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Penetration enhancer containing vesicles as carriers for dermal delivery of tretinoin

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ABSTRACT

The ability of a recently developed novel class of liposomes to promote dermal delivery of tretinoin (TRA) was evaluated. New penetration enhancer-containing vesicles (PEVs) were prepared adding to conventional phosphatidylcholine vesicles (control liposomes) different hydrophilic penetration enhancers: Oramix® NS10 (OrNS10), Labrasol® (Lab), Transcutol® P (Trc), and propylene glycol (PG). Vesicles were characterized by morphology, size distribution, zeta potential, incorporation efficiency, stability, rheological behaviour, and deformability. Small, negatively charged, non-deformable, multilamellar vesicles were obtained. Rheological studies showed that PEVs had fluidity higher than conventional liposomes.

The influence of the obtained PEVs on (trans)dermal delivery of tretinoin was studied by *ex vivo* diffusion experiments through new born pig skin using formulations having the drug both inside and outside the vesicles, having TRA only inside, in comparison with non-incorporated drug dispersions of the same composition used to produce the studied vesicles. Main result of these experiments was an improved cutaneous drug accumulation and a reduced transdermal TRA delivery (except for PG-PEVs). TRA deposition provided by PEVs was higher for dialysed than for non-dialysed vesicles. Further, the accumulation increased in the order: control liposomes < PG-PEVs < Trc-PEVs ≤ Or-PEVs < Lab-PEVs. SEM analysis of the skin gave evidence of PEVs' ability to strongly interact with the intercellular lipids causing an enlargement of this region.

1. Introduction

Liposomal formulations have been extensively studied to enhance efficiency of (trans)dermal drug delivery, since they offer many advantages over traditional topical dosage forms such as enhancement of drug effectiveness and bioavailability as well as reduction of side effects. However, the function of vesicles as transdermal or dermal delivery systems is controversial. Therefore, in the last two decades, as an alternative to liposomes and niosomes, innovative vesicular systems have been proposed (Paolino et al., 2008; Sinico and Fadda, 2009; Tavano et al., 2010). Several authors have investigated the ability of different molecules to modify vesicular bilayer properties, increasing their deformability and capability of delivering drugs to and through the skin. Although most studies have reported that high deformable liposomes are especially able to enhance transdermal drug delivery, this class of lipid vesicles has also been reported to improve *ex vivo* cutaneous drug deposition with a higher effectiveness than conventional vesicles (Cevc and Blume, 1992; van den Bergh, 1999; Touitou et al., 2000; Bouwstra and Honeywell-Nguyen, 2002; Paolino et al., 2005; Elsayed et al., 2006). Recently, different penetration enhancer (PE) molecules have been tested as "edge activator" in the formulation of new deformable vesicles by several authors (Verma, 2002; El Maghraby et al., 2004; Elsayed et al., 2007; Dragicevic-Curic et al., 2008; Manconi et al., 2009). In previous studies, we introduced the acronym PEVs to identify the penetration enhancer containing vesicles as carriers for dermal delivery of minoxidil and diclofenac (Manconi et al., 2009, 2011; Mura et al., 2009, 2011). These vesicles have shown to possess the advantage to combine liposome potential as carriers and PE ability to modify the order of stratum corneum (SC) packing (Mura et al., 2009, 2011), thus promoting skin delivery. Moreover, during last years the benefits of *trans*-retinoic acid (TRA) incorporation in liposomes and niosomes on its dermal delivery have also been extensively studied (Trapasso et al., 2009). *Trans*-retinoic acid or tretinoin (TRA) is a natural retinoid widely used in proliferative and inflammatory skin diseases, such

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as psoriasis, acne, and epithelial skin cancer. Unfortunately, this drug is very unstable in the presence of air, light and heat and, in addition, its topical application may cause irritation and peeling of the treated area (Manconi et al., 2006). Our previous works demonstrated that TRA-incorporated liposomes and niosomes are able to improve *ex vivo* drug localization in the superficial skin layers (Sinico et al., 2005; Manconi et al., 2006). Indeed, especially niosomes were shown to dramatically enhance the drug cutaneous retention, mostly if compared to the commercial formulation RetinA® and phosphatidylcholine liposomes. Furthermore, liposomes saturated with TRA demonstrated to be significantly more efficient in promoting drug accumulation than the corresponding non-saturated vesicles (Manconi et al., 2006).

Taking into account these results, with the aim of finding new formulations capable of improving TRA cutaneous retention, new PEVs were studied. In particular, in this work, PEVs were prepared using soy phosphatidylcholine (Phospholipon 50, P50) and one of four selected hydrophilic PEs: decylpolyglucoside (Oramix® NS10, OrNS10), caprylocaproyl macrogol 8-glyceride (Labrasol®, Lab), 2-(2-ethoxyethoxy)ethanol (Transcutol® P, Trc), and propylene glycol (PG). As a consequence of the results obtained in previous studies with Transcutol, in this work we evaluated only the influence of hydrophilic PEs. In fact, the further aim of this study is to compare Transcutol containing PEVs' properties to those of vesicles obtained with PEs with similar hydrophilic characteristic but different chemical structure. Therefore, all prepared formulations were obtained using the same PE concentration (i.e. 0.6%, w/v). Moreover, in an attempt to understand the PE capability of interacting with vesicle bilayer and affecting its properties, a rheological study was carried out. To shed some light on the possible mechanism by which these formulations act, *ex vivo* skin permeation and deposition behaviour of the PEVs was also studied using new born pig skin. To this purpose, formulations having the drug both inside and outside the vesicles (non-dialysed, ND), having TRA only inside (dialysed, D), and non-incorporated drug dispersions of the same composition used to produce the studied vesicles (MIX) were tested. Furthermore, to elucidate the effect and interaction between PEVs and skin, Scanning Electron Microscopy (SEM) was used to visualize the new born pig skin structure after the liposomal administration.

2. Materials and methods

2.1. Materials

Soy phosphatidylcholine (Phospholipon 50, P50) was kindly supplied by AVG S.r.l. (Garbagnate Milanese, Milan, Italy) and Lipoid GmbH (Ludwigshafen, Germany). Decylpolyglucoside (Oramix® NS10, OrNS10) was kindly donated by Seppic (Milan, Italy). 2-(2-Ethoxyethoxy)ethanol (Transcutol® P, Trc), caprylocaproyl macrogol 8-glyceride (Labrasol®, Lab) were a gift from Gattefossè (Saint Priest, France). Propylene glycol (PG), *trans*-retinoic acid (TRA), and all the other products were of analytical grade and were purchased from Sigma–Aldrich (Milan, Italy).

2.2. Vesicle preparation

Multilamellar liposomes (control) and PEVs were prepared according to the thin film hydration method but with a slight modification (hydration in two steps) that allowed us to obtain more homogeneous vesicular populations (polydispersity index < 0.4). P50 (1.2 g), TRA (22 mg) and PE (0.06 g), when appropriate, were dissolved in chloroform. The excess of drug (22 mg) was used to obtain saturated vesicles, which have shown to better promote drug accumulation in pig skin (Manconi et al., 2006). The lipid mixture was deposited as a thin film in a round-bottom flask by roto-evaporating (Rotavapor Büchi R110, Switzerland) the chloroform under vacuum, which was applied for 1 h to ensure total removal of solvent traces. Hydration of the film was performed in two steps: first, 5 ml of phosphate buffer solution (PBS, pH 7) was added to the flask and the concentrated dispersion was mechanically shaken for 1 h at room temperature. Then, a second 5 ml aliquot of PBS was added and the dispersion shaken for another hour. PE final concentration was 0.6% (w/v). All suspensions were prepared under yellow light and kept in the dark at all times. The presence of TRA crystals, which was used as an indicator of vesicle saturation with the drug, was checked using light microscopy. Each vesicle suspension was then purified from the non-incorporated drug by centrifugation (5000 rpm for 5 min), followed by exhaustive dialysis, avoiding any sample dilution and allowing a more precise dosage of tretinoin-loaded vesicles. Dispersions were loaded into dialysis tubing (Spectra/Por® membranes: 12–14 kDa MW cut-off, 3 nm pore size; Spectrum Laboratories Inc., USA) and dialysed against PBS at 4 ± 1 °C. Dialysis of each sample (2 ml) was carried out in 1000 ml of PBS that was changed 3 times during 24 h. Incorporation efficiency (*E*%), expressed as the percentage of the amount of TRA initially used, was determined by high performance liquid chromatography (HPLC) after disruption of vesicles with 0.025% non-ionic Triton X-100. TRA content was quantified at 350 nm using a chromatograph Alliance 2690 (Waters, Italy). The column was a Simmetry C18 (3.5 μm, 4.6 × 100 mm, Waters). The mobile phase was a mixture of acetonitrile, water and acetic acid (84.5:15:0.5, v/v), delivered at a flow rate of 1.2 ml/min.

2.3. Vesicle characterization

Vesicles were characterized by Transmission Electron Microscopy (TEM) for vesicle formation and morphology. A drop of the vesicular dispersion was applied to a carbon film-covered copper grid and stained with a 1% phosphotungstic acid. Then, samples were examined with a JEM-1010 (Jeol Europe, France) transmission electron microscope equipped with a digital camera MegaView III and Software "AnalySIS", at an accelerating voltage of 80 kV.

The average diameter and polydispersity index (P.I.) of the samples were determined by Photon Correlation Spectroscopy (PCS) using a Zetasizer nano-ZS (Malvern Instrument, UK). Samples were backscattered by a helium–neon laser (633 nm) at an angle of 173° and a constant temperature of 25 °C. The instrument systematically and automatically adapts to the sample by adjusting the intensity of the laser and the attenuator of the photomultiplier, thus ensuring reproducibility of the experimental measurement conditions. The P.I. was used as a measure of the width of the size distribution. P.I. less than 0.4 indicates a homogenous and monodisperse population. Zeta potential was estimated using the Zetasizer nano-ZS by means of the M3-PALS (Phase Analysis Light Scattering) technique, which measures the particle electrophoretic mobility in a thermostated cell. All the samples were analysed 24 h after their preparation.

A long-term stability study was performed by monitoring the vesicle average size, polydispersity, and surface charge over 90 days at 4 ± 1 °C. Tretinoin retention and chemical stability were also checked by measuring the amount of drug retained during storage, after dialysis, by HPLC.

2.4. Vesicle viscosity measurements

Just before the analyses, P50 dispersions were frozen and defrosted to break vesicle bilayer, in order to avoid their reconstitution prior to viscosity and shear rate measurements. The rheology study was carried out on a Bohlin Visco 88 rheometer (Bohlin Instruments, Cranbury, NJ) equipped with eight rotational speed

settings in geometric progression from 20 to 1000 rpm and a coaxial cylinder C-30 geometry. Temperature control of the samples was done at 25 ± 1 °C. Shear stress measurements were performed by increasing shear rate from 15 to 507 s^{-1}, and then decreasing it to 15 s^{-1}. The cycle was continuously repeated, six times for each sample. The apparent viscosity measurements were made at a constant shear rate of 325 s^{-1}. All the viscosity measurements were repeated three times. The shear stress against shear rate, as well as the viscosity against shear rate plots were made by "ViscoSoft" software.

2.5. Deformation index determination

Comparative measurement of deformability of liposome and PEV bilayers was carried out by the extrusion method. Each vesicle dispersion was extruded at constant pressure (4 bar) through 19-mm polycarbonate filters of definite pore size (50 nm), using the extrusion device Liposofast® basic installed on a pneumatic actuator (Avestin, Canada) connected to a nitrogen supplier. The vesicle deformability was expressed in terms of deformation index (DI) according to Eq. (1):

$$DI = J\left(\frac{d_0}{p}\right)\left(\frac{d_0}{|d_0 - d_1|}\right) \quad (1)$$

where J is the fraction of suspension recovered after extrusion (ranging from 0 to 1, with 1 representing 100% of the dispersion loaded in the extruder 0.5 ml syringe); d_0 and d_1 are vesicle mean diameters before and after extrusion; p is the pore size of the extruder membrane.

2.6. Ex vivo skin penetration and permeation studies

Experiments were performed non-occlusively using Franz diffusion vertical cells with an effective diffusion area of 0.785 cm^2, and new born pig skin. One-day-old Goland–Pietrain hybrid pigs (~1.2 kg) were provided by a local slaughterhouse. The skin, stored at −80 °C, was pre-equilibrated in physiological solution (0.9%, w/v of NaCl) at 25 °C, 2 h before the experiments. Skin specimens (n = 6 per formulation) were sandwiched securely between donor and receptor compartments of the Franz cells, with the stratum corneum (SC) side facing the donor compartment. The receptor compartment was filled with 5.5 ml of physiological solution, which was continuously stirred with a small magnetic bar and thermostated at 37 ± 1 °C throughout the experiments to reach the physiological skin temperature (i.e. 32 ± 1 °C).

Prior to (trans)dermal delivery experiments, dispersions (both non-dialysed and dialysed) were diluted (with PBS) to obtain the TRA concentration of 0.25 mg/ml. One hundred microliters of the tested vesicle suspensions were placed onto the skin surface. At regular intervals, up to 8 h, the receiving solution was withdrawn and replaced with an equivalent volume of pre-thermostated (37 °C) physiological fresh solution, to ensure sink conditions. Withdrawn receiving solutions were analysed by HPLC for drug content (as described in Section 2.2).

After 8 h, the skin surface of specimens was gently washed (3 times) with 1 ml of distilled water, then dried with filter paper. The SC was removed by stripping with adhesive tape Tesa® AG (Hamburg, Germany). The method was previously validated by histological examination of stripped skin (Manconi et al., 2005). Each piece of the adhesive tape was firmly pressed on the skin surface and rapidly pulled off with one fluent stroke. Ten stripping procedures were performed consecutively. Epidermis was separated from dermis with a surgical sterile scalpel. Tape strips, epidermis, and dermis were cut and placed each in a flask with methanol and then sonicated for 4 min in an ice bath to extract the drug. The tapes and tissue suspensions were centrifuged for 10 min at 10000 rpm, and then the supernatants were filtered and assayed for drug content by HPLC (see Section 2.2). TRA recovery was always higher than 98% of the amount applied onto the skin.

Studies with non-incorporated drug were also carried out using suspensions (MIX) of the same composition of the tested vesicles. MIX were prepared by simply blending, at 1:1 ratio, TRA and P50 dispersions in order to obtain the same final TRA, lipids, and PE concentrations tested in the vesicular formulations. TRA (0.50 mg/ml) dispersion in PBS was obtained by probe sonication in a Soniprep 150 apparatus (MSE Crowley, UK) for 5 min. Just before the skin permeation experiments, P50 (240 mg/ml) was gently dispersed in PBS that also contained each PE (12 mg/ml) when necessary. Fifty microliters of the two dispersions were mixed just before applying 100 μl of each mixture onto the skin surface. The permeation study was carried out for 8 h as described above.

2.7. Scanning electron microscopy

At the end of the skin permeation experiments, the epidermis was surgically separated from the dermis and immediately immersed in 10% buffered formalin. After fixation, samples were dehydrated in a set of ethanolic solutions (70, 90, 95 and 100%, v/v), and frozen with liquid nitrogen. The blocks were then cracked into four pieces and coated with gold. The coated samples were visualized under a scanning electron microscope (SEM) Hitachi S4100 (Madrid, Spain).

2.8. Statistical analysis of data

Data analysis was carried out with the software package R, version 2.10.1. Results are expressed as the mean ± standard deviation. Multiple comparisons of means (Tukey test) were used to substantiate statistical differences between groups, while Student's t-test was applied for comparison between two samples. Significance was tested at the 0.05 level of probability (p).

3. Results and discussion

In the present work, four different PEV formulations were prepared using P50 and one of four hydrophilic penetration enhancers that are largely employed in topical formulations. In particular, Labrasol (Lab) is a safe, non-ionic hydrophilic surfactant (HLB = 14). It is a well-defined mixture of mono-, di- and triglycerides and mono- and di-fatty acid esters of polyethyleneglycol, with caprylic and capric acids being the predominant ones (Koga et al., 2006). Decylpolyglucoside (OrNS10; HLB = 12) is a commercial mixture of alkylpolyglucosides, a class of hydrophilic non-ionic surfactants that have shown to be biodegradable, non-toxic and thus largely used in pharmaceutical and cosmetic formulations. OrNS10 has been shown to be capable of forming niosomes in the presence of appropriate amounts of cholesterol (Manconi et al., 2006).

Transcutol (diethylene glycol monoethylether) is a well known non-toxic, biocompatible with skin, penetration enhancer. It is known to act by swelling the stratum corneum intercellular lipids without altering their multiple bilayer structure (Panchagnula and Ritschel, 1991; Godwin et al., 2002). It has been already tested in PEV formulations (Manconi et al., 2009; Mura et al., 2009, 2011). Propylene glycol (PG), widely used as a PE and adjuvant in topical formulations, has been already assayed as a component of lipid vesicles by Elsayed et al. (2007) who tested the PG-liposomes as carriers for skin delivery of cinchocaine.

The aim of the present work was to investigate whether the selected hydrophilic penetration enhancers could positively affect

Table 1
Characteristics of empty and TRA-loaded PEVs and conventional liposomes, before (ND) and after dialysis (D): mean diameter (MD), polydispersity index (P.I.), zeta potential (ZP) and entrapment efficiency (*E*%). Each value represents the mean ± S.D., $n = 6$.

		MD (nm)	P.I.	ZP (mV)	*E* (%)
P50	Empty	116 ± 3	0.25	−55 ± 1	
	ND	118 ± 7	0.26	−54 ± 4	
	D	125 ± 6	0.35	−58 ± 7	87 ± 4
P50/Or	Empty	120 ± 3	0.28	−47 ± 2	
	ND	°123 ± 4	0.29	−54 ± 5	
	D	°133 ± 6	0.29	−65 ± 8	91 ± 3
P50/Lab	Empty	127 ± 2	0.24	−58 ± 1	
	ND	*120 ± 6	0.23	−56 ± 4	
	D	*137 ± 8	0.23	−68 ± 10	84 ± 5
P50/Trc	Empty	142 ± 2	0.24	−55 ± 2	
	ND	150 ± 6	0.29	−56 ± 5	
	D	164 ± 7	0.34	−66 ± 8	82 ± 4
P50/PG	Empty	142 ± 3	0.32	−57 ± 2	
	ND	145 ± 5	0.27	−56 ± 7	
	D	157 ± 7	0.29	−69 ± 8	86 ± 6

° $p < 0.05$.
* $p < 0.05$.

the physico-chemical properties of phospholipid vesicles, and synergically improve the dermal delivery of loaded tretinoin. Since higher quantities (1%) of Oramix or Labrasol produced vesicles with unfavourable characteristics in terms of size, homogeneity, and morphology, for a comparative study, all PEs were tested at the same concentration (0.6%, w/v).

Empty, non-dialysed, and dialysed PEVs and liposomes were characterized in terms of structure, size distribution, charge, and incorporation efficiency (Table 1). TEM observation confirmed the formation of multilamellar vesicles (MLVs, Fig. 1). Since all the obtained MLVs exhibited a small size, the sonication procedure was not performed. A difference in size, related to the composition of the samples, was evident only for vesicles containing Trc and PG, which were larger (∼150 nm) than the control liposomes and Or- and Lab-containing PEVs (∼120 nm). All dialysed PEVs showed slightly larger size than the corresponding non-dialysed vesicles. As known, dialysis can induce an increase of vesicle size as a consequence of the osmotic stress during this purification procedure (Mura et al., 2007). However, the increase in size was statistically significant only in the case of P50/Or and P50/Lab formulations. On the contrary, dialysed liposome mean size was quite similar to that of the corresponding non-dialysed preparations ($p > 0.05$). Zeta potential values were always highly negative (from −47 to −69 mV), indicative of a good stability against vesicle aggregation and fusion. All empty and TRA non-dialysed PEVs showed the same zeta potential values (∼−52 mV), which decreased to ∼−63 mV for TRA dialysed vesicles. The higher negative zeta potential of the dialysed vesicles could be due to a redistribution of phospholipids during dialysis.

TRA incorporation into vesicles at a percentage ranging from 82 to 91 (*E*%; Table 1) was achieved by all the prepared formulations, showing their extremely high loading capability, which was not significantly affected by the investigated compositions.

Stability studies during storage were carried out to check the physico-chemical properties of the prepared formulations, in terms of average size and zeta potential, over a period of 90 days at 4 ± 1 °C. Fig. 2 shows the mean size of the different TRA-loaded vesicle formulations measured during this period. The size of control liposomes increased four times compared to the initial value, especially in the last 60 days. No relevant size changes affected TRA-loaded PEVs, except for the formulations prepared with Or and PG, whose mean diameter increased by 40%. Vesicles with Lab and Trc were the most stable, suggesting a stabilizing effect of these PEs, in particular Lab, against aggregation and fusion of the vesicles. The zeta potential values were constant during all the period. The

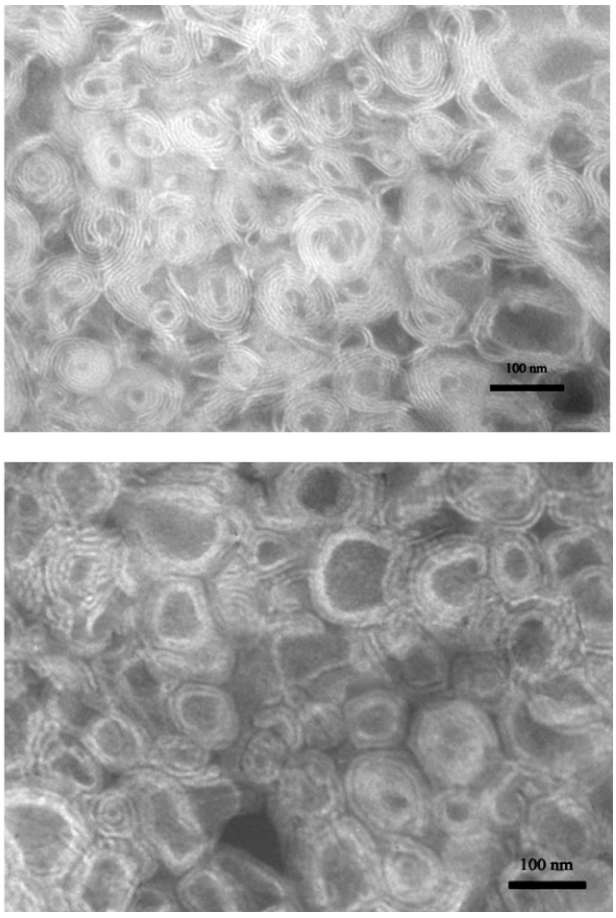

Fig. 1. Negative stain electron micrographs of tretinoin-loaded PEVs prepared with Oramix (A, P50/Or) and Labrasol (B, P50/Lab).

amount of loaded tretinoin did not significantly change during storage (*E*% ≥ 80), and no features indicative of trans/cis isomerization (i.e. peak shift, broadening, shoulders or new peaks) were detected.

In order to verify the ability of the used PEs to act as edge activators (Cevc et al., 2002), the deformability of the prepared PEVs was studied by extruding them through polycarbonate membranes with pores smaller (50 nm) than their mean size. The deformation index (DI, data not shown) of PEVs was very close to that of conventional liposomes, thus showing that the former cannot be considered deformable vesicles.

In a previous work, an investigation of the rheological properties of diclofenac loaded-PEVs showed that glycols interacted, in a concentration-dependent manner, with bilayer packing facilitating its curvature in closed vesicle and decreasing the energy for vesicle reconstitution (Manconi et al., 2009). Moreover, it was observed that the presence of glycols in soy phosphatidylcholine vesicular dispersions assisted vesicle reformation from the lamellar phase, leading to a reduction of the hysteresis loop area and apparent viscosity (Manconi et al., 2006). In the present work, in order to elucidate the viscoelastic features of liposomes and PEVs and, therefore, the different PE ability to interact with the vesicular bilayers, the rheological study was also performed. Vesicle dispersions were slowly frozen to break the vesicle bilayer and their rheological behaviour was studied by means of shear stress vs shear rate curves. Shear force is the external force necessary to induce the transition from lamellar phases to closed vesicles. The shear rate, measured by progressively increasing the shear stress, is different from that measured when shear stress is progressively decreased, thus obtaining a hysteresis loop area (HLA). This area is

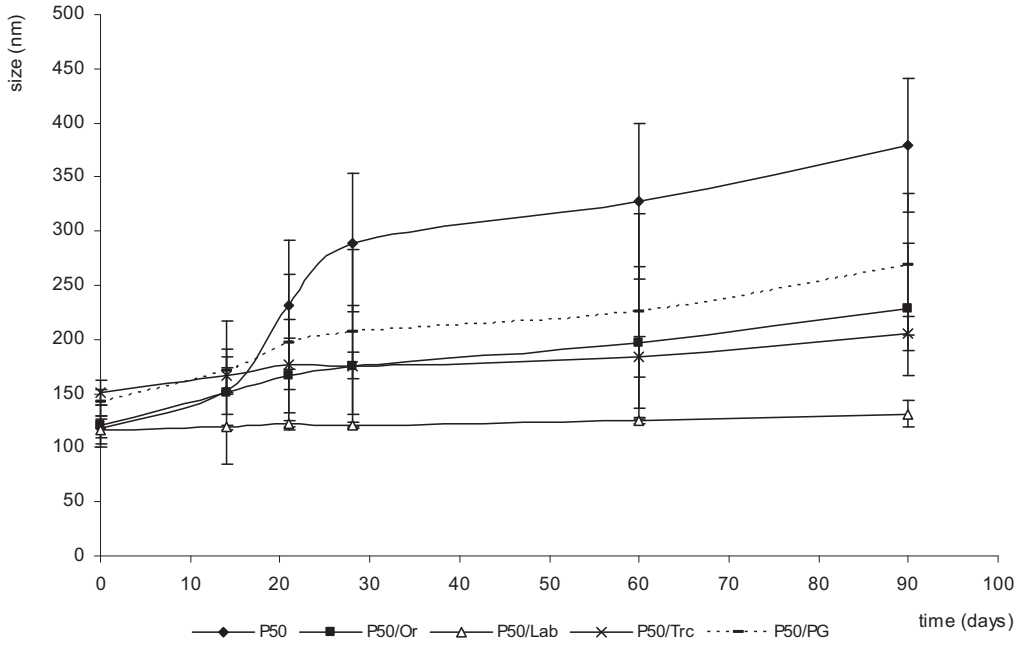

Fig. 2. Mean size of liposomes and PEVs monitored during a 90-day period on storage at 4 ± 1 °C. The error bars are the standard deviations of six independent samples.

related to the energy involved in the vesicle reconstitution process under shear.

In Fig. 3 the curves of the up and down shear stress versus shear rate for non-frozen and frozen-defrosted (first and third cycles are shown) P50 liposomes (control) are reported as representative of PEVs too, as they showed the same trend. For non-frozen vesicles, the hysteresis loop area did not exist because intact vesicles could easily flow in the shear direction. For defrosted liposomes in the first cycle, the HLA was evident and quantifiable due to the segregation of vesicles from fragments of the lamellar phase by shear stress, while HLA disappeared after the third cycle, once the vesicles had formed. The HLA of defrosted PEVs decreased in the presence of Trc and PG and disappeared using Or and Lab (Table 2). The apparent viscosity of the samples was measured at constant shear rate: 325 s^{-1} (Table 2). Before freezing-defrosting treatment, the apparent viscosity was identical for all the tested samples (~6.2 mPas).

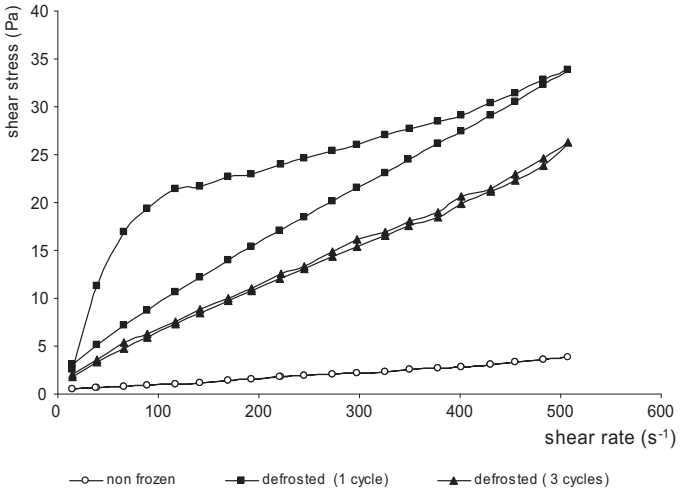

Fig. 3. Shear stress versus shear rate for soy phosphatidylcholine dispersions, non-frozen and defrosted, after 1 or 3 cycles of shear. Shear rate was increased from 15 to 507 s^{-1} and then decreased to 15 s^{-1}. Measure cycle was repeated continuously six times for each sample.

The apparent viscosity of non-frozen PEVs was always lower than that of the corresponding defrosted samples. In comparison to control, the apparent viscosity of PEVs decreased in the presence of the PE molecules, especially when Or and Lab were used (Table 2). This effect could be related to the different PE ability to interact with the bilayer structure, causing a molecular rearrangement and increased membrane fluidity. This led to a reduction of the energy required for bilayer deformation, thus, permitting the formation of closed vesicles, under a shear stress. In the presence of PE, broken vesicles reformed immediately under shear (without HLA), and reconstituted vesicles flowed in the shear direction, causing a decrease of the system viscosity. Trc and PG are water-miscible solvents that, at the concentration used for PEVs' preparation (0.6%), are mainly dissolved in the aqueous phase. Therefore, they poorly interact with the lipid bilayer, leading to a small decrease in segregation energy and viscosity. Higher Trc and PG concentrations could probably allow these PEs to interact more with lipid packing (Manconi et al., 2009). On the other hand, Or and Lab, hydrophilic surfactants with long hydrocarbon chains, strongly cooperate with the vesicular bilayer, making it more fluid and leading to a significant decrease of the apparent viscosity, as well as the energy necessary for the transition from planar lamellar phase to closed multilamellar vesicles.

Ex vivo (trans)dermal delivery of TRA-loaded liposomes and PEVs using Franz cells and new born pig skin was investigated. The amount of TRA accumulated into SC, epidermis, and dermis is expressed as the percentage of the drug applied onto the skin (Fig. 4A). To clarify the role of the studied vesicles in (trans)dermal drug delivery, and in particular to elucidate if they act as carriers or simply as penetration enhancers, in this study *ex vivo* TRA permeation and skin deposition was tested using formulations with TRA both inside and outside the vesicles (non-dialysed, ND), having TRA only inside (dialysed, D), and with simple suspensions of the same composition used to produce vesicles (MIX). As written above, all the tested samples were diluted (with PBS) to obtain the TRA concentration of 0.25 mg/ml (Manconi et al., 2006).

As shown in Fig. 4A, the main result obtained from the *ex vivo* (trans)dermal experiments was the cutaneous deposition of tretinoin into the new born pig skin. Indeed, with both control and

Table 2
Apparent viscosity variations (shear rate: 325 s^{-1}) and hysteresis loop area (HLA) of non-frozen and defrosted liposomes and PEVs. Each value represents the mean ± S.D., $n = 3$.

Sample	Measurement	P50	P50/Or	P50/Lab	P50/Trc	P50/PG
Non-frozen	Viscosity (mPas)	5.8 ± 0.2	6.1 ± 0.3	6.1 ± 0.3	6.6 ± 0.2	6.3 ± 0.3
Defrosted	Viscosity (mPas)	82.9 ± 2.3	12.3 ± 1.3	18.0 ± 3.1	28.6 ± 3.8	30.8 ± 4.3
Defrosted	HLA (a.u.)	1.2 ± 0.2	–	–	0.9 ± 0.2	0.5 ± 0.2

PEVs the amount of TRA detected in the receptor fluid was always lower than that accumulated, although some differences related to the vesicle composition can be identified. In particular, except for PG-PEVs, transdermal TRA delivery was negligible if compared to cutaneous accumulation, and it was higher for D formulations than for the corresponding ND.

In addition, cutaneous TRA accumulation provided by D and ND control liposomes was very similar: low in SC and epidermis, nil in dermis. On the contrary, the TRA deposition provided by PEVs in all skin strata was always higher for D vesicles than for ND. More specifically, dialysed PEVs provided the highest TRA accumulation in SC and epidermis, while Or-PEVs delivered similar high TRA

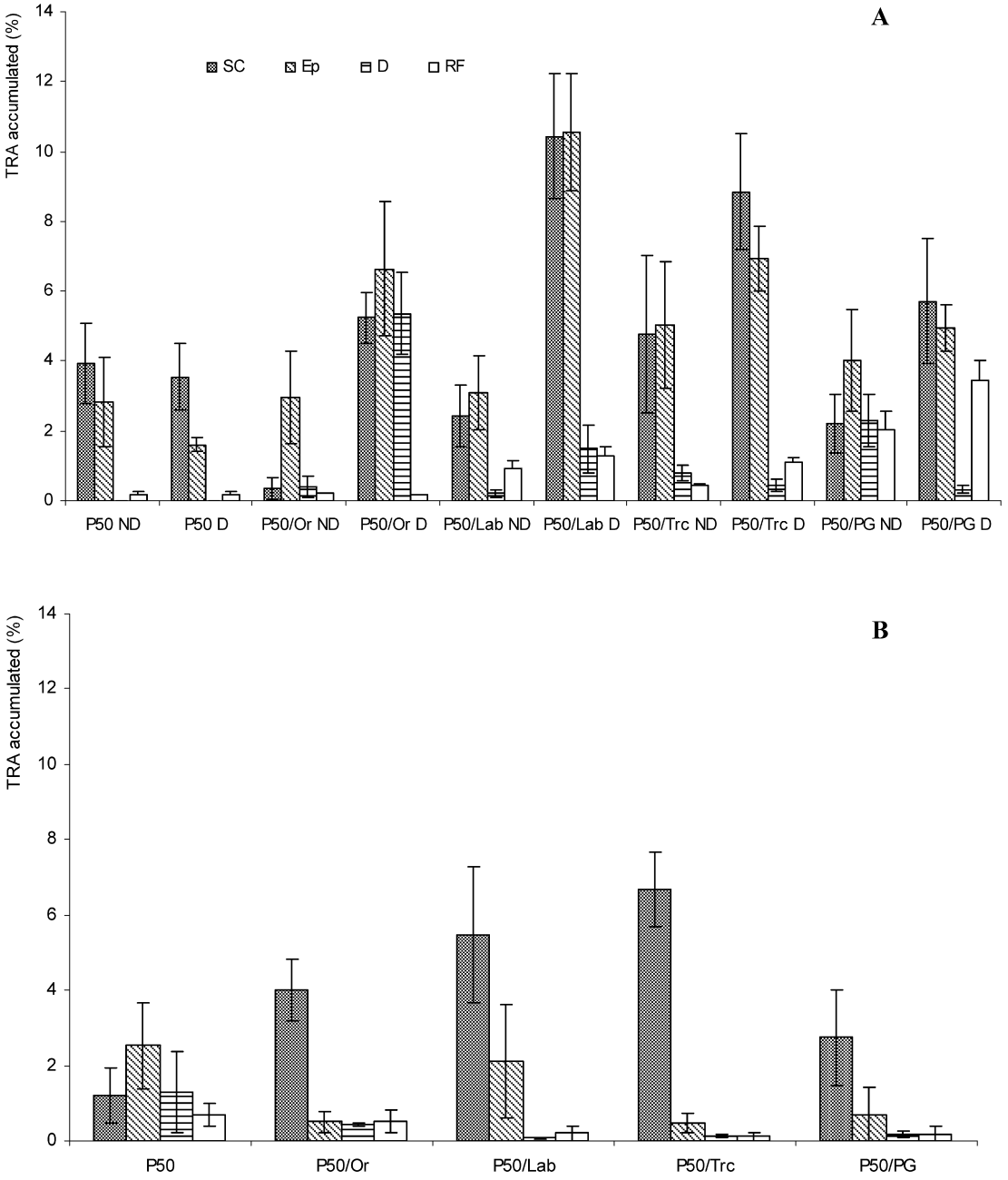

Fig. 4. Determination of TRA deposition into pig skin layers (SC, stratum corneum; Ep, epidermis; D, dermis.) after 8-h non-occlusive treatment; (A) control liposomes and PEV suspensions, before (ND) and after dialysis (D); (B) coarse dispersions of P50, PE and non-entrapped drug. Each value is the mean ± S.D. of at least six experimental determinations.

Table 3
Results of *ex vivo* permeation study obtained using: TRA-loaded PEVs and control liposomes, non-dialysed (ND) and dialysed (D); TRA in a coarse dispersion of P50 and each PE (MIX). Amount of TRA accumulated into the whole skin and permeated through the skin at the end of the experiment (8 h); Local Accumulation Efficiency (LAC) values: drug accumulated into the skin/drug permeated through the skin ratio; transdermal flux (*J*).

Composition	Accumulated TRA ($\mu g/cm^2$)	Permeated TRA ($\mu g/cm^2 \pm SD$)	LAC	J ($\mu g/cm^2/h \pm SD$)
P50 ND	5.6	0.14 ± 0.01	39	17 ± 3
P50 D	4.1	0.14 ± 0.01	30	18 ± 3
P50 MIX	3.1	0.44 ± 0.09	7	34 ± 4
P50/Or ND	2.9	0.17 ± 0.02	16	17 ± 2
P50/Or D	12.1	0.13 ± 0.02	96	14 ± 2
P50/Or MIX	3.1	0.33 ± 0.03	10	38 ± 4
P50/Lab ND	4.3	0.71 ± 0.01	6	88 ± 6
P50/Lab D	14.6	0.84 ± 0.10	17	101 ± 8
P50/Lab MIX	4.8	0.13 ± 0.01	38	15 ± 4
P50/Trc ND	7.8	0.33 ± 0.10	23	43 ± 5
P50/Trc D	13.4	0.76 ± 0.01	14	95 ± 9
P50/Trc MIX	4.6	0.07 ± 0.01	60	11 ± 2
P50/PG ND	6.8	1.64 ± 0.08	4	195 ± 14
P50/PG D	8.7	2.70 ± 0.20	3	315 ± 13
P50/PG MIX	2.3	0.12 ± 0.01	18	12 ± 3

amount to all the three skin layers (~6% each). Moreover, as shown in Table 3, the total amount of TRA delivered to the skin was ~3-fold higher with dialysed PEVs than with dialysed control, except for PG-PEVs (only 2-fold). The TRA cutaneous accumulation increased in the order: Control < PG-PEVs < Trc-PEVs ≤ Or-PEVs < Lab-PEVs.

Results of these experiments might have been affected by the different lipid concentration of the ND and D formulations. As written above, to compare vesicles saturated with tretinoin at the same drug concentration, both the ND and the D preparations were diluted (with PBS) to achieve 0.25 mg/ml of TRA in each of them. Therefore, after dilution, lipid concentrations were accordingly lower in ND formulations as compared with the corresponding diluted D ones. However, obtained TRA incorporation efficiency was high (ranging between 82 and 91%, Table 1) and, thus, dilution needed to achieve this TRA concentration was not much higher for non-dialysed formulations than for the dialysed ones. Therefore, we believe that the slightly higher lipid dilution of ND formulations cannot alone explain the 2–3-fold higher TRA deposition found with D vesicles. This is further supported by the fact that diluted ND conventional liposomes (control) gave higher drug deposition than corresponding D ones.

Therefore, all these results show a different performance of PEVs in comparison to conventional liposomes. As already recognized by most authors, conventional liposomes act as penetration enhancers improving both entrapped and non-entrapped drug delivery to the skin (Verma et al., 2003; Manconi et al., 2006; Elsayed et al., 2006; Sinico and Fadda, 2009). On the other hand, results obtained here indicate that PEVs seem to behave as true carriers: the drug must be incorporated into the vesicles to be delivered to and through the skin. As a further trial, *ex vivo* (trans)dermal experiments were also performed with suspensions (MIX) prepared by mixing appropriate amounts of TRA and P50 dispersions in order to obtain the same final concentration of TRA, phospholipids, and PE (when necessary) of the vesicles. TRA dispersion was prepared under sonication to improve the amount of dissolved drug, while the P50 was only gently shaken. During this procedure it is likely that

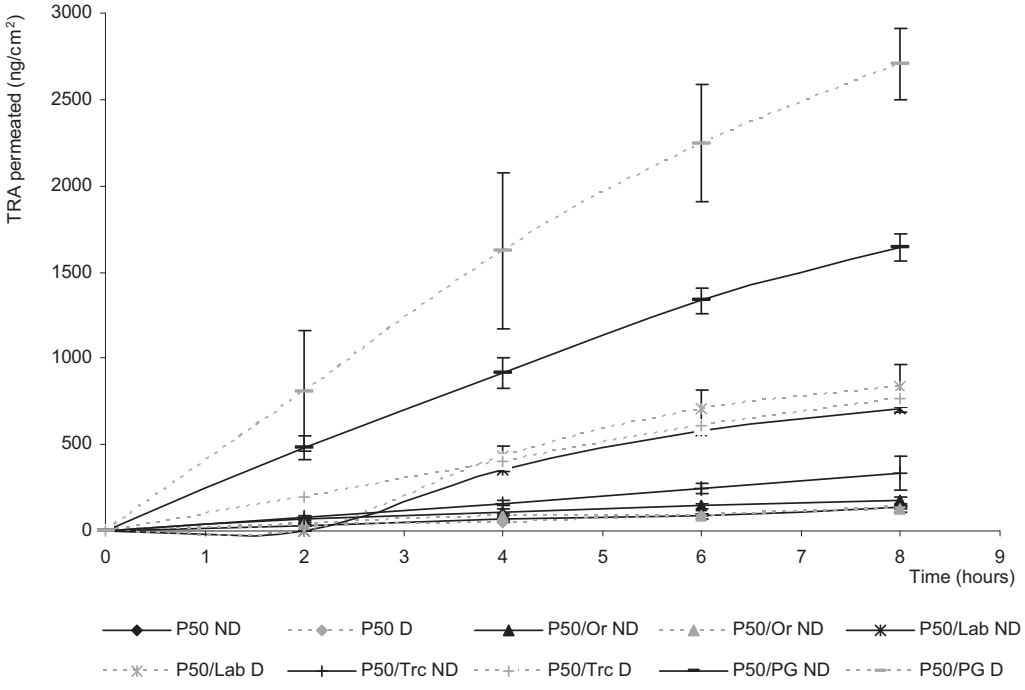

Fig. 5. *Ex vivo* diffusion of TRA through pig skin during 8-h non-occlusive treatment from control liposomes and PEVs, before (ND) and after dialysis (D). Data represent the mean ± standard deviation of at least six experimental determinations.

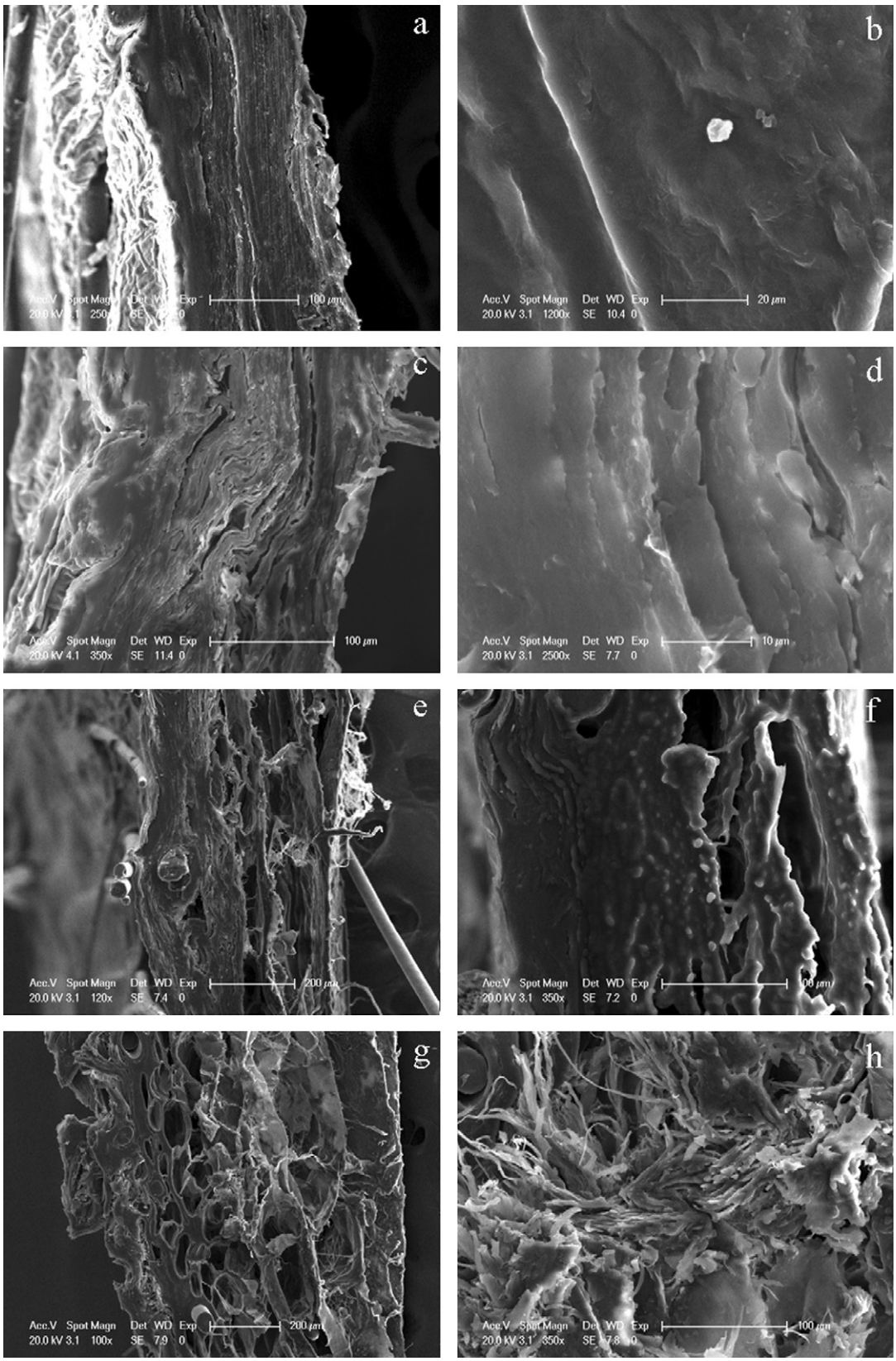

Fig. 6. Scanning electron microscopy micrographs of pig skin treated with phosphate-buffered dispersion of TRA (a and b), TRA-loaded liposomes (c and d), TRA-loaded Or-PEVs (e and f), and TRA-loaded Trc-PEVs (g and h) after 8 h of application. Cross-sectional images taken perpendicular to the treated skin surface, and morphology of skin surface are shown (left and right column, respectively).

phospholipids formed empty vesicles and, thus, the MIX applied onto the pig skin contained TRA partially dissolved in the solvent, in part electrostatically bound to the vesicle surface, and somewhat suspended. Nevertheless, the drug was only outside the formed vesicles. Results of these experiments are shown in Fig. 4B and Table 3. As can be seen, when the MIX formulations were tested, the total amount of drug accumulated in the skin strata was always lower than that obtained with the corresponding drug-loaded vesicular formulations. Moreover, the drug was especially deposited into the SC, with the only exception of the suspension (MIX) without PE, which led to a higher drug deposition in the epidermis. Therefore, these results further support our hypothesis regarding an active role played by PEVs in carrying the drug into and through the skin.

Therefore, comparison of the results from the three sets of TRA-containing formulations (D, ND, and MIX) confirms that PEVs are able to act as true carriers and not simply as penetration enhancers (Mura et al., 2009). In the light of these findings, PEVs seem to be able to penetrate intact the skin reaching the lowest SC layers where they form a depot from which the drug can be released (Bouwstra and Honeywell-Nguyen, 2002; Honeywell-Nguyen et al., 2006).

Permeation profiles (cumulative amounts of drug permeated versus time) of TRA-loaded liposomes and PEVs (Fig. 5) did not show the typical trend with a steady state phase, because the flux was nearly constant from 2 to 8 h, at all sampling times. TRA release occurred rapidly when the drug was loaded in PG-PEVs, both dialysed and non-dialysed, and provided a flux of 315 and 195 $\mu g/cm^2/h$, respectively. The extent of TRA permeation from liposomes was lower than from PEVs, quite similar for dialysed and non-dialysed vesicles. Permeation profiles obtained from the MIX formulation were even lower (data not shown).

Local Accumulation Efficiency (LAC; Table 3) of TRA was estimated as a useful dimensionless parameter for the evaluation and comparison of the ability of vesicular formulations as carriers for cutaneous targeting of TRA. LAC values were obtained from the TRA accumulated/TRA delivered ratio. TRA-loaded liposomes showed a good LAC (~35), while among PEVs, only those with Or had higher LAC, because Lab- and Trc-PEVs allowed not only a high TRA deposition into the skin strata, but also an elevated flux that reduced LAC. Altogether these results show that Or-PEVs are better carriers than liposomes and other PEVs for the cutaneous delivery of tretinoin.

SEM observation of cross-sections and surface parts of epidermis specimens after permeation experiments was carried out to elucidate the effect of PEVs topical application and their interaction with the skin. The understanding of the mechanisms of vesicle-SC interaction, being SC the main barrier to drug diffusion, is important for an appropriate evaluation of vesicles as drug carriers in dermatological preparations. In previous works, TEM analysis showed that TRA-incorporated liposomes might strongly affect the ultrastructure of the intra- and intercellular regions of the horny layer causing an enlargement of intercellular regions (Sinico et al., 2005), and a confocal microscopy study proved that PEVs were able to reach intact the epidermis due to a synergistic mechanism among the penetration enhancer (Trc), vesicles, and intercellular pig skin lipids (Manconi et al., 2011).

Fig. 6 shows representative SEM photomicrographs of pig skin epidermis treated with: TRA dispersion in PBS (a and b), TRA loaded in liposomes (c and d), in Or-PEVs (e and f) and in Trc-PEVs (g and h). The skin incubated with TRA dispersion appears compact, with very thin intracellular structures in SC and total thickness of the epidermis is about 150 μm. The surface of the horny layer is regularly smooth and composed of a mosaic of close, long, and thin corneocytes (b). Skin treated with TRA liposomes shows signs of increased hydration with evident intercellular spaces in the cross-section and in the surface photomicrographs, as previously reported (Sinico et al., 2005). Total thickness of the epidermis is about 200 μm. All skin samples treated with the different PEVs show similar features. In comparison to skin treated with TRA in PBS or liposomes (a and b, c and d), cross-sections and surface of PEV-treated skin (e and f, g and h) show a greater hydration. Indeed, in the cross-sections the corneocyte structure may be easily distinguished from the intercellular region. The inner SC layers show a different organization with intercellular lipid regions larger than in the samples treated with TRA dispersion or liposomes. The skin surface incubated with Or-PEVs appears irregularly undulant, with large junction region very hydrated and elastic (f). Further, skin surface incubated with Trc-PEVs shows desquamated corneocytes distant one from the other (h). Total thickness of the epidermis is about 350 μm.

SEM analysis did not show any evidence of vesicular structures into the pig skin, but provided evidence of PEVs' ability to strongly interact with the intercellular lipids causing an enlargement of this region. PEVs facilitated the drug crossing through the skin, as well as enhanced TRA bioavailability, since they allowed the diffusion of free TRA molecules into the deeper layers of the epidermis.

4. Conclusions

This work shows that PEVs are eligible for the use as suitable carriers for tretinoin in skin disease treatment. Indeed, *ex vivo* penetration and permeation results have shown that the presence of a hydrophilic PE molecule in the formulation of P50 liposomes is crucial for improving dermal delivery of a lipophilic drug such as tretinoin. In particular, drug deposition and permeation in the skin was found to be affected by PEVs composition. Or-containing vesicles seem to be the most appropriate formulations for tretinoin dermal delivery.

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