Liposomes for (trans)dermal drug delivery: the skin-PVPA as a novel *in vitro stratum corneum* model in formulation development

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Abstract:

Penetration potential of vesicles destined for trans(dermal) administration remains to be of great interests both in respect to drug therapy and cosmetic treatment. This study investigated the applicability of the phospholipid vesicle-based permeation assay (PVPA) as a novel *in vitro* skin barrier model for screening purposes in preformulation studies. Various classes of liposomes containing hydrophilic model drug were examined, including conventional liposomes (CLs), deformable liposomes (DLs) and propylene glycol liposomes (PGLs). The size, surface charge, membrane deformability and entrapment efficiency were found to be affected by the vesicle lipid concentration, the presence of the surfactant and propylene glycol. All liposomes exhibited prolonged drug release profiles with an initial burst effect followed by a slower release phase. The permeation of the drug from all of the tested liposomes, as assessed with the mimicked *stratum corneum* - PVPA model, was significantly enhanced as compared to the permeability of the drug in solution form. Although the DLs and the PGLs exhibited almost the same membrane elasticity, the permeability of the drug delivered by PGLs was higher (6.2×10^{-6} cm/s) than DLs (5.5×10^{-6} cm/s). Therefore, this study confirmed both the potential of liposomes as vesicles in trans(dermal) delivery and potential of the newly developed skin-PVPA for the screening and optimization of liposomes at the early preformulation stage.

1. INTRODUCTION

Transdermal drug delivery provides many advantages over other administration routes, including the avoidance of gastrointestinal side-effects, hepatic first-pass effects and variability in absorption as well as lower fluctuations in plasma drug levels (El Maghraby et al., 2006). Moreover, the transdermal route is noninvasive, painless, easy and well accepted by patients. Unfortunately, the barrier nature of *stratum corneum* limits the penetration of most drugs (Honeywell-Nguyen and Bouwstra, 2005). Different strategies have been used to improve transdermal delivery, including the optimization of the drug and vehicle properties, the modification of *stratum corneum* by chemical penetration enhancers, and electrical/external force methods such as iontophoresis, electroporation and sonophoresis (Delgado-Charro and Guy, 2001). Considerable research has focused on developing nanopharmaceuticals, including nanoparticles (nanospheres, nanocapsules) derived from natural or synthetic polymers, nanoemulsions, solid lipid nanoparticles, nanostructured lipid carriers, dendrimers and vesicular nanosystems, such as niosomes and liposomes, to enhance the transport of drugs through the skin (Cereda et al., 2013; Cevc and Vierl, 2010; Prow et al., 2011; Subongkot et al., 2012). The use of nanopharmaceuticals enables the alteration of the pharmacokinetic properties of a given drug and increases its bioavailability. Liposomes have received particular attention among nanopharmaceuticals. Their phospholipid nature, non-toxicity and ability to encapsulate different compounds (hydrophilic, lipophilic and amphiphilic) make liposomes a promising option for improved skin drug delivery. However, most of the reports on conventional liposomes (CLs) describe localized effects as a result of vesicles accumulation in the *stratum corneum* or upper layers of the epidermis (Barry, 2001). To overcome these limitations, new classes of lipid vesicles with pronounced membrane elasticity, such as deformable (flexible, elastic) liposomes (DLs) (Cevc et al., 1998), ethosomes (Touitou et al., 2000), invasomes (Dragicevic-Curic et al., 2008) and propylene

glycol-containing liposomes (PGLs) (Elsayed et al., 2007a), have been investigated. These novel types of liposomes demonstrate superior skin penetration ability and seem to transport active substances more efficaciously through *stratum corneum* into the deeper layers of the skin and even transdermally. In order to optimize their composition and desired outcome, the drug permeability studies, performed on robust and reliable models, are essential for the successful development of liposomes for (trans)dermal drug delivery.

In vitro studies using human skin are ideal for monitoring drug delivery and evaluating the drug formulation because these studies ensure an accurate representation of the processes involved. However, the complex nature of biological tissue, the inter- and intra-individual variability of skin samples and ethical issues limit the use of human skin in the early stages of formulation assessment. Most *in vitro* investigations use pig ear skin because of its structural equivalence to human skin (Herkenne et al., 2007; Salerno et al., 2010); however, measurements using Franz diffusion cell and animal skin are only partially standardized, leading to large variations in permeability results (Chilcott et al., 2005). Artificial model membranes, such as silicone membranes (Oliveira et al., 2010), the ceramide-derived parallel artificial membrane permeability assay (Sinko et al., 2012) and the phospholipid vesicle-based permeation assay (PVPA) (Engesland et al., 2013), represent simple and reproducible methods for studying the fundamental mechanisms of drug permeation.

The skin-PVPA is a novel *in vitro* skin barrier model based on the tightly packed phospholipid vesicles chosen to mimic *stratum corneum*. This assay provides a valuable method for evaluating the skin permeation of different drugs (Engesland et al., 2013) and has the potential to be used to estimate (trans)dermal formulations. Skin-PVPA has not yet been examined for the assessment of skin-targeting nanopharmaceuticals. Therefore, the present study evaluated the applicability of this novel skin barrier in determining the drug penetration abilities of various liposomes. CLs, DLs and PGLs containing a hydrophilic model drug

(diclofenac sodium) were prepared, and their physicochemical properties were evaluated. It has been shown that the lipid concentration and composition, presence of edge activators and/or penetration enhancers, lamellarity, particle size, surface charge, entrapment efficiency and mode of application affect the degree of (trans)dermal drug penetration and, consequently, the efficacy of the liposomes as a drug delivery system (Cevc and Blume, 1992; Gillet et al., 2011a,b).

2. MATERIALS AND METHODS

2.1. Materials

Soy lecithin (Lipoid S75) and egg phosphatidylcholine (Lipoid E80) were generous gifts from Lipoid GmbH (Ludwigshafen, Germany). Diclofenac sodium (DCS) (Mw 318.13) was kindly provided by Pliva Croatia Ltd. (Zagreb, Croatia). Ceramides from bovine spinal cord, cholesterol, cholesteryl sulfate, palmitic acid, Sepharose CL-4B, Sephadex G-50 and sodium deoxycholate (SDCh) were purchased from Sigma-Aldrich Company (St. Louis, USA). Chloroform was purchased from Merck (Darmstadt, Germany). Ethanol, methanol and propylene glycol (PG) were of analytical grade and were obtained from Kemika (Zagreb, Croatia). Filter inserts (Transwell, d = 6.5 mm) and plates were purchased from Corning Inc. (Corning, New York, USA). 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). Nucleopore filters (0.4 μm pore size) were purchased from Whatman (part of GE Healthcare, Oslo, Norway).

Phosphate buffer was prepared by dissolving KH_2PO_4 (0.6 g), $Na_2HPO_4 \times 12H_2O$ (6.4 g), NaCl (7.24 g) and NaN_3 (0.2 g) in distilled water up to 1000 ml, and the pH was adjusted to 7.4 by adding HCl or NaOH.

2.2. Preparation of liposomes

CLs, DLs and PGLs of different lipid concentrations (26 or 52 mM total lipids) were prepared by a film hydration method (Vanić et al., 2012). Briefly, Lipoid S75 (for the CLs and the PGLs) or Lipoid S75/SDCh at an 85/15% weight ratio (for the DLs) was dissolved in concentrated ethanol in a round-bottomed flask. The ethanol was removed completely using a rotary vacuum evaporator (Büchi Rotavapor R-200, Büchi Labortechnik AG, Flawil, Switzerland), and the thin lipid layer was hydrated by adding an aqueous solution of DCS (150 mg/10 ml) for the preparation of CLs and DLs. For the preparation of PGLs, 10% or 30% (w/v) of PG was added to the aqueous solution of the DCS. All of the liposome preparations were extruded three times through the 400-nm pore size polycarbonate membranes (LiposoFast, Avestin, Canada).

The phospholipid contents in all of the liposome formulations were determined as the total amount of phosphorous according to Bartlett (1959). The recoveries were found to be in the range between 93 and 98.3% of the starting amounts.

2.3. Size measurements

The mean diameter and the polydispersity index (PI) of the liposomes were determined by photon correlation spectroscopy (PCS) on a Zetasizer 3000HS (Malvern Instruments, Malvern, UK) 24 h after preparation. Measurements were performed at a scattering angle of 90° and a temperature of 25°C. The liposome samples were diluted with 1 mM NaCl, which was previously filtered through 200-nm Minisart filters, to achieve a count rate of between

100 and 300 Kcps. Prior to the measurements, all of the prepared liposomes were sonicated for 15 s in an ultrasonication bath (Branson 1210, Branson Ultrasonics Corp., Danbury, USA) (Vanić et al., 2013).

2.4. Zeta potential measurements

Zeta potential measurements were performed on a Zetasizer 3000HS (Malvern Instruments, Malvern, UK) using a capillary cell with the optical modulator operating at 1000 Hz. To ensure the validity of the measurements, the instrument was calibrated throughout the measurements using the Malvern Zeta Potential Transfer Standard (-50 ± 5 mV). Liposomal samples were prepared by diluting the liposome suspension with an appropriate volume of 1 mM NaCl to achieve the proper count rate. All measurements were performed at 25°C (Vanić et al., 2013).

2.5. Entrapment efficiency determination

To determine the entrapment of DCS in liposomes, unentrapped (free) DCS was separated from the liposomes by four different methods: gel chromatography, minicolumn centrifugation, ultracentrifugation and dialysis. Gel chromatography was performed on Sepharose CL-4B (Vanić et al., 2012), and minicolumn centrifugation was conducted with Sephadex G-50 minicolumns according to a previously described procedure (Vanić et al., 2013). For the ultracentrifugation method, 0.5 ml of each liposome suspension was diluted with 1.5 ml of demineralized water and ultracentrifuged (Beckman Optima LE-80 K Ultracentrifuge, Beckman Coulter Inc., Fullerton, USA) for 1 h at 100 000 *g* (20°C). The supernatant (unentrapped drug) was removed, and the pellet was washed with 2 ml of water under the same conditions. To separate the unentrapped drug by dialysis, the samples of liposome suspensions (100 µl) were placed in a dialysis bag (Dialysis Tubing Visking,

Medicell International Ltd., London, UK, Mw cut-off 12-14000 Da) and extensively dialyzed in 100 ml of demineralized water for 1 h during constant stirring at 50 rpm. The receptor medium was completely replaced with fresh medium continuing dialysis for the next hour. The concentrations of both the unentrapped (free) and the liposome-entrapped drug were determined spectrophotometrically (Ultrospect Plus, Pharmacia LKB, Cambridge, UK). The concentration of the free DCS was measured at 276 nm (demineralized water), and the loaded drug was detected at 282 nm after dissolving the liposomes in methanol.

The entrapment of the drug was expressed as the entrapment efficiency (%), and the drug/lipid ratio according to the following equations:

Entrapment efficiency (%) = [drug in liposomes/(drug in liposomes + unentrapped drug)] x 100

Drug/lipid ratio (µg/mg) = µg of the drug in liposomes/mg of lipid recovered

Drug recovery (%) = [(drug in liposomes + unentrapped drug)/total drug] x 100

The amount of the total drug was determined by using an aliquot of the initial liposome suspension and adding methanol to dissolve lipids in the liposomes.

The recovery of the drug was determined for all of the samples and was found to be between 89.7 and 98.5%.

2.6. Measurements of liposome elasticity

The membrane elasticity of the liposomes was determined with a home-made device, as previously reported (Vanić et al., 2014). In brief, an external pressure of 5 bar was used to drive the flux of extruded liposome suspensions through a membrane with a pore size of 100 nm. The amount of liposome suspension that was extruded in 5 min was measured, and the mean diameter and polydispersity index (PI) were monitored by PSC measurements before

and after extrusion. The degree of membrane elasticity of the liposomes (E) was calculated with the following equation:

$$E = J(r_v/r_p)^2,$$

where J is the amount of suspension (g) extruded in 5 min, r_v is the mean diameter of the liposomes after extrusion and r_p is the membrane pore size (Jain et al., 2003; Ferderber et al., 2009).

The measurements were performed with liposomes that were prepared with the lower lipid concentration (CL-A, DL-A, PGL-10-A, PGL-30-A) and extruded three times through a membrane with a pore size of 400 nm.

2.7. Storage stability study

Samples of CL-A, DL-A and PGL-10-A (extruded through 400-nm pore size filters) were stored for a period of ten months at 4°C, and the mean diameter, PI and zeta potential were analyzed as described above.

2.8. In vitro release studies

The release of DCS from liposomes that were previously separated from unentrapped drug was performed on a Franz cell diffusion system under sink conditions using cellulose membranes and a heating circulator set to 32°C (Hurler et al., 2012). The acceptor chamber (16 ml) was filled with demineralized water. Aliquots of the liposomes (200 µl) or a control solution (an aqueous solution of DCS at a concentration that corresponds to the liposome formulations) were added to the donor chamber onto a presoaked membrane, and the system was adequately sealed. The receptor medium was constantly mixed with a small magnetic stirrer. Samples (1 ml) were withdrawn from the acceptor compartment at certain time

intervals and replaced with fresh medium. The amount of released DCS was determined spectrophotometrically as described above.

2.9. Permeability studies on the novel artificial stratum corneum barrier (skin-PVPA)

The DCS permeability of various liposomes (CL-A, DL-A, PGL-10-A) was evaluated on the recently developed skin-PVPA model (Engesland et al., 2013). Phospholipid vesicles composed of materials similar to those found in human *stratum corneum* were prepared and used to build an artificial *stratum corneum* barrier.

2.9.1. Preparation of vesicles for PVPA

The vesicles were prepared using the film hydration method (Flaten et al., 2006a). Two different lipid compositions were used:

1.   E-80 (77%, w/w) and cholesterol (23%, w/w) for the preparation of phospholipid vesicles 1 (PV-1);

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) for the preparation of phospholipid vesicles 2 (PV-2).

The lipids were dissolved in chloroform (for PV-1) or in a mixture of chloroform and methanol (1:1, volume ratio, for PV-2). The organic solvents were removed by evaporation, and the dried lipid films were hydrated with phosphate buffer, pH 7.4, followed by the addition of 10% (v/v) ethanol to achieve 6% (w/v) dispersions. The PV-1 were then extruded by nitrogen-driven extrusion (Lauda Dr R Wobster GmbH Königshofen, Germany) using 400- and 1200-nm pore size filters, and the PV-2 were extruded through 1200-nm filters using Liposofast (Avestin Europe GmbH, Mannheim, Germany). The different strategies for

extrusion of the two types of vesicles were chosen because we were unable to extrude the less flexible PV-1 by hand using the easy Liposofast-based method.

The size measurements of all of the phospholipid vesicles used to prepare the PVPA were determined by PCS using a Submicron Particle Sizer 370 (PSS Nicomp Particle Sizing Systems, Santa Barbara, California) (Flaten et al., 2006b).

2.9.2. Preparation of PVPA barriers

The modified PVPA barriers designed to study the permeability of DCS from different liposomal formulations were prepared based on the recently developed skin-PVPA models (Engesland et al., 2013). $PVPA_c$ denotes the PVPA barrier prepared with PV-1, while $PVPA_s$ denotes the more complex PVPA barrier mimicking *stratum corneum* that was prepared with PV-2. The procedure was as follows: the small vesicles (extruded through 400-nm pores) were distributed into the pores of the filter support (650 nm) using repeated centrifugation (950 g) for 15 min followed by heating at 50°C for 45 min. Then, the large vesicles (extruded through 1200-nm pores) were deposited on top of the filter support as follows:

• 35 μl of PV-1 (for preparation of $PVPA_c$) was deposited on the top layer of the filter support and centrifuged at 1030 g for 30 min. Invert centrifugation of inserts at 25 g was then used to remove the supernatant, followed by freezing at −70°C for at least 1 h to promote the fusion of the vesicles. The barrier was thawed at 30°C for 2 h prior to the permeability experiment.

• 35 μl of PV-2 (for preparation of $PVPA_s$) was placed onto the filter support and centrifuged at 950 g for 15 min (to align the vesicles on the surface). After evaporation at 50°C for 30 min (15 min in a closed container and 15 min in an open container), the barrier was frozen at −70°C for 1 h. The barrier was thawed at 30°C for 2 h prior to the permeability experiment.

2.9.3. Permeation experiments with liposomal formulations using the PVPA models

Permeation studies with various classes of liposomes containing DCS, namely, CL-A, DL-A and PGL-10-A (extruded 3x400 nm and separated from the unentrapped drug), were preformed according to the procedure described previously for the permeability testing of hydrophilic compounds (Flaten et al., 2006a). In brief, the inserts were loaded with 100 μl of the liposomal formulations, all containing the same amount of the drug (donor compartment). At certain time intervals (every 1 h in the first 3 h and every 0.5 h for the next 2 h), the inserts were moved to wells containing pH 7.4 phosphate buffer (600 μl) as an acceptor medium to maintain sink conditions. At the end of experiment (after 5 h), 200 μl of samples from each acceptor compartment were transferred into 96-well titer plates for spectrophotometric analysis (Spectramax 190; Molecular Device Corporation, Sunnyvale, California). Control trials with DSC aqueous solutions with the same concentration of DSC as in the liposomal samples were performed under the same conditions. All of the trials were performed at least in triplicate.

To test the integrity of the barriers, the electrical resistance of the PVPA barriers was measured (Millicell-ERS; Millipore) immediately after finishing the permeability studies.

The apparent permeability coefficients (P_{app}) were calculated from the steady-state flux according to the following equation:

$$P_{app} \text{ (cm/s)} = J/AC_d,$$

where 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 DCS in the donor compartment (nmol/ml).

2.10. Statistical analysis

The statistical data analyses of the two groups were performed using Student's t-test, and the means were considered significantly different when $p < 0.05$. When comparing three or more groups, a one-way ANOVA and Tukey's multiple comparison test with $p < 0.05$ set as the minimal level of significance were applied. Calculations were performed with the GraphPad Prism program (GraphPad Software, Inc., San Diego, CA).

3. RESULTS AND DISCUSSION

3.1. Physicochemical properties of liposomes

To effectively deliver the hydrophilic drug through the skin, different classes of liposomes were prepared and investigated, including CLs, DLs and PGLs. Composition of the liposomes used as drug carrier is known to influence their physicochemical properties and therefore their efficacy as drug delivery systems (Šentjurc et al., 1999). Therefore, the first objective of this study was to evaluate and optimize the liposome formulations based on the effect of the lipid composition and concentration on the size, charge, membrane elasticity (deformability) and, most importantly, entrapment efficiency. All liposomes were prepared by the film hydration method using two different lipid concentrations (26 and 52 mM) and keeping the DCS concentration constant in all formulations (47 mM).

3.1.1. Liposome size and charge

As shown in Table 1, the liposomes prepared with the lower lipid concentration (formulations denoted as CL-A, DL-A, PGL-10-A and PGL-30-A) had a mean diameter ranging from 239 nm for the DL-A to 390 nm for the PG liposomes containing 30% (w/v) of PG in formulation (PGL-30-A). The mean diameter of the CL-A liposomes was between these two formulations

(278 nm). Increasing the lipid concentration from 26 to 52 mM (formulations denoted as CL-B, DL-B, PGL-10-B and PGL-30-B) resulted in a larger size of CL-B (312 nm) and PGL-30-B (405 nm), and a broader size distribution for CL-B liposomes (PI enhanced to 0.73). However, these properties remained unchanged for DL-B liposomes (t-test, $p > 0.05$), suggesting that the lipid concentration increase did not influence the size. The smaller size of the DLs compared to the CLs has been confirmed in a recent study by our group (Vanić et al., 2013) and might be ascribed to the increased flexibility and reduced surface tension of the vesicles due to the presence of SDCh, as previously reported (Chen et al., 2009). The PGLs prepared with lower lipid and PG concentration (PGL-10-A) were found to be smaller than CL-A, while PGLs-30 were the largest. This finding might be explained by an osmotic effect of the PG within the liposome leading to vesicle swelling upon the influx of water during the sample dilutions for the PSC measurements.

To obtain liposomes with diameters less than 180 nm, which are known to increase dermal drug delivery (Verma et al., 2003), all of the liposome formulations were extruded through polycarbonate membranes. The mean diameters of all of the extruded liposomes were in the range of 147 (DL-A) to 165 nm (PGL-30-B), with lower PIs for CLs and PGLs. However, the DLs had a larger PI (0.29), likely as a result of the presence of the edge activator (SDCh), which contributes the morphological varieties to the vesicle population (elongated forms) (Cevc et al., 2002) that are reflected in the size measurements resulting in an increasing PI.

The zeta potential data followed the characteristics of the material used for the preparation of liposomes, primarily soy lecithin S 75 (Table 1). Strongly negative values of approximately -60 mV for CLs and DLs and –70 mV for PGLs indicate the formation of physically stable liposomal suspensions. Negative surface charges on the liposomes are also favorable for skin applications because recent studies have shown that negatively charged liposomes enhance

the skin penetration of a drug to a greater extent than positively charged and neutral liposomes (Gillet et al., 2011a).

3.1.2. Entrapment efficiency

The desirable therapeutic effect by using liposomes can be achieved if the liposomes are loaded with a sufficient amount of the drug that should reach the target site. Hence, the appropriate entrapment efficiency, in addition to the appropriate size and membrane properties, is of great importance. To determine the drug content entrapped in the liposomes, unentrapped DCS had to be successfully separated from the liposomes. Different separation methods were applied (gel chromatography, dialysis, minicolumn centrifugation and ultracentrifugation) to ensure that all unentrapped drug was removed, and the methods were compared to optimize the separation. Moreover, we investigated the possible influence of the lipid concentration used in the liposome preparation on the entrapment efficiency. The entrapment results were expressed in two ways: as the entrapment efficiency (%) and as the drug per lipid ratio. Although both modes appeared to yield the same entrapment profiles for all of the samples (Figures 1 and 2), differences were observed for the liposomes prepared with higher lipid concentrations. Increasing the lipid concentration enhanced the apparent entrapment efficiency for all of the liposomes (Figure 1), although the drug/lipid ratio decreased (Figure 2). Therefore, the lower lipid concentration was optimal for preparing liposomes.

Liposome extrusion has been shown to reduce the original liposome size and PI (Table 1) while simultaneously significantly reducing the encapsulation efficiency (Figures 1 and 2). Regardless of the separation method, the extruded CLs were able to encapsulate more than 100 μg of DCS per mg of lipid at lower lipid concentrations. Among the tested separation methods, gel chromatography (the most accurate and stress-free method) yielded significantly

higher values of entrapped DCS in CLs (ANOVA, $p < 0.05$) as compared to other methods (Figure 2). Similar patterns of higher entrapment using gel chromatography were observed for PGLs containing 10% PG (PGL-10-A).

The evaluation of the different liposome classes revealed the high entrapment of DCS in the PGLs, especially in PGL-30-A (>160 µg/mg). This increased entrapment could be attributed to the presence of PG, a well-known co-solvent that is often used in pharmaceutical formulations to improve the water solubility of drug. The increased drug incorporation in the PGLs, compared to the CLs, has been observed by several research groups (Elmoslemany et al. 2012; Manconi et al., 2009). In contrast, the lowest DCS entrapment was obtained in DLs, and similar results were confirmed by all separation methods (Figure 2). The addition of SDCh has been demonstrated to significantly decrease the liposome size and, accordingly, the entrapment of hydrophilic drugs, as recently reported (Gillet et al., 2011b; Vanić et al., 2013).

3.1.3. Liposome elasticity

The essential feature of DLs is the high, stress-dependent adaptability (elasticity, deformability) of their bilayers, which allows DLs to squeeze between narrow pores that are smaller than the size of the DLs and to transport encapsulated drugs deeper through the skin following an *in vivo* transcutaneous hydration gradient under non-occlusive conditions (Cevc et al., 1998, 2002). Recently introduced PGLs have been shown to improve skin drug delivery (Manconi et al., 2009), although the exact mechanism is still under investigation. Because PG is able to solubilize phospholipids during liposome preparation (Kikuchi et al., 1994; Pavelić et al., 2005), it has been suggested that PG intercalates into the bilayers (Zhao et al., 2013), thus contributing to the flexibility of vesicles. Therefore, the membrane elasticity is an important parameter in the characterization of liposomes that are intended for (trans)dermal delivery. To determine the degree of deformability (E) of the liposomes containing DCS, all

of the vesicles were passed through polycarbonate membranes (100 nm) using a constant external pressure of 5 bar. The size of vesicles after the passage through the membrane at a constant pressure is important for the calculation of E; however, E appears to be mostly dependent on J (the amount of the extruded liposome suspension) in this study, as shown by Figure 3. DL-A was calculated to have an E of 5.59, which is more than five-fold higher than the E value for CL-A (1.08). An approximately equal E value (5.21) was attained for PG liposomes with lipid composition that were identical to the conventional liposomes, which additionally contained 10% w/v of propylene glycol (PGL-10-A). This result suggests that PG is responsible for the improved membrane elasticity of the PGLs. Although one would expect a further increase in E with enhanced PG content, the opposite effect was observed: the degree of elasticity decreased to 3.69. Therefore, in spite of the ability of PGL-30-A to entrap a larger amount of DCS than PGL-10-A, we have excluded PGL-30-A from further studies because of its tendency to reduce liposome elasticity (Figure 3).

3.1.4. Storage stability evaluation

The physical stability of the liposomes was determined by monitoring the changes in the size (mean diameter, PI) and the zeta potential during storage at 4°C for ten months. The results presented in Figure 4 demonstrate the increased size of all of the liposomes compared to the initial sizes. The mean diameter increased approximately 20% for PGL-10-A, and the highest increase was obtained for CL-A. The PI increased for DL-A and CL-A; however, the PI remained unchanged for PGL-10-A. The zeta potential values remained the same as those of the initial formulations, suggesting good physical stability for all of the examined liposome suspensions.

3.2. In vitro release studies

The predictable and controllable release of the drug from the liposomes is an important property considering the application of liposomes as a drug delivery system. To evaluate various liposomes, we performed *in vitro* release studies. As shown in Figure 5, all of the liposomal formulations were characterized by the sustained release profiles compared to the control (a solution of the free drug), although all of the liposomes exhibited rapid release rates during the first 2 h. This initial burst effect may be important for attaining higher levels of the drug at the target site. The release of DCS was markedly influenced by the composition of vesicles (Figure 5). The slowest drug release was obtained from CL-A, and the presence of PG enhanced drug release, most likely as a consequence of the intercalation of PG into the bilayers, causing the vesicles to be more permeable to the entrapped drug. These observations are also in an agreement with the determined degrees of membrane elasticity for the tested formulations (Figure 3). Among the investigated liposomes, the DCS release rate was the highest for DL-A. These results are expected because of the presence of the edge activator in the bilayer, which destabilizes the membranes of the DLs and makes the membranes more permeable for the entrapped drug, as previously observed (Cevc and Blume 1992; Kawano et al., 2009). Our results also corroborate the latest reports for the faster initial drug kinetic profile of DLs compared with that of CLs (Cadena et al., 2013).

3.3. Permeability studies

Evaluating the drug permeation through the skin is essential in the development of (trans)dermal delivery systems. Recently developed skin-PVPA, a model for monitoring the transdermal permeation of drug candidates at an early development stage (Engesland et al., 2013), was originally introduced as an artificial membrane model for the intestinal epithelia (Flaten et al., 2006a,b, 2007). The appropriate selection of the lipid composition for the PVPA could create an artificial *stratum corneum* barrier model with high reproducibility, which

could eliminate the variability in human and animal *in vitro* skin studies. Moreover, this model reduces the use of animals during the early development phases of topical formulations (Engesland et al., 2013).

To enable the (trans)dermal delivery of the model hydrophilic drug DCS, different liposomal formulations were evaluated for drug permeability on a novel skin-PVPA model. This artificial barrier used to mimic the human *stratum corneum* was prepared via the tight depositing of different-sized phospholipid vesicles, composed of skin-like lipid components, on filter supports in Transwell inserts. Because the initial trials with the DCS liposomal formulations on the original skin-PVPA model (Engesland et al., 2013) exhibited difficulties in differentiating between the permeation of the model drug from different liposome formulations (data not shown), small modifications in the preparation of the skin-PVPA barrier were required. Changing the manufacturing conditions significantly influenced the properties of the barrier. Thus, to prepare a model that enabled the differentiation of DCS permeation from various liposomal formulations, the thickness of the skin-PVPA barrier was reduced by adding fewer aliquots of large vesicles on the top of the barrier (for details, see experimental section).

The assessment of the penetration ability of various liposomes containing DCS was performed using two PVPA barriers: a simple $PVPA_c$ with a top layer consisting of E-80 and cholesterol and more skin-like $PVPA_s$ prepared with phospholipid vesicles composed of lipids normally found in *stratum corneum* as the top layer. Steady-state permeation values of DCS were attained from all of the tested samples. A mean value for P_{app} of DCS was calculated for each liposomal formulation and for the control. Moreover, to validate the permeability experiments, the barrier integrity was tested by measuring the electrical resistance across the barriers throughout the study.

Figure 6 shows the slight improvement in the DCS permeation profiles using DL-A and PGL-10-A on $PVPA_c$. However, when experiments were performed on the $PVPA_s$ with a lipid composition closer to the lipid composition in *stratum corneum* and a more loosely packed top layer of large phospholipid vesicles, the overall permeation was higher (Figure 7). All of the liposomal formulations exhibited significantly higher permeabilities than the DCS aqueous solution (ANOVA, $p < 0.05$) due to the penetration-enhancing effect of the phospholipids (Elsayed et al., 2007b), especially soy lecithin, which contains a higher portion of unsaturated fatty acids. The highest P_{app} of DCS was attained with PGL-10-A, followed by DL-A, and the lowest values were obtained with CL-A. The obtained values for the electrical resistance across the barriers were in an agreement with the calculated drug permeability. An inversely proportional relationship was observed, demonstrating a decrease of electrical resistance as a result of the increased leakiness in the barrier caused by the liposomes, which consequently increased the drug permeation (Figure 7). The differences in the amount of permeated drug among the liposome formulations examined were in accordance with the findings of previous study (Elsayed et al., 2007a,b). The presence of the edge activator (SDCh) in the bilayers of the DLs enhanced the flexibility of the vesicles and permitted the vesicles to squeeze between the cells in *stratum corneum* and transport the entrapped drug deeper into/through the skin (Cevc et al., 1992, 1998). Although the PGLs were of the same lipid composition as the CLs, the PGLs contained PG, a known skin penetration enhancer (Lane, 2013) that, in addition to phospholipids, notably enhanced the permeation of the drug. These results are consistent with the high elasticity of PGL-10-A and DL-A (Figure 3) and with the results of the release studies (Figure 5), in which similar release profiles were observed for both types of vesicles.

Our findings demonstrate the increased permeation of hydrophilic drug when delivered through elastic vesicles. However, from the performed studies on the PVPA barriers we

cannot confirm that the transport mechanism of elastic liposomes is indeed by squeezing between the "pores" in the skin as reported in the studies by Cevc et al. (1998, 2002), since we have not determined lipid/vesicles content in the receiver compartment.

A greater skin permeation of DCS using deformable liposomes (transferosomes) has been shown in recent investigations (El Zaafarany et al., 2010; Ghanbarzadeh et al., 2013), although studies have used different *ex vivo* skin models and the experiments differed in the composition and physicochemical properties of the investigated liposomes. The enhancement ratios found in previous studies, calculated as the ratio of the permeability coefficients obtained for the deformable liposomes and the control formulation, were between 2.4 (El Zaafarany et al., 2010) and 5.2 (Ghanbarzadeh et al., 2013), and in our study on the PVPA$_s$, the enhancement ratio was 2.65. Therefore, we believe that the skin-PVPA model is a valuable *in vitro* screening method for the optimization of liposome formulations for (trans)dermal drug delivery. However, we are aware of the necessity of conducting *ex vivo* skin permeation studies, preferably on human skin, to confirm the value of the novel *in vitro* barrier model.

The phospholipid vesicle-based barriers have already been proven to be useful for evaluating the drug permeation of oral and skin drug candidates (Engesland et al., 2013; Flaten et al., 2006a,b, 2007). In addition, intestinal PVPA has been shown to be suitable for permeability screening and formulation optimization of complex formulations like solid dispersions (Kanzer et al., 2010). However, the present study is the first to demonstrate the potential of skin-PVPA for screening the skin-penetration abilities of various liposomes as drug nanocarriers without using animals at an early development stage. The degree of the leakiness of the barrier can be finely adjusted depending on the composition of barrier vesicles and the preparation conditions to differentiate the tested formulations. The high reproducibility and

stability of the barriers at determined storage conditions provide the opportunity for possible up-scaling of the model.

CONCLUSION

A novel skin barrier model based on PVPA as a mimicking *stratum corneum* barrier was modified and applied in the screening of the penetration abilities of different liposome formulations containing a model hydrophilic drug. The permeation of the hydrophilic drug from the liposomes was affected by their physicochemical properties, which were influenced by the lipid composition and the presence of the edge activator or penetration enhancer. The value of the developed artificial skin barriers would be supported through verification for the evaluation of liposomes containing lipophilic and amphiphilic drugs, including those of higher molecular weight.

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DECLARATION OF INTEREST

The authors report no declaration of interest.

REFERENCES

Barry BW. (2001). Novel mechanisms and devices to enable successful transdermal drug delivery. *Eur J Pharm Sci*, 14:101-14.

Bartlett G.R. (1959). Phosphorus assay in column chromatography. *J Biol Chem*, 234:466-68.

Cadena P.G., Pereira M.A., Cordeiro R.B., Cavalcanti I.M., Barros Neto B., Pimentel Mdo C., Lima Filho J.L., Silva V.L., Santos-Magalhaes N.S. (2013). Nanoencapsulation of quercetin and resveratrol into elastic liposomes. *Biochim Biophys Acta*, 1828:309-16.

Cereda, C.M., Franz-Montan, M., da Silva, C.M., Casadei, B.R., Domingues, C.C., Tofoli, G.R., de Araujo, D.R., de Paula, E. (2013). Transdermal delivery of butamben using elastic and conventional liposomes. *J Liposome Res*, 23:228-34.

Cevc G., Blume G. (1992). Lipid vesicles penetrate into intact skin owing to the transdermal osmotic gradients and hydration force. *Biochim Biophys Acta*, 1104:226-32.

Cevc G., Gebauer D., Stieber J., Schatzlein A., Blume G. (1998). Ultraflexible vesicles, Transfersomes, have an extremely low pore penetration resistance and transport therapeutic amounts of insulin across the intact mammalian skin. *Biochim Biophys Acta*, 1368:201-15.

Cevc G., Schatzlein A., Richardsen H. (2002). Ultradeformable lipid vesicles can penetrate the skin and other semi-permeable barriers unfragmented. Evidence from double label CLSM experiments and direct size measurements. *Biochim Biophys Acta*, 1564:21-30.

Cevc G., Vierl U. (2010). Nanotechnology and the transdermal route: a state of the art review and critical appraisal. *J Control Release*, 141:277-99.

Chen Y., Lu Y., Chen J., Lai J., Sun J., Hu F., Wu W. (2009). Enhanced bioavailability of the poorly water-soluble drug fenofibrate by using liposomes containing a bile salt. *Int J Pharm*, 376:153-60.

Chilcott R.P., Barai N., Beezer A.E, Brain S.I., Brown M.B., Bunge A.L., Burgess S.E., Cross S., Dalton C.H., Dias M., Farinha A., Finnin B.C., Gallagher S.J., Green D.M., Gunt H., Gwyther R.L., Heard C.MJarvis., C.A., Kamiyama F., Kasting G.B., Ley E.E., Lim S.T., McNaughton G.S., Morris A., Nazemi M.H., Pellett M.A., Du Plessis J., Quan Y.S., Raghavan S.L., Roberts M., Romonchuk W., Roper C.S., Schenk D., Simonsen L., Simpson A., Traversa B.D., Trottet L., Watkinson A., Wilkinson S.C., Williams F.M., Yamamoto A., Hadgraft J. (2005). Inter- and intralaboratory variation of *in vitro* diffusion cell measurements: an international multicenter study using quasi-standardized methods and materials. *J Pharm Sci*, 94:632-38.

Delgado-Charro M.B., Guy R.H. (2001).Transdermal Drug Delivery. In: Hillery A.M., Lloyd A.W., Swarbrick J., eds. Drug Delivery and Targeting for Pharmacists and Pharmaceutical Scientists. London-New York: Taylor & Francis, 208-236.

Dragicevic-Curic N., Scheglmann D., Albrecht V., Fahr A. (2008). Temoporfin-loaded invasomes: development, characterization and *in vitro* skin penetration studies. *J Control Release*, 127:59-69.

El Maghraby G.M., Williams A.C., Barry B.W. (2006). Can drug-bearing liposomes penetrate intact skin?. *J Pharm Pharmacol*, 58:415-29.

El Zaafarany G.M., Awad G.A., Holayel S.M., Mortada N.D. (2010). Role of edge activators and surface charge in developing ultradeformable vesicles with enhanced skin delivery. *Int J Pharm*, 397:164-72.

Elmoslemany R.M., Abdallah O.Y., El-Khordagui L.K., Khalafallah N.M. (2012). Propylene glycol liposomes as a topical delivery system for miconazole nitrate: comparison with conventional liposomes. *AAPS PharmSciTech*, 13:723-31.

Elsayed M.M.A., Abdallah O.Y., Naggar V.F., Khalafallah N.M. (2007a). PG-liposomes: novel lipid vesicles for skin delivery of drugs. *J Pharm Pharmacol*, 59:1447-50.

Elsayed M.M.A., Abdallah O.Y., Naggar V.F., Khalafallah N.M. (2007b). Lipid vesicles for skin delivery of drugs: Reviewing three decades of research. *Int J Pharm*, 332:1-16.

Engesland A., Skar M., Hansen T., Škalko-Basnet N., Flaten G.E. (2013). New applications of phospholipid vesicle-based permeation assay: permeation model mimicking skin barrier, *J Pharm Sci*, 102 1588-1600.

Ferderber, K., Hook, S., Rades, T. (2009). Phosphatidyl choline–based colloidal systems for dermal and transdermal drug delivery. *J Liposome Res*, 19:267-77.

Flaten G.E., Bunjes H., Luthman K., Brandl M. (2006a). Drug permeability across a phospholipid vesicle-based barrier 2. Characterization of barrier structure, storage stability and stability towards pH changes. *Eur J Pharm Sci*, 28:336-43.

Flaten G.E., Dhanikula A.B., Luthman K., Brandl M. (2006b). Drug permeability across a phospholipid vesicle based barrier: a novel approach for studying passive diffusion. *Eur J Pharm Sci*, 27:80-90.

Flaten G.E., 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*, 30:324-32.

Ghanbarzadeh S., Arami S. (2013). Enhanced transdermal delivery of diclofenac sodium via conventional liposomes, ethosomes, and transferosomes. *BioMed Res Int*, 2013:1-7.

Gillet A., Compere P., Lecomte F., Hubert P., Ducat E., Evrard B., Piel G. (2011a). Liposome surface charge influence on skin penetration behavior. *Int J Pharm*, 411:223-31.

Gillet A., Lecomte F., Hubert P., Ducat E., Evrard B., Piel G. (2011b). Skin penetration behaviour of liposomes as a function of their composition. *Eur J Pharm Biopharm*, 79:43-53.

Herkenne C., Naik A., Kalia Y.N., Hadgraft J., Guy R.H. (2007). Ibuprofen transport into and through skin from topical formulations: *in vitro-in vivo* comparison. *J Invest Dermatol*, 127:135-42.

Honeywell-Nguyen P.L., Bouwstra J.A. (2005). Vesicles as a tool for tansdermal and dermal delivery. *Drug Discov Today Technol*, 2:67-74.

Hurler J., Berg O.A., Skar M., Conradi A.H., Johnsen P.J., Škalko-Basnet N. (2012). Improved burns therapy: liposomes-in-hydrogel delivery system for mupirocin. *J Pharm Sci*, 101:3906-15.

Jain S., Jain P., Umamaheshwari R.B., Jain N.K. (2003). Transfersomes - a novel vesicular carrier for enhanced transdermal delivery: Development, characterization, and performance evaluation. *Drug Dev Ind Pharm*, 29:1013-26.

Kanzer, J., Tho, I., Flaten, G.E., Mägerlein, M., Hölig, P., Fricker, G., Brandl, M. (2010). In-vitro permeability screening of melt-estrudate formulations containing poorly water-soluble drug compounds using the phospholipid vesicle-based barrier. *J Pharm Pharmacol*, 62:1591-98.

Kawano K., Onose E., Hattori Y., Maitani Y. (2009). Higher liposomal membrane fluidity enhances the *in vitro* antitumor activity of folate-targeted liposomal mitoxantrone. *Mol Pharm*, 6:98-104.

Kikuchi H., Yamauchi H., Hirota S. (1994). A polyol dilution method for mass production of liposomes. *J Liposome Res*, 4:71-91.

Lane M.E. (2013). Skin penetration enhancers. Int J Pharm, 447:12-21.

Manconi M., Mura S., Sinico C., Fadda A.M., Vila A.O., Molina F. (2009). Development and characterization of liposomes containing glycols as carriers for diclofenac. *Colloid Surface A*, 342:53-58.

Oliveira G., Beezer A.E., Hadgraft J., Lane M.E. (2010). Alcohol enhanced permeation in model membranes. Part I. Thermodynamic and kinetic analyses of membrane permeation. *Int J Pharm*, 393:61-67.

Pavelić Ž., Škalko-Basnet N., Filipović-Grčić J., Martinac A., Jalšenjak I. (2005). Development and *in vitro* evaluation of a liposomal vaginal delivery system for acyclovir. *J Control Release*, 106:34-43.

Prow T.W., Grice J.E., Lin L.L., Faye R., Butler M., Becker W., Wurm E.M., Yoong C., Robertson T.A., Soyer H.P., Roberts M.S. (2011). Nanoparticles and microparticles for skin drug delivery. *Adv Drug Deliv Rev*, 63:470-91.

Salerno C., Carlucci A.M., Bregni C. (2010). Study of *in vitro* drug release and percutaneous absorption of fluconazole from topical dosage forms. *AAPS PharmSciTech*, 11:986-93.

Sinko B., Garrigues T.M., Balogh G.T., Nagy Z.K., Tsinman O., Avdeef A., Takacs-Novak K. (2012). Skin-PAMPA: a new method for fast prediction of skin penetration, *Eur J Pharm Sci*, 45:698-707.

Subongkot, T., Duangjit, S., Rojanarata, T., Opanasopit, P., Ngawhirunpat, T. (2012). Ultradeformable liposomes with terpenes for delivery of hydrophilic compound. *J Liposome Res*, 22:254-62.

Šentjurc 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*, 59:87-97.

Touitou E., Dayan N., Bergelson L., Godin B., Eliaz M. (2000) Ethosomes - novel vesicular carriers for enhanced delivery: characterization and skin penetration properties. *J Control Release*, 65:403-18.

Vanić Ž., Barnert S., Süss R., Schubert R. (2012). Fusogenic activity of PEGylated pH-sensitive liposomes, *J Liposome Res*, 22:148-57.

Vanić Ž., Hafner A., Bego M., Škalko-Basnet N. (2013). The characterization of various deformable liposomes with metronidazole. *Drug Dev Ind Pharm*, 39:481-88.

Vanić Ž., Hurler J., Ferderber K., Golja Gašparović P., Škalko-Basnet N., Filipović-Grčić J. (2014). Novel vaginal drug delivery system: deformable propylene glycol liposomes-in-hydrogel. *J Liposome Res*, 24:27-36.

Verma D.D., Verma S., Blume G., Fahr A. (2003). Particle size of liposomes influences dermal delivery of substances into skin. *Int J Pharm*, 258:141-51.

Zhao Y.Z., Dai D.D., Lu C.T., Chen L.J., Lin M., Shen X.T., Li X.K., Zhang M., Jiang X., Jin R.R., Li X., Lv H.F., Cai L., Huang P.T. (2013). Epirubicin loaded with propylene glycol liposomes significantly overcomes multidrug resistance in breast cancer. *Cancer Lett*, 330:74-83.

Table 1. The composition and physicochemical properties of different classes of liposomes containing DCS

Formulation code	Composition			Mean diameter (nm)		Polydispersity index, PI		Zeta potential (mV)
	S 75 (mg)	SDCh (mg)	PG (g)	Non-extruded	Extruded (3x400 nm)	Non-extruded	Extruded (3x400 nm)	
CL-A	200	-	-	278 ± 3	157 ± 4	0.602 ± 0.084	0.186 ± 0.021	-60.7 ± 0.9
CL-B	400	-	-	312 ± 9	161 ± 3	0.733 ± 0.147	0.208 ± 0.057	-58.8 ± 0.4
DL-A	170	30	-	239 ± 9	147 ± 4	0.442 ± 0.024	0.285 ± 0.025	-58.6 ± 0.8
DL-B	340	60	-	240 ± 5	153 ± 1	0.452 ± 0.067	0.296 ± 0.031	-57.3 ± 0.3
PGL-10-A	200	-	1	269 ± 5	153 ± 1	0.583 ± 0.176	0.189 ± 0.022	-67.8 ± 0.6
PGL-10-B	400	-	1	293 ± 2	156 ± 4	0.688 ± 0.135	0.210 ± 0.049	-69.3 ± 1.4
PGL-30-A	200	-	3	390 ± 11	161 ± 4	0.689 ± 0.122	0.198 ± 0.018	-70.0 ± 0.8
PGL-30-B	400	-	3	405 ± 17	166 ± 1	0.454 ± 0.156	0.220 ± 0.041	-67.9 ± 0.6

CL – conventional liposomes; DL – deformable liposomes; PGL – propylene glycol liposomes; A - liposomes prepared with lower lipid concentration (26 mM total lipids); B – liposomes prepared with higher lipid concentration (52 mM, total lipids)
The amounts of S 75, SDCh and PG correspond to 10 ml of liposome suspension. The values denote the mean ± S.D. (n=3).
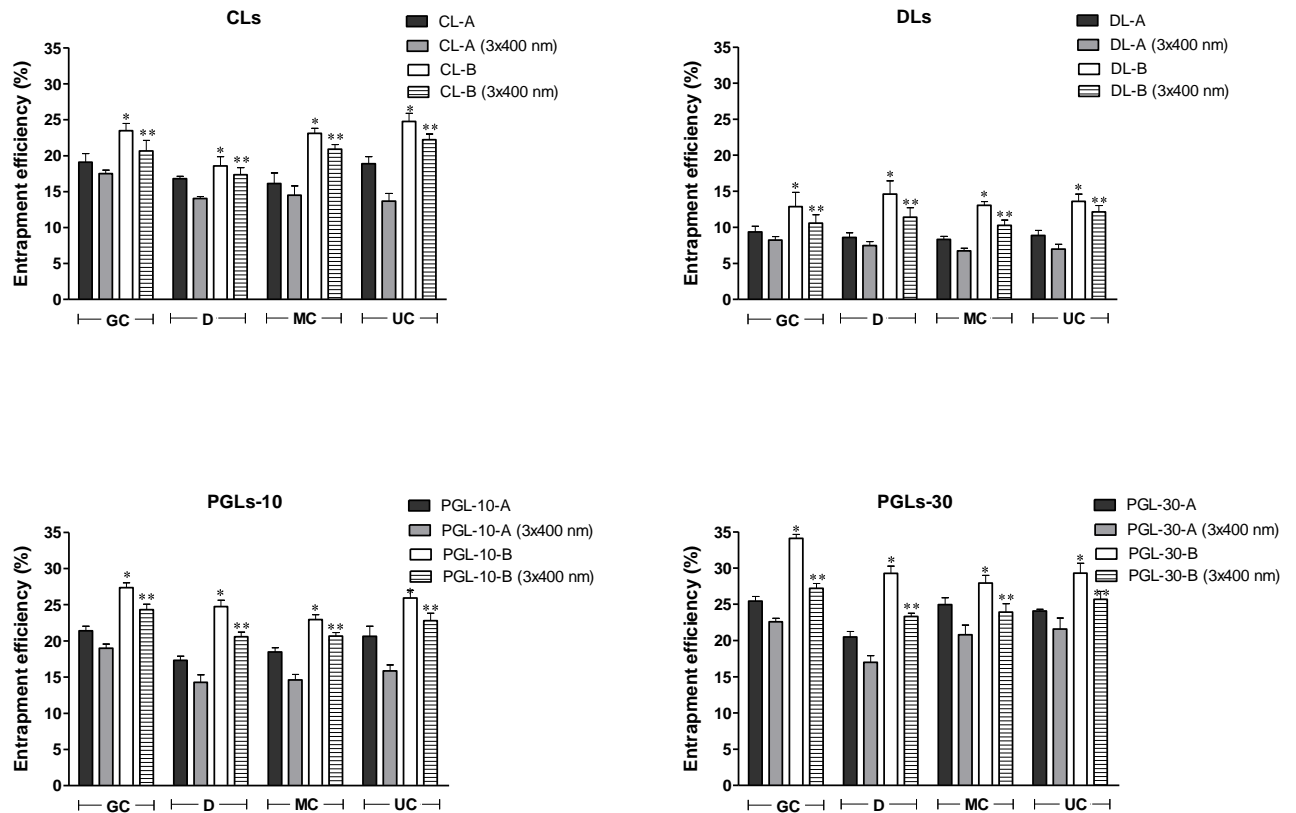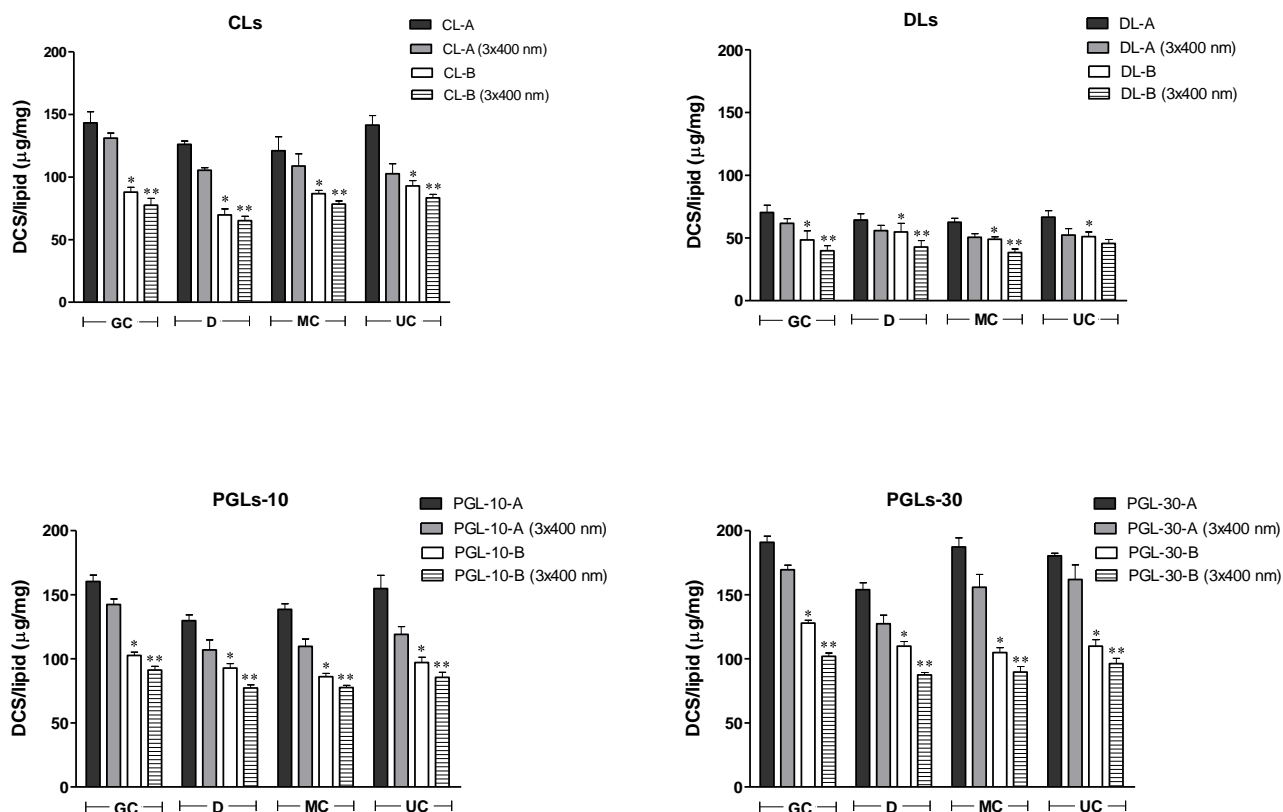
Figure 1. The entrapment of DCS (%) in various liposomes in relation to different separation methods (GC – gel chromatography; D – dialysis; MC – minicolumn centrifugation; UC – ultracentrifugation). All of the liposomes were prepared in two different concentrations of lipids: 26 mM (CL-A, DL-A, PGL-10-A and PGL-30-A) and 52 mM (CL-B, DL-B, PGL-10-B and PGL-30-B). Data are expressed as the mean ± S.D. (n = 3). *Differs from the liposomes of the lower lipid concentration (t-test, $p < 0.05$). **Differs from the extruded liposomes of the lower lipid concentration (t-test, $p < 0.05$).

Figure 2. The entrapment of DCS (drug/lipid ratio) in various liposomes in relation to different separation methods (GC – gel chromatography; D – dialysis; MC – minicolumn centrifugation; UC – ultracentrifugation). All of the liposomes were prepared in two different concentrations of lipids: 26 mM (CL-A, DL-A, PGL-10-A and PGL-30-A) and 52 mM (CL-B, DL-B, PGL-10-B and PGL-30-B). Data are expressed as the mean ± S.D. (n = 3). *Differs from the liposomes of the lower lipid concentration (t-test, $p < 0.05$). **Differs from the extruded liposomes of the lower lipid concentration (t-test, $p < 0.05$).
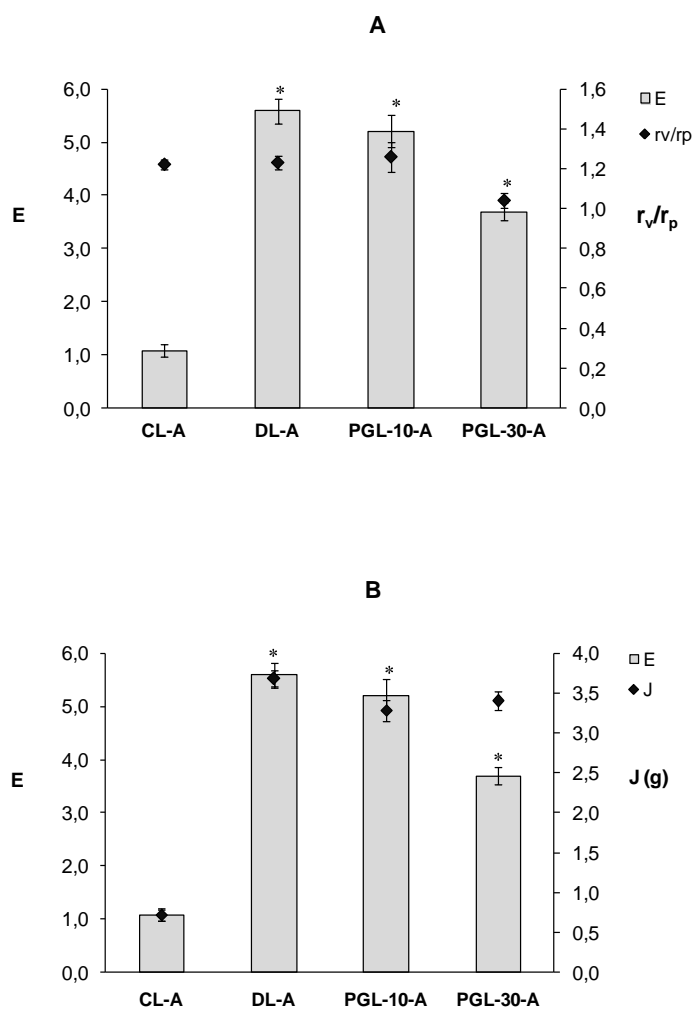
Figure 3. The degree of liposome elasticity (E) as a function of r_v/r_p (A) and J (B). E was determined for extruded liposomes (3x400 nm) at a constant pressure of 5 bar (for details, see Section 2.6.). The values denote the mean ± S.D. (n = 3). *Statistically significant difference of the E compared to the E of CL-A (ANOVA, $p < 0.05$).
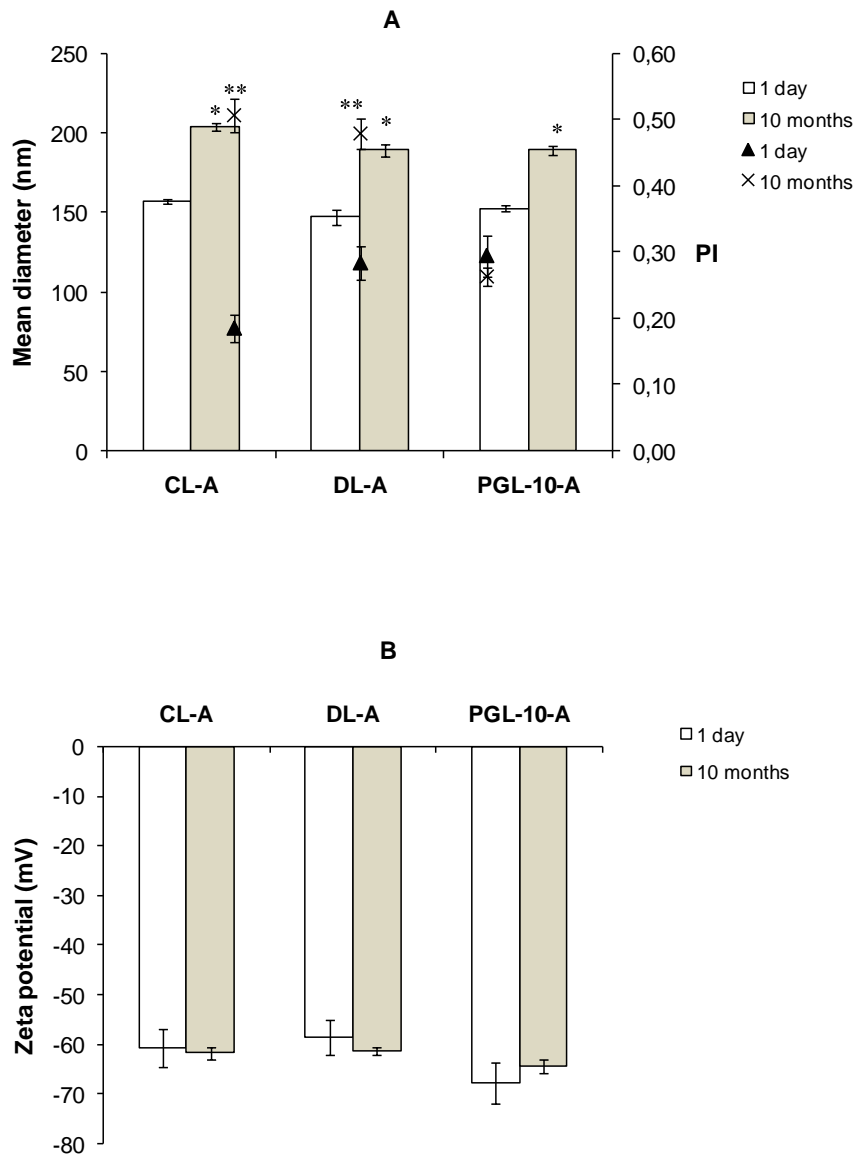
Figure 4. The storage stability of the extruded liposome formulations after ten months of storage at 4°C: mean diameters and PI (A); zeta potentials (B). The indicated values are the mean ± S.D. (n = 3). *Mean diameters were significantly different (t-test, $p < 0.05$) after ten months. **PI was significantly different (t-test, $p < 0.05$) after ten months.

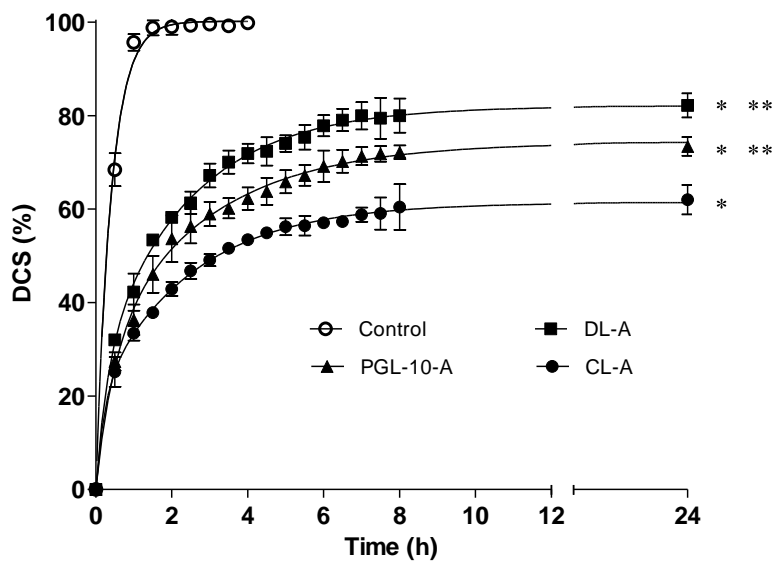Figure 5. The cumulative release of DCS from liposome formulations performed on a Franz cell diffusion system under sink conditions using cellulose membranes and a heating circulator set to 32°C. The values denote the mean ± S.D. (n = 3). *Statistically significant difference (ANOVA, $p < 0.05$) compared to the control. **Statistically significant difference (ANOVA, $p < 0.05$) compared to CL-A.
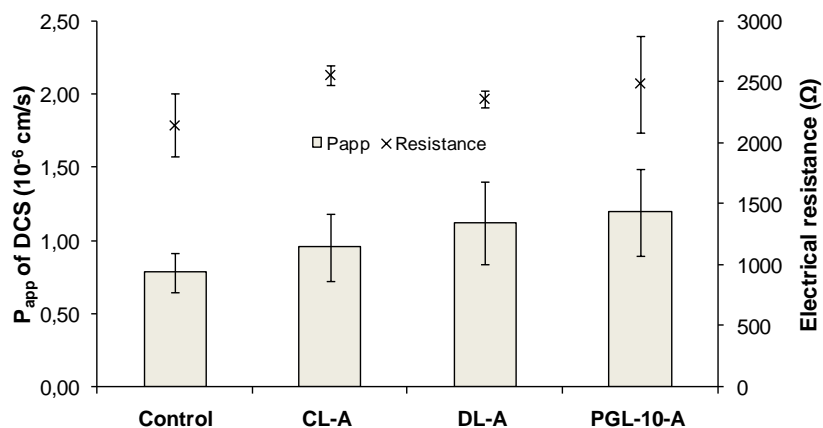
Figure 6. The permeability of DCS from the liposomal formulations on PVPA$_c$ barrier. The values denote the mean ± S.D. (n = 3).

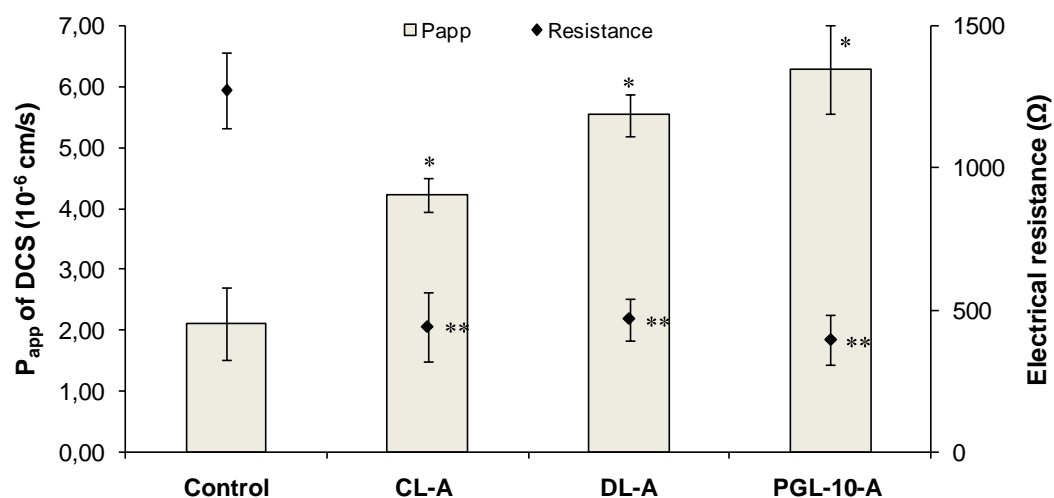

Figure 7. The permeability of DCS from the liposomal formulations using a PVPA$_s$ barrier. The values denote the mean ± S.D. (n = 6). *P$_{app}$ is significantly different (ANOVA, $p < 0.05$) from the control. **Electrical resistance is significantly different (ANOVA, $p < 0.05$) from the control.