New Applications of Phospholipid Vesicle-Based Permeation Assay: Permeation Model Mimicking Skin Barrier

ANDRÉ ENGESLAND,[1] MERETE SKAR,[1] TERKEL HANSEN,[2] NATAŠA ŠKALKO-BASNET,[1] GØRIL EIDE FLATEN[1]

[1]Drug Transport and Delivery Research Group, Department of Pharmacy, University of Tromsø, Tromsø N-9037, Norway

[2]Natural Products and Medicinal Chemistry Research Group, Department of Pharmacy, University of Tromsø, Tromsø N-9037, Norway

Received 20 December 2012; revised 28 January 2013; accepted 27 February 2013

Published online 25 March 2013 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jps.23509

ABSTRACT: The phospholipid vesicle-based permeation assay (PVPA), based on a tight barrier composed of liposomes mimicking cells, is providing an opportunity to predict passive drug permeability through biological membranes. Although it was originally developed to mimic the intestinal epithelia, this study focuses on its potential as a simple and affordable skin model for transdermal permeation of drug candidates and evaluation of various drugs and formulations at an early development stage. The changes induced in lipid composition of the lipid-based barriers to better mimic the *in vivo* stratum corneum lipid composition required optimization of liposomal properties and manufacturing conditions applied in barrier formation. The preparation conditions could be modified to prepare lipid-based barriers of different degrees of leakiness, potentially representing different degree of intact and compromised skin. The different PVPA models developed in this study appeared to be able to distinguish between drugs with different degrees of lipophilicity and penetration potential. Moreover, the PVPA can be produced in controlled and reproducible manner with different degree of leakiness. The model could therefore be applied in both pharmaceutical and cosmeceuticals manufacturing and also has the potential to provide deeper insight on safety of nanodelivery systems administered onto the skin. © 2013 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 102:1588–1600, 2013

Keywords: skin; permeability; liposomes; lipids; *in vitro* models

INTRODUCTION

Transdermal delivery of drugs provides an appealing alternative to other modes of drug administration.[1] The number of new transdermal delivery systems and drugs administered transdermally is hence continuously increasing. As a result, penetration of drug into/through skin has gained high interest in the pharmaceutical as well as cosmetic industries, both in respect to desired therapeutic efficacy and potential toxicity issues.[2]

The main barrier of the skin, the *stratum corneum* (*SC*), is located in the outermost layer of the skin and consists of corneocytes surrounded by lipid regions.[1] As most lipophilic drugs applied onto the skin permeate along the lipid domains, the lipid organization is considered to be of high importance for the skin barrier function. This is also the reason why the lipid organization has been investigated quite extensively.[3–5] Because of the exceptional lipid composition found in *SC*, dominated by ceramides, free fatty acids, cholesterol (Chol), and cholesteryl sulfate (Cholsul), the lipid organization within *SC* differs greatly from that in other biological membranes.[3]

The alteration in the lateral packing and in the lamellar organization observed in the *SC* of patients suffering from skin disorders accounts for the aberrations in barrier function.[6,7] As a result, the formulation is facing skin barriers with increased leakiness.

Abbreviations used: Chol, cholesterol; Cholsul, cholesteryl sulfate; EtOH, ethanol; FITC–dextran, fluorescein isothiocyanate–dextran (MW: 4000 Da); PA, palmitic acid; PAMPA, parallel artificial membrane permeability assay; PSA, polar surface area; PVPA, phospholipid vesicle-based permeation assay; *SC*, *stratum corneum*.

Additional Supporting Information may be found in the online version of this article. Supporting Information

Correspondence to: Gøril Eide Flaten (Telephone: +47-776-46169; Fax: +47-776-46151; E-mail: Goril.Flaten@uit.no)

Journal of Pharmaceutical Sciences, Vol. 102, 1588–1600 (2013)

With the development of nanotechnology and nanocosmeceuticals, the concerns related to their potential toxicity and penetration into/through skin highlight additional need for the investigation of differences in penetration into/through intact and compromised skin.[8]

Most of the models used to mimic healthy skin, and to a lesser extent compromised skin, rely either on various types of diffusion cells or phospholipid mixture models.[9] In the last several years, attempts were made to develop perfusion cell culture systems.[10] Quantitative structure–permeability relationship models, immobilized artificial membrane, biopartitioning micellar chromatography, and silicone membranes have also been used as the simple alternatives predicting dermal absorption.[11–15]

The parallel artificial membrane permeability assay (PAMPA) system with synthetic ceramide derivatives mimicking *SC* composition[2,16] and model membranes designed to study the impact of ceramide species on drug diffusion and permeation have been proposed.[17]

The phospholipid vesicle-based permeation assay (PVPA) was first introduced as a medium-throughput screening method to mimic the intestinal epithelia and thus render rapid screening of a large number of drug candidates for oral administration possible.[18,19] Such a simple and straightforward permeation model has the potential to provide insight into barrier function and serve as a tool to predict transdermal permeation in the development of novel, advanced treatments for both healthy and diseased skin. The PVPA barriers are prepared by placing the liposomes, by means of centrifugation, onto a filter support, followed by solvent evaporation and freeze–thaw cycling to promote liposome fusion, finally resulting in a tight barrier. By using the cell-like liposomal structures as main building block, the PVPA represents more *in vivo*-like structure.[18] The PVPA also differs from other permeability models in that agitation does not lead to an increase in permeability, not even for highly lipophilic drugs such as testosterone.[20] The reason why agitation and hence reduction of the thickness of the unstirred water layers unlike in other models, that is, the PAMPA,[21] does not influence permeability is explained by the characteristic morphology of the vesicle-based barrier, which is containing a layer of mostly aqueous compartments immobilized within a matrix of phospholipids vesicles.

The PVPA has been further used to test the permeability of both new active substances as well as drugs in various formulations.[22–24] Additionally, the PVPA has been shown to be suitable for automation using a robotic system with a connected plate reader, which is a very important feature in industrial screening of drug candidates.[25]

Because passive diffusion is a process that occurs across all biological barriers,[26] models developed to enable studying the process across one type of barrier should, in principle, be a good starting point for the development of a model to study other types of barriers.

By changing the lipid composition to better mimic the composition found in human *SC*, the original PVPA could be developed into a model for studying skin permeation. Even more interesting is the possibility to correlate the degree of membrane leakiness with the degree of damage in original barrier properties in compromised skin. The development of an *in vitro* model for healthy and skin with compromised *SC* barrier properties would directly reduce the excessive use of animals and human studies in early phase development of topical formulations.

The focus of this study was thus to develop a simple and affordable skin model as a medium-to-high-throughput screening tool for potential transdermal penetration of drug candidates, drugs in topical formulations, and cosmeceuticals.

MATERIALS AND METHODS

Materials

Egg phosphatidylcholine, Lipoid E-80, was obtained from Lipoid (Ludwigshafen, Germany). Chloroform was purchased from Merck (Darmstadt, Germany). Chol, ceramides from bovine spinal cord, Cholsul, calcein, ethanol (EtOH), flufenamic acid, fluorescein isothiocyanate–dextran (FITC–dextran), ibuprofen, indomethacin, methanol, and palmitic acid (PA) were the product of Sigma–Aldrich Company (St. Louis, California). Salicylic acid was purchased from Apotekproduksjon AS (Oslo, Norway). Filter inserts (Transwell, $d = 6.5$ mm) and plates were purchased from Corning Inc., (Corning, New York). The mixed cellulose ester filters (0.65 μm pore size) and the isopore filters (0.8 and 1.2 μm pore size) were obtained from Millipore (Billerica, Massachusetts). The nucleopore filters (0.4 μm pore size) were obtained from Whatman (part of GE Healthcare, Oslo, Norway).

Calculations of Physicochemical Properties and Skin Penetration

Schrödinger's QikProp application running on Meastro software 9.1 (Schrodinger, New York) was used to calculate the log K_p, log P, polar surface area (PSA), and molecular weight for the drugs and the hydrophilic fluorescing markers calcein and FITC–dextran. All the substances except FITC–dextran were within the structural limits of the program.

Liposome Preparation

The liposomes were prepared by the film hydration method.[18] Three different lipid compositions were used:

(1) E-80 (100%);
(2) E-80 (77.1%, w/w) and Chol (22.9%, w/w);
(3) E-80 (50%, w/w), ceramides (27.5%, w/w), Chol (12.5%, w/w), Cholsul (2.5%, w/w), and PA (7.5%, w/w).

Lipids were dissolved in chloroform or a mixture of chloroform and methanol. The organic solvents were removed before hydration with phosphate buffer (pH 7.4), containing 10% (v/v) EtOH, if not stated otherwise, and 6% (w/v) liposomal dispersion prepared as described earlier.[18]

The liposomes were then extruded either by hand using syringe filter holders or Lipofast (Avestin Europe GmbH, Mannheim, Germany), or by nitrogen-driven extrusion (Lauda Dr R Wobster Gmbh, Königshofen, Germany), depending on the rigidity of vesicles.

The liposome size distributions were measured by photon correlation spectroscopy using a Submicron Particle Sizer 370 (PSS Nicomp Particle Sizing Systems, Santa Barbara, California). Sample preparation and measuring conditions were the same as described earlier.[27] Three cycles of 15-min measurements were performed.

The Original Preparation Method for the PVPA Barriers

The original PVPA barriers ($PVPA_o$) were prepared according to the procedure described earlier[18] and were stored for up to 2 weeks at −70°C in accordance with the previous stability studies.[19] In brief, liposome dispersions extruded through filters with a pore size of 400 and 800 nm, respectively, were deposited on a filter support by the use of centrifugation. The liposomes were added in consecutive steps, first the smaller liposomes, followed by the larger ones. Freeze–thaw cycling was then used to promote the fusion of liposomes and produce a tight barrier (flow chart shown in Fig. 1). Earlier characterization of the PVPA barriers has shown that the amount of lipid on the filter mainly originates from the addition of larger liposomes to settle on the top of the filter support, and

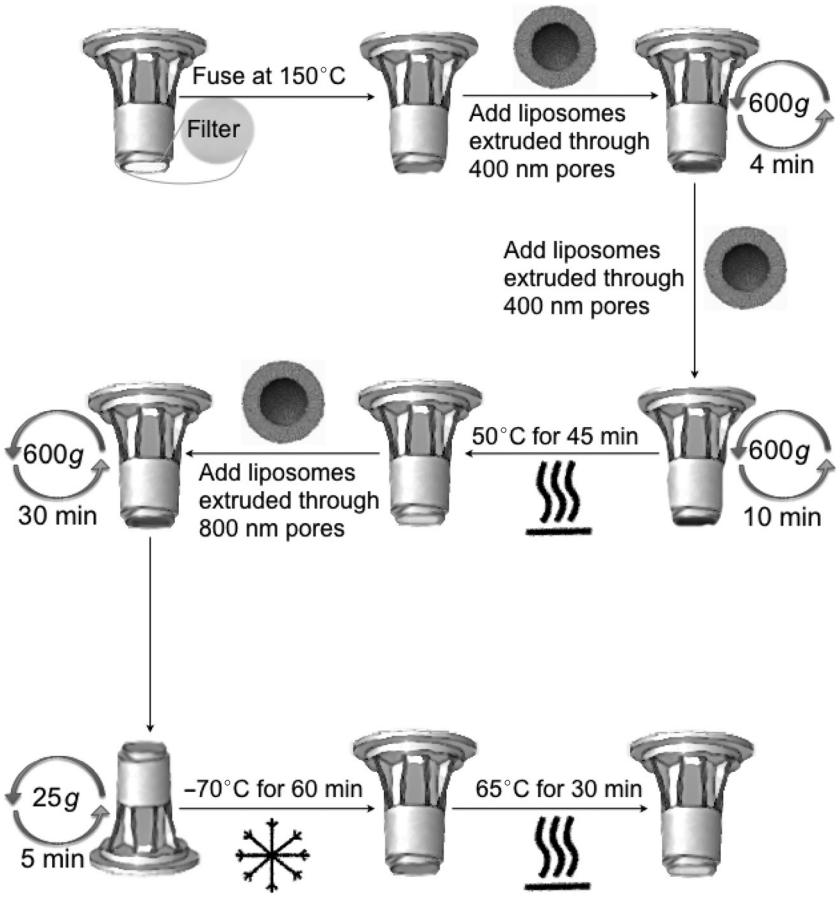


Figure 1. A flowchart for the preparation of the PVPA barriers with E-80.

 DOI 10.1002/jps

that this represents the major diffusion barrier lies of the barrier. However, the liposomes in the pores have also shown to significantly contribute to the permeability properties of the barriers, and their presence is thus needed to produce tight barriers.[19]

The Permeation Experiments with Drugs or Hydrophilic Markers

Permeation studies were performed using solutions of drugs or hydrophilic markers in phosphate buffer (pH 7.4) according to the procedure described earlier.[18] In short, the inserts were loaded with donor solution (100 μL) containing drugs or hydrophilic markers (see Table S1 in Supporting information for details on drug concentrations, acceptor volumes, and wave length used in analysis). The inserts were moved to wells containing acceptor solution of phosphate buffer, at certain time intervals to maintain sink conditions. At the end of the permeation experiment, samples (200μL) from each acceptor compartment were transferred into 96-well titer plates for analysis. The drug concentrations were measured spectrophotometrically (Spectramax 190; Molecular Device Corporation, Sunnyvale, California), and fluorescence measurements of the hydrophilic markers were performed with a Polarstar fluorimeter (Fluostar; BMG Technologies, Offenburg, Germany).

The resistance of the lipid barriers was measured (Millicell-ERS; Millipore) immediately after the completion of permeation studies. The mean values and standard deviations are reported. The experiments were performed at least in triplicate.

The permeability experiments were performed under sink conditions as described earlier by us.[18] A lag phase was observed in the beginning of the experiment, so the flux was calculated only from the linear part of the curve representing steady-state conditions. Equation 1 was used to calculate the apparent permeability coefficient (P_{app}). J is the observed flux rate (nmol/s), A is the surface area of the insert (cm^2), and C_d is the concentration of the donor (nmol/mL).

$$P_{app}(\text{cm/s}) = \frac{J}{AC_d} \quad (1)$$

Modification of the PVPA Barriers by Changing the Lipid Composition and Preparation Process Parameters

The Effect of EtOH Concentration in the Liposome Dispersion on the Barrier Properties

To investigate the effect of EtOH concentration on the barrier properties, permeation barriers were prepared according to the original preparation method described earlier using E-80 liposome dispersions containing different concentrations (0%, 1%, 5%, 10%, 15%, or 20%; v/v) of EtOH. Permeability experiments with calcein and FITC–dextran solution were performed as described earlier to reveal the differences in tightness of the barriers in correspondence to the EtOH concentration in the liposome dispersion.

Modifications of the PVPA Barrier Lipid Composition to Better Mimic Skin

Inclusion of Chol

Permeation barriers were prepared from liposomes containing 23% (w/w) Chol and 77% (w/w) E-80 ($PVPA_c$) to produce barriers that could mimic the *in vivo*/skin-like lipid composition to a greater extent, as well as to provide a more robust system able to withstand harsher procedure conditions.[28]

To be able to prepare tight permeation barriers with the new lipid composition, changes had to be made in the original preparation process.

The different parameters investigated in the preparation process were (1) the centrifugation speeds after addition of small and large liposomes, (2) the centrifugation time after addition of liposomes to settle on top of the filter, (3) temperature and duration during freeze–thaw cycling, and (4) the number of freeze–thaw cycles (see Table S2 in Supporting information for further details). Permeability of the hydrophilic marker calcein as well as electrical resistance across the barriers were used to monitor the effect of changing of different parameters on the barrier properties.[18]

Inclusion of Ceramides, PA, Chol, and Cholsul

To mimic the lipid composition in the healthy human *SC* closely, the permeation barriers were prepared from liposomes containing E-80 (50%, w/w), ceramides (27.5%, w/w), Chol (12.5%, w/w), Cholsul (2.5%, w/w), and PA (7.5%, w/w) ($PVPA_s$).[29] The reason for using E-80 in the barrier composition was that the original PVPA contained only E-80, and we intended to develop this into a model for the human *SC*.

The more complex lipid composition required additional changes in the original preparation process.

The following parameters in the preparation process were investigated: (1) the centrifugation speed and time after addition of large liposomes on top of the filter, (2) the time and temperature during evaporation of buffer/EtOH, and (3) the time and temperature during freeze–thaw cycling (see Table S3 in Supporting Information for further details). The membrane integrity and resistance were checked as for $PVPA_c$.[18]

Comparison of Drug Permeabilities Obtained from the $PVPA_o$, $PVPA_c$, and $PVPA_s$ with Reported Permeabilities in Animal Skin

Permeability experiments using a selection of standard drugs, chosen based on their lipophilicity,

were performed in three different PVPA models: (1) the original E-80 PVPA ($PVPA_o$), (2) the newly developed E-80–Chol PVPA ($PVPA_c$), and (3) the E-80–ceramide–PA–Chol–Cholsul PVPA ($PVPA_s$). The selected drugs included indomethacin, salicylic acid, ibuprofen, and flufenamic acid and the permeation experiments were performed as described earlier. The findings were further compared with literature values on permeation properties of selected drugs based on Franz diffusion cell experiments using animal skin of different origin as reported by Stahl et al.[30]

Statistical Methods

The Student's *t*-test was used for comparison of two means. A significance level of p below 0.05 was considered significant.

RESULTS AND DISCUSSION

Comparison of P_{app} Values Obtained from the Original PVPA with Calculated log K_p

The permeability data for a selection of 20 marketed drugs obtained earlier with the original PVPA,[18,25,31] in addition to the newly obtained permeability data for four drugs with the original PVPA model, were compared with their calculated log P and log K_p values to evaluate to which extent is the model able to distinguish and rank the selected drugs in a manner expected from the skin penetration data. Both calculated log P and log K_p correlated well with values of log P_{app} obtained with the PVPA, as shown in Figures 2a and 2b, respectively.

Although one could expect that the skin can represent a tighter barrier than intestine, the comparison is not as simple, as we need to consider several other factors related to the properties of the drug such as the size and shape of the molecule, log D, and charge. The higher permeability with increased log P of the drug is expected, but is not always the case. We have observed higher permability for the lipophillic drugs ibuprofen and indomethacin in the skin PVPA. According to the work published by Johnson et al.,[32] our findings seem to be unexpected, indicating certain degree of selectivity connected to *SC* barrier model, not observed for intestinal membrane. This has to be further evaluated for different types of drug molecules. However, the data in Figure 2 clearly demonstrate that the drugs showing the highest permeation in the PVPA also exhibited high calculated log P and log K_p values. In summary, the original PVPA is able to distinguish between highly and poorly transdermally absorbed drugs to the same extent as calculated log K_p values and better than calculated log P values.

Comparison of P_{app} Values from the Original PVPA with log P_e Values from Franz Diffusion Cell Experiments

Further, we compared the permeability data from the original PVPA with experimentally obtained *in vitro* transdermal permeability values from literature using rat skin segments in the Franz diffusion cells.[33] Both P_{app} and log P_{app} from the PVPA were compared with log P_e values as shown in Figures 3a and 3b, respectively. The comparison with the experimentally obtained transdermal permeability values showed that the original PVPA was able to distinguish between highly and poorly transdermally absorbed drugs to the same extent as in *in vitro* tests using rat skin. The PVPA thus seemed to be a good basis for the development of an artificial *in vitro* skin model.

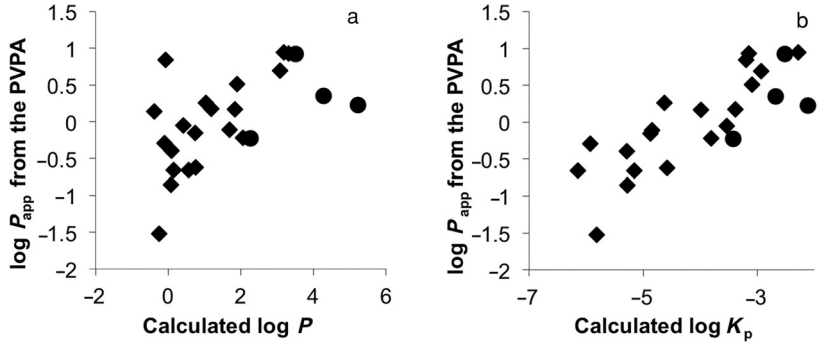


Figure 2. Correlation between log P_{app} values from the original PVPA and calculated log P values (a) and log K_p values (b). Experimentally obtained permeability values from literature[18,25,31] for 20 drugs (acebutolol, alprenolol, atenolol, amiloride, caffeine, chloramphenicol, chlorothiazide, cimetidine, enalapril, hydrochlorothiazide, metoprolol, nadolol, naproxen, propranolol, ranitidine, sulphasalazine, sulpiride, testosterone, timolol, and terbutaline) are shown as squares, whereas newly obtained experimental permeability values for flufenamic acid, ibuprofen, indomethacin, and salicylic acid are represented by circles.

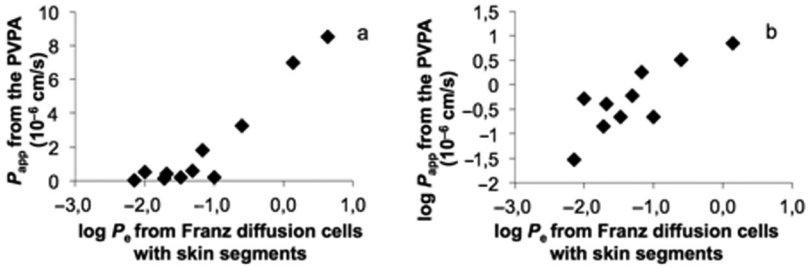


Figure 3. Correlation between log P_e values from Franz diffusion cell experiments[30] and P_{app} values (a) and log P_{app} values (b) from the original PVPA for the following drugs: atenolol, caffeine, chloramphenicol, chlortiazide, enalapril, hydrochlorothiazide, metoprolol, sulfasalazine, sulpiride, terbutline, and testosterone.

Regulation of the Barrier Properties in Response to Changes in the Preparation Process

Effect of the EtOH Concentration in the Liposome Dispersion

Ethanol was originally added to the liposome dispersion to induce higher degree of fusion during freeze–thaw steps in the preparation process.[18] Addition of EtOH resulted in extensively less permeable barriers, and our hypothesis was that by reducing the concentration of EtOH the leakiness of the barrier could be controlled.

The results presented in Figure 4a indicate a strong correlation between concentration of EtOH in the liposome dispersions and permeability of the small hydrophilic marker calcein. By increasing the EtOH concentration up to 10% (v/v), the barriers become both tighter and the permeability values more reproducible. However, by increasing the EtOH concentration above 10% (v/v), the increased permeability and decreased reproducibility were again detectable (Fig. 4a).

To determine whether the same trend as observed for calcein (MW = 600 Da) also applies to the larger molecules, the permeability of FITC–dextran (MW = 4000 Da) was investigated through the barriers made from liposomal dispersions containing 1%, 5%, or 10% EtOH, respectively. The results presented in Figure 4b showed that the trend observed for the FITC–dextran permeability was the same as seen for calcein, that is, decreasing permeability with increasing concentration of EtOH at least up to 10% (v/v).

The results presented in Figure 4 confirmed our hypothesis that the presence as well as the concentration of EtOH is significantly influencing the barrier properties. When preparing barriers of different degrees of leakiness mimicking skin with reduced *SC* barrier properties, optimization of the EtOH concentration could be one of the simplest parameters to utilize.

Modification of the Lipid Composition of the PVPA Barriers to Develop an *In Vitro* Permeation Model Mimicking *SC*

Inclusion of Chol in the Lipid Composition of PVPA Barriers

Cholesterol was included in the liposome dispersion used to prepare the permeation barriers to mimic the *SC* closely. By using the original preparation procedure, the barriers with a mean calcein permeability of 0.38×10^{-6} cm/s were obtained as compared with 0.08×10^{-6} cm/s for the original PVPA barriers. The results clearly showed that changes in lipid composition required an adjustment of the parameters in the preparation process.

Trends observed in permeability of calcein and electrical resistance, which are both the measures of the

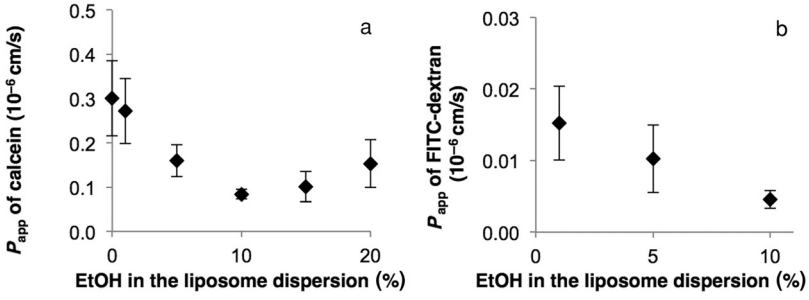


Figure 4. The effect of increasing EtOH concentration in the original liposomal dispersion on the permeability values (P_{app}) of calcein (a) and FITC–dextran (b). Error bars represent standard deviations.

tightness/integrity of the barrier, with changing parameters, are presented and discussed below.

Extrusion of Liposomes to Obtain Desired Size Distribution

Extruded liposomes (800 nm filter) were used for the original PVPA as large liposomes to settle on top of the filter. However, in $PVPA_c$, we had to increase the filter pore size to 1200 nm to be able to obtain liposomes large enough not to pass through the filter support during centrifugation. The size distribution of E-80–Chol liposomes extruded through 400 nm filters was 278 ± 87 nm and the extruded through 1200 nm liposomes was 756 ± 100 nm.

Deposition of Small Liposomes into the Pores of the Filter

For the centrifugation used to deposit the small liposomes into the pores of the filter support, different centrifugation speeds in the range 608–1369 *g* were tested. The results (displayed in Fig. S1 in Supporting Information) showed that centrifugation at 608 *g* during the first two steps, as in the original PVPA, resulted in barriers with a high permeability value and low resistance, representing leaky barriers, and was thus rejected from further testing. There were no significant differences among 951, 1109, and 1369 *g*, thereof 951 *g* was chosen because this involved most gentle conditions in respect to the filters.

Deposition of Large Liposomes on Top of the Filter

The parameters tested here were the time and speed of centrifugation. The results (Fig. S2 in Supporting Information) indicate that no significant change in permeation of calcein with prolonged centrifugation time from 30 to 50 min was observed.

Further, when minimum centrifugation time was set to 30 min, maximum centrifugation speed without inducing leakage of the filters was found to be 1279 *g*. No significant change in permeation of calcein could be observed because of increasing the speed above 1028 *g*. Therefore, 1028 *g* was chosen for further procedures.

Freeze–Thaw Cycling to Induce Fusion of Liposomes

Freeze–thaw cycling used to induce fusion of the liposomes was studied last. The effect of thawing temperature ranging from 30°C to 90°C can be seen in Figure 5a. Thawing at 30°C and 40°C resulted in barriers with acceptable calcein permeability and electrical resistance values. The increased permeability with increasing temperatures could be a result of decomposition/melting of the lipids in the barrier. During heating at 40°C for a longer period, discoloration of the membranes was observed; therefore, temperature of 30°C was chosen for further studies.

When the optimal temperature for the freeze–thaw cycling was established, we optimized the duration of the thawing/heating cycles. The calcein permeability through the resulting barriers and the electrical resistance are shown in Figure 5b. Decreasing permeation of calcein and better reproducibility with increasing duration of the thawing step were observed. The thawing time of 120 min was used in the further studies.

Finally, we examined the number of freeze–thaw cycles applied. The results (shown in Fig. S3 in Supporting Information) confirmed that increasing numbers of freeze–thaw cycles resulted in tighter barriers. However, increasing the number of freeze–thaw cycles resulted in more complex and time-consuming preparation process. In the final procedure, one freeze–thaw cycle was used to compromise between efficiency and required tightness of the resulting barrier.

To summarize the effects of changes made in the barrier preparation process, the final preparation procedure for the $PVPA_c$ is given as following:

- Addition of small (extruded through 400 nm pore filter) liposomes of E-80–Chol (6%, w/v) to fill the pores of the filter: 100 μL added and centrifuged at 950*g* for 15 min, performed twice with change of direction of inserts in between.
- Heating at 50°C for 45 min.
- Addition of large (extruded through 1200 nm pore filter) liposomes of E-80–Chol (6%, w/v) to settle on top of the filter: 100 μL added and centrifuged at 1030*g* for 60 min.
- Removal of supernatant by invert centrifugation of inserts: 25 *g*.
- Freezing at −70°C for minimum 60 min.
- Heating at 30°C for 120 min.

This approach resulted in barriers exhibiting a calcein permeability of $0.081 \times 10^{-6} \pm 0.041 \times 10^{-6}$ cm/s, whereas the FITC–dextran permeability was $0.010 \times 10^{-6} \pm 0.007 \times 10^{-6}$ cm/s. This revealed tightness comparable to the $PVPA_o$ barriers. In summary, the main differences between $PVPA_c$ and the $PVPA_o$ are that higher centrifugation force over longer period of time was required to settle the liposomes, and that the thawing step was performed at lower temperature with longer duration.

Inclusion of Ceramides, PA, Chol, and Cholsul in Barrier Lipid Composition

Ceramides, free fatty acid (PA), Chol, and Cholsul are the major lipid classes found in the skin.[3,29] The lipid composition we used in the preparation of the

barriers is the same as used earlier by Abraham and Downing.[29]

The type of ceramide and amount of lipids, especially Chol, is crucial for the packing and organization of the skin and skin properties.[6] The aim of this study was to use the main lipid classes found in skin as constituents in the barrier. Bouwstra et al.[34] investigated the ceramide composition of different samples taken from a number of pigs, revealing quite varying ceramide type and content. This suggests that the ceramides in *SC* may vary in both type and amount between individuals, and the need for a more general use of ceramides can be useful.

When the original preparation procedure for $PVPA_o$ barriers was used for the new lipid composition, the permeability of the selected marker calcein differed in order of magnitude as compared with $PVPA_o$ barrier (5.1×10^{-6} and 0.08×10^{-6} cm/s, respectively). The original procedure thus obviously required modification when liposomal composition included ceramides, PA, Chol, and Cholsul, in addition to E-80 as basic lipid.

Deposition of Small Liposomes into the Pores of the Filter

Small liposomes (400 nm filters) were prepared from E-80–Chol and deposited into the pores as described for the $PVPA_c$. Using E-80–Chol liposomes to fill the pores of the filter was performed in an attempt to use less of the expensive ceramide-containing liposomes to keep the assay as affordable as possible.

Deposition of Large Liposomes on Top of the Filter

The larger liposomes containing E-80–Ceramide–PA–Chol–Cholsul were extruded by hand using the Lipofast device (Avestin Europe GmbH). By using a filter pore size of 1200 nm, liposomes with a size distribution of 907 ± 82 nm was prepared, which were large enough to settle on top of the filter support.

Two different centrifugation speeds, namely 608 and 1028 *g*, were used in an attempt to settle liposomes on top of the filter. Both speeds resulted in inadequate barriers; therefore, we decided to add the liposome dispersion (100 μL) on top of the filter

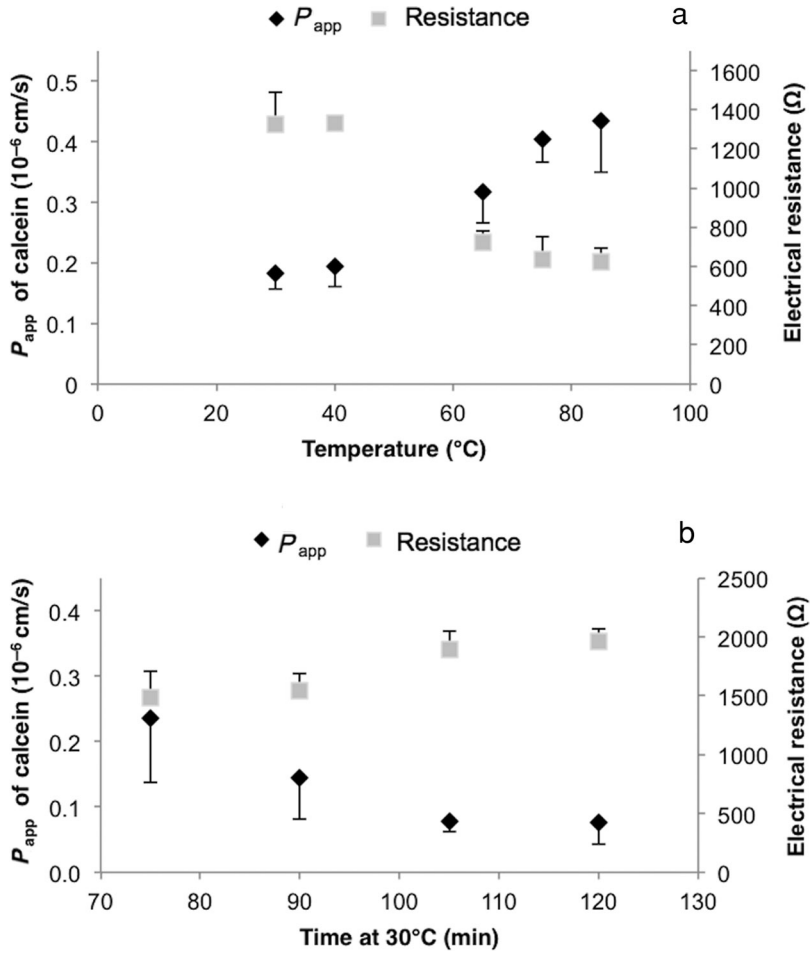


Figure 5. The effect of increasing temperature during the freeze–thaw cycles (a) and duration of heating during the freeze–thaw cycles (b) on the permeability values (P_{app}) of calcein and electrical resistance. Error bars show standard deviations.

support and let the liquid evaporate in the incubator leaving the lipids/liposomes on the filter, as an alternative to centrifugation. This approach was in accordance to Abraham and Downing[29] who used drop-wise addition of liposome dispersion made from *SC* lipids onto a filter support to make barriers for a specially designed diffusion cell. For evaporation of the buffer and EtOH, the temperature of 50°C was selected to avoid lipid decomposition.

After the barriers were dry, accumulation of lipids at the insert wall was visually detectable, and an uneven distribution of lipids on the filter surface could also be observed because of a transparent spot in the middle of the filter. The barriers also demonstrated high permeability of calcein and very low reproducibility (results not shown). To solve the problem of uneven distribution of lipids on the filter surface, two additions of 50 μL of liposomes, with evaporation of the buffer/EtOH in between, were applied. After addition of first aliquot of liposomes, the inserts were heated for 40–45 min, allowing the water and EtOH to evaporate. The liposome layer on the filter support was however still moist when adding the second aliquot of liposomes and heating the inserts for up to 80 min. The moist barriers with a visually improved distribution of liposomes were then subjected to freeze–thaw cycling. The reason to terminate the evaporation before complete dryness was to allow the freeze–thaw step to be as efficient as possible.

Freeze–Thaw Cycling to Promote Fusion of the Liposomes

The filters heated at 60°C (Fig. 6, number 1 and 2) were dark and transparent, indicating possible decomposition/melting of the lipids in the barriers. The barriers heated at 40°C (Fig. 6, number 3) and 30°C (Fig. 6, number 4 and 5) were whitish with a flat finish after incubation. For number 5 in Figure 6, 100 μL buffer was added on top after incubation and centrifuged at 608 *g* for 30 min to add more force on the liposomes layer. However, this did not give any significant change in permeability of calcein and was not further investigated. The 30°C was chosen as the thawing temperature for the $PVPA_c$ because of less variation between barriers and gentler handling of the lipids. This method resulted in barriers that produced liposome layer visually similar to the original PVPA.

The effect of the duration of the thawing step was also investigated, and the conditions represented as number 3 in Figure 7 appeared to be the most promising one; thereof, we finalized the heating procedure at 30°C for 120 min, followed by increasing the heat to 50°C for 15 min to remove excess moisture.

The final preparation procedure for $PVPA_s$ barriers is summarized as following:

- Addition of small (extruded through 400 nm pore filter) liposomes of E-80–Chol (6%, w/v) to fill the pores of the filter: 100 μL added and centrifuged at 950 *g* for 15 min, performed twice with change of direction of inserts in between.
- Heating at 50°C for 45 min.
- Addition of large (extruded through 1200 nm pore filter) liposomes of E-80–ceramide–PA–Chol–Cholsul (6%, w/v) to settle on top of the filter support:

- 50 μL liposomes added, solvent allowed to evaporate in incubator at 50°C for 40 min: 20 min in closed container, 20 min in open.

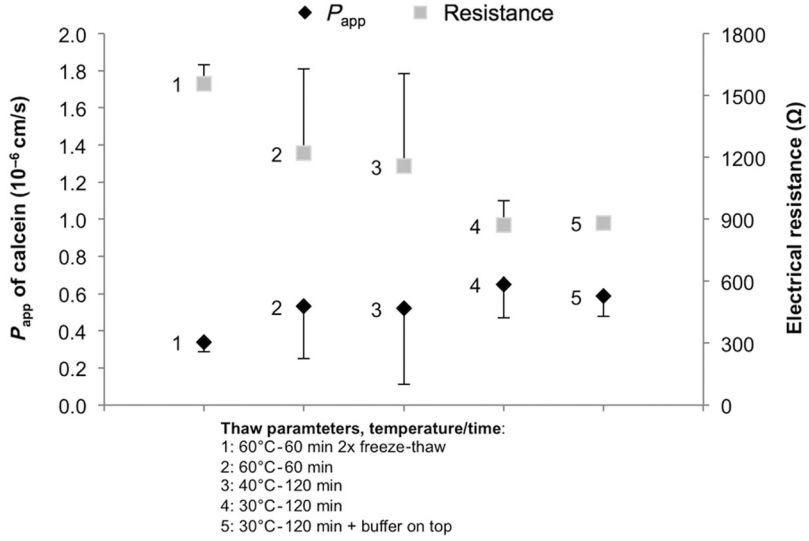


Figure 6. The effect of thawing temperatures and time on the permeability values (P_{app}) of calcein and electrical resistance. Error bars show standard deviations.

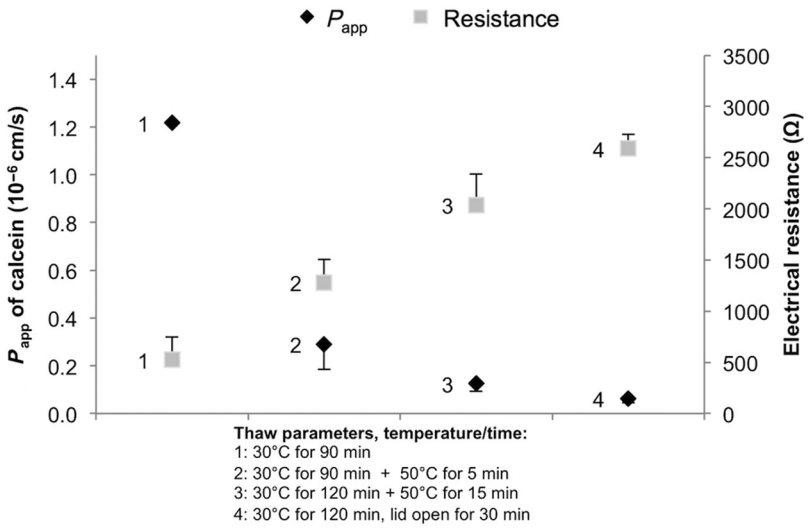


Figure 7. The effect of thawing time during the freeze–thaw cycle on the permeability values (P_{app}) of calcein and electrical resistance. Error bars show standard deviations.

- 50 μL liposomes added, solvent allowed to evaporate in incubator at 50°C for 60 min: 20 min in closed container, 40 min in open
- Freezing at −70°C for minimum 60 min.
- Heating at 30°C for 120 min and 50°C for 15 min.

The barriers prepared by this procedure exhibited calcein permeability of $0.126 \times 10^{-6} \pm 0.033 \times 10^{-6}$ cm/s and FITC–dextran permeability of $0.026 \times 10^{-6} \pm 0.009 \times 10^{-6}$ cm/s.

In summary, the major changes from the $PVPA_o$ were that centrifugation to settle the large liposomes on top of the filter support was exchanged with evaporation and that the thawing step was performed at lower temperature during longer period of time.

To summarize the differences/advantages of these novel PVPA models over other comparable *in vitro* skin permeation models already on the market, we could state that in contrast to the PAMPA models,[2,16] the PVPA consists of all the important lipid components of human skin. The main advantage can further be attributed to the fact that PVPA consists of liposomes, which could be seen as simple models for the cells, and therefore represent a more *in vivo*-like structure compared with the continuous lipophilic environment in the PAMPA models.[18] This has also been seen earlier from the partition between liposomes and buffer to be a better predictor for permeation than partition between a bulk solvent such as octanol and water.[35,36] However, even though liposomes mimic cell structure to a greater extent than the other proposed models, the complexity of the corneocytes, as more elongated and flattened geometrical structures, should be taken into account when evaluating the penetration of chemical entities through model membranes. Ochalek et al.[17] developed and characterized very sophisticated model membranes designed to study the impact of ceramide species on drug diffusion and permeation. To study the effect of different types of ceramides on permeation of drugs through the model membrane was, however, not the aim of this model development.

The additional advantage and strength of the PVPA is that it is easy to perform, has shown to be suitable for automation, and could thus be run in a medium-to-high-throughput format making screening of the drug candidates feasible.[25] Furthermore, probably the most unique characteristic of the PVPA is that the preparation steps of the PVPA barrier could be controlled to produce barriers with different degree of leakiness, thus mimicking compromised skin of different degree.

Evaluation of Drug Permeabilities Obtained from the $PVPA_o$, $PVPA_c$, and $PVPA_s$ as Compared with Reported Permeabilities in Animal Skin

Three different PVPA models, namely $PVPA_o$, $PVPA_c$, and $PVPA_s$, showed permeability values for the hydrophilic markers calcein and FITC–dextran in the same range.

Comparison of the permeability values of salicylic acid, ibuprofen, indomethacin, and flufenamic acid (see Fig. S4 in Supporting Information for molecular structures of the drugs) through the three different PVPA barriers (see Table 1) showed that the substances were ranked in the same order according to their permeability values, namely salicylic acid < flufenamic acid < indomethacin < ibuprofen, for all the three models.

To evaluate the applicability of our model, we compared permeability data from our PVPA models with

Table 1. Permeability Values for Flufenamic Acid, Ibuprofen, Indomethacin, Salicylic Acid, FITC–Dextran, and Calcein Across Different PVPA Models and Different Types of Animal Skin in Franz Diffusion Cell Experiments Together with Calculated Physicochemical Properties and Literature Values on Experimental log *D* and p*K*a

	$PVPA_o$		$PVPA_c$		$PVPA_s$		Franz Diffusion Cell[a]								
	P_{app}	SD	P_{app}	SD	P_{app}	SD	P_{app} (10^{-6} cm/s)				$\log K_p$	PSA	$\log P$	MW	$\log D^b$	p*Ka*[c]
Drugs	(10^{-6} cm/s)		(10^{-6} cm/s)		(10^{-6} cm/s)		Rat	Cattle	Dog	Pig						
Flufenamic acid	1.68	0.59	0.40	0.06	0.59	0.15	1.60	0.85	0.81	0.59	−2.11	57.7	5.23	281.2	2.03	3.9
Ibuprofen	8.42	0.86	2.80	0.60	7.95	0.69	1.20	0.61	0.38	0.18	−2.52	48.8	3.50	206.3	0.68-1.02	4.6
Indomethacin	2.23	0.37	1.42	0.36	4.48	0.79	0.81	0.57	0.32	0.14	−2.68	86.3	4.27	357.8	0.93-1.00	4.5
Salicylic acid	0.59	0.03	0.31	0.07	0.41	0.03	0.77	0.39	0.31	0.14	−3.42	70.4	2.26	138.1	−1.44	3.0
FITC–dextran	0.006	0.003	0.010	0.007	0.026	0.009	–	–	–	–	<−13[e]	–	–	4000[d]	–	
Calcein	0.084	0.011	0.081	0.041	0.126	0.033	–	–	–	–	−11.8	286.6	−1.71	622.5	–	1.8/9.2

[a] From Ref. 30.
[b] From Refs. 37 and 38.
[c] From Refs. 18,39–41
[d] From the producer.
[e] Because of the limitation of maximum number of non-H-atoms one could possibly have in one structure in QikProp, the program is able to calculate only up to seven glucose compared with 20 that is the number found in one FITC–dextran molecule
–, Literature values not available or calculations could not be performed.

literature data obtained from Franz diffusion cell experiments using different types of animal skin.[30] The rank order of the tested drugs according to penetration through animal skin was salicylic acid < indomethacin < ibuprofen < flufenamic acid. It could be seen that flufenamic acid showed a relatively lower permeation through the PVPA barriers as compared with skin obtained from rat, dog, pig, and cattle. One reason for this might be that flufenamic acid also showed a higher degree of membrane retention compared with the other drugs (data not shown), and this could again result in a lower than expected apparent permeability. However, one interesting observation is that the permeation values obtained for flufenamic acid was the same as those found in the Franz diffusion studies. For flufenamic acid, the same permeation values were obtained for $PVPA_o$ and rat skin and for $PVPA_s$ and pig skin. Literature indicates that investigations on the ceramide composition of samples taken from different animals revealed quite varying ceramide type and content.[34] Differences in lipid composition of the different skin types could possibly partly be the reason for the deviation in the penetration through different skin types. Further, a 10-fold difference could be observed in permeation for ibuprofen when comparing the values from rat and pig skin; therefore, it is clear that different types of animal skin lead to larger variations than when comparing the permeability values obtained in the different PVPA models. Here, it should be pointed out that animal skin varies not only based on originating species, but also on the site of the body from where the skin was taken.[42,43] Extrapolation of dermal absorption data between species is thus difficult because of the differences in epidermal anatomy and physiology.[43] Use of animal skin as model for the human skin is thus not optimal, but was found to be useful in establishing the PVPA for skin. The next step, where preliminary experiments are under way in our group, will be of course comparison of animal and human skin to confirm the potential of PVPA.

Further, the calculated log K_p values have been evaluated, and the same rank order as reported for the diffusion through animal skin was seen. However, the program used to calculate the log K_p values has certain limitations, and for the FITC–dextran with a molecular weight over 4000 Da, it was far outside the program's limits. This shows that *in vitro* testing is still very important not only to obtain high-quality results to base the *in silico* models on, but also to cover the drug molecules outside the size range of the program, for example, proteins.

We here, thus, propose a setup that can easily be adapted to several types of skin research. The PVPA can thus be used for rapid classification of drug candidates and topical formulations as well as toxicological studies on, for example, nanocosmeceuticals.

DOI 10.1002/jps

CONCLUSIONS

Permeation barriers able to mimic human *SC* lipid composition were developed by changing the lipid composition of the liposomes used to prepare the PVPA barriers as well as the preparation procedure compared with the original PVPA. The barriers were found to be reproducible showing low permeability of hydrophilic markers, comparable to the original PVPA, and be able to distinguish between substances with different degree of transdermal absorption.

The most remarkable finding was that we were able to prepare the barriers where the degree of leakiness can be fine-tuned depending on composition of the liposomes and procedures during production of the barriers, possibly representing different level of compromised *SC* barrier of the skin. This opens broad application potentials in the development and optimization of topical formulations for skin administration, both in respect to efficacy of active ingredients as well as toxicity related to the possible penetration through damaged as well as healthy skin.

The model could be applied in both pharmaceutical and cosmeceuticals manufacturing and has the potential to provide deeper insight on the safety of nanotechnology and nano delivery systems. In this way, the model could avoid excessive use of animals and human testing in early phase development of topical formulations.

ACKNOWLEDGMENTS

The authors thank The Norwegian Animal Protection Fund for financial support and Julia Rubner for assistance with the permeability experiments.

The authors declare that they have no conflict of interest to disclose.

REFERENCES

1. Bouwstra JA, Honeywell-Nguyen PL, Gooris GS, Ponec M. 2003. Structure of the skin barrier and its modulation by vesicular formulations. Prog Lipid Res 42(1):1–36.
2. Sinko B, Garrigues TM, Balogh GT, Nagy ZK, Tsinman O, Avdeef A, Takacs-Novak K. 2012. Skin-PAMPA: A new method for fast prediction of skin penetration. Eur J Pharm Sci 45(5):698–707.
3. Bouwstra JA, Ponec M. 2006. The skin barrier in healthy and diseased state. Biochim Biophys Acta 1758(12):2080–2095.
4. Gorcea M, Hadgraft J, Moore DJ, Lane ME. 2012. Fourier transform infrared spectroscopy studies of lipid domain formation in normal and ceramide deficient stratum corneum lipid models. Int J Pharm 435(1):63–68.
5. Groen D, Poole DS, Gooris GS, Bouwstra JA. 2011. Is an orthorhombic lateral packing and a proper lamellar organization important for the skin barrier function? Biochim Biophys Acta 1808(6):1529–1537.
6. Bouwstra J. 2002. Structure of stratum corneum lipid layers and interactions with lipid liposomes. In Cosmetic lipids and the skin barrier. Cosmetic Science and Technology series; Förster T, Jungermann E, Eds. Vol. 24, 1st ed. New York: Marcel Dekker Inc, pp 37–73.
7. Ogiso T, Hirota T, Iwaki M, Hino T, Tanino T. 1998. Effect of temperature on percutaneous absorption of terodiline, and relationship between penetration and fluidity of the stratum corneum lipids. Int J Pharm 176(1):63–72.
8. DeLouise LA. 2012. Applications of nanotechnology in dermatology. J Invest Dermatol 132(3 Pt 2):964–975.
9. 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(5):951–960.
10. Hayashi M, Matsuzaki Y, Shimonaka M. 2009. Impact of plasminogen on an in vitro wound healing model based on a perfusion cell culture system. Mol Cell Biochem 322(1–2):1–13.
11. Moss GP, Dearden JC, Patel H, Cronin MT. 2002. Quantitative structure–permeability relationships (QSPRs) for percutaneous absorption. Toxicol In Vitro 16(3):299–317.
12. Barbato F, Cappello B, Miro A, La Rotonda MI, Quaglia F. 1998. Chromatographic indexes on immobilized artificial membranes for the prediction of transdermal transport of drugs. Farmaco 53(10–11):655–661.
13. Escuder-Gilabert L, Martinez-Pla JJ, Sagrado S, Villanueva-Camanas RM, Medina-Hernandez MJ. 2003. Biopartitioning micellar separation methods: Modelling drug absorption. J Chromatogr B Analyt Technol Biomed Life Sci 797(1–2):21–35.
14. Oliveira G, Beezer AE, Hadgraft J, Lane ME. 2010. Alcohol enhanced permeation in model membranes. Part I. Thermodynamic and kinetic analyses of membrane permeation. Int J Pharm 393(1-2):61–67.
15. Oliveira G, Beezer AE, Hadgraft J, Lane ME. 2011. Alcohol enhanced permeation in model membranes. Part II. Thermodynamic analysis of membrane partitioning. Int J Pharm 420(2):216–222.
16. Sinko B, Kokosi J, Avdeef A, Takacs-Novak K. 2009. A PAMPA study of the permeability-enhancing effect of new ceramide analogues. Chem Biodivers 6(11):1867–1874.
17. Ochalek M, Heissler S, Wohlrab J, Neubert RH. 2012. Characterization of lipid model membranes designed for studying impact of ceramide species on drug diffusion and penetration. Eur J Pharm Biopharm 81(1):113–120.
18. Flaten GE, Dhanikula AB, Luthman K, Brandl M. 2006. Drug permeability across a phospholipid vesicle based barrier: A novel approach for studying passive diffusion. Eur J Pharm Sci 27(1):80–90.
19. Flaten GE, Bunjes H, Luthman K, Brandl M. 2006. Drug permeability across a phospholipid vesicle-based barrier 2. Characterization of barrier structure, storage stability and stability towards pH changes. Eur J Pharm Sci 28(4):336–343.
20. Flaten GE, Skar ML, Luthman K, Brandl M. 2007. Drug permeability across a phospholipid vesicle-based barrier: 3. Characterization of drug–membrane interactions and the effect of agitation on the barrier integrity and on the permeability. Eur J Pharm Sci 30:324–332.
21. Avdeef A, Nielsen PE, Tsinman O. 2004. PAMPA—A drug absorption in vitro model 11. Matching the in vivo unstirred water layer thickness by individual-well stirring in microtitre plates. Eur J Pharm Sci 22(5):365–374.
22. Flaten GE, Kottra G, Stensen W, Isaksen G, Karstad R, Svendsen JS, Daniel H, Svenson J. 2011. In vitro characterisation of human peptide transporter hPEPT1 interactions and passive permeation studies of short cationic antimicrobial peptides. J Med Chem 54(14):2422–2432.
23. Hansen T, Ausbacher D, Flaten GE, Havelkova M, Strom MB. 2011. Synthesis of cationic antimicrobial beta(2,2)-amino acid

derivatives with potential for oral administration. J Med Chem 54(3):858–868.
24. Kanzer J, Tho I, Flaten GE, Magerlein M, Holig P, Fricker G, Brandl M. 2010. In-vitro permeability screening of melt extrudate formulations containing poorly water-soluble drug compounds using the phospholipid vesicle-based barrier. J Pharm Pharmacol 62(11):1591–1598.
25. Flaten GE, Awoyemi O, Luthman K, Brandl M, Massing U. 2009. The phospholipid vesicle-based permeability assay: 5. Development towards an automated procedure for high throughput permeability screening. J Lab Automat (14):12–21.
26. Brandl M, Flaten GE, Bauer-Brandl A. 2009. Passive diffusion across membranes. Wiley Encycl Chem Biol 3:541–550.
27. Ingebrigtsen L, Brandl M. 2002. Determination of the size distribution of liposomes by SEC fractionation, and PCS analysis and enzymatic assay of lipid content. AAPS PharmSciTech 3(2):E7.
28. Wertz PW. 2000. Lipids and barrier function of the skin. Acta DermVenereol Suppl (Stockh) 208:7–11.
29. Abraham W, Downing DT. 1989. Preparation of model membranes for skin permeability studies using stratum corneum lipids. J Invest Dermatol 93(6):809–813.
30. Stahl J, Niedorf F, Kietzmann M. 2011. The correlation between epidermal lipid composition and morphologic skin characteristics with percutaneous permeation: An interspecies comparison of substances with different lipophilicity. J Vet Pharmacol Ther 34(5):502–507.
31. Flaten GE, Luthman K, Vasskog T, Brandl M. 2008. Drug permeability across a phospholipid vesicle-based barrier 4. The effect of tensides, co-solvents and pH changes on barrier integrity and on drug permeability. Eur J Pharm Sci 34(2-3): 173–180.
32. Johnson ME, Berk DA, Blankschtein D, Golan DE, Jain RK, Langer RS. 1996. Lateral diffusion of small compounds in human stratum corneum and model lipid bilayer systems. Biophys J 71(5):2656–2668.
33. Lian GP, Chen LJ, Han LJ. 2008. An evaluation of mathematical models for predicting skin permeability. J Pharm Sci 97(1):584–598.
34. Bouwstra JA, Dubbelaar FE, Gooris GS, Weerheim AM, Ponec M. 1999. The role of ceramide composition in the lipid organisation of the skin barrier. Biochim Biophys Acta 1419(2):127–136.
35. Hellwich U, Schubert R. 1995. Concentration-dependent binding of the chiral beta-blocker oxprenolol to isoelectric or negatively charged unilamellar vesicles. Biochem Pharm 49(4):511–517.
36. Kramer SD. 2001. Liposome/water partitioning: Theory, techniques and applications. In Pharmacokinetic optimization in drug research; Testa B, van de Waterbeemd H, Folkers G, Guy R, Eds. Zürich: Wiley-VCH Inc., pp 420–428.
37. Alelyunas YW, Pelosi-Kilby L, Turcotte P, Kary MB, Spreen RC. 2010. A high throughput dried DMSO log *D* lipophilicity measurement based on 96-well shake-flask and atmospheric pressure photoionization mass spectrometry detection. J Chromatogr A 1217(12):1950–1955.
38. Zhu C, Jiang L, Chen T, Hwang K. 2002. A comparative study of artificial membrane permeability assay for high throughput profiling of drug absorption potential. Eur J Med Chem 37(5):399–407.
39. Avdeef A. 2003. Absorption and drug development: Solubility, permeability and charge state. 1st ed. New Jersey: Wiley-Interscience, pp 116–246.
40. Hansen S, Henning A, Naegel A, Heisig M, Wittum G, Neumann D, Kostka KH, Zbytovska J, Lehr CM, Schaefer UF. 2008. In-silico model of skin penetration based on experimentally determined input parameters. Part I: Experimental determination of partition and diffusion coefficients. Eur J Pharm Biopharm 68(2):352–367.
41. Ermondi G, Lorenti M, Caron G. 2004. Contribution of ionization and lipophilicity to drug binding to albumin: A preliminary step toward biodistribution prediction. J Med Chem 47(16):3949–3961.
42. Elliott NT, Yuan F. 2011. A review of three-dimensional in vitro tissue models for drug discovery and transport studies. J Pharm Sci 100(1):59–74.
43. Magnusson BM, Walters KA, Roberts MS. 2001. Veterinary drug delivery: Potential for skin penetration enhancement. Adv Drug Deliv Rev 50(3):205–227.

Supporting Information

New applications of PVPA:

Permeation model mimicking skin barrier

André Engesland[1], Merete Skar[1], Terkel Hansen[2], Nataša Škalko-Basnet[1], Gøril Eide Flaten[1]

[1]Drug Transport and Delivery Research Group, Department of Pharmacy, University of Tromsø, N-9037 Tromsø, Norway

[2]Natural Products and Medicinal Chemistry Research Group, Department of Pharmacy, University of Tromsø, N-9037 Tromsø, Norway

*Correspondence to: Gøril Eide Flaten (Telephone: +47-776-46169; Fax. +47-776-46151; Email: Gøril.Flaten@uit.no)

Table 1: Overview of the drug concentrations in donor compartment, acceptor volumes and wavelengths used for tested drugs.

Drug/marker	Concentration mg/mL	Molarity mM	Acceptor volume µL	Wavelength nm
Flufenamic acid	1.10	3.91	600	287[1)]
Ibuprofen	1.03	5	600	220[1)]
Indomethacin	0.15	0.42	500/600	220[1)]
Salicylic acid	32.20	233	600	300[1)]
Calcein	3.11	5	600	485/520[2)]
FITC-dextran	20	5	600	485/520[2)]

1) UV spectrophotometer wavelength
2) Fluorescence measurements of the hydrophilic markers were performed with excitation and emission wavelengths of 485 and 520 nm, respectively

Table 2: Overview of the different preparation parameters investigated in $PVPA_c$ (E80/Chol PVPA) barriers.

Parameters examined	Range tested
Centrifugation speed for the small liposomes to enter the filter pores *	2000, 2500, 2700, 3000 rpm
Centrifugation speed to settle the large liposomes on top of the filter *	2000, 2500, 2600, 2700, 2800, 2900, 3000, 4000, 5000 rpm
Centrifugation time to settle the large liposomes on top of the filter	30, 40, 50, 60 min
Heating temperature	23, 30, 40, 50, 65, 75, 85 °C
Heating time	30, 60, 75, 90, 105, 120 min
Number of freeze-thaw cycles	1, 2 cycles

*With the centrifugation setup used, 2000 rpm equals to 610 *g*, 2500 rpm = 950 *g*, 2600 rpm = 1030 *g*, 2700 rpm = 1110 *g*, 2800 rpm = 1190 *g*, 2900 rpm = 1280 *g*, 3000 rpm = 1370 *g*, respectively.

Table 3: Overview of the different preparation parameters investigated in the $PVPA_s$ (E-80/Ceramide/PA/Chol/Cholsul PVPA) barriers.

Parameters examined	Range tested
Centrifugation time to settle the large liposomes on top of the filter*	2000 and 2600 rpm
Evaporation time in incubator	45 min + 80 min 40 min + 60 min [1)]
Evaporation temperature	50 °C
Heating time during freeze-thaw cycling	60, 90, 95 min[2)], 120, 135 min[3)]
Heating temperature during freeze-thaw cycling	30, 40, 60 °C

*With the centrifugation setup used, 2000 rpm equals to 610 *g*, 2600 rpm = 1030 *g*

3) First evaporation: 20 min with closed container, then open for 20 min.
Second evaporation: 20 min with closed container, then open for 40 min.

4) After 90 min at 30 °C, the temperature was increased to 50 °C and left for 5 more min to relieve all moisture from the inserts.

5) After 120 min at 30 °C, the temperature was increased to 50 °C and left for 15 more min to relieve all moisture from the inserts.

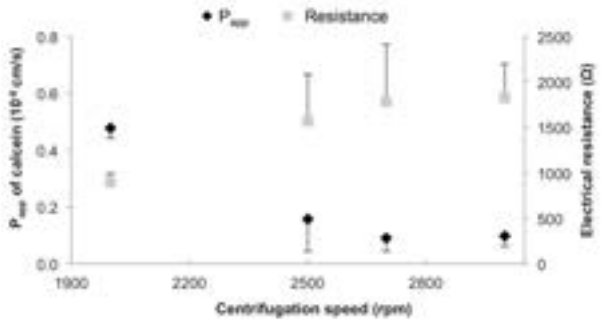


Figure 1. The effect of different centrifugation speed after addition of small liposomes on the calcein permeability values (P_{app}) and electrical resistance. Error bars represent standard deviations.

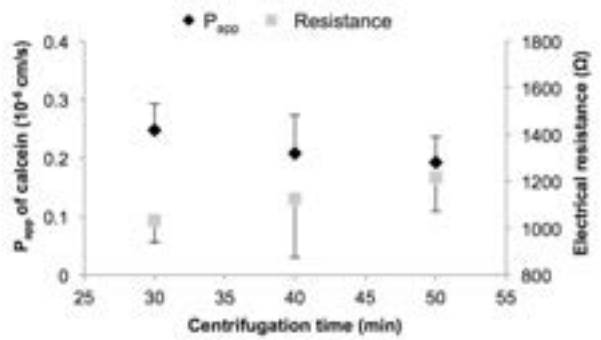


Figure 2. The effect of centrifugation time on the permeability values (P_{app}) of calcein and electrical resistance for liposomes staying on top of the filter. Error bars represent standard eviations.

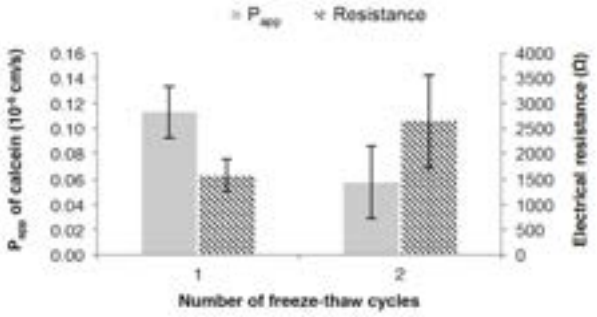
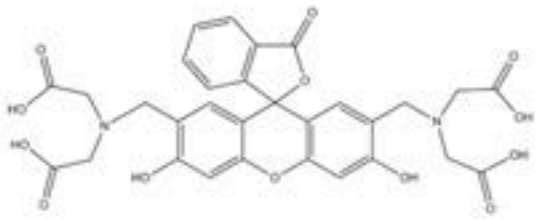
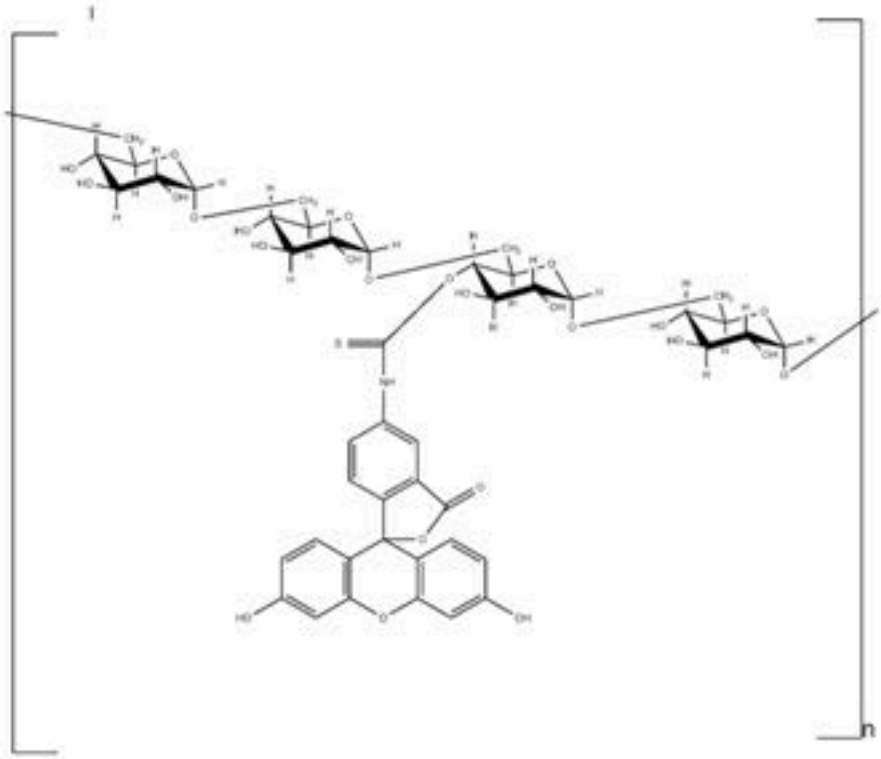


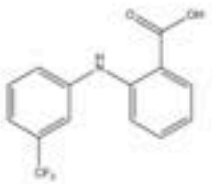
Figure 3. The effect of the number of freeze-thaw cycles on the permeability values (P_{app}) of calcein. Error bars show standard deviations.



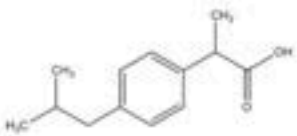
Calcein



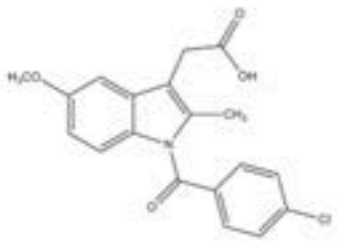
FITC-dextran, FD-4, (Mw. 4000). FITC attached randomly to hydroxyl group.



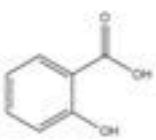
Flufenamic acid



Ibuprofen



Indomethacin



Salicylic acid

Figure 4. The molecular structures for the drug compounds and markers used in the study.