Regular article AATEX

Utilization of reconstructed cultured human skin models as an alternative skin for permeation studies of chemical compounds

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Abstract

Two reconstructed human skin models, Episkin^{SM} and EpiDerm^{TM}, have been approved as alternative membranes for skin corrosive/irritation experiments due to their close correlation with animal skin. Such reconstructed human skin models were evaluated as alternative membranes for skin permeation experiments. Seven drugs with different lipophilicities and almost the same molecular weight were used as test penetrants. Relationships were investigated between permeability coefficients (*P* values) of the seven drugs through six kinds of reconstructed cultured human skin models and human skin. A fairly good relationship in *P* values was observed between TESTSKIN^{TM} LSE-high (LSE-high) or EpiDerm^{TM} and human skin, suggesting that these reconstructed human skin models could be used as alternative membranes for skin permeation experiments. However, the partition parameter, *KL*, and diffusion parameter, DL^{-2} in these reconstructed human skin models were different to those of human skin. Especially, *KL* values in reconstructed human skin models were very different to those in human skin, even for LSE-high and EpiDerm^{TM}. Therefore, suitable reconstructed human skin models should be carefully selected on a case by case basis.

Key Words: reconstructed human skin models; skin permeation; alternative membrane; skin corrosive/irritation;

1. Purpose

Excised human cadaver skin and experimental animal skins have been widely used for *in vitro* skin permeation- and skin corrosive/irritation-experiments with chemical compounds. Recently, reconstructed cultured human skin models (cultured skin models) have been seen as a promising alternative membrane for human and animal skins. The European Center for the Validation of Alternative Methods (ECVAM) approves Episkin^{SM} and EpiDerm^{TM} as alternative animal membranes in skin corrosive experiments. Now, various kinds of cultured skin models such as living human skin equivalent (TESTSKIN^{TM} LSE-high), LabCyte EPI-Model, Vitrolife-skin and Neoderm-E, are available in Japan in addition to Episkin^{SM} and EpiDerm^{TM}. Many reports have revealed the usefulness of such cultured human skin models in skin permeation experiments with chemical compounds, as well as skin corrosive/irritation experiments (Netzlaff *et al.*, 2007; Schmook *et al.*, 2001; Gabbanini *et al.*, 2009; Lotte *et al.*, 2002; Hammell *et al.*, 2005; Asbill *et al.*, 2000; Wagner *et al.*, 2001). These reports, however, only showed different skin permeation-time profiles or permeability coefficient (P) values of chemical compounds between excised human cadaver skin or excised animal skin and a cultured skin model. We have already shown a 1:1 relationship in the P values of chemical compounds for excised human cadaver skin and

hairless rat skin (Watanabe *et al.*, 2001; Morimoto *et al.*, 1992).

Generally, the *P* value of chemical compounds is the product of partition (*KL*) and diffusion parameters (DL^{-2}). The permeability parameters *KL* and DL^{-2} are closely related to the lipophilicity or transfer potential of chemical compounds, respectively, into the stratum corneum. Therefore, the *KL* and DL^{-2} profiles of different compounds represent the characteristics of each cultured human skin model in permeation experiments. In our previous report (Watanabe *et al.*, 2001), log *P* values of chemical compounds through LSE-high, a cultured human skin model, showed a fairly good relationship to those through excised cadaver human skin when seven kinds of chemicals with different physicochemical characteristics were applied to their skin. In addition, *P* values of chemical compounds in LSE-high were about ten-fold higher than those in excised human cadaver skin. This was due to a ten-fold higher DL^{-2} and almost the same *KL* values to those in excised human cadaver skin (Watanabe *et al.*, 2001). Thus, the skin permeation parameters *KL* and DL^{-2} could aid understanding of the characteristics of cultured human skin models as an alternative membrane for skin permeation experiments.

In the present study, epidermis and dermis models, TESTSKIN™ LSE-high, Vitrolife-skin and epidermis models, EpiDerm™ 606X, LabCyte EPI-model,

Neoderm-E and Episkin^{SM} were selected as cultured human skin models. *P* value and skin permeation parameters of chemical compounds were compared to those through excised human cadaver skin using seven kinds of chemical compounds with different lipophilicities and almost the same molecular weight. Understanding the characteristics of each cultured human skin model using *P* values would be useful to predict *P* values in human skin. Thus, this experiment could directly lead to a reduction in animal skin permeation experiments.

2. Theoretical

2.1. The relationship between *P* values and the *n*-octanol/water partition coefficient ($K_{o/w}$) of chemical compounds in excised human cadaver skin.

We (Morimoto *et al.*, 1992, Watanabe *et al.*, 2001) investigated the relationship between *P* values and the *n*-octanol/water coefficient (*K*o/w) of 30 chemical compounds. Figure 1 illustrates the relationship in excised human cadaver skin (Morimoto *et al.*, 1992, Watanabe *et al.*, 2001). A linear increase in the log *P* values in excised human cadaver skin was found by an increase in log *K*o/w of chemical compounds from log *K*o/w =1.

(Figure 1)

2.2. Relationship between *KL* or DL^{-2} and *Ko/w* of chemical compounds in excised human cadaver skin.

Figure 2 illustrates double logarithmic plots of skin permeation parameters (DL^{-2} or *KL*) and the *Ko/w* of chemical compounds. Log DL^{-2} was almost constant over the present log *Ko/w* of chemical compounds. On the other hand, log *KL* increased with an increase in log *Ko/w* and the relationship between log *Ko/w* and log *KL* was similar to that between log *P* and log *Ko/w* as shown in Fig. 1.

(Figure 2)

2.3. Calculation of skin permeation parameters

Each flux was calculated from the time profiles of the cumulative amount of the chemical compounds that permeated through skin obtained in skin permeation experiments, and the *P* value was calculated by a division of the steady state-flux by applied chemical compound concentration. The skin permeation-time profile was expressed by eq. 1, which comes from Fick's second law of diffusion (Scheuplein, 1967) and the lag time was calculated from the regression line from the steady state-flux region of the permeation curve. Theskin permeation parameters DL^{-2} and *KL* were calculated from eq. 1 and the lag time (t_{lag}) (eq. 2), which was obtained from eq. 1 (Jaymin, 1993).

$$Q = KLCv\left[\frac{D}{L^2}t - \frac{1}{6} - \frac{2}{\pi^2}\sum_{n=1}^{\infty}\frac{(-1)^n}{n^2}\exp\left(-\frac{D}{L^2}n^2\pi^2 t\right)\right] \quad (1)$$

$$t_{Lag} = \frac{L^2}{6D} \quad (2)$$

$$P = \left(DL^{-2}\right)\cdot\left(KL\right) \quad (3)$$

where Q is the cumulative amount of chemical compounds permeated, K is the partition coefficient, L is the thickness of the barrier membrane, Cv is the chemical compound concentration in the vehicle, D is diffusivity and t is time after application of the chemical compound on the skin. The P value is expressed as a product of DL^{-2} and KL (eq. 3).

3. Materials and Methods

Antipyrine (ANP), caffeine (CAF), aminopyrine (AMP) and benzoic acid (BA) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Isosorbide dinitrate (ISDN) was from Sigma Aldrich Corporation (St. Louis, MO, U.S.A.) and isosorbide mononitrate (ISMN) was from Tokyo Chemical Industry Co., Ltd. (Toyko, Japan). Flurbiprofen (FP) was gifted from Lead Chemical Co., Ltd. (Toyama, Japan). Other chemicals and solvents were of reagent or HPLC grade and used without further purification. Table 1 shows the molecular weight and log *Ko/w*

values of these chemical compounds.

(Table 1)

3.1. Reconstructed cultured human skin models

EpiDerm™ Epi606X (EpiDerm), Neoderm-E, Vitrolife-skin, LabCyte EPI-model and Episkin℠ (Episkin) were purchased from Kurabo Industries Ltd. (Osaka, Japan), Tego Science Inc. (Gumcheon-gu, Seoul, Korea), Gunze Ltd. (Kyoto, Japan), Japan Tissue Engineering Co., Ltd. (Gamagori, Aichi, Japan) and Skinethic Laboratories (St. Philippe, France), respectively. All of these models were used within 3 days of receipt.

3.2. Skin permeation experiment

Each cultured human skin model was carefully removed from 6 or 12 well transwells and the stratum corneum side was well rinsed with pH7.4 phosphate buffered saline (PBS). The obtained EpiDerm, Neoderm-E and Vitrolife-skin were mounted in a side by-side diffusion cell (effective diffusion area 0.95 cm^2) (Okumura *et al*., 1989) and LabCyte EPI-model and Episkin were mounted in a Franz type diffusion cell (effective diffusion area 0. 50 cm^2). The cultured skin was hydrated with PBS over 1 hour. Then, PBS was removed and a chemical compound solution or suspension in

PBS (chemical compound concentration: about two or three times solubility) was applied on the stratum corneum side and and the same volume of PBS for hydrophilic compounds (antipyrine, ISMN and caffeine) or 40% polyethylene glycol 400 for lipophilic compounds (aminopyrine, ISDN, benzoic acid and flurbiprofen) was applied to the dermal side. Skin permeation of each chemical compound was followed at 32°C by periodic sampling of 1 mL from the receiver solution. The same volume of the solvent was added to keep the volume constant. The chemical compound concentration in each sample was assayed by HPLC. Permeation results through excised human cadaver skin and TESTSKIN^{TM} LSE-high (LSE-high) were referred to our previous report (Watanabe *et al.*, 2001).

3.3. HPLC conditions

Each sample was mixed with acetonitrile containing an internal standard. The solution was centrifuged (15000 rpm, 5 min, 4°C) and 20 μL of the supernatant was injected into an HPLC system composed of an auto injector (SIL-10A, Shimadzu, Kyoto, Japan), a pump (LC-10AD, Shimadzu), a detector (SPD-10AV, Shimadzu), an integrator (SCL-10A, Shimadzu) and a reverse-phase column (Wakopak Wakosil-2 5C18HG, 4.6 mm i.d.×250 mm, Wako Pure Chemical Industries, Ltd., Osaka, Japan).

The f1ow rate was 1.0 mL/min and the column temperature was maintained at 40°C by an oven (CTO-10AC, Shimadzu). Each data analysis was performed by Smart Chrom (KYA Technologies Corp., Tokyo, Japan). The mobile phase, internal standard and UV wavelength for detection were as follows. AMP; acetonitrile : 0.1% phosphoric acid containing 5 mM SDS=10 : 90, butyl *p*-hydoxy benzoate、245 nm, ISDN; acetonitrile : water=55 : 45, butyl *p*-hydoxy benzoic acid, 245 nm, BA; acetonitrile : 50 mM potassium dihydrogen phosphate=45 : 55, ethyl *p*-hydoxy benzoate, 245 nm, FP; acetonitrile : 0.1% phosphoric acid=50 : 50, isopropyl *p*-hydoxy benzoate, 245 nm, respectively. ANP and CAF were detected using an absolute calculation method and mobile phase and UV wavelength for detection were as follows. ANP; acetonitrile : water=30 : 70, 254 nm, ISMN and CAF; acetonitrile : water=10 : 90, 220 nm and 254 nm, respectively.

3.4. Statistical analysis

Spearman's rank correlation coefficient was used to evaluate whether a good relationship was obtained between log *P* values of chemical compounds in human skin and those in cultured human skin at a 5% significance level.

4. Results

4.1. Relationship between log *P* values in cultured skin model and those in excised human cadaver skin.

Figure 3a-f shows a double logarithmic plot of *P* values through each cultured skin model and *K*o/w of chemical compounds. Log *P* values in human skin are also shown in the Fig. 1 and represented by open circles. As shown in Fig. 3a, an increase in log *K*o/w increased log *P* in LSE-high. All of the obtained log *P* values in LSE-high were about 10 times higher than those in human skin. Almost the same results were found in our previous study (Watanabe *et al.*, 2001). In Epiderm, the log *P* values of chemical compounds except for FP were almost the same values as those in human skin (Fig. 3b). The log *P* values of chemical compounds in Vitrolife-skin and Neoderm-E (Fig. 3c, d) were constant independent of log $K_{o/w}$ values, although log *P* values of BA and FP corresponded to those in excised human cadaver skin. On the other hand, the log *P* values of chemical compounds in LabCyte EPI-model and Episkin (Fig. 3e, f) were very different and no order rule was observed. The *P* value of CAF in Episkin was similar to that reported by Netzlaff *et al*. (2007).

Figure 4 shows the relationships between the log *P* values of chemical compounds in cultured skin models (x-axis) and those in excised human cadaver skin (y-axis). As seen in Fig. 4a, a fairly good correlation (slope 1.0; 1:1 relationship) was

observed between LSE-high and excised human cadaver skin, although log *P* values in LSE-high were about 10-folds higher than those in the excised human skin. A similar result was obtained in our previous study (Watanabe *et al.*, 2001). A fairly good correlation, Log P_{human}=1.022×Log $P_{Epiderm}$-0.202 （r=0.9401） (p<0.05), was also observed between EpiDerm and excised human cadaver skin as shown in Fig. 4b. Different to LSE-high, the log *P* values in EpiDerm were almost the same as those in excised human cadaver skin. On the other hand, in Fig. 4c-f, the relationships between Vitrolife-skin, Neoderm-E, LabCyte EPI-model and Episkin and excised human cadaver skin in *P* values of chemical compounds were represented by the following equations; log P_{human}=2.929 × log $P_{vitrolife\text{-}skin}$ + 7.871 (r=0.8035) (no significant correlation), log P_{human}=1.688 × log $P_{Neoderm\text{-}E}$ + 2.32 (r=0.8162) (no significant correlation), log P_{human}=0.937 × log $P_{LabCyte\ EPI\text{-}model}$ - 0.759 (r=0.3894) (no significant correlation) or log P_{human}=0.768 × log $P_{episkin}$ - 1.17 (r=0.8162) (no significant correlation), respectively. These cultured skins did not show good correlations with log *P* values in excised human cadaver skin.

(Figures 3 and 4)

4.2. Comparison of *KL* and DL^{-2} between each cultured skin model and excised human cadaver skin

Figure 5 shows double logarithm plots of *KL* and *Ko/w* of chemical compounds. Although the log *KL* of ANP in LSE-high was slightly higher than that in excised human skin, the other log *KL* in LSE-high was almost equal to that in excised human skin as shown in Fig. 5a. A similar set of results were obtained in our previous study (Watanabe *et al.*, 2001). The log *KL* values in EpiDerm increased by an increase in log *Ko/w* of the chemical compound as shown in excised human skin. All of the log *KL* values in EpiDermis, however, were lower than those in excised human cadaver skin and the differences were especially marked by an increase in lipophilicity of chemical compounds (Fig. 5b). In Neoderm-E, Vitrolife-skin, LabCyte EPI-model and Episkin, the increment behaviors of log *KL* were not the same as those in excised human skin.

Figure 6 shows double logarithmic plots of DL^{-2} and *Ko/w* between each cultured human skin model and excised human skin. Although the log DL^{-2} in cultured human skin models was higher than in excised human skin, the log DL^{-2} values over the present log *Ko/w* values were almost constant, as shown in excised human skin.

(Figures 5 and 6)

5. Discussion

P values of chemical compounds in cultured skin models except for EpiDerm were much higher than that through excised human skin. Other reports also revealed

that cultured skin models showed higher *P* values of chemical compounds because of a low barrier function (Netzlaff *et al.*, 2007). Evaluation of cultured skin models as skin permeation models is mainly focused on the comparison of permeation curves and/or *P* values of chemical compounds between the cultured skin model and excised human skin. A 1:1 relationship in *P* values between human skin and cultured skin model would be best to predict *P* values in excised human skin from that of a cultured skin model. Thus, evaluation of the correlation slope of *P* values would be necessary to use a cultured skin model as an alternative membrane for skin permeation experiments. When the correlation slope in log *P* values between excised human skin and a cultured skin model is larger or smaller than slope 1.0 (1:1 relationship), a slight difference in *P* value through a cultured skin model would lead to a much larger difference in the estimated *P* value through human skin. Therefore, this type cultured skin model would be unattractive as an alternative membrane for skin permeation experiments.

We (Watanabe *et al.*, 2002, Kano and Sugibayashi, 2006, Sugibayashi *et al.*, 2002) have already reported that the extent of skin irritation by chemical compounds was related to their concentration in viable skin tissues. Thus, a good correlation was observed between skin irritation (MTT assay result) and chemical compound

concentration in the skin. We also reported recently that skin concentration of chemical compounds can be determined by the *KL* and *P* determined by obtained values from skin permeation experiments (Sugibayashi *et al.*, 2010).

The log *KL* increased with an increase in the lipophilicity of the chemical compound applied. The obtained log *KL* values in cultured skin models, however, were different to those in excised human cadaver skin, except for LSE-high. When a chemical compound is applied, it rapidly distributes into skin. The extent of distribution of the chemical compound into skin depends on its partition coefficient into skin. The outmost layer of skin, the stratum corneum, is a highly lipophilic membrane. The structure of the stratum corneum is thought to be like "bricks and mortar" (Williams *et al.*, 1987): the corneocytes and intracellar lipids are the bricks and mortar, respectively. Therefore, the composition in lipids (extracellular layer of corneocytes) and lipid structure may be important determinants of the partition coefficient of chemical compounds from the applied vehicle into skin. A comparison of total extracellular lipid compositions among cultured skin models such as EpiDerm and Episkin and human skin were reported by Ponec *et al.* (2000). They mentioned that the lipid compositions in cultured skin were different to those in human skin. The constitution ratio of total lipids in a cultured skin model is necessary to understand the

features and extent of lipophilicity of the stratum corneum barrier. The reason for the *KL* differences among cultured skin models has not yet been fully explained. Further investigation will be needed to clarify the differences.

The DL^{-2} in both a cultured skin model and excised human cadaver skin were almost constant independent of the *Ko/w* of applied chemical compounds. Every cultured skin model has ahigher DL^{-2} than excised human cadaver skin. The intrinsic diffusion coefficient (D_t) in solvent can be obtained by the Stokes-Einstein equation and is a function only of the molecular weight. The D in DL^{-2} represents the apparent diffusion coefficient (D_a) (Young and Ball, 1998; Desai *et al.*, 1965) and the D_a composed of toutosity, effective permeation area and truth diffusion coefficient. Intracellular (stratum corneum; brick) and extracellular (intracellular spaces in the stratum corneum; mortar) pathways are well known as the diffusion route in skin permeation for chemical compounds and the predominant permeation route of chemical compounds is an extracellular route (Naik *et al.*, 2000). Thus, a different DL^{-2} suggests differences in permeation pathways in cultured human skin.

Although total intracellular lipid constitution and X-ray small angle scattering analysis (Ponec *et al.*, 2000; Hatta *et al.*,2001) were not performed to evaluate cultured skin models in the present study, the results suggested that the comprehension of

analyses such as the 1:1 relationship, KL and DL^{-2} between human skin and cultured human skin will help clarify the characteristics of cultured skin models in skin permeation experiments.

6. Conclusion

Evaluation of a 1:1 relationship in P values between excised human cadaver skin and cultured skin model is crucial if we want to use a cultured human skin model as an alternative membrane for skin permeation experiments. A few cultured human skin models showed a fairly good relationship in P values compared to human skin. The difference in P and KL values in a cultured skin model compared to human skin may lead a false positive or negative in skin corrosion/irritation experiments because the chemical compound concentration in skin is determined by the parameterd (Sugibayashi *et al*., 2009). The reason for the difference in P values compared to excised human skin remains unclear, so further experiments will need to clarify not only skin permeation experiments, but also total extracellular lipid constitution determined by X-ray small angle scattering analysis (Ponec *et al.*, 2000; Hatta *et al.*, 2001) and transmission electron microscopy observation.

Acknowledgement

A part of this research was supported by the first Mandom International Research Grants on Alternative to Animal Experiments.

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Table 1 Physicochemical properties of the chemical compounds

Drug	Abbreviation	Log *K*o/w	M.W.
Antipyrine	ANP	-1.507	188
Isosorbide-5-mononitrate	ISMN	-0.151	191
Caffeine	CAF	-0.123	194
Aminopyrine	AMP	1.065	231
Isosorbide dinitrate	ISDN	1.225	236
Benzoic acid	BA	1.410	122
Flurbiprofen	FP	2.179	224

Figure captions

Figure 1 Relationship between log *P* values in excised human cadaver skin and log *K*o/w values of chemical compounds. Semi-solid line represents the calculated value: P_{human} (cm/s) = 1.77×10^{-7} $K_{ow}^{0.780}$+2.37×10^{-8} (Morimoto *et al.*, 1992), Symbol represents observed value. Symbols:▲; values cited from Morimoto *et al.*, 1992, ○: values cited from Watanabe *et al.*, 2001.

Figure 2 Relationships between log *P* values in cultured skin models and log *K*o/w values of chemical compounds. (a): LSE-high, (b): EpiDerm, (c): Vitrolife-skin, (d): Neoderm-E (e) : LabCyte EPI-model and (f): Episkin. Each point represents the mean ± S.E. (n=4-6)

Figure 3 Relationships between log *P* values in cultured skin models and log *K*o/w values of chemical compounds. (a): LSE-high, (b): EpiDerm, (c): Vitrolife-skin, (d): Neoderm-E (e) LabCyte EPI-model and (f): Episkin. Symbols: ○; human skin, □; cultured skin model. Each point represents the mean ± S.E. (n=4-6).

Figure 4 Relationships between log *P* values in excised human cadaver skin and log *P* values in cultured skin models. (a): LSE-high versus excised human cadaver skin, (b): EpiDerm versus excised human cadaver skin, (c): Vitrolife-skin versus excised human cadaver skin, (d): Neoderm-E versus excised human cadaver skin, (e): LabCyte EPI-model versus excised human cadaver skin, and (f): Episkin versus excised human cadaver skin. Each point represents the mean ± S.E. (n=4-6).

Figure 5 Relationships between log *KL* values in cultured skin models and log *K*o/w values of chemical compounds. (a): LSE-high, (b): EpiDerm, (c): Vitrolife-skin, (d): Neoderm-E, (e): LabCyte EPI-model and (f): Episkin. Symbols: ○; human skin, □; cultured skin model. Each point represents the mean ± S.E. (n=4-6).

Figure 6 Relationships between log DL^{-2} values in cultured skin models and log *K*o/w values of chemical compounds. (a): LSE-high, (b): EpiDerm, (c): Vitrolife-skin, (d): Neoderm-E, (e): LabCyte EPI-model and (f): Episkin. Symbols: ○; human skin, □; cultured skin model. Each point represents the mean ± S.E. (n=4-6).

Figure 1

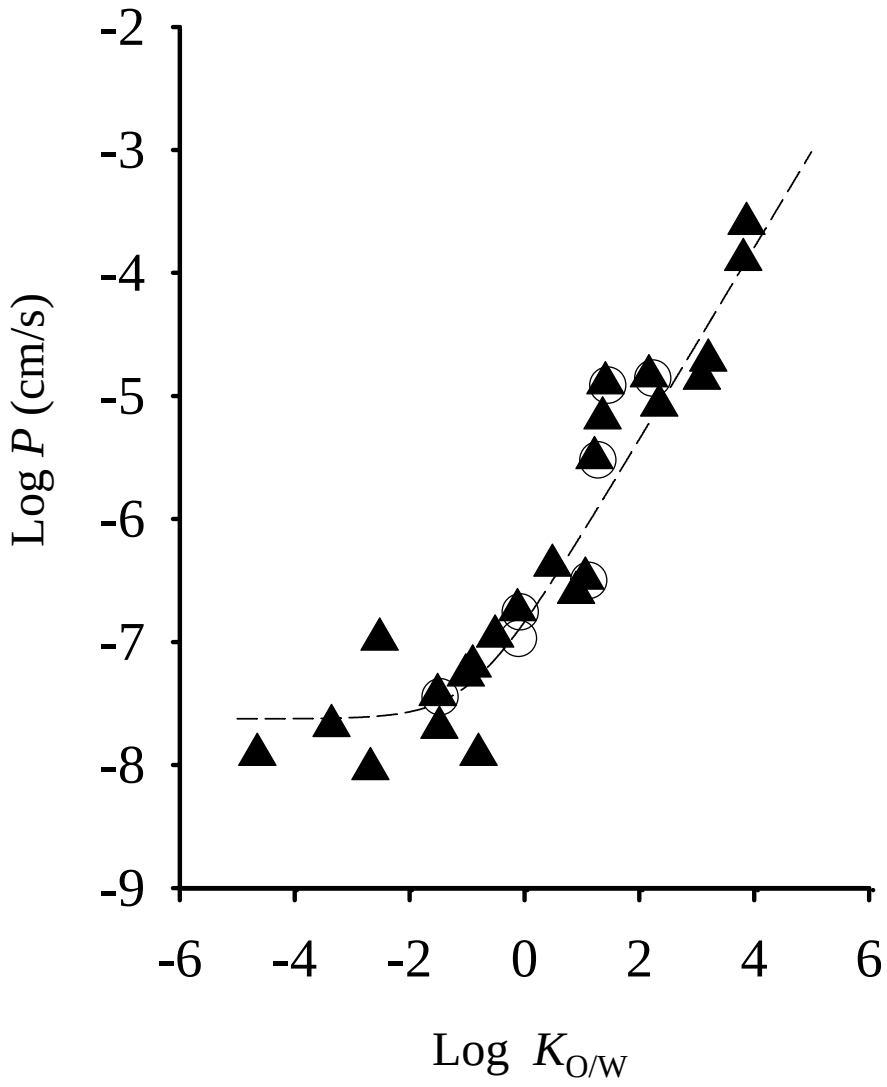
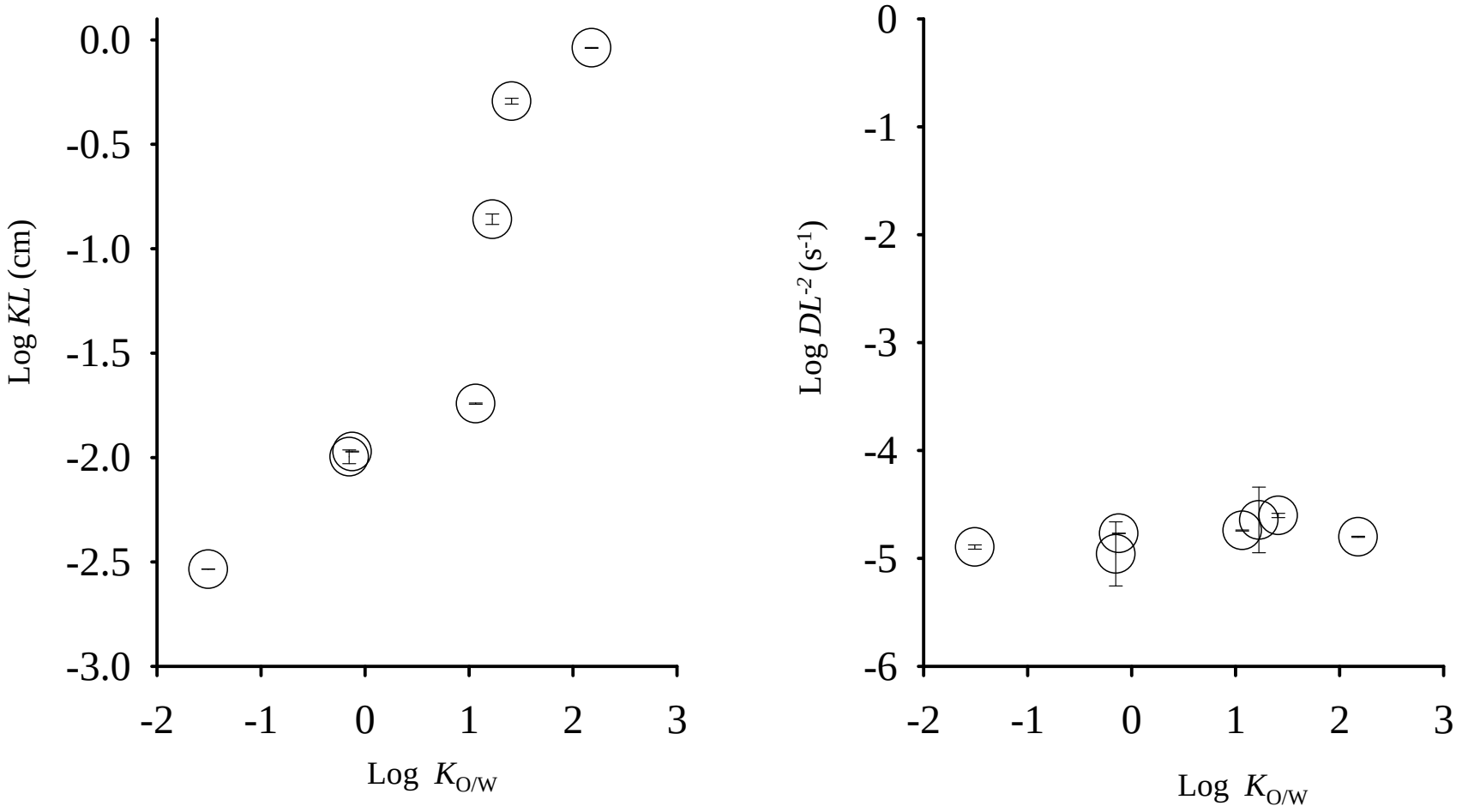


Figure 2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-0.5
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Log KL (cm)
-2
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1
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3
Log K_O/W
0
-1
-2
-3
-4
-5
-6
Log DL^-2 (s^-1)
-2
-1
0
1
2
3
Log K_O/W

Figure 3

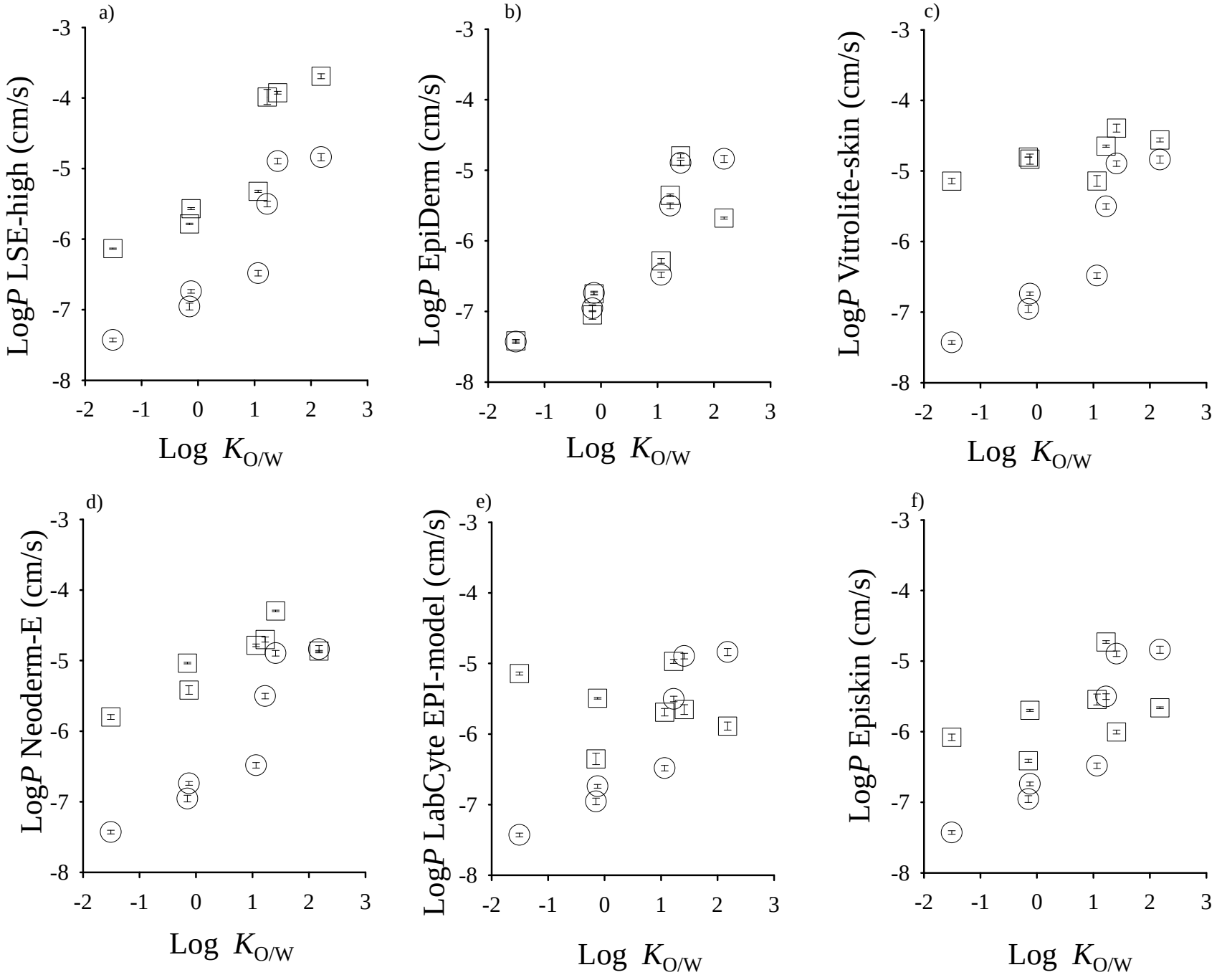

a)
b)
c)
d)
e)
f)
Log*P* LSE-high (cm/s)
Log*P* EpiDerm (cm/s)
Log*P* Vitrolife-skin (cm/s)
Log*P* Neoderm-E (cm/s)
Log*P* LabCyte EPI-model (cm/s)
Log*P* Episkin (cm/s)
Log $K_{O/W}$
-3
-4
-5
-6
-7
-8
-2
-1
0
1
2
3

Figure 4

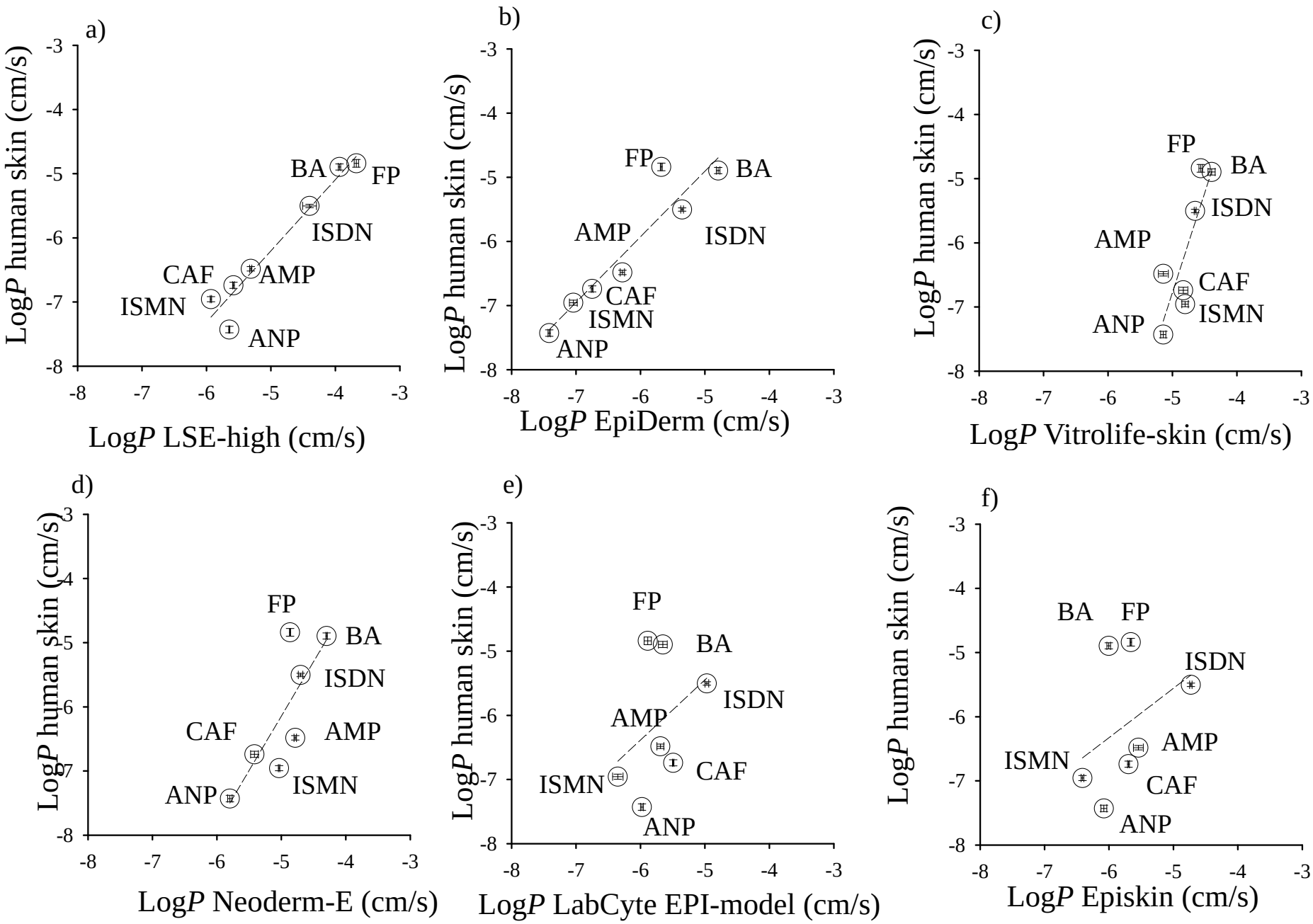

a)
b)
c)
d)
e)
f)
Log*P* human skin (cm/s)
Log*P* LSE-high (cm/s)
Log*P* EpiDerm (cm/s)
Log*P* Vitrolife-skin (cm/s)
Log*P* Neoderm-E (cm/s)
Log*P* LabCyte EPI-model (cm/s)
Log*P* Episkin (cm/s)
FP
BA
ISDN
AMP
CAF
ISMN
ANP
-3
-4
-5
-6
-7
-8

Figure 5

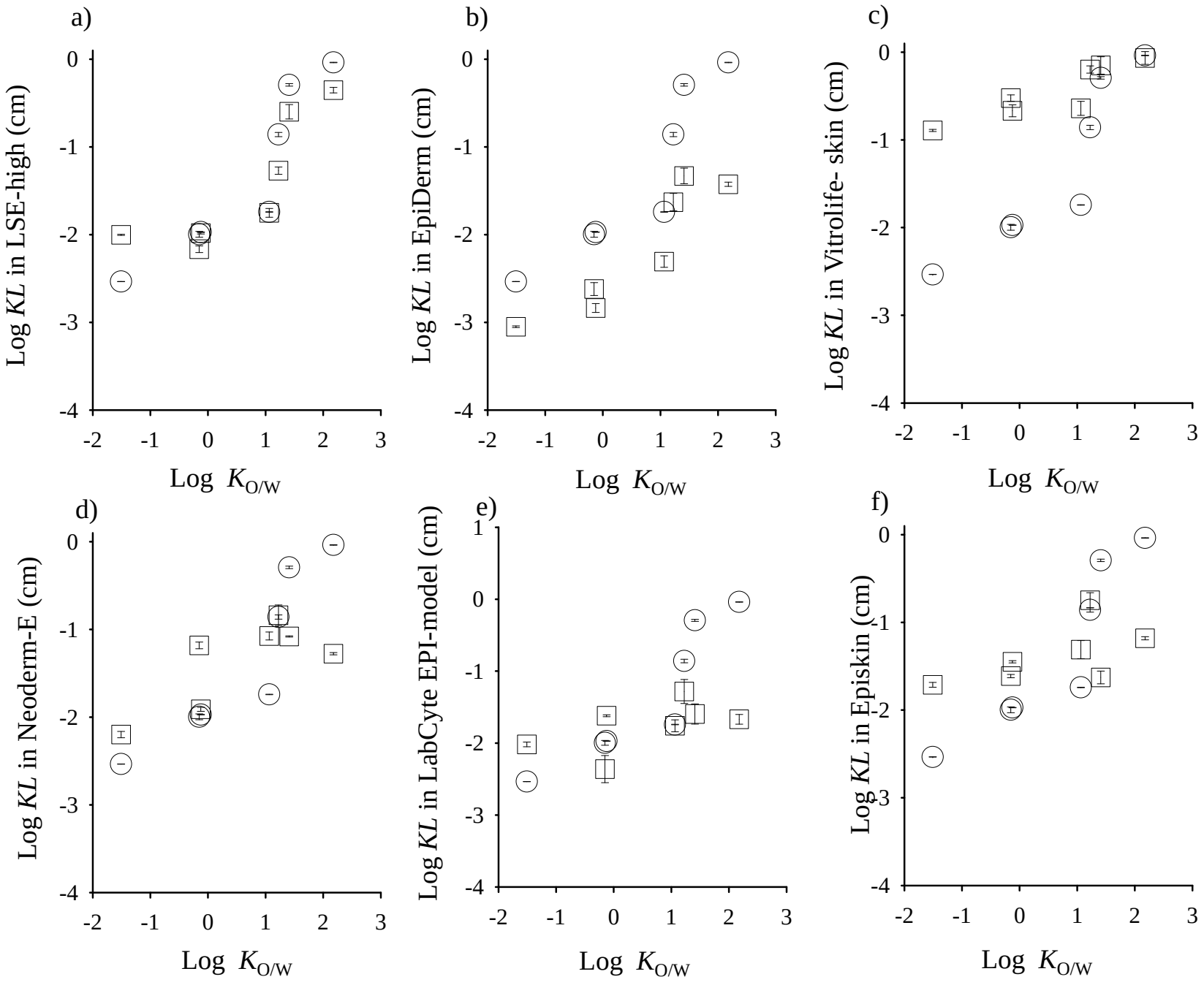
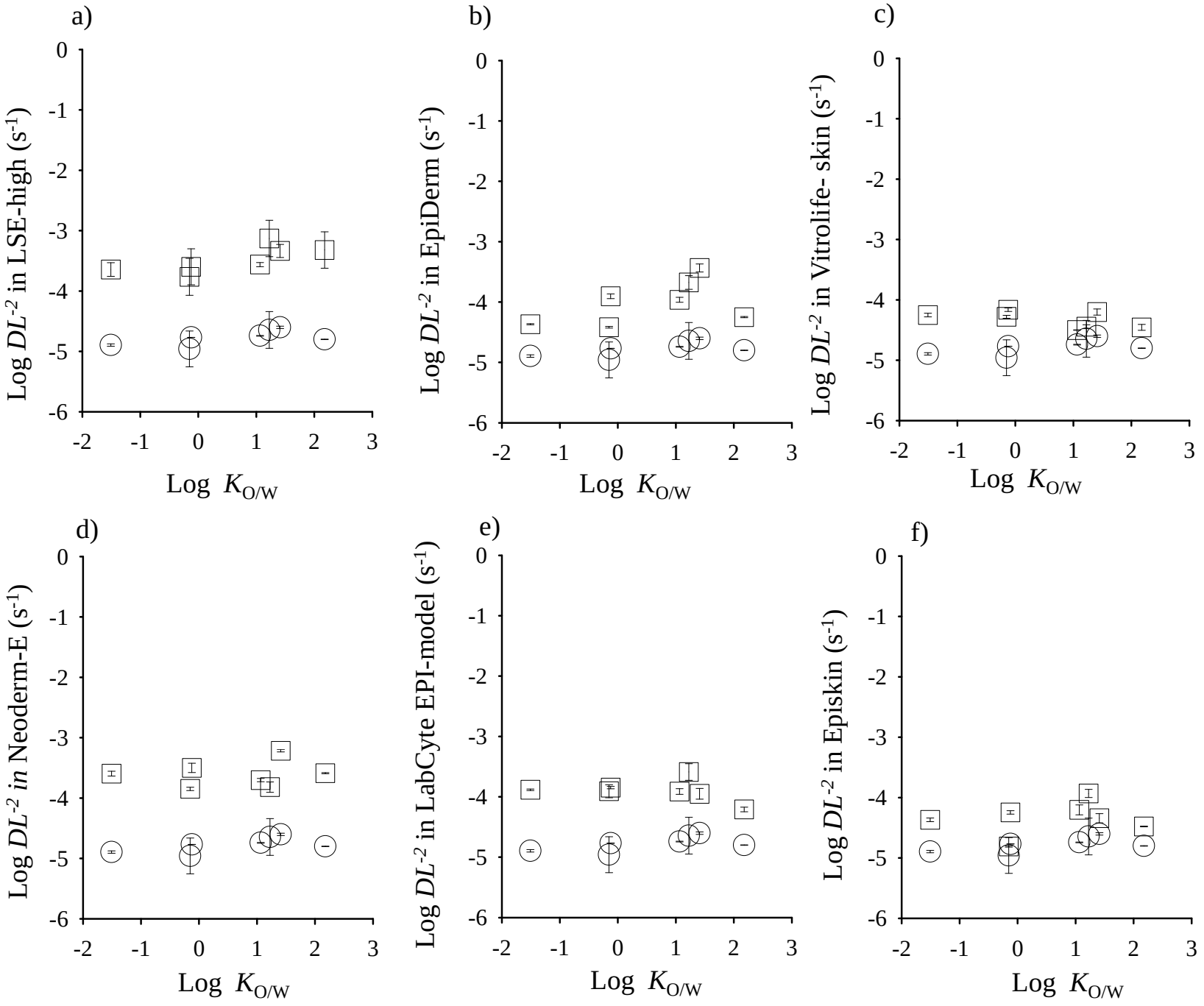

a)
b)
c)
d)
e)
f)
Log KL in LSE-high (cm)
Log KL in EpiDerm (cm)
Log KL in Vitrolife- skin (cm)
Log KL in Neoderm-E (cm)
Log KL in LabCyte EPI-model (cm)
Log KL in Episkin (cm)
Log K_O/W

Figure 6


a)
b)
c)
d)
e)
f)
Log DL^{-2} in LSE-high (s^{-1})
Log DL^{-2} in EpiDerm (s^{-1})
Log DL^{-2} in Vitrolife- skin (s^{-1})
Log DL^{-2} *in* Neoderm-E (s^{-1})
Log DL^{-2} in LabCyte EPI-model (s^{-1})
Log DL^{-2} in Episkin (s^{-1})
Log $K_{O/W}$
0
-1
-2
-3
-4
-5
-6
-2
-1
0
1
2
3