PVPA and EpiSkin® in Assessment of Drug Therapies Destined for Skin Administration

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ABSTRACT

Cost-effective and efficient methods for permeability screening are crucial during early development of drugs, drug formulations and cosmeceuticals. Alternatives to animal experiments are impelled for both economical and ethical reasons. The aim of this study was to determine the ability of the phospholipid vesicle-based permeation assays (PVPA) to assess the effect of different formulations on drug permeability and thus establish its utility in formulation development. Three model drugs were tested in solutions and as liposomal formulations. The permeability results for the PVPA models were compared with the results for the reconstructed human skin model, EpiSkin®. The drugs were ranked based on their estimated penetration potentials, and the results were in accordance with what was expected considering the physicochemical properties of the drugs. $PVPA_S$ was able to distinguish between drug solutions and liposomal formulations; however, EpiSkin® detected only small differences between the drugs in solution and in formulations. In contrast with EpiSkin®, which is limited by a three-day testing window, PVPA barriers can be stored frozen for up to two weeks or even up to 16 months, depending on their compositions. The PVPA models are thus more cost-effective and efficient than the EpiSkin® model for permeability screening during early drug development.

Keywords

Permeability; Reconstructed human epidermis; PVPA; EpiSkin®; Skin; Liposomes; Lipids; *In vitro*

List of Abbreviations

ACV, acyclovir; ACV-PC, liposomes of PC (S 100) and acyclovir, ACV-PC/PG; liposomes of PC (S 100), EPG-Na and acyclovir; CF, caffeine; CAM, chloramphenicol; CAM-PC, liposomes of PC (S 100) and CAM; PVPA, phospholipid vesicle-based permeation assay; $PVPA_o$, (E-80); $PVPA_c$, (E-80 and cholesterol); $PVPA_s$, (E-80, ceramide, cholesterol, cholesteryl sulfate, and palmitic acid); and PBS, Dulbecco's phosphate-buffered saline

INTRODUCTION

Topical skin drug delivery is attractive, non-invasive, and painless and usually involves less adverse reactions compared to systemic delivery. In addition, the skin is an easily accessible administration site.[1] The choice of formulation can influence whether a drug will exert local or systemic transdermal effects. In addition to affecting the delivery of a drug, formulations can also affect the epidermis by providing lubrication, hydration, occlusion, and barrier protection and even causing it to dry out.[2] Liposomes have been extensively studied for decades as carriers for dermal drug delivery and as active carriers in cosmeceuticals. They have been shown to fuse with skin lipids and sometimes dehydrate the skin after topical administration.[3,4]

During topical formulation development, different characteristics of liposomes as drug carriers need to be controlled and different strategies must be applied to either enhance the penetration of compounds through skin or to promote their deposition into the skin for local drug effects.[3,5] Toxicity and pharmacokinetic studies are also important for the optimization of topical formulations. Therefore, during the early phase of formulation development, reliable and cost-effective screening methods are

crucial. To optimize the composition of drug carriers/vehicles and drug-in-liposome characteristics, models for permeability screening will help to identify the best candidates for further development. The *stratum corneum* is the main barrier of the skin,[6] and the lipids found in corneocytes and their arrangements are important for the barrier function.[7] Several *in vitro* models have been proposed that mimic the *stratum corneum*, for example, silicon model membranes, although they lack crucial lipids found in the *stratum corneum.*[8,9] Skin-PAMPA models, on the other hand, contain crucial skin lipids but lack cell-like structures.[10] Animal skin in Franz diffusion cells is often used as a model for estimating skin penetration using either full-thickness skin samples or isolated *stratum corneum.* However, difficulties during skin sample preparation and biological variations can generate complications during data interpretation, and a substantial number of replicates is needed to generate reliable data.[11] Although animal models can offer some useful data when assessing topical formulations, their costs, as well as new regulations and a progressing consensus between government/regulatory, research, teaching, industry and animal welfare organizations to promote the 3 R's (replace, reduce and refine) is limiting their utility. Recently, the phospholipid vesicle-based permeation assay ($PVPA_S$), which includes a barrier containing the main lipid classes found in the skin, was introduced as a simple and reproducible model for predicting skin permeability.[12] The PVPA model was originally developed as a robust, high-to-medium throughput permeability screening model for estimating intestinal permeability [13,14] and was later used for both lead compound selection[15] and formulation optimization.[16,17] PVPA barriers consist of liposomes on a filter support and therefore mimic biological cells and membranes. The fact that different lipids can be incorporated into PVPA barriers to closely resemble various biological barriers makes this model promising for the assessment of

drug administration at various sites. For example, our PVPA models mimicking the *stratum corneum* have also been used as *in vitro* models in the development of (trans)dermal formulations.[2] In the present study, PVPA models were used for estimating the *stratum corneum* penetration of model drugs and their liposomal formulations.

As an alternative, reconstructed human skin models, such as EpiSkin®, Skinethic® and EpiDerm®, have been proposed for permeability studies as well as for phototoxicity, irritancy and corrosiveness testing.[18,19] The barrier in the EpiSkin® model consists of a reconstructed epidermis on a collagen support. EpiSkin® kits are prepared in Costar® well plates.[11,20] The protocol is rather complex, and the inclusion of *stratum corneum* cells from human donors makes this model expensive. EpiSkin® is optimized for safety testing,[21] and several applications have been described and recommended by the Organization for Economic Co-operation and Development (OECD).[22] Moreover, EpiSkin® can be used for the testing of irritants and skin metabolism as well as skin absorption.[20,21] It has also been used to test various topical formulations and vehicle effects.[11,23,24] Therefore, due to its resemblance to human epidermis and its ability to be used directly in Costar® well plates, the EpiSkin® model was chosen in this study for a comparison with our *in vitro* PVPA *stratum corneum* models.

The aim of this study was to demonstrate the abilities of PVPA models to assess the effects of different drug formulations to establish their utility in drug development. This was done by testing three different model drugs in solutions or as liposomal formulations and comparing the permeability results from the $PVPA_c$ and $PVPA_s$ models with those from the much more complex and expensive reconstructed human

skin model, EpiSkin®. Acyclovir (ACV), chloramphenicol (CAM) and caffeine (CF) were chosen as model drugs to cover broad ranges of lipophilicity and molecular size.

MATERIAL AND METHODS

Material

ACV, CF, calcein, ceramides from the bovine spinal cord, CAM, cholesterol, cholesteryl sulfate, ethanol, methanol, palmitic acid, Dulbecco's phosphate-buffered saline (PBS) and sodium hydroxide (NaOH) were obtained from Sigma–Aldrich Company (St. Louis, MO, USA). Acetic acid (glacial) and chloroform were purchased from Merck (Darmstadt, Germany). Hydrochloric acid (37 %, HCl; w/w) was purchased from VWR International (Leuven, Belgium). Egg phospholipid Lipoid E-80, soy phosphatidylcholine Lipoid S 100 (PC) and egg phosphatidylglycerol sodium Lipoid EPG-Na (PG) were obtained from Lipoid (Ludwigshafen, Germany). Mixed cellulose ester filters (0.65-µm pore size) and isopore filters (0.8- and 1.2-µm pore sizes) were purchased from Millipore (Billerica, Massachusetts). Filter inserts (Transwell, d = 6.5 mm) and plates were purchased from Corning Inc. (New York, USA). Nucleopore filters (0.4-µm pore size) were obtained from Whatman (part of GE Healthcare, Oslo, Norway). EpiSkin® (aged 13 days, large/1.07 cm^2 surface area) was purchased from SkinEthic Laboratories (Lyon, France).

Methods

Preparation of Liposomes Containing ACV or CAM

Liposomes containing ACV or CAM were prepared by the film hydration method. Three different formulations were prepared, two of which contained PC as the only lipid and one of which contained a mixture of PC and PG. PC (200 mg) was dissolved

together with either CAM or ACV (20 mg) in chloroform. PC (180 mg), PG (20 mg) and ACV (20 mg) were dissolved in chloroform and MeOH (1:10, v/v). Organic solvents were removed under vacuum, and lipid films were hydrated with distilled water (10 mL) to form the liposomal dispersions CAM-PC, ACV-PC and ACV-PC/PG, respectively. Liposomes were stored at 2-8 °C for at least 24 hours before further use. They were extruded three times through 0.8-μm filters by nitrogen-driven extrusion (Lauda Dr. R. Wobser Gmbh, Königshofen, Germany).

Size Distribution and Zeta Potential Measurements

The size distribution and polydispersity indices (PIs) of liposomes were determined by photon correlation spectroscopy using Particle Sizer 370 (PSS Nicomp Particle Sizing Systems, Santa Barbara, CA). The sample preparation and measuring conditions were the same as previously described.[17] The measurements were performed in three cycles of 10 min each. The zeta potential measurements of the liposomal dispersions ACV-PC, ACV-PC/PG and CAM-PC were performed with a Malvern Zetasizer Nano Z (Malvern, Worcestershire, UK). The liposome dispersions were diluted 1:40 in MilliQ water prior to the measurements to achieve proper count rates, and the results reported were obtained from at least three measurements.[17]

Entrapment Efficiency Determination

To separate free drugs from liposomally entrapped drugs, extruded liposomes were centrifuged in a Beckman model L8-70M ultracentrifuge with an SW 60 Ti rotor (Beckman Instruments, Palo Alto, CA, USA). The samples were centrifuged at 216000 *g* for 60 min at 10 °C. Pellets were resuspended in distilled water and further

diluted in methanol to dissolve the lipids before the concentration of CAM or ACV was quantified by HPLC as described below. The supernatants were measured by PCS to verify that they contained no vesicles and then diluted in methanol before quantification by HPLC. The experiments were performed in triplicate, and the entrapment efficiency was expressed as the drug/lipid ratio.

Quantifications of ACV, CAM, CF and Calcein

The concentrations of ACV and CAM were determined by high-performance liquid chromatography (HPLC). A reversed-phase column (Waters XTerra® C18; 5 µm; 3.9 × 150 mm; Waters, Milford, MA) installed in a Waters e2795 separations module equipped with a UV 2489 detector was used. Wavelengths of 258 nm for ACV and 280 nm for CAM, respectively, were used.[17,25] mobile phase for ACV detection was MeOH/MilliQ water 50:50, pH 2.5 (HCl), and that for CAM was MeOH/MilliQ water 45:55, pH 2.5 (glacial acetic acid).[17,25] The run time was 7 min, and the sample injection volume was 10 µL. The column temperature during the quantification of ACV was 25 °C, and it was 30 °C for CAM.

The flow rates were 0.2 mL/min for the samples from the PVPA experiments and 0.4 mL/min for those from the EpiSkin® experiments. Different conditions were applied for the samples from the PVPA and EpiSkin® models to obtain satisfactory separation while concurrently maintaining acceptable column pressure.

The concentrations of CF in all experiments as well as those of CAM in the $PVPA_c$ and $PVPA_s$ experiments with the CAM solution were determined by measuring UV absorbance (Spectramax 190; Molecular Device Corporation, Sunnyvale, CA) as

previously described, and wavelengths of 270 nm for CF and 285 nm for CAM were used.[13]

Calcein was quantified by fluorescence according to a previously described procedure using a Polarstar fluorimeter (Fluostar; BMG Technologies, Offenburg, Germany), with excitation and emission wavelengths of 485 and 520 nm, respectively.[13]

Preparation of the PVPA Barriers

The PVPA barriers were prepared as previously described.[12] Briefly, the barriers were made of liposomes containing the following:

composition 1: E-80 (77 %, w/w) and cholesterol (23 %, w/w); and

composition 2: E-80 (50 %, w/w), ceramides (27.5 %, w/w), cholesterol (12.5 %, w/w), cholesteryl sulfate (2.5 %, w/w), and palmitic acid (7.5 %, w/w).

Liposomes were prepared by the film hydration method using phosphate buffer (pH 7.4).[13] The liposomes in composition 1 were extruded through filters with pore sizes of 1200 and 400 nm to produce liposome dispersions of two different size distributions, while the liposomes in composition 2 were extruded through filters with a pore size of 1200 nm only. The small liposomes (400-nm filter extrusion) were forced into the filter pores by repeated centrifugation (100 μL of dispersion was added, and the inserts were centrifuged for 15 min x 2) followed by heating (50 °C for 45 min), whereas the large liposomes (1200-nm filter extrusion) from composition 1 and composition 2 were deposited onto the filter support in two different manners. The barriers for $PVPA_c$ were prepared by the addition of liposomes from composition 1 (100 μL) and centrifugation for 60 min to settle the liposomes on top of the filter, followed by inverted centrifugation to remove the supernatant. The barriers for

$PVPA_s$ were prepared by adding liposomes from composition 2 (50 µL x 2), and the liposomes were settled on top of the filter support by evaporation of the solvent at 50 °C for 40 min (20 min in a closed and 20 min in an open container) for the first addition and 50 °C for 60 min (20 min in a closed and 40 min in an open container) for the second addition. The PVPA barriers were immediately frozen at -70 °C for a minimum of 60 min before further use.[12]

Permeation Experiments Using the PVPA Models

Permeation experiments were executed according to previously described procedures.[12,17] Briefly, the inserts containing the PVPA barriers were loaded with donor solutions (100 µL) containing the drugs CAM (10 mM), ACV (2.22 mM), CF (10 mM), the hydrophilic marker calcein or the liposomal dispersions ACV-PC, CAM-PC and ACV-PC/PG, respectively. The inserts were placed into wells containing phosphate buffer (pH 7.4)[13] as an acceptor solution and moved to new wells at specific time intervals for a total of 5 hours to maintain sink conditions. Immediately after the permeation experiments, the electrical resistance across the barriers was measured followed by the quantification of the drugs in both the donor and acceptor compartments. The experiments were performed at least in quintuplicate.

Permeation Experiments Using the EpiSkin® Model

The EpiSkin® reconstructed human epidermis was immediately incubated in EpiSkin® medium (37 °C; 5 % CO_2; 24 hours) upon arrival. Prior to the permeation experiments, the EpiSkin® inserts were washed with sterile PBS buffer to remove traces of EpiSkin® medium, and placed in new Costar® (12 wells) plates containing 2

mL of sterile PBS buffer as the acceptor phase. Drug solutions containing CAM (10 mM), ACV (2.22 mM) or CF (10 mM), or the liposomal dispersions (ACV-PC and CAM-PC) were added to the donor compartment (200 µL). Permeability experiments using the EpiSkin® are often performed for 4, 6 and/or 24 hours.[11,20,26] A timeframe of 5 hours was chosen to implement similar conditions as the PVPA models. Aliquots of 500 µL were collected from the acceptor compartment every hour and replaced with 500 µL of PBS buffer. The drug concentrations in both the acceptor and donor compartments were quantified by HPLC, as described above. The experiments were performed at least in triplicate.

Mass Balance Calculations after experiments with the $PVPA_s$ and EpiSkin® models

After the quantifications of the amounts of drugs in the donor and acceptor compartments, the total amounts in the acceptor compartments were summarized, and the amount left in the barrier at the end of the permeability experiment was calculated as follows:

$$B = D_{start} - (D_{end} + A_{end})$$

B: amount in barrier

D_{start}: amount in donor compartment at start of experiment

D_{end}: amount in donor compartment at end of experiment

A_{end}: amount in acceptor compartment at end of experiment

Storage Stabilities of the $PVPA_c$ and $PVPA_s$ Barriers

To confirm the stabilities of the $PVPA_c$ and $PVPA_s$ barriers during storage at -70 °C,

they were stored for various time intervals before the permeability experiments were performed. To evaluate stability, the permeabilities of the hydrophilic marker calcein and the drug caffeine were assessed, and electrical resistance across the barriers was measured.

Statistical Methods

SPSS Statistics (version 21/22, IBM software, IBM, New York, NY) was used for statistical evaluation. Student's *t*-test was used for the comparison of two means. ANOVA and Tukey's post hoc tests were used to compare variance between more than two means. A $p \leq 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

The characteristics of liposomes used as carrier systems for topical drug delivery as well as the choice of vehicle for administering liposomes are known to influence the physicochemical characteristics of the system and the penetration potential of the active ingredient.[3,27] Liposomes can effectively entrap and deliver drugs to the upper part of the skin, the *stratum corneum*, for sustained localized effects.[3] Liposomes modified with edge activators or surfactants can produce transfersomes, which can greatly increase the penetration of drugs through viable skin.[28] Liposomes with strong negative surface charges have been reported to exhibit increased delivery to the skin as opposed to neutral or positively charged liposomes.[29] The choice of liposomal composition in this study was based on our previous study, in which liposomes were used to improve the permeability of acyclovir, as assessed by the original $PVPA_o$.[17]

Characterization of Liposomal Formulations

We aimed to prepare simple liposome formulations of large multilamellar vesicles (MLVs) containing the model drugs with a main size distribution in the range of 500-800 nm.

The results of the liposomal characterization are presented in Table 1.

Table 1.

All liposomal formulations exhibited a bimodal size distribution, i.e., two clear vesicle populations could be detected. The mean size of the main fraction, which contained more than 90 % of the liposomes, was approximately 650 nm, and that of the smaller fraction, containing less than 10 % of the liposomes, was approximately 150 nm. The neutral liposomes tended to be slightly larger than the negatively charged liposomes composed of PC and PG. The liposome formulations had PI values of between 0.2 and 0.35 and were expected to be MLVs.

The zeta potential of the PC liposomes was, as expected, closer to neutral compared to the negatively charged PC/PG liposomes, and these liposomes were thus more susceptible to the formation of larger aggregates.[30] The negatively charged PC/PG liposomes are expected to exhibit enhanced penetration compared to the neutral PC liposomes.[29]

The entrapment efficiency of the CAM-PC formulation was approximately twice those of the ACV-PC and ACV-PC/PG formulations (Table 1), which could influence its delivery to the skin. CAM had a log *P* value of 1.14, thus favoring lipophilic liposomes more than ACV, which had a log *P* value of -1.56; hence, the higher

entrapment of CAM was expected. Our results are in agreement with entrapment efficiencies of ACV and CAM reported by previous studies.[17,31]

Permeation Experiments Using the $PVPA_c$, $PVPA_s$ and EpiSkin® Models

During the early phase of drug development for topical delivery, a cost-effective permeability screening method that produces reliable and reproducible permeability data is crucial. When comparing drug carriers or formulations, reproducible and reliable permeability results as well as the ability of a model to detect small changes in penetration potentials are of great importance. To establish the utility of the PVPA models in drug development and formulation optimization, three different drugs in solution and their corresponding liposomal formulations were tested. The permeability results for the $PVPA_c$ and $PVPA_s$ models were compared with the results obtained with the EpiSkin® model.

Figure 1.

The permeability results for the $PVPA_c$, $PVPA_s$ and EpiSkin® models are presented in Figure 1A, 1B and 1C, respectively. The drugs were ranked based on their permeability values for both the $PVPA_c$ and $PVPA_s$ models in increasing order as follows: ACV, CAM and CF. Both ACV and CAM exhibited low permeabilities, but that of CAM was higher than that of ACV. CF demonstrated the overall highest permeability value, which was more than five times higher than those of ACV and CAM for $PVPA_s$. A previous report of permeability results obtained with $PVPA_o$, in which it mimicked a general biological barrier, described similar drug rankings as those obtained for $PVPA_c$ and $PVPA_s$ in the current study.[13,17]

The drugs were ranked based on their permeability values for the EpiSkin® model in increasing order as follows: CAM, ACV and CF (Figure 1C). Both ACV and CAM exhibited low permeabilities, with CAM showing a slightly lower permeability than ACV. The permeability of CF observed with the EpiSkin® model was the highest among the tested drugs, which is in agreement with previous reports in the literature.[11,32]

The lipophilicity and molecular mass of a drug are considered to be the most important parameters when predicting its potential for dermal penetration. The log *P* value should be in the range of 1-3, and the molecular mass should be below 500 Da for a drug to be able to pass through the skin.[33] ACV has the lowest log *P* value at -1.56; thus, it is more hydrophilic than CAM and CF, which have log *P* values of 1.14 and -0.07, respectively.[33] CF is the smallest molecule (194.2 Da) compared with ACV (225.2 Da) and CAM (323.1 Da). Small lipophilic molecules are expected to pass through the *stratum corneum* barrier more effectively than larger hydrophilic molecules.[34] CF was thus expected to exhibit the highest permeability values because it had the smallest mass.[33] However, the more lipophilic characteristic of CAM compared to ACV was expected to contribute to its higher permeability. This has also been observed in previous experiments using PVPA models.[13,17]

The $PVPA_s$ model more closely resembles skin than the $PVPA_c$ model; therefore, it was chosen together with EpiSkin® for the evaluation of the liposome formulations in this study. Previous studies using $PVPA_c$ and $PVPA_s$ have also demonstrated that the $PVPA_s$ model is better than the $PVPA_c$ model in distinguishing between the permeabilities of drugs in different liposome formulations.[2]

Three different liposome formulations were tested using the $PVPA_s$ model. The permeability results for ACV-PC and ACV-PC/PG indicated significant ($p<0.01$) increases in drug permeation for both formulations compared to ACV in solution. In addition, ACV-PC/PG exhibited a higher permeability of ACV compared with ACV-PC. CAM-PC, on the other hand, did not result in any significant changes in the permeability of CAM compared with the CAM solution. Previous *in vitro* experiments assessing ACV in liposomes using the $PVPA_o$ model mimicking a general absorption barrier have also indicated the enhanced permeation of ACV.[17]

Two different liposome formulations were tested in the EpiSkin® model, and the permeability results are presented in Figure 1C. Due to experimental error, the permeability results for ACV-PC/PG are unfortunately missing. The results demonstrated only small changes in permeability when comparing the drugs in solution with those in liposomes. A significant ($p<0.05$) decrease in the permeability of ACV in liposomes (ACV-PC) compared to that in solution was observed. It is evident that EpiSkin® functions better as a skin model, whereas $PVPA_s$ can be considered as more of a *stratum corneum* model. In skin models, the dermis contributes to the barrier properties, especially for lipophilic molecules.[23] The deposition of drugs in the different layers of EpiSkin® can be determined by separating the epidermis from the collagen and further extracting and quantifying the amounts in the different layers.[23] This process is time-consuming, and this model has limited use as a screening model. In a previous study, EpiSkin® has been used to assess the depositions of vitamin C and E from various microemulsions into the barrier as well as their permeation through the barrier,[23] revealing that vitamins in microemulsions show enhanced percutaneous absorption compared to those in

aqueous solutions.[23] Simple formulations of benzoic acid, caffeine and mannitol in glycerol have also been studied with EpiSkin®.[35] The penetration of the drugs in the model was compared with penetration in the human skin as well as another reconstructed skin model, the EpiDerm®. The reconstructed models gave similar results, but showed higher penetration than the human skin. Different cosmetic preparations containing caffeine and α-tocopherol have also been studied, however less pronounced vehicle effects were observed in the reconstructed skin models (EpiSkin® and EpiDerm®) as compared to human skin *ex vivo*. [24] The authors concluded that effects of cosmetic vehicles on bioavailability were less predictable using the reconstructed human skin models as compared to human skin. The penetration of drugs using this model was compared with their penetration into human skin as well as an additional reconstructed skin model, EpiDerm®. The reconstructed models gave similar results but showed higher penetration compared with human skin. Different cosmetic preparations containing caffeine and α-tocopherol have also been studied; however, less pronounced vehicle effects have been observed in the reconstructed skin models (EpiSkin® and EpiDerm®) compared to human skin *ex vivo*.[24] The authors concluded that the effects of cosmetic vehicles on bioavailability are less predictable using reconstructed human skin models compared to human skin.[24]

It is further known that occlusion and hydration can produce unexpected effects during permeability experiments.[3] Non-occlusive experiments can be performed with EpiSkin® to assess permeability, which further complicates and prolongs experimental setup. In our study, the EpiSkin® barriers were visually hydrated after the permeability experiments. EpiSkin® is a multipurpose skin model that can be used for phototoxicity, irritancy and corrosivity as well as permeability experiments.[19,21] Its

use as a permeability model is associated with many of the same limitations as other simpler and cost-effective models.[18,19,21] Most *in vitro* models have some degree of decreased barrier function compared to the human skin barrier.[26,32] The *stratum corneum* intercellular lipid compositions and organizations of human skin models also differ to some extent from those of the human *stratum corneum ex vivo*, which contributes to the less pronounced barrier properties together with the increased hydration of the outermost *stratum corneum* layers of the models.[24]

To further investigate the faith of the drugs as well as their effects following their inclusion into liposomes, the amounts of drugs present in the barriers were calculated based on the amounts in the donor and acceptor compartments after the experiments were completed. These calculations were performed for the $PVPA_s$ and EpiSkin® models, which were used to test the liposome formulations, and the resulting mass balances are presented in Figure 2A and 2B.

Figure 2.

The results indicated evident effects of the formulations on drug distribution to the $PVPA_s$ barrier. A greater amount of ACV was deposited into the barrier as well as the acceptor compartments for ACV-PC and ACV-PC/PG compared to ACV in solution. For the more lipophilic CAM, the opposite results were observed; more of the CAM in solution accumulated in the barrier after the experiments compared with that in liposomes (CAM-PC). For EpiSkin®, more of the drugs were found in the barrier after the permeability experiments with the drug solutions compared to the liposome formulations (Figure 2B). The CAM in solution and CAM-PC exhibited the lowest

permeabilities of the tested drugs. However, the high level of CAM in the barriers compared to the respective acceptor compartments indicated that this finding could be partially due to barrier retention.

PVPA models have been shown to be able to serve as efficient screening tools in the early development of drug candidates and formulations by mimicking different biological barriers. Table 2 summarizes the different PVPA models and their areas of application in pharmaceutical development.

Stability of the PVPA Barriers

The stability of the PVPA barriers was assessed as an important factor in determining cost-effectiveness with regard to screening during drug development. If barriers are stable over a long period of time, larger batches can be produced, which can further reduce costs and the need for rigid planning during permeability screening.

Figure 3.

The results from the stability experiments with $PVPA_c$ showed that the permeability of calcein was not significantly different from that of the control (1 day of storage), even after 16 months of storage at -70 °C (Figure 3). However, a significant ($p<0.05$) decrease in the permeability of calcein was evident after 29 months of storage. Moreover, the resistance values (results not shown) revealed elevations over time, which indicated the increasing tightness of the barriers. In an earlier study, $PVPA_o$ barriers were stored for up to four weeks at three different temperatures (-80 °C, 2-8 °C and 20 °C), revealing that -80 °C is the optimal storage temperature and that these

barriers can be stored for up to two weeks without significant changes occurring in the permeability of calcein or electrical resistance.[14] $PVPA_c$ with cholesterol incorporated into the liposomes thus exhibited a drastic increase in storage stability compared to $PVPA_o$, which contained only E-80 in the barriers. Cholesterol is known to have a stabilizing effect on liposomes,[27,36] and its incorporation into liposomes for $PVPA_c$ barriers resulted in consistent and reproducible permeability results for calcein following storage for up to 16 months.

For $PVPA_s$, with a more complex lipid composition, the permeability results of calcein did not significantly differ from those of the control (1 day) after up to two weeks of storage at -70 °C (Figure 3). However, a relatively large decrease in the permeability of calcein as well as an increase in standard deviations after one month of storage at -70 °C was clear. After prolonged storage, the time needed for the $PVPA_s$ barriers to thaw/dry was longer, indicating the increased hydration of the liposomes in the barriers. The results of the stability experiments assessing $PVPA_s$ using CF indicated a similar permeability compared with the control following up to two months of storage (Figure 3). CF is a small lipophilic molecule with increased barrier permeability in contrast with calcein. Small lipophilic molecules are not expected to be as affected by the tightness of the barrier compared to more hydrophilic molecules, and their permeabilities are thus not expected to be greatly influenced following prolonged storage. The results from the stability experiments of $PVPA_s$ showed that these barriers can be stored for up to two weeks. After two weeks of storage, variations in permeability can occur, at least for larger hydrophilic molecules such as calcein. The results of the assessment of $PVPA_s$ using calcein are in accordance with previously reported stability results for $PVPA_o$, which also indicated

a limit of two weeks of storage.[13] The PVPA models can thus be prepared in larger batches and used any time during the recommended period of storage.

In contrast, experiments using the EpiSkin® model need to be thoroughly planned and follow a strict time schedule after ordering. The EpiSkin® barriers require special attention and more labor-intensive follow up before and during experiments due to the presence of cells, and this model is limited to a testing period after arrival of up to three days.[21] If differences between formulations are observed and need to be further verified, the ordering and preparation for new experiments will be both expensive and time consuming, whereas the PVPA models can be used immediately due to their longer storage stabilities compared to the EpiSkin® model. The $PVPA_c$ model can be produced in large batches at a low cost compared to the EpiSkin® model and stored for months before performing screening experiments. During research and development, $PVPA_c$ barriers can be stored in a freezer and be ready to use after 2 hours of thawing, on any day during the 16 months of storage time. When a more complex lipid composition closer to the *stratum corneum* barrier is needed, $PVPA_s$ barriers can be prepared and used at any time for up to two weeks of storage, and they are also much lower in cost than EpiSkin®.

Table 2 summarizes the different PVPA models, their shelf lives and their areas of application in pharmaceutical development. In summary, we have shown that the PVPA models are able to mimic different biological barriers and serve as efficient tools for permeability screening purposes in early drug development. The $PVPA_c$ and $PVPA_s$ models that were used in this study mimicked the *stratum corneum* and were shown to be time-efficient and cost-effective screening tools for the early selection of lead compounds, drug candidates and the evaluation of drug formulations or cosmeceuticals intended for dermal application.

Table 2.

The EpiSkin® model is more skin-like and has been optimized for safety testing but has limited potential for permeability experiments. The cost of EpiSkin® and the limited time window for performing experiments further restricts its applicability in early permeability screening experiments. This model is more suitable for the efficient testing of the irritancy or corrosion of chemicals or for specific studies of skin metabolism.

CONCLUSIONS

The *in vitro stratum corneum*-mimicking $PVPA_s$ model was able to detect permeability differences when the drugs were present in formulations, whereas only small alterations in permeability between the drug formulations and drugs in solution were detecting using EpiSkin®. In contrast with EpiSkin®, which is known to have a three-day testing window, $PVPA_c$ can be stored for 16 months, and $PVPA_s$ can be stored for two weeks. Hence, the PVPA models were found to be superior to EpiSkin® according to their ease of use, efficiencies, cost-effectiveness and abilities to identify the effects of formulations on permeability in early drug development.

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REFERENCES

1. Schafer-Korting M, Mehnert W, Korting HC 2007. Lipid nanoparticles for improved topical application of drugs for skin diseases. Adv Drug Deliv Rev 59(6):427-443.
2. Palac Z, Engesland A, Flaten GE, Skalko-Basnet N, Filipovic-Grcic J, Vanic Z 2014. Liposomes for (trans)dermal drug delivery: the skin-PVPA as a novel in vitro stratum corneum model in formulation development. J Liposome Res 10.3109/08982104.2014.899368.
3. Barry BW 2001. Novel mechanisms and devices to enable successful transdermal drug delivery. Eur J Pharm Sci 14(2):101-114.
4. El Maghraby GM, Barry BW, Williams AC 2008. Liposomes and skin: from drug delivery to model membranes. Eur J Pharm Sci 34(4-5):203-222.
5. El Maghraby GM, Williams AC, Barry BW 2006. Can drug-bearing liposomes penetrate intact skin? J Pharm Pharmacol 58(4):415-429.
6. 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.
7. Bouwstra JA, Ponec M 2006. The skin barrier in healthy and diseased state. Biochim Biophys Acta 1758(12):2080-2095.
8. 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.
9. 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.
10. 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.
11. Gregoire S, Patouillet C, Noe C, Fossa I, Benech Kieffer F, Ribaud C 2008. Improvement of the experimental setup for skin absorption screening studies with reconstructed skin EPISKIN. Skin Pharmacol Physiol 21(2):89-97.
12. Engesland A, Skar M, Hansen T, Skalko-Basnet N, Flaten GE 2013. New applications of phospholipid vesicle-based permeation assay: permeation model mimicking skin barrier. J Pharm Sci 102(5):1588-1600.
13. 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.
14. 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.
15. 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.
16. 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.
17. Naderkhani E, Erber A, Skalko-Basnet N, Flaten GE 2014. Improved permeability of acyclovir: optimization of mucoadhesive liposomes using the phospholipid vesicle-based permeation assay. J Pharm Sci 103(2):661-668.

18. Godin B, Touitou E 2007. Transdermal skin delivery: predictions for humans from in vivo, ex vivo and animal models. Adv Drug Deliv Rev 59(11):1152-1161.
19. Netzlaff F, Lehr CM, Wertz PW, Schaefer UF 2005. The human epidermis models EpiSkin, SkinEthic and EpiDerm: an evaluation of morphology and their suitability for testing phototoxicity, irritancy, corrosivity, and substance transport. Eur J Pharm Biopharm 60(2):167-178.
20. Schafer-Korting M, Bock U, Diembeck W, Dusing HJ, Gamer A, Haltner-Ukomadu E, Hoffmann C, Kaca M, Kamp H, Kersen S, Kietzmann M, Korting HC, Krachter HU, Lehr CM, Liebsch M, Mehling A, Muller-Goymann C, Netzlaff F, Niedorf F, Rubbelke MK, Schafer U, Schmidt E, Schreiber S, Spielmann H, Vuia A, Weimer M 2008. The use of reconstructed human epidermis for skin absorption testing: Results of the validation study. Altern Lab Anim 36(2):161-187.
21. Mathes SH, Ruffner H, Graf-Hausner U 2014. The use of skin models in drug development. Adv Drug Deliv Rev 69-70:81-102.
22. Alepee N, Grandidier MH, Cotovio J, Organisation for Economic C-o, Development 2014. Sub-categorisation of skin corrosive chemicals by the EpiSkin reconstructed human epidermis skin corrosion test method according to UN GHS: revision of OECD Test Guideline 431. Toxicol In Vitro 28(2):131-145.
23. Rozman B, Gasperlin M, Tinois-Tessoneaud E, Pirot F, Falson F 2009. Simultaneous absorption of vitamins C and E from topical microemulsions using reconstructed human epidermis as a skin model. Eur J Pharm Biopharm 72(1):69-75.
24. Dreher F, Fouchard F, Patouillet C, Andrian M, Simonnet JT, Benech-Kieffer F 2002. Comparison of cutaneous bioavailability of cosmetic preparations containing caffeine or alpha-tocopherol applied on human skin models or human skin ex vivo at finite doses. Skin Pharmacol Appl Skin Physiol 15 Suppl 1:40-58.
25. United States Pharmacopeial Convention Inc 2014. United States Pharmacopeia and National Formulary. USP 37-NF 32, online version, USP Monographs: Chloramphenicol, Rockville, MD: p 2277.
26. Schafer-Korting M, Mahmoud A, Lombardi Borgia S, Bruggener B, Kleuser B, Schreiber S, Mehnert W 2008. Reconstructed epidermis and full-thickness skin for absorption testing: influence of the vehicles used on steroid permeation. Altern Lab Anim 36(4):441-452.
27. Sentjurc M, Vrhovnik K, Kristl J 1999. Liposomes as a topical delivery system: the role of size on transport studied by the EPR imaging method. J Control Release 59(1):87-97.
28. Cevc G 1996. Transfersomes, liposomes and other lipid suspensions on the skin: permeation enhancement, vesicle penetration, and transdermal drug delivery. Crit Rev Ther Drug Carrier Syst 13(3-4):257-388.
29. Gillet A, Compere P, Lecomte F, Hubert P, Ducat E, Evrard B, Piel G 2011. Liposome surface charge influence on skin penetration behaviour. Int J Pharm 411(1-2):223-231.
30. Pavelic Z, Skalko-Basnet N, Filipovic-Grcic J, Martinac A, Jalsenjak I 2005. Development and in vitro evaluation of a liposomal vaginal delivery system for acyclovir. J Control Release 106(1-2):34-43.
31. Pavelic Z, Skalko-Basnet N, Jalsenjak I 2004. Liposomal gel with chloramphenicol: characterisation and in vitro release. Acta Pharm 54(4):319-330.
32. Netzlaff F, Kaca M, Bock U, Haltner-Ukomadu E, Meiers P, Lehr CM, Schaefer UF 2007. Permeability of the reconstructed human epidermis model Episkin in comparison to various human skin preparations. Eur J Pharm Biopharm 66(1):127-134.
33. Wishart DS, Knox C, Guo AC, Cheng D, Shrivastava S, Tzur D, Gautam B, Hassanali M 2008. DrugBank: a knowledgebase for drugs, drug actions and drug targets. Nucleic Acids Res 36(Database issue):D901-906.

34. Van Gele M, Geusens B, Brochez L, Speeckaert R, Lambert J 2011. Three-dimensional skin models as tools for transdermal drug delivery: challenges and limitations. Expert Opin Drug Deliv 8(6):705-720.
35. Dreher F, Patouillet C, Fouchard F, Zanini M, Messager A, Roguet R, Cottin M, Leclaire J, Benech-Kieffer F 2002. Improvement of the experimental setup to assess cutaneous bioavailability on human skin models: dynamic protocol. Skin Pharmacol Appl Skin Physiol 15 Suppl 1:31-39.
36. Harvey RD, Ara N, Heenan RK, Barlow DJ, Quinn PJ, Lawrence MJ 2013. Stabilization of distearoylphosphatidylcholine lamellar phases in propylene glycol using cholesterol. Mol Pharm 10(12):4408-4417.

Table 1. Liposomal characteristics, with size distribution, polydispersity index (PI), entrapment efficiency and zeta potential (n=3)

Formulation	Size distribution				PI	Entrapment efficiency mean ± SD (μg/mg lipid)	Zeta potential mean ± SD (mV)
	Peak 1 (nm)	Weight intensity (%)	Peak 2 (nm)	Weight intensity (%)			
ACV-PC	663	96	175	4	0.20	15.5 ± 1.5	- 13.5 ± 3.0
ACV-PC/PG	619	90	124	10	0.35	16.4 ± 0.9	- 31.3 ± 2.0
CAM-PC	667	96	158	4	0.28	32.5 ± 3.8	- 13.8 ± 1.7

Table 2. Summary of the different PVPA models and their use in drug development

PVPA model*)	Mimicking barrier	Storage stability	Uses	References
$PVPA_o$	Intestine/ General biological barrier	2 weeks	Drugs in solution (pH 6.2/7.4)	1-3
			pH range in donor (pH 2-8)	4
			Solubilizers, tensides and co-solvents in donor	5,6
			Solid dispersions with poorly water soluble drugs	7
			Coated and non-coated liposome formulations	8
			Drug candidates	9-12
			FaSSIF	13
$PVPA_c$	Skin	16 months	Drugs in solution (pH 7.4)	14
			(Trans)dermal liposome formulations	15
$PVPA_s$	Skin	2 weeks	Drugs in solution (pH 7.4)	14
			(Trans)dermal liposome formulations	15
$PVPA_{biomimetic}$	Intestine	-	Drugs in solution (pH 6.2/7.4)	2
			Tensides and co-solvents	2
$PVPA_{mod}$	Intestine	-	Combined dissolution and permeability testing	16
			Drugs in solution (pH 7.4)	16

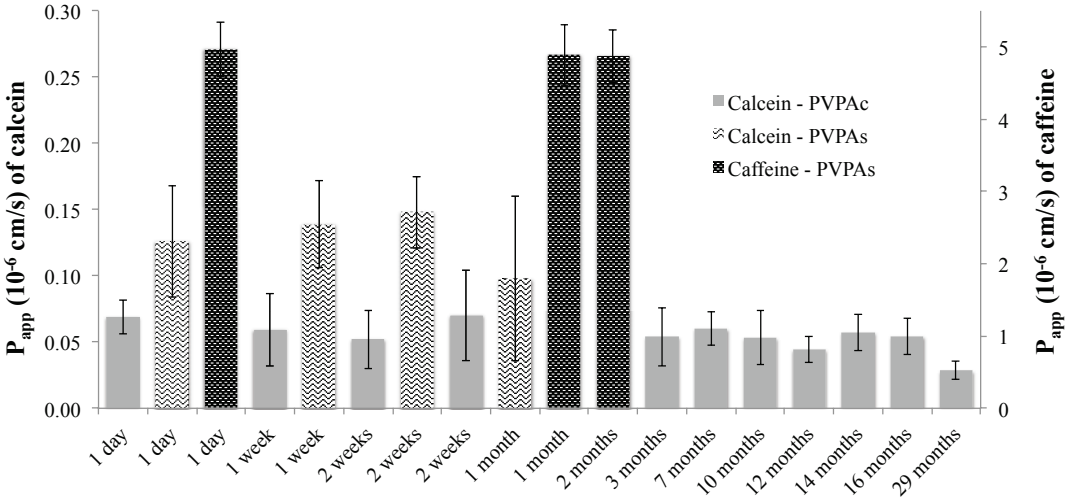
*) lipid composition from different models
$PVPA_o$: E-80
$PVPA_c$: E-80/cholesterol
$PVPA_s$: E-80/ceramide/cholesterol/cholesteryl sulphate/palmitic acid
$PVPA_{biomimetic}$: PC/phosphatidyl ethanolamine/phosphatidyl serine/phosphatidyl inositol/cholesterol
$PVPA_{mod}$: E-80

1. 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.
2. Naderkhani E, Isaksson J, Ryzhakov A, Flaten GE 2014. Development of a biomimetic phospholipid vesicle-based permeation assay for the estimation of intestinal drug permeability. J Pharm Sci 103(6):1882-1890.
3. Flaten GE, Skar M, 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(3-4):324-332.
4. 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.
5. 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.
6. Fischer SM, Flaten GE, Hagesaether E, Fricker G, Brandl M 2011. In-vitro permeability of poorly water soluble drugs in the phospholipid vesicle-based permeation assay: the influence of nonionic surfactants. J Pharm Pharmacol 63(8):1022-1030.
7. 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.
8. Naderkhani E, Erber A, Skalko-Basnet N, Flaten GE 2014. Improved permeability of acyclovir: optimization of mucoadhesive liposomes using the phospholipid vesicle-based permeation assay. J Pharm Sci 103(2):661-668.
9. Svenson J, Karstad R, Flaten GE, Brandsdal BO, Brandl M, Svendsen JS 2009. Altered activity and physicochemical properties of short cationic antimicrobial peptides by incorporation of arginine analogues. Mol Pharm 6(3):996-1005.
10. 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.
11. Flaten GE, Kottra G, Stensen W, Isaksen G, Karstad R, Svendsen JS, Daniel H, Svenson J 2011. In vitro characterization of human peptide transporter hPEPT1 interactions and passive permeation studies of short cationic antimicrobial peptides. J Med Chem 54(7):2422-2432.
12. Perlovich GL, Proshin AN, Volkova TV, Petrova LN, Bachurin SO 2012. Novel 1,2,4-thiadiazole derivatives as potent neuroprotectors: approach to creation of bioavailable drugs. Mol Pharm 9(8):2156-2167.
13. Fischer SM, Buckley ST, Kirchmeyer W, Fricker G, Brandl M 2012. Application of simulated intestinal fluid on the phospholipid vesicle-based drug permeation assay. Int J Pharm 422(1-2):52-58.
14. Engesland A, Skar M, Hansen T, Skalko-Basnet N, Flaten GE 2013. New applications of phospholipid vesicle-based permeation assay: permeation model mimicking skin barrier. J Pharm Sci 102(5):1588-1600.
15. Palac Z, Engesland A, Flaten GE, Skalko-Basnet N, Filipovic-Grcic J, Vanic Z 2014. Liposomes for (trans)dermal drug delivery: the skin-PVPA as a novel in vitro stratum corneum model in formulation development. J Liposome Res 10.3109/08982104.2014.899368.
16. Gantzsch SP, Kann B, Ofer-Glaessgen M, Loos P, Berchtold H, Balbach S, Eichinger T, Lehr CM, Schaefer UF, Windbergs M 2014. Characterization and evaluation of a modified PVPA barrier in comparison to Caco-2 cell monolayers for combined dissolution and permeation testing. J Control Release 175:79-86.

Figure 1. Permeability of ACV, CAM and CF in solutions (SOL) and liposomal formulations (PC or PC/PG) in the $PVPA_c$ (A, n=5), $PVPA_s$ (B, n=5) and the EpiSkin® (C, n=4; *) n=3) models. Error bars represent SD.

Figure 2. Mass balances of drugs in donor, barrier and acceptor after completed permeability experiments using the $PVPA_s$ (A, n=5) and EpiSkin® (B, n=4, *) n=3).

Figure 3. Effect of storage (-70 °C) on permeability through the $PVPA_c$ and $PVPA_s$ barriers. Error bars represent the standard deviations (n=6).


0.30
0.25
0.20
0.15
0.10
0.05
0.00
P_{app} (10^{-6} cm/s) of calcein
5
4
3
2
1
0
P_{app} (10^{-6} cm/s) of caffeine
Calcein - PVPAc
Calcein - PVPAs
Caffeine - PVPAs
1 day
1 day
1 day
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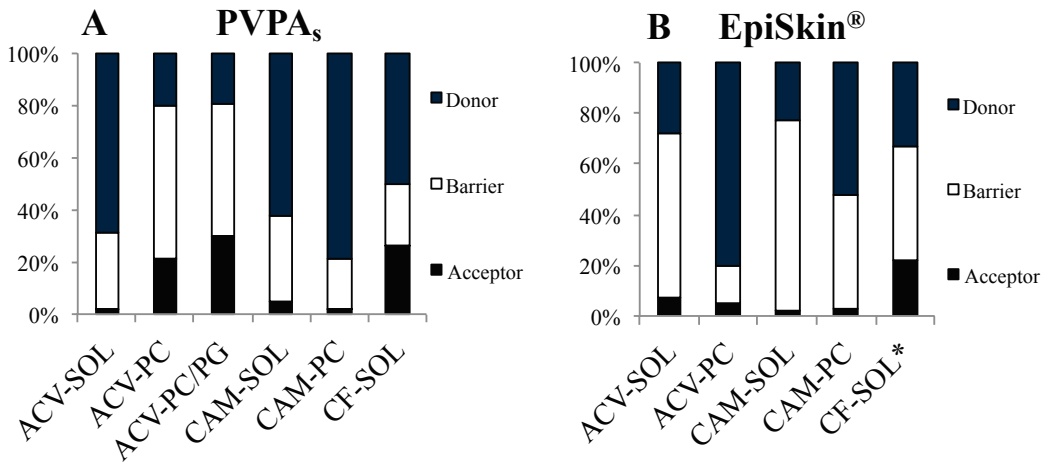
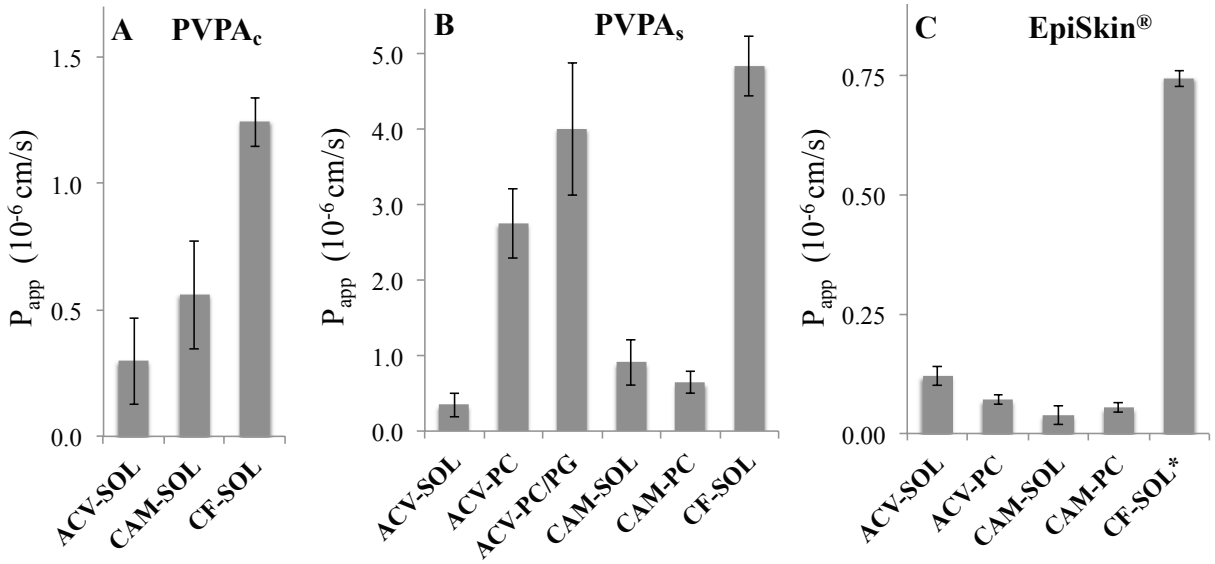
16 months
29 months


A
PVPA$_s$
100%
80%
60%
40%
20%
0%
Donor
Barrier
Acceptor
ACV-SOL
ACV-PC
ACV-PC/PG
CAM-SOL
CAM-PC
CF-SOL
B
EpiSkin®
100%
80%
60%
40%
20%
0%
Donor
Barrier
Acceptor
ACV-SOL
ACV-PC
CAM-SOL
CAM-PC
CF-SOL*


A
PVPA$_c$
B
PVPA$_s$
C
EpiSkin®
P_{app} (10^{-6} cm/s)
1.5
1.0
0.5
0.0
5.0
4.0
3.0
2.0
1.0
0.0
0.75
0.50
0.25
0.00
ACV-SOL
CAM-SOL
CF-SOL
ACV-SOL
ACV-PC
ACV-PC/PG
CAM-SOL
CAM-PC
CF-SOL
ACV-SOL
ACV-PC
CAM-SOL
CAM-PC
CF-SOL*