.

$$\log P_{mem.\ B} = 0.698 \log K_{O/W} - 5.961 \qquad \text{(Eq. 1)}$$

(Fig. 3a, determination coefficient (R^2) = 0.859, $p < 0.05$, $n = 9$)

$$\log P_{mem.\,A} = 0.520 \log K_{O/W} - 5.272 \quad \text{(Eq. 2)}$$

(Fig. 3b, $R^2 = 0.843$, $p < 0.05$, $n = 9$)

The slopes and R^2 values of the regression lines were similar to each other (Eq. 1 and 2). We used a physicochemical approach to differentiate between the compounds with log $K_{O/W} \geq -2.0$ and the compounds with log $K_{O/W} < -2.0$. The lipophilic compounds might permeate through a lipophilic region in the skin or polymer membranes depending on their $K_{O/W}$ values. However, the permeation of highly hydrophilic compounds with log $K_{O/W} < -2.0$ across such lipophilic regions may be difficult because these regions would act as a potent barrier. These hydrophilic compounds permeate easily through the hydrophilic regions in the skin or polymer membranes, which are regarded as water-filled pores (Hatanaka et al., 1990). When the MW of the compounds does not vary, their permeation through hydrophilic pathways is assumed to be almost constant and to be independent of their $K_{O/W}$ values. The values of log $P_{mem.\,B}$ and log $P_{mem.\,A}$ increased with an increase in the $K_{O/W}$ values for the lipophilic compounds, and the slopes and R^2 values of the regression lines were similar (Fig. 3 and Eq. 1 and 2). These results indicated that both membranes A and B had lipophilic regions with similar properties. This might be related to the fact that membranes A and B contained a high amount of PDMS units

(Table 3). Thus, the permeability of these membranes was thought to be dependent on the indicators of the polarity of the compounds, such as their $K_{O/W}$ values (Geinoz et al., 2002; Hatanaka et al., 1990).

The log $P_{mem.\,A}$ values of the compounds with a log $K_{O/W}$ < -2.0 were almost constant (log $P_{mem.\,A}$: -6.72 to -6.44 cm/s). Further, we observed a trend for hydrophilic compounds similar to that observed in our previous studies on human and hairless rat skin (Morimoto et al., 1992). This trend is thought to originate from the presence of a small proportion of hydrophilic regions in the skin. In our previous study, we synthesized MMA/GMA/PEG 6000 copolymers and noted the possibility that PEG 6000 units formed a hydrophilic microdomain in the copolymer membranes (Miki et al., 2010). Therefore, a hydrophilic region that consists of PEG 6000 units is considered to exist separately from the lipophilic region in membrane A, which indicates that membrane A has a microphase-separated structure.

3.2.2. Correlative evaluation between the permeability of the impregnated membranes and the human skin

The R^2 and slope values in the regression analysis of the log $P_{mem.}$–log $P_{hum.}$ correlation were used as indicators for evaluating alternatives to the human skin. The

relationships between the log $P_{mem.\,B}$ and log $P_{hum.}$ for the lipophilic compounds is shown in Fig. 4a, and the relationship between the log $P_{mem.\,A}$ and log $P_{hum.}$ for the lipophilic and hydrophilic compounds is shown in Fig. 4b. The regression lines obtained are shown in Eq. 3 and 4.

$$\log P_{hum.} = 0.846 \log P_{mem.\,B} - 1.544 \quad \text{(Eq. 3)}$$

(Fig. 4a; $R^2 = 0.913$, $p < 0.05$, $n = 9$)

$$\log P_{hum.} = 1.067 \log P_{mem.\,A} - 0.899 \quad \text{(Eq. 4)}$$

(Fig. 4b; $R^2 = 0.930$, $p < 0.05$, $n = 12$)

Eq. 3 and 4 indicated good linear relationships; the R^2 values were 0.913 and 0.930, and the slopes were 0.846 and 1.067, respectively. Although good correlation was observed between log $P_{mem.\,B}$ and log $P_{hum.}$ for the compounds with a log $K_{O/W} \geq -2.0$ (Fig. 4a and Eq. 3), we could not determine the permeability of membrane B for L-DP. Therefore, these results confirmed that the membrane composed of PDMS units alone cannot be used to predict accurately the permeability of the human skin for highly hydrophilic compounds with log $K_{O/W} < -2.0$. A good correlation was observed between log $P_{mem.\,A}$ and log $P_{hum.}$ not only for the lipophilic but also for the hydrophilic compounds, and the slope of the regression line was close to 1.0, which is the ideal value (Fig. 4b and Eq. 4).

3.3. Correlative evaluation between the permeability obtained using the PAMPA model and that of the human skin

As mentioned above, the presence of a microdomain, which consists of PEG 6000 units, in membrane A works as a permeation pathway for compounds with a log $K_{O/W}$ < -2.0. In addition, a good relationship was observed between the log $P_{mem.\,A}$ and log $P_{hum.}$ values for lipophilic and hydrophilic compounds. Because polymer A would be a good candidate for preliminary evaluation of the human skin permeability for lipophilic and hydrophilic compounds, we used polymer A as the PAMPA model for development of a HTS system. Further, we performed permeation studies using the PAMPA model of polymer A and evaluated the permeation behaviours of the compounds. The values of J_{PAMPA}, P_{PAMPA}, and log P_{PAMPA} of the compounds permeated through the PAMPA model are shown in Tables 7-8. We determined the concentrations of the six compounds in the receiver phase by using a microplate reader. Further, we evaluated the P_{PAMPA} values of six compounds over a permeation period of 0.5–5.0 h. Thus, the use of polymer A in the PAMPA enabled rapid and easy evaluation of the permeability of the compounds. The relationship between the log P_{PAMPA} and log $P_{hum.}$ values for the six compounds is shown in Fig. 5. The regression line obtained is shown in Eq. 5.

$$\log P_{hum.} = 1.046 \log P_{PAMPA} - 0.360 \quad \text{(Eq. 5)}$$

(Fig. 5; $R^2 = 0.927$, $p < 0.05$, $n = 6$)

We obtained a good linear relationship; the R^2 value was 0.927, and the slope was 1.046, respectively. An ideal correlation between log P_{PAMPA} and log $P_{hum.}$ was obtained including NR, which is relatively hydrophilic (log $K_{O/W}$; -1.02; Table 1, Fig. 5 and Eq. 5). This result suggested that PEG 6000 units in polymer A worked as a hydrophilic region similar to that observed using the 2-chamber diffusion cell. The PAMPA model using polymer A enabled rapid and easy prediction of the permeability of the human skin not only for lipophilic compounds but also for hydrophilic compounds. The SC is a heterogeneous membrane, which has several pathways for permeation (Mitragotri, 2003). Therefore, the barrier properties of the skin cannot be perfectly mimicked from a chemical and biological point of view by artificial materials. However, a physicochemical approach to determine the permeation behaviour of the skin indicates that the skin has at least two pathways: a lipophilic pathway, which is present to a large extent, and a hydrophilic pathway, which is present in a small extent. Ottaviani et al. (2006) reported a PAMPA model for skin permeation (PAMPA-skin) that could be easily prepared by mixing silicone oil and isopropyl myristate as an impregnated membrane. This model was a simple physical mixture, and thus, it had a homogeneous structure. Sinkó et al. (2012)

reported another PAMPA-skin that was prepared by using several ceramides, cholesterol, stearic acid, and silicone oil. Several studies have reported a solid copolymer membrane or composite membrane for use as an alternative to the skin for studies on skin permeability; these membranes have lipophilic and hydrophilic pathways (Hatanaka et al., 1992; Miki et al., 2010; Yamaguchi et al., 1997). However, since these were solid membranes, application of these polymers to a 96-well plate would be difficult. Polymer A synthesized in our study was a liquid rubber. These polymers had a single-stranded structure and consisted of a large part of a silicone component, which had a low glass-transition point (typically -125 to -127°C) (Morishita et al., 2009; Rutnakornpituk et al., 2005). Although polymer A contained PEG 6000 units having a crystalline structure at ambient temperature (Zheng et al., 2005), the content of the PEG 6000 units was only 3.5 wt% (Table 3). This structure, composition, and thermal property resulted in a liquid rubber polymer at ambient temperature. This liquid rubber property was suitable for application on a PAMPA model. In addition, our model has several practical advantages, in that it is easy to prepare and to handle in any laboratory and that it is physically and chemically more stable than the lipid components. Moreover, this polymer is more cost-effective than the three-dimensional cultured human skin model (Schreiber et al., 2005) and the artificial lipid membrane (De Jager et al., 2006).

4. Conclusions

Our aim was to develop a HTS system for predicting the permeability of human skin by using synthesized polymers with permeation characteristics for lipophilic and hydrophilic compounds similar to those of the human skin. Our findings were as follows:

1) The presence of a microdomain, which consists of PEG 6000 units, in membrane A works as a permeation pathway for hydrophilic compounds.

2) The 2-chamber diffusion cell study showed a good relationship between the permeability of membrane A and that of human skin, not only for lipophilic compounds but also for hydrophilic compounds.

3) The relationship between the permeability of the PAMPA model of polymer A and that of human skin is good for both lipophilic and hydrophilic compounds.

Polymer A can be used to predict the permeability of the human skin for lipophilic and hydrophilic compounds. Moreover, the PAMPA model using polymer A can enable rapid and easy prediction of the permeability of the human skin. Thus, the PAMPA model

using polymer A seems to be a good membrane model for preliminary evaluations of the permeability of human skin.

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References

Avdeef, A., Bendels, S., Di, L., Faller, B., Kansy, M., Sugano, K., Yamauchi, Y., 2007. PAMPA - Critical factors for better predictions of absorption. J. Pharm. Sci. 96, 2893–2909.

De Coensel, N., Desmet, K., Górecki, T., Sandra, P., 2007. Determination of polydimethylsiloxane-air partition coefficients using headspace sorptive extraction. J. Chromatogr. A 1150, 183–189.

De Jager, M., Groenink, W., Bielsa I Guivernau, R., Andersson, E., Angelova, N., Ponec, M., Bouwstra, J., 2006. A novel in vitro percutaneous penetration model: Evaluation of barrier properties with *p*-aminobenzoic acid and two of its derivatives. Pharm. Res. 23, 951–960.

Dias, M., Hadgraft, J., Lane, M.E., 2007. Influence of membrane-solvent-solute interactions on solute permeation in model membranes. Int. J. Pharm. 336, 108–114.

Geinoz, S., Rey, S., Boss, G., Bunge, A.L., Guy, R.H., Carrupt, P.A., Reist, M., Testa, B., 2002. Quantitative structure - Permeation relationships for solute transport across silicone membranes. Pharm. Res. 19, 1622–1629.

Hamurcu E. E., Baysal B. M., 1996. Preparetion and characterization of block and graft copolymers using macroazoinitiators having siloxane units. J. Appl. Polym. Sci. 62, 1415–1426.

Hatanaka, T., Inuma, M., Sugibayashi, K., Morimoto, Y., 1990. Prediction of skin permeability of drugs. I. Comparison with artificial membrane. Chem. Pharm. Bull. 38, 3452–3459.

Hatanaka, T., Inuma, M., Sugibayashi, K., Morimoto, Y., 1992. Prediction of skin permeability of drugs. II. Development of composite membrane as a skin alternative. Int. J. Pharm. 79, 21–28.

Hesse M., Meier H., Zeeh B., 2000. Spectroscopic Methods In Organic Chemistry. Kagaku Dojin, Kyoto.

James-Smith, M.A., Hellner, B., Annunziato, N., Mitragotri, S., 2011. Effect of surfactant mixtures on skin structure and barrier properties. Ann. Biomed. Eng. 39, 1215–1223.

Kansy, M., Senner, F., Gubernator, K., 1998. Physicochemical high throughput screening: parallel artificial membrane permeation assay in the description of passive absorption processes. J. Med. Chem. 41, 1007–1010.

Laverty J. J., Gardlund Z. G., 1977. Poly(vinyl chloride)-poly(etylene oxide) block copolymers: Synthesis and characterization. J. Polym. Sci., Polym. Chem. Ed. 15, 2001–2011.

Miki, R., Kimura, S., Ueda, H., Shimamura, T., Numajiri, S., Seki, T., Juni, K., Kitano, H., Morimoto, Y., 2010. Suitability of synthetic copolymer membranes as a human skin alternative for in vitro skin permeation testing. Altern. Anim. Test. Exp. 15, 14–27.

Mitragotri, S., 2003. Modeling skin permeability to hydrophilic and hydrophobic solutes based on four permeation pathways. J. Control. Release 86, 69–92.

Mojsiewicz-Pieńkowska, K., Jamrógiewicz, Z., Łukasiak, J., 2003. Determination of polydimethylsiloxanes by ^{1}H-NMR in wine and edible oils. Food Addit. Contam. 20, 438-444.

Morimoto, Y., Hatanaka, T., Sugibayashi, K., Omiya, H., 1992. Prediction of skin permeability of drugs: comparison of human and hairless rat skin. J. Pharm. Pharmacol. 44, 634–639.

Morishita, T., Matsushita, M., Katagiri, Y., Fukumori, K., 2009. Synthesis and properties of macromer-grafted polymers for noncovalent functionalization of multiwalled carbon nanotubes. Carbon N. Y. 47, 2716–2726.

Okumura, M., Sugibayashi, K., Ogawa, K., Morimoto, Y., 1989. Skin permeability of water-soluble drugs. Chem. Pharm. Bull. 37, 1404–1406.

Ottaviani, G., Martel, S., Carrupt, P.A., 2006. Parallel artificial membrane permeability assay: A new membrane for the fast prediction of passive human skin permeability. J. Med. Chem. 49, 3948–3954.

Rutnakornpituk, M., Ngamdee, P., Phinyocheep, P., 2005. Synthesis, characterization and properties of chitosan modified with poly(ethylene glycol)–polydimethylsiloxane amphiphilic block copolymers. Polymer. 46, 9742–9752.

Schreiber S., Mahmoud A., Vuia A., Rübbelke M. K., Schmidt E., Schaller M., Kandárová H., Haberland A., Schäfer U. F., Bock U., Korting H. C., Liebsch M., Schäfer-Korting M., 2005. Reconstructed epidermis versus human and animal skin in skin absorption studies. Toxicol. Vitr. 19, 813–822.

Sinkó, B., Garrigues, T.M., Balogh, G.T., Nagy, Z.K., Tsinman, O., Avdeef, A., Takács-Novák, K., 2012. Skin-PAMPA: A new method for fast prediction of skin penetration. Eur. J. Pharm. Sci. 45, 698–707.

Sugano, K., Nabuchi, Y., Machida, M., Aso, Y., 2003. Prediction of human intestinal permeability using artificial membrane permeability. Int. J. Pharm. 257, 245–251.

Tamura, Z., 1991. Analytical Chemistry Handbook. Maruzen, Tokyo.

Wasdo, S.C., Juntunen, J., Devarajan, H., Sloan, K.B., 2009. A correlation of flux through a silicone membrane with flux through hairless mouse skin and human skin in vitro. Int. J. Pharm. 373, 62–67.

Yamaguchi, Y., Usami, T., Natsume, H., Aoyagi, T., Nagase, Y., Sugibayashi, K., Morimoto, Y., 1997. Evaluation of skin permeability of drugs by newly prepared polymer membranes. Chem. Pharm. Bull. 45, 537–541.

Zheng, L., Wang, Y.-Z., Yang, K.-K., Wang, X.-L., Chen, S.-C., Li, J., 2005. Effect of PEG on the crystallization of PPDO/PEG blends. Eur. Polym. J. 41, 1243–1250.

Figures and Tables

Figure captions

Fig. 1. Scheme of polymer synthesis.

The superscript symbols represent the functional group whose chemical shifts are shown in Table 4. The molecular weights of the poly(dimethylsiloxane) (PDMS) units, poly(ethylene glycol) (PEG) 6000 units, and poly(polyoxyethylene-azobiscyanopentanoate) (VPE-0601) were approximately 724 Da, 6 and 50 kDa, respectively. x = 8, m = 136, and n = 8.

Fig. 2. The cumulative amounts of isosorbide dinitrate (ISDN) and nicorandil (NR) diffused across the impregnated membranes in 2-chamber diffusion cells.

The data represent the mean ± standard deviation (S.D.) (n = 4).

Fig. 3. The relationships between the octanol/water partition coefficients (log $K_{O/W}$) of the compounds, and their ability to permeate through the impregnated membranes in 2-chamber diffusion cells (log $P_{mem.}$).

Fig. 4. The relationships between the ability of the compounds to permeate through the impregnated membranes (log $P_{mem.}$) and through the human skin (log $P_{hum.}$) in 2-chamber diffusion cells.

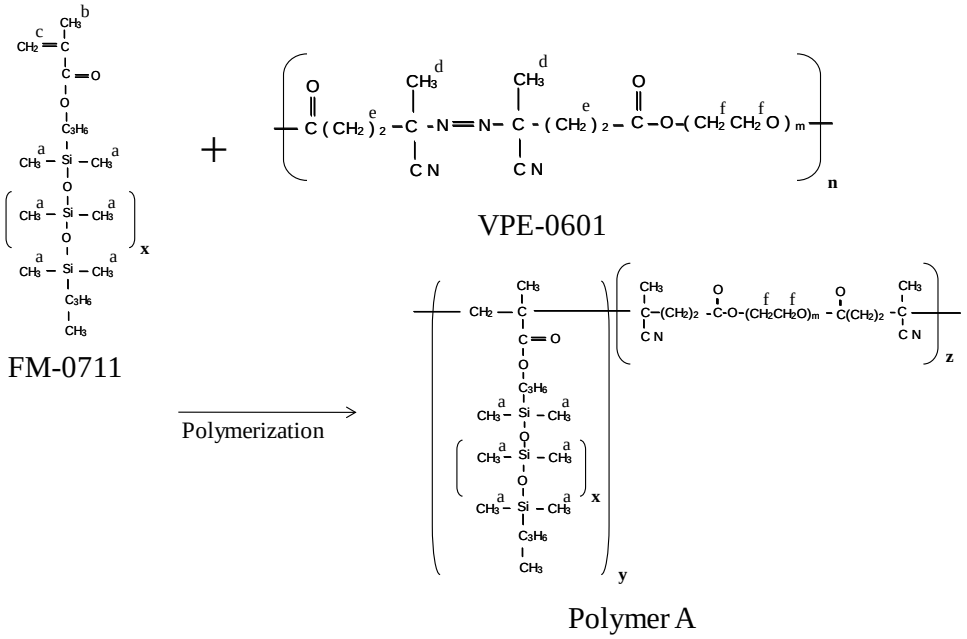
*The data were obtained from a previous study (Morimoto et al., 1992).

Fig. 5. The relationships between the ability of the compounds to permeate through the parallel artificial membrane permeation assay (PAMPA) model of polymer A (log P_{PAMPA}) and through the human skin.

*The data were obtained from a previous study (Morimoto et al., 1992).

KP, ketoprofen; LC, lidocaine; ISDN, isosorbide dinitrate; DC-Na, diclofenac sodium; AMP, aminopyrine; NR, nicorandil.

(a) Polymer A



(b) Polymer B

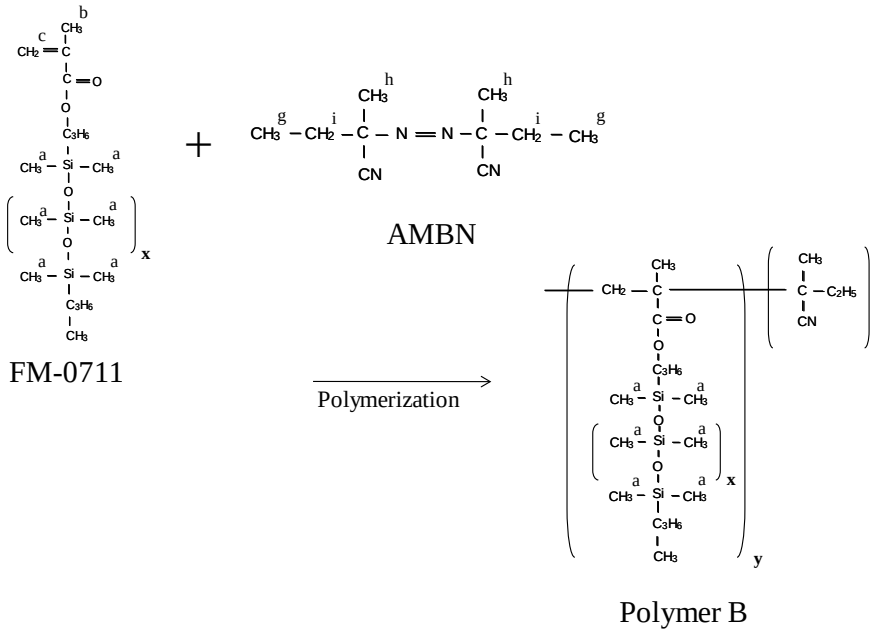


Fig. 1

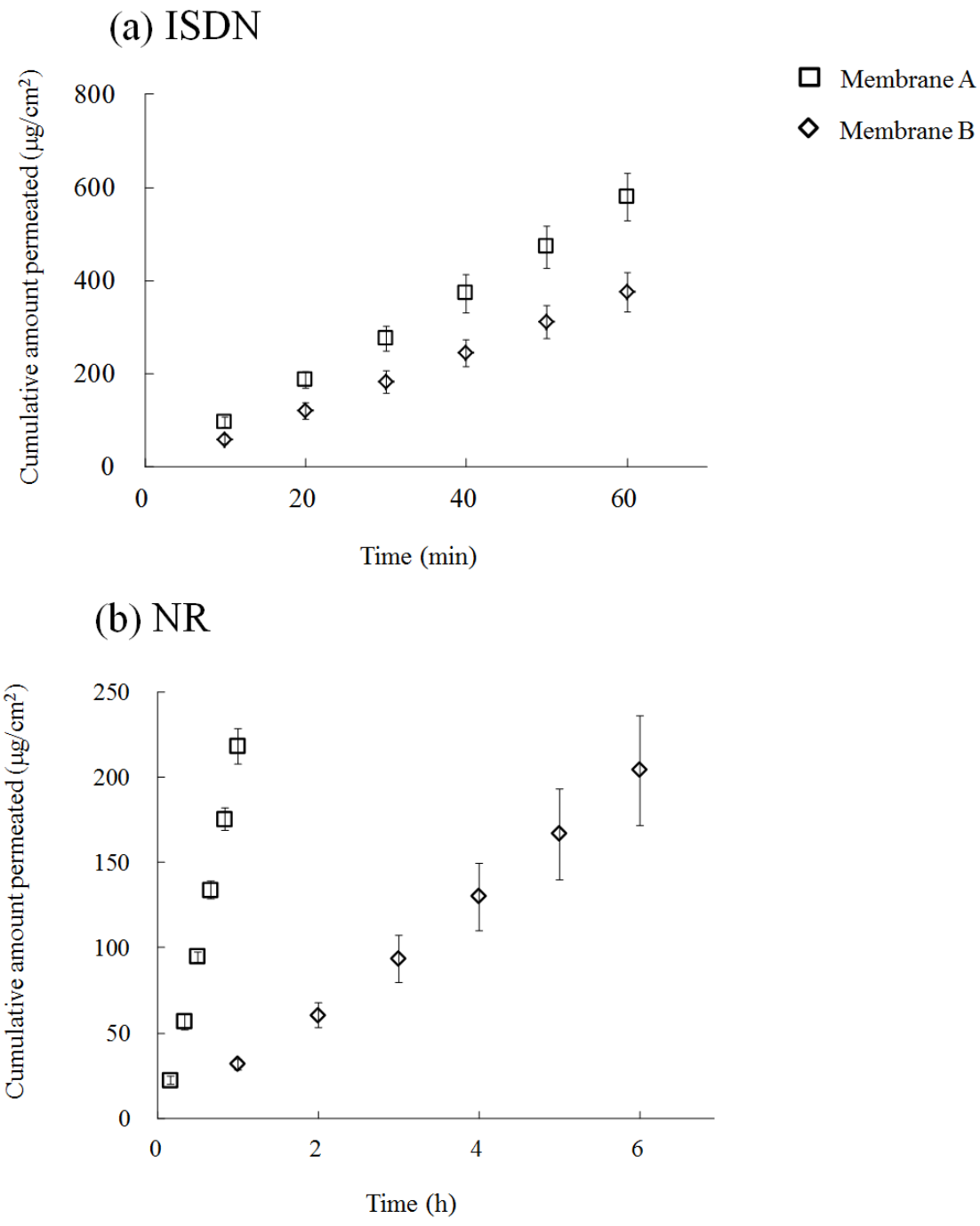

(a) ISDN
Membrane A
Membrane B
Cumulative amount permeated (μg/cm²)
800
600
400
200
0
0
20
40
60
Time (min)
(b) NR
Cumulative amount permeated (μg/cm²)
250
200
150
100
50
0
0
2
4
6
Time (h)

Fig. 2

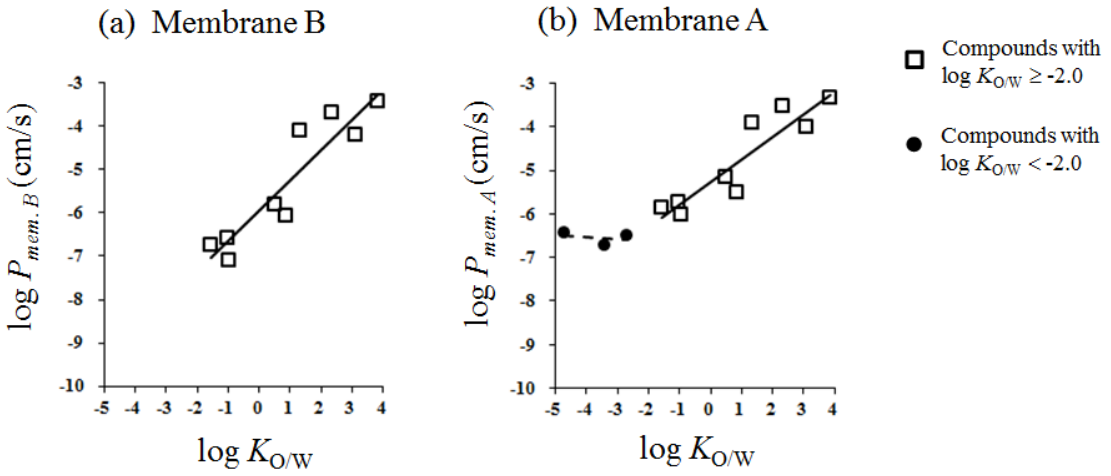

(a) Membrane B
(b) Membrane A
log $P_{mem.B}$ (cm/s)
log $P_{mem.A}$ (cm/s)
log $K_{O/W}$
log $K_{O/W}$
Compounds with log $K_{O/W} \geq$ -2.0
Compounds with log $K_{O/W}$ < -2.0

Fig. 3

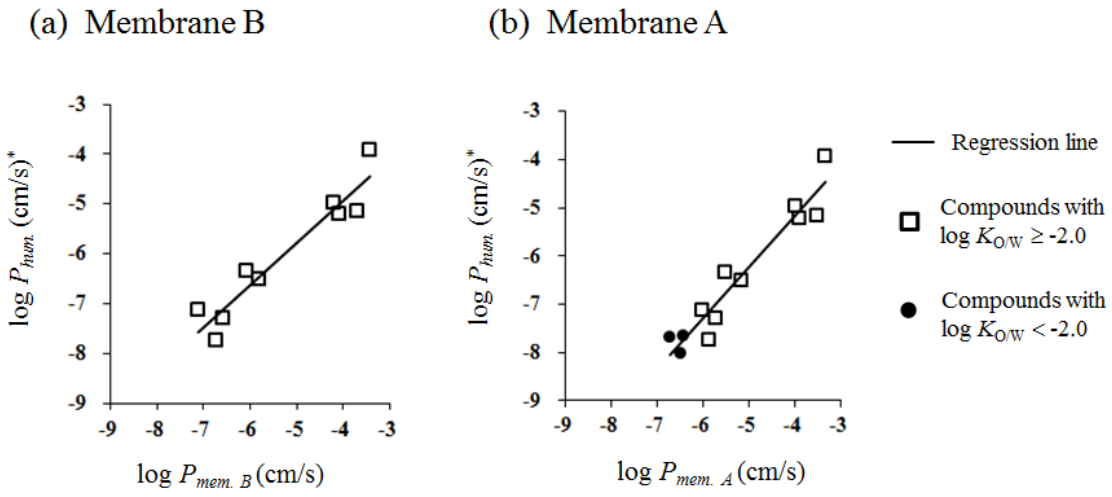

(a) Membrane B
(b) Membrane A
log P_{mm} (cm/s)*
log $P_{mem.\ B}$ (cm/s)
log $P_{mem.\ A}$ (cm/s)
-3
-4
-5
-6
-7
-8
-9
Regression line
Compounds with log $K_{O/W} \geq$ -2.0
Compounds with log $K_{O/W}$ < -2.0

Fig. 4

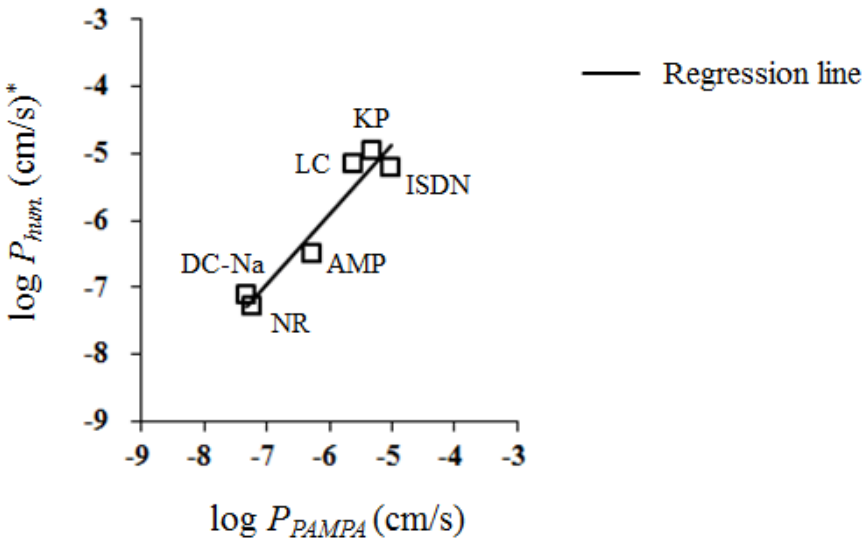

Regression line
KP
LC
ISDN
DC-Na
AMP
NR
-3
-4
-5
-6
-7
-8
-9
-9
-8
-7
-6
-5
-4
-3
log $P_{hum.}$ (cm/s)*
log P_{PAMPA} (cm/s)

Fig. 5

Tables

Table 1. The physicochemical properties of the compounds tested using 2-chamber diffusion cells.

Compound	MW (Da)	Solubility in water (mg/ml)[a]	log $K_{O/W}$[b]	UV Detection (nm)
Flurbiprofen	244	0.0277	3.86	245
Ketoprofen	254	0.185	3.11	254
Lidocaine	234	3.03	2.34	245
Isosorbide dinitrate	236	1.34	1.34	220
Cyclobarbital	236	3.07	0.873	205
Aminopyrine	231	55.9	0.497	254
Diclofenac sodium	318	32.0	-0.962	286
Nicorandil	211	39.6	-1.02	254
Antipyrine	188	816	-1.55	245
Isoproterenol hydrochloride	247	345	-2.69	280
Dopamine hydrochloride	189	520	-3.40	280
L-Dihydroxyphenylalanine	197	5.00	-4.70	280

[a]Solubility in water at 37°C (Morimoto et al., 1992).

[b]Logarithm of the octanol/water partition coefficient (log $K_{O/W}$) at 37°C from a previous study (Morimoto et al., 1992).

Table 2. The pH of aqueous suspension and the pK_a of the compounds tested.

	pH[a]	p*K*a[a]			
Flurbiprofen	4.70	3.73			
Ketoprofen	3.72	3.90			
Lidocaine	6.82	7.86			
Cyclobarbital	3.58	7.50			
Aminopyrine	7.94	5.00			
Diclofenac sodium	7.96	4.00			
Antipyrine	7.60	1.50			
Isoproterenol hydrochloride	2.75	8.57	10.1	12.0	
Dopamine hydrochloride	3.26	8.74	10.3		
L-Dihydroxyphenylalanine	5.42	2.31	8.71	9.74	13.4

[a]From a previous study (Morimoto et al., 1992).

Table 3. The feed ratio, composition, and average molecular weight of the polymers synthesized.

	Feed ratio (wt%)			Product				
				Composition (wt%)		Molecular weight distribution[c]		
Code	FM-0711	AMBN	VPE-0601	PDMS	PEG 6000	Mn (kDa)	Mw (kDa)	Mw/Mn
A	97.00	0	3.00	96.5[a]	3.5[a]	157	209	1.32
B	99.69	0.31	0	100[b]	0[b]	52	106	2.03

[a]The weight ratios in polymer A were calculated using proton nuclear magnetic resonance (^{1}H-NMR).

[b]We assumed that the synthesized polymer B consisted only of poly(dimethylsiloxane) (PDMS) units.

[c]The average molecular weight (MW) was determined using gel permeation chromatography (GPC) calibrated with poly(styrene) standards.

Table 4. Assignment of the resonance signals of the recorded proton nuclear magnetic resonance (^{1}H-NMR) spectra.

*Symbols are shown as superscript in Fig. 1.

	Chemical shift (δ), ppm	Symbol*	Literature data
			Chemical shift (δ), ppm
FM-0711	–0.1 to 0.2	a	0.072 (Hamurcu et al., 1996), 0.0~0.5 (Mojsiewicz-Pieńkowska et al., 2003)
	1.90~1.95	b	1.97 (Hesse et al., 2000)
	5.5, 6.1	c	5.45 (Tamura, 1991), 5.72 (Hesse et al., 2000), 6.14 (Tamura, 1991), 6.30 (Hesse et al., 2000)
VPE-0601	1.6~1.7	d	1.65 (Laverty et al., 1977), 1.62~1.67 (Hamurcu et al., 1996)
	2.3~2.7	e	2.45 (Laverty et al., 1977), 2.33~2.68 (Hamurcu et al., 1996)
	3.2~4.0	f	3.58 (Hesse et al., 2000), 3.60 (Laverty et al., 1977)
AMBN	0.98~1.04	g	—
	1.66~1.68	h	—
	2.0~2.2	i	—
Polymer A	–0.1~0.2	a	—
	3.60~3.65	f	—
Polymer B	–0.1~0.2	a	—

Table 5. The permeation parameters of the compounds permeated through the impregnated membranes in 2-chamber diffusion cells.

(a) Membrane A

Compound	J (μg/cm^2/h)			P (cm/s)			Lag time (h)	n
FP	46.37	±	5.626	4.65×10^{-4}	±	0.56×10^{-4}	0.02	4
KP	67.70	±	6.066	1.02×10^{-4}	±	0.09×10^{-4}	0.11	4
LC	3238	±	315.5	2.97×10^{-4}	±	0.29×10^{-4}	0.01	3
ISDN	606.3	±	62.05	1.26×10^{-4}	±	0.13×10^{-4}	0.05	4
CB	33.40	±	1.895	3.02×10^{-6}	±	0.17×10^{-6}	0.11	4
AMP	1411	±	380.0	7.01×10^{-6}	±	1.89×10^{-6}	0.09	4
DC-Na	113.4	±	30.75	9.84×10^{-7}	±	2.67×10^{-7}	0.45	4
NR	262.9	±	29.94	1.84×10^{-6}	±	0.21×10^{-6}	0.16	4
ANP	482.3	±	178.8	1.34×10^{-6}	±	0.50×10^{-6}	0.08	4
IPH	116.9	±	74.00	3.25×10^{-7}	±	2.06×10^{-7}	0.54	3
DPH	69.04	±	29.64	1.92×10^{-7}	±	0.82×10^{-7}	0.54	4
L-DP	6.559	±	3.242	3.64×10^{-7}	±	1.80×10^{-7}	0.79	3

(b) Membrane B

Compound	J (μg/cm^2/h)			P (cm/s)			Lag time (h)	n
FP	36.63	±	6.133	3.67×10^{-4}	±	0.62×10^{-4}	0.14	4
KP	42.37	±	9.198	6.36×10^{-5}	±	1.38×10^{-5}	0.21	4
LC	2251	±	139.7	2.06×10^{-4}	±	0.13×10^{-4}	0.02	3
ISDN	386.2	±	37.92	8.01×10^{-5}	±	0.79×10^{-5}	0.03	4
CB	9.420	±	1.654	8.52×10^{-7}	±	1.50×10^{-7}	0.19	4
AMP	319.4	±	45.35	1.59×10^{-6}	±	0.23×10^{-6}	0.01	4
DC-Na	8.960	±	0.8724	7.78×10^{-8}	±	0.76×10^{-8}	1.00	3
NR	36.92	±	5.796	2.59×10^{-7}	±	0.41×10^{-7}	0.48	4
ANP	65.45	±	7.921	1.82×10^{-7}	±	0.22×10^{-7}	0.23	4
IPH	—		—	—		—	—	—
DPH	—		—	—		—	—	—
L-DP	—[a]		—	—[a]		—	—	3

[a]The concentration of the receiver phase was below the detection limit of 0.05 μg/ml until 48 h, which suggested that the P of L-dihydroxyphenylalanine (L-DP) through membrane B was below 1.58×10^{-10} cm/s. FP, flurbiprofen; KP, ketoprofen; LC, lidocaine; ISDN,

isosorbide dinitrate; CB, cyclobarbital; AMP, aminopyrine; DC-Na, diclofenac sodium; NR, nicorandil; ANP, antipyrine; IPH, isoproterenol hydrochloride; DPH, dopamine hydrochloride; L-DP, L-dihydroxyphenylalanine.

Table 6. Permeability through the human skin and impregnated membranes in 2-chamber diffusion cells.

Compound	Human skin	Membrane A	Membrane B
	log $P_{hum.}$ (cm/s)[a]	log $P_{mem.\ A}$ (cm/s)	log $P_{mem.\ B}$ (cm/s)
Flurbiprofen	–3.928	–3.333	–3.435
Ketoprofen	–4.967	–3.993	–4.196
Lidocaine	–5.154	–3.527	–3.685
Isosorbide dinitrate	–5.213	–3.901	–4.097
Cyclobarbital	–6.355	–5.520	–6.069
Aminopyrine	–6.510	–5.154	–5.799
Diclofenac sodium	–7.127	–6.007	–7.109
Nicorandil	–7.295	–5.734	–6.587
Antipyrine	–7.733	–5.873	–6.740
Isoproterenol hydrochloride	–8.021	–6.488	—
Dopamine hydrochloride	–7.695	–6.717	—
L-Dihydroxyphenylalanine	–7.662	–6.438	—[b]

[a]The data were obtained from a previous study (Morimoto et al., 1992).

[b]The concentration of the receiver phase was below the detection limit of 0.05 μg/ml until 48 h.

Table 7. The permeation parameters of compounds permeated through the parallel artificial membrane permeability assay (PAMPA) model of polymer A.

Compound	J_{PAMPA} (μg/cm^2/h)			P_{PAMPA} (cm/s)			*n*
Ketoprofen	0.6407	±	0.02877	4.81E-06	±	2.16E-07	4
Lidocaine	5.381	±	1.504	2.47E-06	±	6.89E-07	4
Isosorbide dinitrate	9.146	±	2.769	9.48E-06	±	2.87E-06	4
Aminopyrine	21.36	±	3.029	5.31E-07	±	7.53E-08	4
Diclofenac sodium	1.151	±	0.1489	5.00E-08	±	6.46E-09	4
Nicorandil	1.700	±	0.4431	5.96E-08	±	1.55E-08	4

Table 8. Permeability through the human skin and the parallel artificial membrane permeability assay (PAMPA) model of polymer A.

Compound	Human skin	Membrane A
	log $P_{hum.}$ (cm/s)[a]	log P_{PAMPA} (cm/s)
KP	-4.967	-5.318
LC	-5.154	-5.608
ISDN	-5.213	-5.023
AMP	-6.510	-6.275
DC-Na	-7.127	-7.301
NR	-7.295	-7.225

[a]The data were obtained from a previous study (Morimoto et al., 1992).

KP, ketoprofen; LC, lidocaine; ISDN, isosorbide dinitrate; AMP, aminopyrine; DC-Na, diclofenac sodium; NR, nicorandil.