European Journal of Pharmaceutical Sciences 216 (2026) 107363

Contents lists available at ScienceDirect

European Journal of Pharmaceutical Sciences

journal homepage: www.elsevier.com/locate/ejps

Design and optimisation of phospholipid vesicles for topical betamethasone dipropionate

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ARTICLE INFO

Keywords:
Betamethasone dipropionate
Phospholipid vesicles
Ultrastructural analysis
Keratinocytes proliferation
Skin delivery

ABSTRACT

Betamethasone dipropionate is a topical corticosteroid widely used to treat common dermatoses and skin disorders. However, the poor skin penetration of traditional betamethasone dipropionate formulations can impair therapeutic outcomes. In the present study, betamethasone dipropionate was formulated in phospholipid vesicles to maximise its benefits and minimise its side effects. Liposomes and transfersomes were developed using the direct sonication method and thoroughly characterised by light scattering, cryo-transmission electron microscopy (TEM), small-angle x-ray scattering (SAXS), rheological analysis and skin permeation studies. Human skin epithelial-like cells were used to study how the phospholipid vesicles affected cell proliferation and morphology. Both the liposomes and transfersomes were nanosized, spherical and predominantly unilamellar, but the transfersomes were smaller, more homogeneous and stable on storage due to the presence of Tween® 80. The latter was also responsible for a higher viscosity, which decreased at 32 °C, the temperature of the skin surface, as determined by rheological analysis. Furthermore, tests of the formulations in skin cells showed the absence of cytotoxicity and increased skin permeation. Phospholipid vesicles represent a promising approach for betamethasone dipropionate delivery, as they are characterised by high stability and cytocompatibility. These properties may improve the therapeutic outcomes and minimise the side effects of betamethasone dipropionate, encouraging further investigation to confirm the effective and safe use of betamethasone dipropionate nanoformulations on the skin.

1. Introduction

Corticosteroids are among the most commonly prescribed medications worldwide because of their strong immune-modulatory effects (Ramamoorthy and Cidlowski, 2016). Regarding dermatological disorders, corticosteroids can be used systemically for various conditions, including acute hypersensitivity reactions – such as urticaria, erythema multiforme, allergic contact dermatitis, and drug-induced skin eruptions, allergic vasculitis, bullous diseases and other skin related conditions (Jackson et al., 2007; Williams and Nesbitt, 2001). Although corticosteroids are effective in treating inflammatory and immune disorders systemically, their long-term or high-dose use is restricted due to significant adverse effects. These include abnormalities of fat distribution, skin and muscle atrophy, cardiovascular complications, gastrointestinal side effects, water/electrolyte imbalances, and increased susceptibility to infections (Schäcke et al., 2002; Yu et al., 2018). Therefore, in long-term treatments, topical administration is generally preferred to minimise side effects (Brazzini and Pimpinelli, 2002). Topical corticosteroids are an essential tool for treating inflammatory skin conditions. They are classified by the strength and risk of adverse effects, which increase with prolonged use, large area of application, higher potency, occlusion and application on thinner skin. Betamethasone, a powerful topical corticosteroid, is well known for its immunosuppressive, anti-inflammatory, and anti-proliferative effects (Wang et al., 2025). Common betamethasone salts, such as betamethasone dipropionate (BD), can amplify betamethasone's anti-inflammatory potency by up to 450 times (Toledo et al., 2024). However, this potency is associated with a high risk of topical and systemic adverse effects. Betamethasone dipropionate is available in several formulations, including ointments, creams, lotions and gels. A major limitation of

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https://doi.org/10.1016/j.ejps.2025.107363
Received 11 August 2025; Received in revised form 8 October 2025; Accepted 3 November 2025
Available online 4 November 2025
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these traditional topical formulations is a reduced skin penetration and permeation into deeper layers, failing to provide optimal results due to inadequate drug absorption, potential side effects and reduced patient adherence (Cláudia Paiva-Santos et al., 2022). Therefore, a key challenge in developing topical formulations is the improvement of drug penetration through the skin, especially the thickened *stratum corneum* that is frequent in some skin disorders, while ensuring that the skin barrier function remains intact in the long term (Baboota et al., 2011). To increase the skin penetration/permeation of drugs, innovative delivery systems have been explored in recent decades, such as phospholipid vesicles, cyclodextrins, solid lipid nanoparticles, lyotropic liquid crystals and nanosuspensions (Chantaburanan et al., 2023; De Luca et al., 2024; Shaikh and Kale, 2020; Wang et al., 2022; Zvonar Pobirk et al., 2024). They can be administered alone or incorporated in gels, creams and other systems (e.g., polymeric microneedles) to by-pass the *stratum corneum* (Anjani et al., 2023; Casula et al., 2023; Zhou et al., 2020). Particular attention has been given to phospholipid-based nanocarriers, since phospholipids occur naturally and are essential components of cellular membranes (Drescher and van Hoogevest, 2020; Liu and Hu, 2007). Comprehensive preclinical studies have revealed that multiple factors, including composition, size, surface properties, and their combinations, influence the delivery and absorption of phospholipid nanocarrier-associated drugs through the skin, ultimately affecting their therapeutic effectiveness (Vanic et al., 2015). Among phospholipid-based nanocarriers, phospholipid vesicles (e.g., liposomes) are well known for the optimal biocompatibility, versatility, and the ability to entrap a variety of drugs, while preventing degradation, regulating release, and promoting penetration through the skin (Carita et al., 2017; Sala et al., 2018).

Focusing on skin delivery, modified phospholipids vesicles containing chemical penetration enhancers, such as transfersomes, ethosomes, and Penetration Enhancer-containing Vesicles (PEVs), can further enhance drug absorption by (i) destabilising the vesicle bilayers and increasing their deformability, and (ii) interacting with the skin components and promoting the penetration of the vesicles and their cargo (Cevc et al., 2002; Elsayed et al., 2006; Pappalardo et al., 2021). The aim of this study was to formulate BD in liposomes and transfersomes (i.e., Tween® 80-modified liposomes) as a strategy to boost skin application of betamethasone dipropionate. Key physico-chemical and technological features of both liposomes and transfersomes, such as size, charge, morphology and lamellarity, storage stability, and rheological behavior, were studied. The effect of the vesicles on cell proliferation and morphology was investigated in human skin epithelial-like cells. Ultimately, to the best of our knowledge, this is the first study that reports on BD liposomes and transfersomes which were produced by a facile, solvent-free method and were tested on the skin for their penetration/permeation capabilities in comparison with a marketed topical cream.

2. Materials and methods

2.1. Materials

Lipoid S100 (soy phosphatidylcholine content ≥ 94 %) was purchased from Lipoid GmbH (Ludwigshafen, Germany). Tween® 80 (polysorbate 80) was purchased from Galeno (Carmignano, Italy). Betamethasone dipropionate (BD; 98.6 % purity) was obtained from Fagron BV (Rotterdam, the Netherlands). Sodium dodecyl sulfate (SDS) was provided by Merck KGaA (Darmstadt, Germany). Cell culture reagents were purchased from Sigma-Aldrich (St. Louis, MO, US).

2.2. Vesicles' production

To produce liposomes, Lipoid S100 was dispersed in ultrapure water, in presence or absence of BD, according to the amounts reported in Table 1, and sonicated at 10 cycles of 5 s on and 2 s off with an ultrasound disintegrator (Soniprep 150 plus; MSE Crowley, London, UK) (Caddeo et al., 2018; De Luca et al., 2024). Transfersomes were produced by applying the same procedure used for liposomes, with the addition of Tween® 80 to the aqueous dispersion (Table 1).

Table 1
Composition of liposomes and transfersomes. S100, phospholipid; BD, betamethasone dipropionate.

Formulation	S100	BD	Tween® 80	H_2O
Empty liposomes	0.48 g			4 ml
BD liposomes	0.48 g	2.56 mg		4 ml
Empty transfersomes	0.48 g		0.2 ml	3.8 ml
BD transfersomes	0.48 g	2.56 mg	0.2 ml	3.8 ml

The formulations were prepared using 0.64 mg/ml of BD (Table 1), which corresponds to 0.50 mg/ml of betamethasone free base, dosage of commercially available topical medications (e.g., Beloderm®, Belupo, Ljubljana, Slovenia).

2.3. Vesicles' size, homogeneity, charge and entrapment efficiency

The mean diameter, polydispersity index, and zeta potential of the vesicles were measured via dynamic and electrophoretic light scattering (Zetasizer nano ZS; Malvern Panalytical, Worcestershire, UK). For the measurements, samples were diluted with ultrapure water (1:100 *v/v*) and analysed at 25 °C.

The BD vesicle dispersions (1 ml; $n = 4$) were dialysed against water (2 l) to allow removal of the non-incorporated BD by loading into 12–14 kDa MW cut-off tubing (Spectrum Laboratories Inc., Breda, The Netherlands). After 2 h under gentle stirring, both non-dialysed and dialysed dispersions were diluted with methanol (1:50 *v/v*) to disrupt the vesicles and allow BD quantification by HPLC (Alliance 2695, Waters, Milan, Italy). The entrapment efficiency of the vesicles was calculated as a percentage of BD in dialysed *versus* non-dialysed samples. The drug loading was calculated as a percentage of the entrapped drug relative to the total amount of entrapped drug plus the lipid content used in the formulation (Caddeo et al., 2018). BD was quantified at $\lambda = 238$ nm using an X-Terra C18 column (Waters) and a mobile phase of acetonitrile:water:acetic acid (69:30.97:0.03 *v/v*) run at 0.7 ml/min (Gonzalez-Paredes et al., 2010).

The stability of the vesicles was studied by measuring their mean diameter, polydispersity index, and zeta potential during 3 months of storage at 4 ± 1 °C.

2.4. Vesicles' ultrastructure

The ultrastructure of the liposomes and transfersomes was investigated via cryogenic-Transmission Electron Microscopy (cryo-TEM) and Small-Angle X-ray Scattering (SAXS). For cryo-TEM analyses, 3 μl of each vesicle dispersion was deposited onto glow-discharged grids, blotted, and vitrified by freezing into ethane using an automatic plunge freezer (EM GP, Leica Microsystems Inc., Deerfield, IL, US). The vitrified samples were observed on a 200 kV electron microscope (Tecnai G2 T20 Sphera, FEI Company/ThermoFisher Scientific, Waltham, MA, US) and images were acquired using a 4 × 4 k CCD camera (TemCam-XF416, TVIPS GmbH, Gilching, Germany) under low electron dose conditions using the camera in binning mode 1 and at a 25,000 × magnification.

For SAXS measurements, a Kratky compact camera system (Anton Paar, Graz, Austria) with a block collimating unit, coupled to an X-ray generator (Bruker AXS, Karlsruhe, Germany) featuring a sealed Cu-anode X-ray tube was used. The X-ray beam, produced with a Ni filter, had a wavelength $\lambda = 0.154$ nm (Cu Kα-line), and the tube operated at 40 kV and 50 mA. The vesicle dispersions were loaded into a quartz capillary placed in a thermally controlled sample holder, aligned in the X-ray beam. Scattering intensities were recorded at 25 and 32 °C using a Mythen 1 K detector (Dectris, Baden, Switzerland). For each sample and

temperature, three SAXS curves were recorded, averaged to ensure reliable statistics, and corrected for solvent scattering. The SAXS profiles report the scattered radiation intensity I as a function of the momentum transfer q, as shown in Eq. (1), where 2θ is the scattering angle.

$$q = 4\pi\sin\theta/\lambda \tag{1}$$

2.5. Vesicles' rheological properties

The rheological behaviour of liposomes and transfersomes, both empty and loaded with BD, was studied using a Physica MCR 301 rheometer equipped with a RheoCompass software (Anton Paar GmbH, Graz, Austria). Measurements were carried out at 25 ± 0.1 °C and 32 ± 0.1 °C using a concentric cylinder measuring system CC27. Rotational tests were performed varying the shear rate from 1 to 100 s^{-1} and the viscosity η was calculated from Eq. (2) where τ is the shear stress and $\dot{\gamma}$ is the shear rate:

$$\eta = \tau/\dot{\gamma} \tag{2}$$

2.6. Vesicles' skin permeation

To assess BD permeation on the skin, BD liposomes, BD transfersomes and a commercial BD cream – having the same BD concentration as the vesicles: 0.64 mg/ml – were tested on full-thickness skin specimens from newborn Goland–Pietrain hybrid piglets (1–1.5 kg), which died of natural causes and were provided by a local slaughterhouse. The skin specimens ($n = 4$ per formulation), previously excised from the piglets and stored at −80 °C, were thawed, equilibrated in saline, and placed on Franz diffusion vertical cells (diffusion area: 0.785 cm^2) with the *stratum corneum* facing the donor compartments. The receptor compartments were filled with 5.5 mL of saline constantly stirred and thermostated at 37 °C, for 8 h. The Franz cells were maintained at 37 °C by water jackets, allowing the formation of a temperature gradient between the receptor medium and the ambient environment (22 ± 1 °C), which resulted in a skin surface temperature of approximately 32 °C.

200 μL of BD liposomes or transfersomes, or 200 mg of BD cream were applied onto the *stratum corneum*, in the donor compartments. No leakage of the formulations from the donor compartments was observed. At predetermined timepoints (5, 15, 30, 45 min and 1, 1.5, 2, 3, 4, 5, 6, 7 and 8 h), the saline in the receptor compartments was completely withdrawn and refreshed to ensure sink conditions. All the obtained samples were freeze-dried using a Buchi Lyovapor L-200 (Milan, Italy), redispersed in 2 mL of methanol and vortexed for 30 s. After 8 h, the skin surface was gently blotted on filter paper. The *stratum corneum* was removed by tape-stripping (Casula et al., 2023) and the remaining skin specimens were cut in small pieces. The samples were soaked in 2 mL of methanol and sonicated for 2 min to extract the accumulated BD. The tapes and tissue suspensions, and the receptor solutions were filtered and analyzed by HPLC to quantify BD (see Section 2.3).

2.7. Vesicles' safety assessment

2.7.1. Cell culture and treatment

Human keratinocytes (NCTC 2544, ICLC, University of Genoa, Genoa, Italy) were cultured as adherent monolayers at 37 °C in a humidified atmosphere with 5 % CO_2. When 80–90 % confluence was reached, the cells were subcultured using trypsin/EDTA (Promega Corporation, Madison, USA) and then maintained in Eagle's Minimal Essential Medium supplemented with fetal bovine serum (10 % *v/v*), 2 mM *L*-glutamine, non-essential aminoacids (1 % *v/v*), and penicillin/streptomycin mixture (1 % *v/v*).

2.7.2. Cell proliferation assay

The keratinocytes were seeded in 96-well plates (0.5 × 10^4 cells/well) and incubated overnight in supplemented medium to adhere. The cells were then treated with the test formulations (liposomes and transfersomes, both empty and loaded with BD). For the purpose of comprehensive interpretation, we also tested the individual components (BD, Lipoid S100, Tween® 80). Supplemented medium was used as a negative control and SDS was used as a positive control.

Volumes of vesicle dispersions were applied to cells, as the formulations are liquid dispersions. Accordingly, the results are presented in terms of the volume of dispersion applied per well, as this provides an accurate representation of the actual amount of formulation the cells were exposed to. For the test formulations, 1, 2, 3, 4, and 5 μl was added to the cells, with the total volume in each well being 100 μl, leading to final concentrations of BD at 6.4, 12.8, 19.2, 25.6 and 32.0 μg/ml, respectively. Each individual component was tested at a concentration corresponding to its content in the test formulations at the highest tested concentration (i.e. 5 μl/100 μl). The mass of the tested SDS corresponded to the total mass of substances forming either BD liposomes (BD, Lipoid S100) or BD transfersomes (BD, Lipoid S100, Tween® 80) for each tested concentration. Following a 24-h incubation, cell proliferation was assessed using the MTS assay (CellTiter 96 Aqueous One Solution Cell Proliferation Assay; Promega, Madison, WI, US). The assay is based on the conversion of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium into a soluble colored formazan product by mitochondrial dehydrogenase enzymes in metabolically active cells. Cell proliferation, which is related to the metabolic activity of cells, was quantified by reading the absorbance of the formazan product at 492 nm using a Safire2 microplate detection system (Tecan, Zürich, Switzerland). Experiments were performed in sextuplicate. The results were expressed as the absorbance ratio of treated to untreated control cells, and the percentage of cell proliferation was calculated according to Eq. (3), where A_S is the absorbance of the treated cells, A_{S0} the absorbance of the sample in cell-free medium, A_C the absorbance of the untreated cells, and A_{C0} the absorbance of the medium alone:

$$\%\ of\ cell\ proliferation = ((As - Aso)/(Ac - Aco) \times 100 \tag{3}$$

2.7.3. Morphological evaluation of keratinocytes

The keratinocytes were seeded in 12-well plates (2 × 10^5 cells/well) and incubated overnight in supplemented medium to adhere. The cells were then treated with the test formulations (liposomes and transfersomes, both empty and loaded with BD), supplemented medium (negative control) or SDS (positive control). The same concentrations as those used in the cell proliferation assay were applied. Following a 24-h incubation, cell morphology was examined using a phase-contrast inverted light microscope (Olympus CKX41, Tokyo, Japan) and photomicrographs were taken at 10 × magnification.

2.8. Statistical analysis

Data are presented as means ± standard deviation (SD). Statistically significant differences ($p < 0.05$) were determined by the Student's *t*-test (pairwise comparisons of groups) using Microsoft Excel (Office 365).

3. Results and discussion

3.1. Vesicles' features

This study aimed to develop an efficient formulation for BD, a corticosteroid with anti-inflammatory and immunosuppressive activity widely used topically to treat a variety of skin conditions, such as dermatitis, eczema and psoriasis, but characterised by both local and systemic adverse effects (Hengge et al., 2006). Hence, there is a need for novel formulation approaches and technologies to develop safe and effective therapies. To this end, two nanoformulations were developed, namely liposomes and transfersomes, which are known to provide

enhanced bioavailability of their cargo in the skin, especially transfersomes by virtue of their deformable bilayer (Dragicevic and Maibach, 2024). A thorough characterisation was carried out to establish key characteristics of the vesicles and their safety in cell cultures. To assess the effect of BD on the vesicles' characteristics, empty vesicles were produced for a proper comparison. Table 2 summarises light scattering results. Empty liposomes were around 120 nm in size, slightly polydispersed (PI ~ 0.4), and negatively charged (−6 mV). The loading of BD significantly affected only the zeta potential, which became more negative (−14 mV; $p < 0.05$). These findings suggest a modification in the arrangement of the phospholipids to accommodate BD, which did not negatively affect the size and the polydispersity.

The impact of Tween® 80 in transfersomes was assessed by comparison with liposomes, empty or loaded with BD. Data in Table 2 show that the transfersomes, regardless of the loading of BD, were smaller (> 70 nm; $p < 0.01$), more homogeneous (PI ~ 0.26; $p < 0.01$), and more negatively charged (~ −20 mV; $p < 0.01$) than liposomes, preventing vesicles' aggregation by electrostatic repulsion (Bhattacharjee, 2016). Presumably, the surfactant, thanks to its amphiphilic character and large hydrophilic head group (HLB 14.9), helped the dissolution of BD and its intercalation within the phospholipid bilayers, which arranged more uniformly in small-sized vesicles (Fernández-García et al., 2020). Despite these differences, both the liposomes and the transfersomes demonstrated a high loading ability, as indicated by the entrapment efficiency (> 80 %; Table 2) and the drug loading levels approaching the theoretical values (~ 0.45 %; Table 2).

The stability of both liposomes and transfersomes was investigated. The formulations were stored for 3 months at 4 ± 1 °C, and the size, homogeneity, and charge were measured. Neither the liposomes nor the transfersomes showed any evidence of relevant physical modification. Liposomes remained around 120 nm with PI > 0.3 (Table 3), and transfersomes remained around 70 nm with PI < 0.3 (Table 4). Only the zeta potential values changed significantly ($p < 0.01$), more pronouncedly in liposomes, which can be correlated with the apparently non-ideal assembly of the phospholipids into inhomogeneous structures.

Table 3
Stability of liposomes, empty and loaded with betamethasone dipropionate (BD), stored at +4 °C and analysed after 2 weeks and 1-2-3 months. Mean diameter (MD), polydispersity index (PI), and Zeta potential (ZP) are presented. Data are means ± standard deviation (SD); $n = 4$.

Formulation	Time	MD nm ± SD	PI ± SD	ZP mV ± SD
Empty liposomes	2 weeks	131 ± 14.8	0.49 ± 0.02	−6 ± 2.3
BD liposomes		147 ± 18.3	0.45 ± 0.05	−18 ± 1.5
Empty liposomes	1 month	114 ± 5.9	0.32 ± 0.04	−15 ± 3.4
BD liposomes		142 ± 27.4	0.44 ± 0.09	−21 ± 8.1
Empty liposomes	2 months	114 ± 4.7	0.31 ± 0.06	−28 ± 3.9
BD liposomes		140 ± 11.9	0.39 ± 0.08	−29 ± 6.3
Empty liposomes	3 months	115 ± 0.4	0.34 ± 0.07	−33 ± 1.6
BD liposomes		130 ± 24.5	0.35 ± 0.07	−26 ± 2.2

Table 4
Stability of transfersomes, empty and loaded with betamethasone dipropionate, (BD), stored at +4 °C and analysed after 2 weeks and 1-2-3 months. Mean diameter (MD), polydispersity index (PI), and Zeta potential (ZP) are presented. Data are means ± standard deviation (SD); $n = 4$.

Formulation	Time	MD nm ± SD	PI ± SD	ZP mV ± SD
Empty transfersomes	2 weeks	71 ± 4.4	0.22 ± 0.02	−20 ± 2.0
BD transfersomes		84 ± 5.3	0.25 ± 0.01	−28 ± 3.0
Empty transfersomes	1 month	72 ± 1.5	0.23 ± 0.03	−22 ± 2.5
BD transfersomes		79 ± 5.5	0.21 ± 0.01	−27 ± 2.8
Empty transfersomes	2 months	71 ± 5.4	0.21 ± 0,05	−25 ± 0.8
BD transfersomes		79 ± 4.9	0.22 ± 0.02	−31 ± 1.3
Empty transfersomes	3 months	71 ± 5.6	0.22 ± 0.03	−26 ± 1.2
BD transfersomes		78 ± 5.4	0.22 ± 0.03	−28 ± 1.2

3.2. Vesicles' ultrastructure

Vesicles' ultrastructure was studied via cryo-TEM and SAXS. Cryo-TEM micrographs (Fig. 1) showed that liposomes were larger and more polydispersed than transfersomes, regardless of the presence of BD, in alignment with light scattering data (Table 2). In addition, the micrographs displayed spherical vesicles, predominantly unilamellar along with few oligo/multilamellar structures, especially in BD vesicles (Fig. 1).

SAXS is a powerful technique for the structural characterisation of self-assembled soft matter materials, including vesicular drug delivery systems. The method provides data on the vesicles' internal organisation, enabling the investigation of aggregation structures and potential structural perturbations induced by guest molecules. Such information is crucial for the development and optimisation of drug delivery systems, as their microstructure and the distribution of the drug within them might influence the therapeutic effectiveness (Di Cola et al., 2016). Considering that our formulations predominantly consist of phospholipids, which possess unique physical properties that allow them to form various supramolecular structures (Gómez-Gaete et al., 2008), SAXS was employed in this study to analyse the vesicular structure, assess the impact of drug loading on phospholipid structural assembly, and investigate the potential effects of temperature. Fig. 2 displays a scaled plot of the SAXS profiles (scattering intensity I *versus* scattering vector q) for our formulations at 25 °C (room temperature). The SAXS scattering curves for liposomes and transfersomes, both empty and loaded with BD, display a broad band in the lower q region, with no distinct peaks, confirming the unilamellar nature of the vesicles and indicating weak interactions between neighbouring vesicles. The scattering profiles show a smooth decay of $I(q)$ with increasing q, which is a clear indication of vesicle formation. In the absence of vesicles, $I(q)$ would remain q-independent, as there would be no electron density fluctuations to produce scattering (Caddeo et al., 2018; Shrestha et al., 2015). These findings suggest that the formulations consist of well-formed unilamellar vesicles with minimal aggregation and interaction between one another. Additionally, it is also important to emphasise that the incorporation of BD did not affect the vesicle structure as scattering curves of empty and BD liposomes and transfersomes were very similar. Noteworthy, the results obtained using the SAXS method are fully consistent with the structural data from cryo-TEM images. Furthermore, SAXS analysis was also conducted at 32 °C (representing skin surface temperature) for liposomes and transfersomes, both empty and loaded with BD. The results obtained at 32 °C (data not shown) were practically identical to those at 25 °C, confirming that the structural integrity of our formulations remains unchanged across temperatures relevant for skin delivery.

Table 2
Characteristics of liposomes and transfersomes, empty and loaded with betamethasone dipropionate (BD): mean diameter (MD), polydispersity index (PI), Zeta potential (ZP), entrapment efficiency (E) and drug loading (DL). Data are means ± standard deviation (SD); $n > 10$.

Formulation	MD (nm ± SD)	PI ± SD	ZP (mV ± SD)	E (% ± SD)	DL (% ± SD)
Empty liposomes	122 ± 14.7	0.41 ± 0.08	−6 ± 3.0		
BD liposomes	124 ± 20.1	0.39 ± 0.03	−14 ± 5.6	83 ± 9.5	0.44 ± 0.05
Empty transfersomes	72 ± 4.4	0.27 ± 0.02	−18 ± 2.0		
BD transfersomes	78 ± 4.6	0.26 ± 0.01	−22 ± 3.5	85 ± 9.8	0.45 ± 0.10

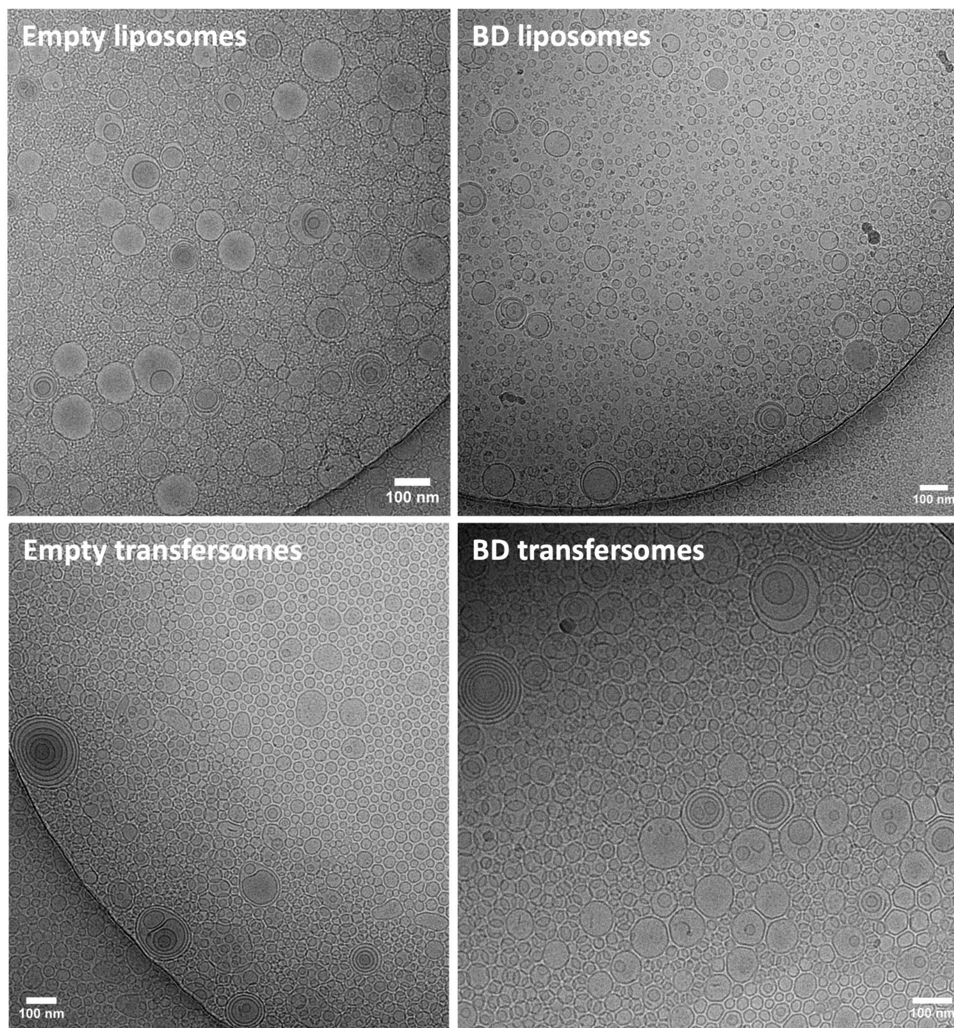

Fig. 1. Cryo-TEM micrographs of liposomes and transfersomes, empty and loaded with betamethasone dipropionate (BD).

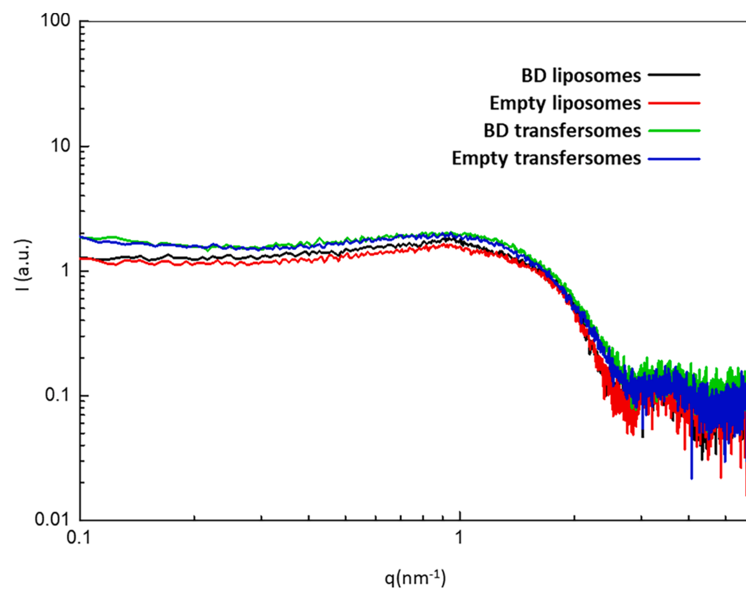

Fig. 2. SAXS scattering curves (25 °C) of liposomes and transfersomes, empty and loaded with betamethasone dipropionate (BD).

3.3. Vesicles' rheological properties

Rheological characterisation plays an important role in the development of skin drug delivery systems, as it reveals structural properties crucial for dermal administration. More specifically, rotational tests assess the system's flow behavior under applied stress, providing insights into structural changes that occur upon skin application. Furthermore, rheological parameters also reveal the impact of drug incorporation on the internal structure of the system, highlighting any potential structural modifications (Volontè et al., 2024; Zylberberg and Matosevic, 2016).

The viscosity of the vesicle formulations, obtained from rotational tests at 25 °C (room temperature), are presented in Fig. 3A. The curves distinctly show that the viscosity of both empty liposomes and transfersomes remained constant regardless of the increasing shear rate. This suggests that our formulations exhibit flow characteristics, classifying them as Newtonian fluids, a behavior typical of vesicle dispersions (Brown et al., 2011; Clares et al., 2014; Hasan et al., 2016). When comparing the viscosity of liposomes and transfersomes measured at the lowest shear rate (1 s^{-1}) at 25 °C, it is five times greater for transfersomes (10.3 mPa·s) compared to liposomes (2.3 mPa·s). A possible explanation is that Tween® 80, a nonionic surfactant, contains a hydrophilic polyoxyethylene segment that facilitates additional water binding and enhances membrane hydration (Simões et al., 2005; Vitek et al., 2023), potentially contributing to the higher viscosity of the dispersion. The supposition of increased membrane hydration of the vesicles also aligns well with the observations from light scattering analysis showing that transfersomes are smaller (> 70 nm) and more homogeneous (PI ~ 0.26) than liposomes (~ 120 nm, PI ~ 0.4). Moreover, due to the presence of the surfactant, which contributes to lowering the interfacial tension, transfersomes exhibit greater elasticity and deformability than conventional liposomes (Kapoor et al., 2019). This allows them to adapt more readily under shear forces and form tighter associations, increasing resistance to flow and consequently raising viscosity.

Upon incorporation of BD into liposomes and transfersomes, the same rheological behavior was observed as in the empty formulations. Namely, for both BD liposomes (2.4 mPa·s) and transfersomes (12.4 mPa·s), the viscosity range and the shape of the curve remained very similar to those of empty vesicles. This indicates that, at the tested concentration, BD does not induce any considerable modifications to the vesicle structure. It seems plausible that, due to its lipophilic nature (Takegami et al., 2008), BD is incorporated into the phospholipid bilayers of the vesicles, with the concentration of BD being so low that it does not lead to relevant change in the bilayers.

Further on, rotational tests for our formulations were subsequently performed at 32 °C, corresponding to skin surface temperature, revealing particularly intriguing results, as can be seen from Fig. 3B The shape of the viscosity curves at this temperature remained unchanged

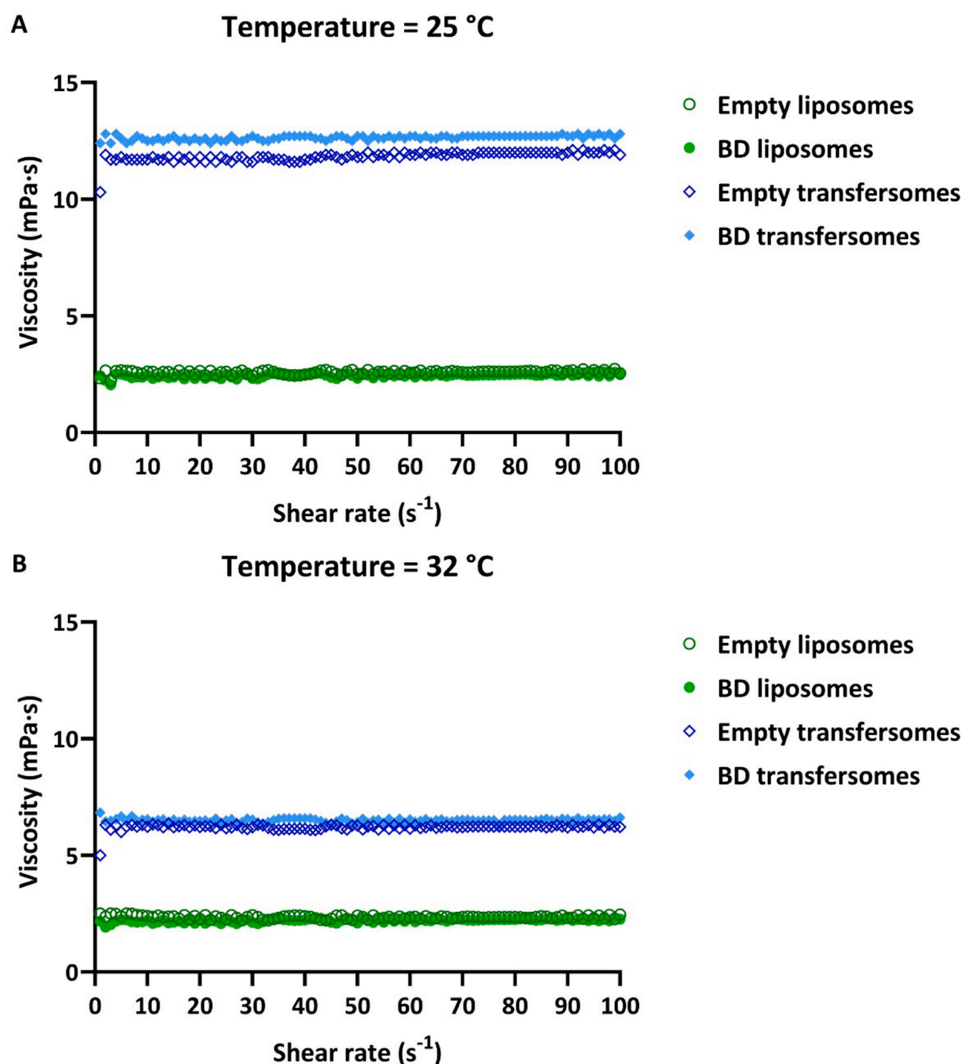

Fig. 3. Viscosity curves of liposomes and transfersomes, empty and loaded with betamethasone dipropionate (BD), determined at (A) 25 °C and (B) 32 °C.

for both liposomes and transferosomes, regardless of BD incorporation, indicating once again Newtonian fluid behavior. The viscosity of liposomes at 32 °C, empty (2.5 mPa·s) and BD-loaded (2.2 mPa·s), stayed approximately the same as at 25 °C. In contrast, for transfersomes, whether empty (5.0 mPa·s) or loaded with BD (6.8 mPa·s), the viscosity at skin temperature decreased by two-fold compared to room temperature. Consistent with published data (Ivanova et al., 2020), at elevated temperatures hydrogen bonding between water molecules and surfactant heads is weakened, resulting in a decreased viscosity. This phenomenon provides a meaningful explanation for the obtained results and, as highlighted above, reinforces the important role of Tween® 80 on the rheological characteristics of the vesicles.

For practical topical application, incorporation of the vesicles into a hydrogel matrix will also be considered in future studies to evaluate enhanced residence time on the skin and improved application convenience. This strategy is widely used in topical vesicular drug delivery and has been successfully applied in various studies (Abdulbaqi et al., 2018; Eroğlu et al., 2020; Ren et al., 2024).

3.4. Vesicles' skin penetration/permeation

Fig. 4 presents the results of BD skin permeation studies involving the application of BD liposomes, BD transfersomes, and a commercial BD cream, all having the same BD concentration (0.64 mg/ml) on newborn pig skin. This skin is widely used in the scientific community for its similarity with human skin and – being obtained from animals that died of natural causes – represents an ethically acceptable alternative for preliminary permeation studies (Cilurzo et al., 2007). In terms of drug accumulation within the skin, BD transfersomes achieved the highest retention, with 23 % of the applied dose, followed by the commercial BD cream (21 %) and BD liposomes (18 %), with the amount recovered upon liposomes application being the only one significantly different from the other two formulations ($p < 0.01$ *versus* transfersomes, $p < 0.05$ *versus* cream). In terms of permeation into the receptor compartment, both liposomes and transfersomes showed high drug permeation (7 and 11 %, respectively), which were both significantly higher ($p < 0.05$) than the cream (4 %). The overall results highlighted an enhanced BD skin penetration/permeation by liposomes, which provided a moderate retention in the skin and a higher permeation than the cream, highlighting a possible modification of the conformational skin structure due to the formulation components that might increase drug permeation (Hasanovic et al., 2011). Transfersomes exhibited the best performance owing to their superior flexibility and deformability, provided by Tween® 80, that facilitate penetration through the *stratum corneum*, by squeezing through tight intercellular spaces and pores, and distribution into deeper skin layers driven by transepidermal osmotic gradient (Dixena et al., 2024; Lai et al., 2020).

3.5. Vesicles' safety assessment

3.5.1. Cell proliferation assay

Biological acceptability was assessed using the MTS assay (Fig. 5), a widely employed method for evaluation of cellular metabolic activity, being indicative of the early cytotoxic effects of the tested compounds and formulations. For liposomes and transfersomes, both empty and loaded with BD, the cell proliferation was assayed by MTS metabolism following long-term (24 h) exposure to the test formulations. To ensure a comprehensive assessment, the individual components were also tested at a concentration corresponding to their content in the test formulations at the highest tested concentration. Additionally, supplemented medium was used as the negative control, while SDS, at the same concentration as substances forming either BD liposomes or transfersomes, represented a positive control.

Exposure of keratinocytes to empty liposomes resulted in consistently high cell proliferation (≥ 93.7 %) across all tested concentrations. Similarly, empty transfersomes maintained high proliferation levels (≥ 85.0 %). To note, no significant differences were detected between empty liposomes and transfersomes, as proliferation values remained comparable and within standard deviations. The minimal effect on keratinocyte proliferation indicates biocompatibility of both empty formulations. These results are in accordance with our expectations, as our formulations are based on phospholipids, which are known for their excellent biocompatibility (Gosenca et al., 2013; Vater et al., 2019). Additionally, our studies on individual components further support this, with Lipoid S100 exhibiting a notably high proliferation rate (94.0 %).

Upon incorporation of BD into the vesicles, more pronounced differences were observed among formulations. For BD liposomes, cell proliferation was slightly lower (≥ 91.7 %) than that of empty liposomes across all tested concentrations. This phenomenon is consistent with our results obtained for BD when tested as an individual component, where cell proliferation decreased by approximately 5 %. This can be attributed to its antiproliferative effect, as reported in scientific literature (Guichard et al., 2015; Vitek et al., 2024). Further, a subtle concentration-dependent trend in proliferation was noted for BD liposomes, suggesting a potential dose-dependent effect of BD. Conversely, BD transfersomes exhibited a relatively noticeable reduction in proliferation (≥ 78.1 %) compared to empty transfersomes across all concentrations, with a clear concentration-dependent decrease in proliferation. Taken together, when comparing BD liposomes and BD transfersomes, an interesting observation in their effects on cell proliferation was noted, with the former showing an advantage over the latter, at all tested concentrations. Based on the assessment of individual components (58.5 %), this difference can be attributed to the presence of Tween® 80 in the transfersomal formulation. Surfactants, in general, are considered a group of substances with potential skin irritating properties that also act as penetration enhancers (Kim et al., 2023; Subongkot and Ngawhirunpat, 2015). Nonionic surfactants, including Tween® 80, are

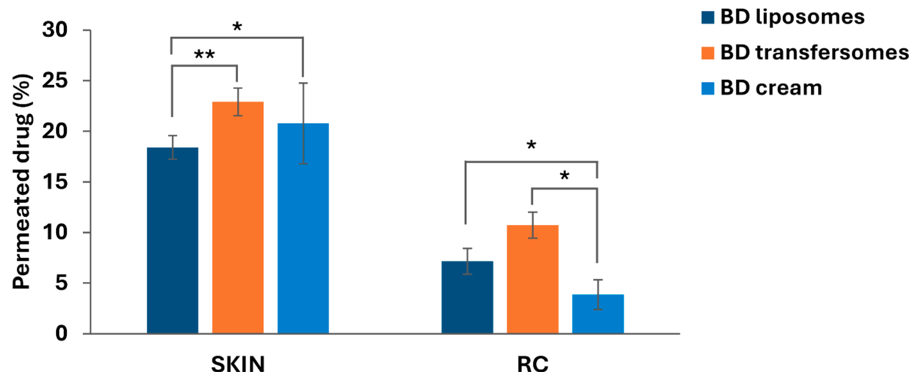

Fig. 4. BD skin permeation test on newborn pig skin. BD accumulated in the whole skin and in the receptor compartment (RC) is reported as a percentage of the BD applied onto the skin surface in liposomes, transfersomes or cream with the same BD concentration (0.64 mg/ml). ($n = 4$; ** $p < 0.01$; * $p < 0.05$).

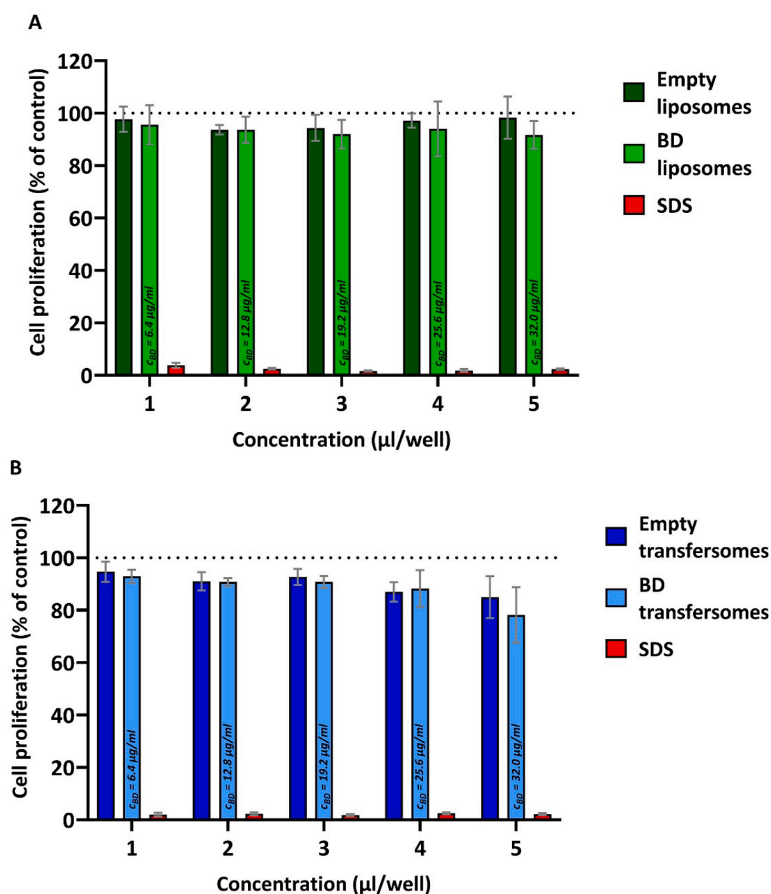

Fig. 5. Proliferation of keratinocytes after 24-h exposure to (A) liposomes, (B) transfersomes, empty and loaded with betamethasone dipropionate (BD), as well as SDS solution used as standard irritant, at different tested concentrations. The results are presented relative to the proliferation of untreated control cells. Data are means ± standard deviation (SD); $n = 6$.

among the least problematic and are considered safe for dermal administration. Nevertheless, their effects largely depend on the formulation itself and the concentration in which the surfactant is present (Effendy and Maibach, 1995). It seems that upon incorporation of BD, a drug with antiproliferative properties, the negative effects of Tween® 80 became more pronounced. To note, this difference was particularly distinct at the highest tested dose, i.e. 5 µl/well, where BD liposomes exhibited significantly ($p < 0.02$) higher proliferation rate than BD transfersomes.

Further, when comparing the results of our formulations to the standard irritant, SDS, the differences were striking. Following exposure to SDS, a well-established positive control in skin irritation testing (Kandárová et al., 2018; Van de Sandt et al., 1995), proliferation values were around 3.8 % or lower across all tested concentrations. In contrast, our empty and BD formulations consistently displayed significantly ($p = 0.00$) higher proliferation values, emphasising their suitability for topical use. These findings highlight the strong potential of our formulations as safe and biocompatible skin delivery systems for BD administration, with liposomes demonstrating superior performance.

3.5.2. Morphological evaluation of keratinocytes

In the next step of biological characterisation, morphological assessment of keratinocytes was performed using inverted phase-contrast microscopy. The cells were treated according to the same protocol, with the same samples and concentrations used as in the cell proliferation assay. Keratinocyte morphology was systematically examined across all concentrations used in the MTS assay as a complementary approach to detect early or subtle cellular changes. This evaluation enabled identification of alterations in cell shape, intercellular contacts, and surface distribution that might not yet be reflected in enzymatic activity measured by the MTS assay. Monitoring these features provided additional insight into potential concentration-dependent cytotoxic or irritative effects.

The resulting photomicrographs are presented in Fig. 6. The observed cellular morphological characteristics align very well with the results from the MTS assay.

Exposure of keratinocytes to empty liposomes resulted in no observable morphological changes across all tested concentrations, confirming their biocompatibility. Similarly, at lower concentrations, empty transfersomes maintained normal cell morphology. However, at doses of 4 µl/well and higher, initial signs of morphological alterations were observed, including a decrease in cell density, reduced intercellular connections, and an increase in the number of non-viable cells. These findings indicate that higher concentrations of empty transfersomes may have a mild effect on keratinocytes.

Upon incorporation of BD into the formulations, the differences among them became more apparent, which is also in good agreement with the results of the MTS assay. BD liposomes exhibited no apparent morphological changes at any tested concentration, maintaining cell shape, density, and intercellular connections comparable to the untreated control. In contrast, BD transfersomes induced morphological alterations starting at a dose of 3 µl/well, where cells exhibited reduced intercellular connectivity and a less uniform distribution. This trend became more pronounced at higher doses, further supporting a dose-dependent effect.

In contrast to the cells exposed to our formulations, completely different photomicrographs were obtained following treatment with SDS. More specifically, after SDS exposure, mostly dead cells were

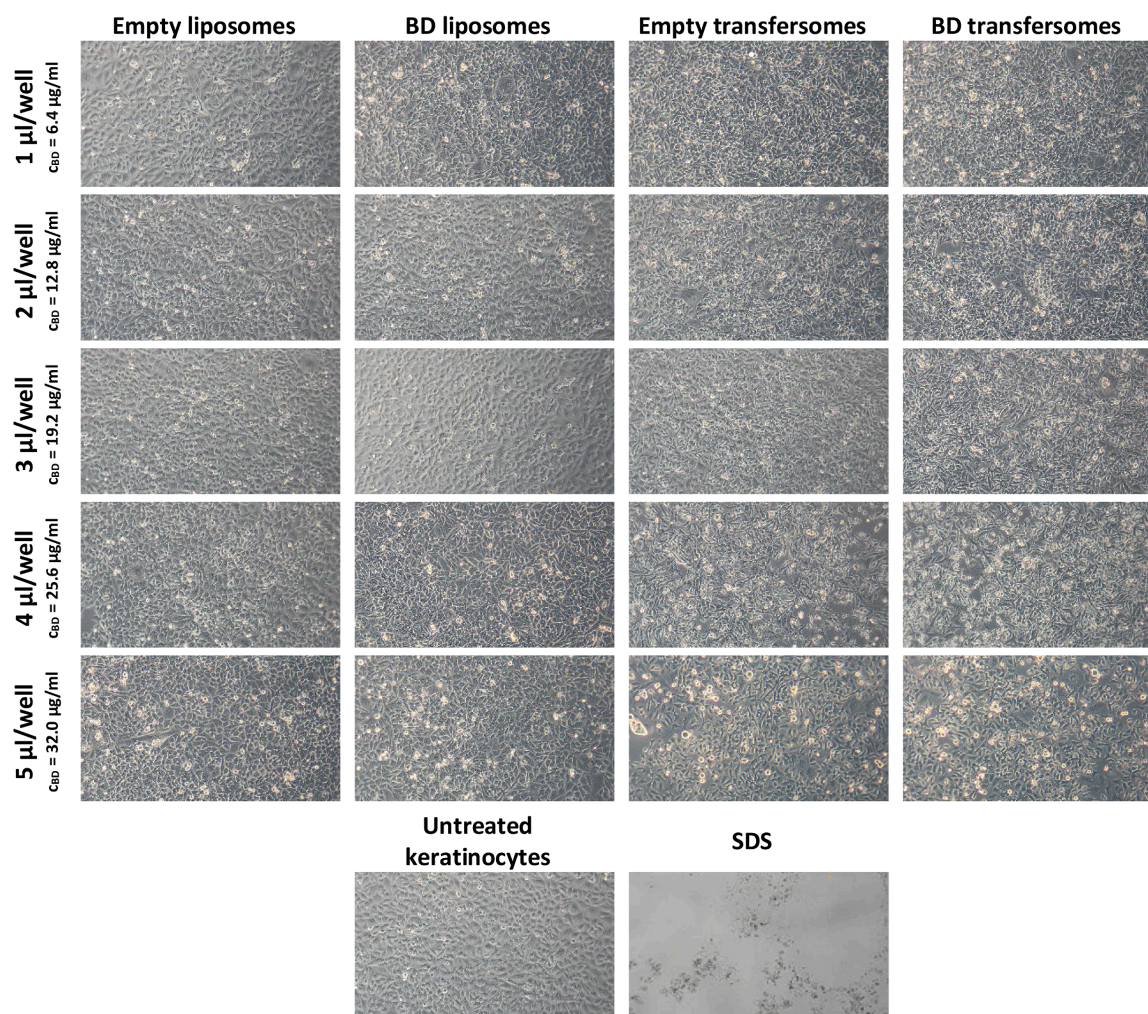

Fig. 6. Inverted phase-contrast microscopy images showing the morphology of keratinocytes after 24-h exposure to liposomes and transfersomes, empty and loaded with betamethasone dipropionate (BD), at different tested concentrations. Untreated cells and cells treated with an SDS solution, used as a standard irritant, are included as controls. The magnification used was 10 ×.

present, along with cell fragments appearing as tiny black dots. The remaining live cells exhibited an elongated shape with minimal intercellular connections. To summarise the results, based on the morphological evaluation of keratinocytes, consistent with the cell proliferation assay, our formulations were proven to be biocompatible, with liposomes standing out as an exceptionally skin-compliant skin delivery system for BD administration.

4. Conclusions

Betamethasone dipropionate was formulated in conventional liposomes and transfersomes to harness the potential of phospholipid-based nanosystems in enhancing its bioavailability upon skin application. Both formulations showed nanosized vesicles with predominantly unilamellar structures and optimal values of entrapment efficiency. The formulations were classified as Newtonian fluids, a behavior usually observed for vesicle dispersions, with higher viscosity values for transfersomes, which was temperature-dependent: the viscosity decreased at the skin surface temperature, which is convenient for a formulation to be applied on the skin. Both formulations led to a high skin penetration/permeation of BD when tested on an *ex vivo* permeation model using newborn pig skin against a commercial BD cream. *In vitro* cell studies highlighted high biocompatibility and no visible morphological changes. Considering these findings, the proposed formulations demonstrated great potential for betamethasone dipropionate skin delivery. Nevertheless, the current results should be supported by additional research, including *in vivo* testing, to determine the penetration/permeation capabilities of liposomes *versus* transfersomes and the anti-inflammatory effectiveness of BD in traditional *versus* phospholipid vesicles-based formulations. Furthermore, an economic analysis of the costs and potential profitability of the vesicle formulations *versus* traditional formulations is required to validate the therapeutic or industrial application of the vesicle formulations.

Funding

This study was supported by the Slovenian Research and Innovation Agency (Research Core Funding No. P1-0189).

CRediT authorship contribution statement

Luca Casula: Writing – review & editing, Writing – original draft, Investigation. **Mercedes Vitek:** Writing – review & editing, Writing – original draft, Investigation. **Alenka Zvonar Pobirk:** Writing – review & editing, Resources, Funding acquisition, Conceptualization. **Mirjam Gosenca Matjaž:** Writing – review & editing, Writing – original draft, Investigation. **Donatella Valenti:** Investigation. **Aurélien Dupont:** Investigation. **Carla Caddeo:** Writing – review & editing, Writing – original draft, Resources, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The EDUC-Share project, funded by the European Union's Horizon 2020 research and innovation program under grant agreement No. 101017526, is gratefully acknowledged for financial support for the cryo-TEM measurements taken at the UNIVREN-BIOSIT-MRic core facility at the University of Rennes (France). The authors would like to express sincere gratitude to Žiga Medoš, PhD, for conducting the SAXS measurements at the Faculty of Chemistry and Chemical Technology at the University of Ljubljana (Slovenia).

Data availability

Data will be made available on request.

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