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Niosomes as carriers for tretinoin III. A study into the in vitro cutaneous delivery of vesicle-incorporated tretinoin

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Abstract

The influence of drug thermodynamic activity and niosome composition, size, lamellarity and charge on the (trans)dermal delivery of tretinoin (TRA) was studied. For this purpose, tretinoin was incorporated at saturated and unsaturated concentrations in both multilamellar (MLV) and unilamellar (UV) vesicular formulations using two different commercial mixtures of alkyl polyglucosides: octyl-decyl polyglucoside and decyl polyglucoside. Positively and negatively charged vesicular formulations were prepared using either stearylamine or dicetylphosphate as a charge inducer. Niosomes made with polyoxyethylene (4) lauryl ether and liposomes made with soy phosphatidylcholine were also prepared and studied. Vesicular formulations were characterised by transmission electron microscopy and optical and light polarized microscopy for vesicle formation and morphology, and by dynamic laser light scattering for size distribution. The effect of the vesicular incorporation of tretinoin on its (trans)dermal delivery through the newborn pig skin was also investigated in vitro using Franz cells, in comparison with a commercial formulation of the drug (RetinA®). The amount of tretinoin delivered through and accumulated in the several skin layers was detected by HPLC. Overall, obtained results showed that tretinoin cutaneous delivery is strongly affected by vesicle composition and thermodynamic activity of the drug. In particular, small, negatively charged niosomal formulations, which are saturated with tretinoin, have shown to give higher cutaneous drug retention than both liposomes and commercial formulation. Moreover, interactions between skin and vesicles seem to depend on physico-chemical properties of the main component of the vesicular bilayer.

Keywords: Niosomes; Non-ionic surfactants; Alkylpolyglucosides; Tretinoin; In vitro skin permeation; In vitro skin delivery

1. Introduction

In recent years, non-ionic surfactant vesicles (NSVs), also referred to as niosomes, have been studied as an alternative to conventional liposomes. In fact, if compared to phospholipids vesicles they offer higher chemical stability, lower costs, and great availability of surfactant classes (Handjani-Vila et al., 1979; Baillie et al., 1985; Hofland et al., 1994; Uchegbu and Florence, 1995; Van Hal et al., 1996; Uchegbu and Vyas, 1998).

Nowadays, vesicular systems, both liposomes and niosomes, play an increasingly important role in drug delivery as they can reduce drug toxicity and modify drug pharmacokinetics and bioavailability. In particular, niosomes seem an interesting drug delivery system in the treatment of dermatological disorders. In fact, topically applied niosomes can increase the residence time of drugs in the stratum corneum and epidermis, while reducing the systemic absorption of the drug. They are thought to improve the horny layer properties, both by reducing transepidermal water loss and by increasing smoothness via replenishing lost skin lipids (Junginger et al., 1991). Since the first reports from L'Oreal laboratories in the seventies (Vanlerberghe and Handjani, 1975; Handjani-Vila et al., 1979), much research has been carried out into the vesicle forming ability of an ever increasing number of amphiphilic lipids, with different chemical structures and different composition of the hydrophilic and hydrophobic moiety (Uchegbu and Florence, 1995; Uchegbu and Vyas, 1998; Gopinath et al., 2002, 2004).

In recent years, attention has been focused on sugar-based surfactants for several types of applications. Increased interest in this surfactant class is the consequence of the demanding

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request for less toxic, highly biodegradable surfactants, which are also produced from renewable raw materials. It has also been suggested that sugar moieties may replace ethylene oxide as the polar head of amphiphiles and that sugar-based amphiphiles may substitute ethylene oxide-based surfactants in several applications. In particular, alkyl polyglucosides (APGs) have been studied for several types of applications. Commercial APGs are a mixture of glucosides, which are obtained from degraded starch fractions. APGs are stable at high pH values, but sensitive to low pH where they hydrolise to glucose and fatty alcohol. The main APGs attractiveness lies in their favourable environmental profile: the rate of biodegradation is usually high while the aqueous toxicity is low. In addition, APGs show favourable dermatological properties, being very mild to the skin and eye. This mildness makes this surfactant class attractive for cosmetic products although APGs have also found a wide range of technical applications. APGs have already shown their capability to form vesicular structures and their properties led us to explore the possibility of using APGs containing niosomes as carriers for the topical delivery of tretinoin (Manconi et al., 2002).

Trans-retinoic acid or tretinoin (TRA) is a natural retinoid widely used in proliferative and inflammatory skin diseases, such as psoriasis, acne, epidermotropic T-cell lymphomas or epithelial skin cancer (Lucek and Colburn, 1985). The use of topical tretinoin in the treatment of skin diseases began with the study of Kligman et al. (1969), which reported its benefits in the treatment of acne vulgaris. At present, this drug is the most effective available topical comedolytic agent for the treatment of acne. Unfortunately, this drug is very unstable in the presence of air, light and heat and tretinoin degradation was reported to occur within 1–2 h after application (Elbaum, 1988). Moreover, its topical application may cause irritation and peeling of the treated area.

Results of our previous studies demonstrated that several vesicle structures (liposomes or niosomes, MLV, LUV or SUV) can incorporate tretinoin (Manconi et al., 2002) and reduce photodegradation of this drug (Manconi et al., 2003). Pursuing our interest in niosomes as carriers for treinoin, in this study, we evaluated the influence of niosomal incorporation on in vitro (trans)dermal delivery of TRA. The aim of this work is to evaluate the potential of APG niosomes as topical delivery systems capable of improving the cutaneous delivery of TRA.

The incorporation of TRA in niosomes would give the same benefits reported for liposomes and, more precisely, the presence of a non-ionic surfactant could improve its skin penetration and increase its accumulation in the superficial skin strata (Sinico et al., 2005). In particular, we investigated how the penetration capacity of vesicular tretinoin can be affected by several parameters (drug thermodynamic activity, vesicle composition, charge, and structure). For this purpose, we prepared multilamellar (MLV) and unilamellar (UV) niosomes using two different commercial mixtures of APGs, which vary for the different grade of polymerisation and the nature of the lipophilic moiety. In fact, we used octyl-decyl polyglucoside (Fig. 1; Oramix CG110®, OrCG110) and decyl polyglucoside (Fig. 1; Oramix NS10®, OrNS10). Positively or negatively charged vesicular formulations were prepared using either stearylamine (SA) or

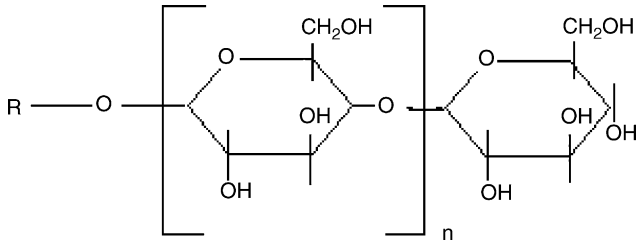

Fig. 1. Structure of the alkyl polyglucoside surfactants: (A) decyl polyglucoside (OrNS10), $n = 1–3$; R = $C_{8–10}$; (B) octyl-decyl polyglucoside (OrCG110), $n = 1–5$; R = $C_{8–10}$.

dicetylphosphate (DCP) as a charge inducer. Niosomes made from polyoxyethylene (4) lauryl ether (Brij 30®) and, for an appropriate comparison, liposomes made with soy phosphatidylcholine (Phospholipon 90, P90) were also prepared and studied. All formulations also contained cholesterol. Two sets of vesicular formulations were prepared and studied: in one of them tretinoin was incorporated at saturated concentrations while the other one was not saturated in tretinoin. The in vitro (trans)dermal delivery of TRA-loaded vesicles and its accumulation in the newborn pig skin was investigated using vertical diffusion Franz cells in occlusive conditions and in comparison with a commercial formulation of TRA (Retin-A®) as a control.

2. Material and methods

2.1. Materials

Oramix®CG110 (OrCG110) and Oramix®NS10 (OrNS10) were kindly obtained from Seppic, Italy. Soy phosphatidylcholine (Phospholipon 90, P90) was a gift from Natterman Phospholipids, Gmb. Brij® 30 (Br30), cholesterol (Chol), trans-retinoic acid (TRA), dicetylphosphate (DCP), stearylamine (SA) and all the other products were analytical grade and were purchased from Aldrich, Milan, Italy. RetinA® is a commercial preparation produced by Janssen-Cilag, Milan, Italy.

2.2. Vesicle preparation

Tretinoin was incorporated into vesicles at saturated and unsaturated concentrations. TRA saturated concentrations were studied to obtain equal thermodynamic activities in a set of the studied formulations. The maximum amount of TRA to be used for obtaining saturated concentrations was established by increasing TRA quantities during the preparation of liposomes and niosomes. The presence of TRA crystals was used as an indicator of vesicle saturation with the drug. Vesicle suspensions were then checked for TRA crystals over a period of 21 days using light microscopy. Vesicle incorporation of TRA at saturated concentrations was obtained using the following molar ratios: OrCG110/Chol/TRA 5:5:1, OrNS10/Chol/TRA 6:4:1, Br30/Chol/TRA 10:6:1 and P90/Chol/TRA 2.5:0.3:1. All vesicle formulations contained a constant amount of DCP (2:1 TRA/DCP molar ratio) or SA (2:1 TRA/SA molar ratio) to obtain a net negative or positive charge on the vesicle surface. More-

over, for an appropriate comparison, vesicle suspensions unsaturated in TRA were also prepared using a fifth of the drug used for the saturated formulations (e.g. P90/Chol/TRA 25:3:1, OrCG110/Chol/TRA 30:30:1, OrNS10/Chol/TRA 33:20:1, Br30/Chol/TRA 53:30:1 molar ratios) and a constant amount of DCP or SA (TRA/DCP o TRA/SA 1:4). Multilamellar vesicles (MLVs) were prepared according to the thin film hydration method. The surfactant, Chol, TRA and DCP or SA in chloroform solution were mixed in the appropriate ratio. The lipid–drug mixture was deposited as a thin film in a round-bottom flask by roto-evaporating the chloroform under vacuum. The vacuum was applied for 1 h to ensure total removal of trace solvents. The film was hydrated with phosphate buffered saline solution (PBS) at room temperature because gel–liquid transition temperature (T_c) of all the amphiphiles is lower than 2 °C (Manconi et al., 2002; Hoffmann et al., 2000). Unilamellar vesicles (UVs) were prepared by sonication of MLV dispersions in a Soniprep 150 apparatus (MSE, Crowley), under a nitrogen stream for 30 min (30 times for 1 min), at a temperature above the T_c. Each vesicle suspension was purified from non-incorporate TRA by gel chromatography on Sephadex G50. All dispersions were diluted with PBS in order to obtain the same TRA concentration (i.e. 0.2 mg/ml). The influence of the dilution process on tretinoin leakage from vesicles was checked by dialysis. Dilute vesicular suspensions were transferred into a Visking tubing (36/32 S.I.C.) and dialysed against PBS for 24 h. Quantitative determination was carried out by HPLC as described below. TRA recovery was always higher than 98% of the dialysed amount. Incorporation efficiencies (*E*%), expressed as a percentage of the total amount of TRA found in the studied formulations at the end of the preparation procedure, were determined by HPLC after disruption of vesicles with Triton X-100. Tretinoin content of samples was analyzed at 350 nm using a liquid chromatograph Alliance 2690 (Waters), equipped with a photodiode array detector and a computer integrating apparatus (Millennium 32). The column was a Nova-Pack C18 (60 Å, 4 μm, Waters). The mobile phase was a mixture of acetonitrile, water and acetic acid (84.5:15:0.5, v/v), at a flow rate of 1.2 ml/min. All suspensions were prepared under yellow light and kept in the dark at all times.

2.3. Vesicle characterisation

Vesicles were characterised by trasmission electron microscopy (TEM) and optical and light polarized microscopy for vesicle formation and morphology and by dynamic laser light scattering (DLLS) for mean size and polydispersivity index. A drop of vesicle dispersion was applied to a carbon film-covered copper grid and was stained with a 1% phosphotungstic acid. Then samples were examined and photographed with Zeiss EM 109 transmission electron microscope at an accelerating voltage of 80 kV.

Optical and light polarized micrographs were obtained with an optical microscope Zeiss Axioplan 2, at 25 °C.

Liposome size distribution was determined by DLLS (N4 plus, Beckman Coulter) at 25 °C. Samples were scattered (633 nm) at an angle of 90°. Data were fitted by the method of inverse "Laplace transformation" and Contin (Provencher, 1982a,b).

2.4. Skin permeation studies

In vitro skin permeation studies were performed using vertical diffusion Franz cells with an effective diffusion area of 0.636 cm^2. The experiments were carried out using new-born pig skin. One-day-old Goland–Pietrain hybrid pigs (1.2 kg) were obtained from a local slaughterhouse. Pigs were sacrificed by cervical dislocation. The skin, previously frozen at −18 °C, was pre-equilibrated in PBS solution at +25 °C 2 h before the experiments. A circular piece of the skin was sandwiched securely between the two halves of cell with the SC side facing the donor compartment. The receiver compartment was filled with 5.5 ml of a hydroalcoholic solution (ethanol/PBS 50:50) which was continuously stirred and thermostated at 37 ± 1 °C throughout the experiments. The tested (1 ml) vesicle suspensions (0.2 mg/ml tretinoin) or control preparation were placed on the membrane surface. A commercial cream, RetinA® (RetA, 0.025%) was used as a control. Before starting the experiments the donor cell was sealed with parafilm and the cells were covered with aluminium foil to prevent exposure to light. At hourly intervals up to 9 h the receiving solution was removed and replaced with an equal volume of prethermostated (37 °C) fresh hydroalcoholic solution. The complete substitution of the receiver compartment was needed to ensure sink conditions and quantitative determination of the small amount of permeated tretinoin. The solutions were analyzed by HPLC. Experiments were performed in triplicate and were repeated three times. Mean values were used for the analysis of the data. Experiments in which the pig skin was pre-treated with SA aqueous solution were also carried out. Pig skin samples, sandwiched securely between the two halves of the Franz cells, were treated with 1 ml of SA solution (1 mg/ml) for 8 h. Then, SA solution was discarded and the skin washed three times with 2 ml of PBS. Afterwards, samples of RetA or TRA loaded vesicular formulations were applied. TRA permeation was studied as described above.

2.5. Skin retention of tretinoin

At the end of the permeation study, the skin was removed and the surface was washed three times with PBS, briefly rubbed with a paper towel and then dried with filter paper. The stratum corneum (SC) was removed by stripping with an adhesive tape (Tesa® film, Beiersdorf, Germany) (Verma et al., 2003; Lindemann et al., 2003; Sinico et al., 2005). The method was previously validated by histological examination of stripped skin. A 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 and the 10 tape strips were placed in a vial and extracted with 20 ml of methanol. The solution was filtered and analyzed by HPLC. The piece of skin was separated into the epidermis (EP) and dermis (D) by pressing the skin surface against a hot plate (60 °C) for 30 s and then peeling off the epidermis (Katahira et al., 1999). The separated

skin samples were each soaked in a flask with 20 ml of methanol for 24 h. Then the methanolic samples were shaken in an ultrasound bath four times for 30 min each, in order to extract all the drug accumulated in the skin pieces.

2.6. Statistical analysis of data

Data analysis was carried out with the software package Microsoft Excel, version 2001. Results are expressed as mean ± standard error (three independent samples). Statistically significant difference was determined using the Student's *t*-test and analysis of variance (Anova) with $P = 0.05$ as a minimal level of significance.

3. Results and discussion

3.1. Vesicle characterisation

In order to evaluate the potential of non-ionic surfactant vesicles as topical delivery systems for the cutaneous delivery of TRA, we prepared and tested niosomes prepared using two different commercial mixtures of APGs, OrCG110 and Or NS10, and with a polyoxyethylene lauryl ether (Br30). For an appropriate comparison, TRA was also incorporated in liposomes made with soy phosphatidylcholine (P90), which we had already studied in the in vitro cutaneous delivery of this drug (Sinico et al., 2005). All amphiphiles used as the main bilayer component have a transition temperature less than 2 °C, and all the prepared formulations contained cholesterol. During this study, we prepared both negatively and positively charged vesicles by using either DCP or SA, respectively, as a charge inducer. Formulations with two different lipids/TRA molar ratios were studied in order to obtain TRA saturated and unsaturated niosomal formulations, which were both diluted to obtain a treinoin concentration of 0.2 mg/ml. The aim of this study was to compare formulations with the same concentration and thermodynamic activity of the incorporated drug (TRA saturated formulations) to others with the same drug concentration but with different thermodynamic activity (TRA unsaturated formulations). This difference in study design is important when comparing drug permeation from vesicles to stratum corneum. In fact, formulations with equal tretinoin thermodynamic activity also present an equal driving force from formulation to stratum corneum (Bouwstra, 2002). Vesicle formation and morphology was studied by light polarized spectroscopy and TEM, which confirmed the production of unilamellar vesicles. Fig. 2 presents two negative stain electron micrographs of tretinoin-loaded OrNS10 vesicles. In particular, Fig. 2A shows multilamellar niosomes (MLVs), while sonicated vesicles (UVs) can be seen in Fig. 2B, where clear evidence of a single layered membrane is shown. Table 1 shows vesicle size and TRA incorporation efficiency for the niosomal dispersions saturated in TRA. Dynamic light scattering analyses showed that some of the samples were polydispersed (PI > 0.35), but reproducibility of vesicle sizes appeared to be good. Moreover, as already observed in our previous related works, the mean size of TRA saturated sonicated vesicles was large despite the preparation method: UVs mean size ranged from 225 to 366 nm.

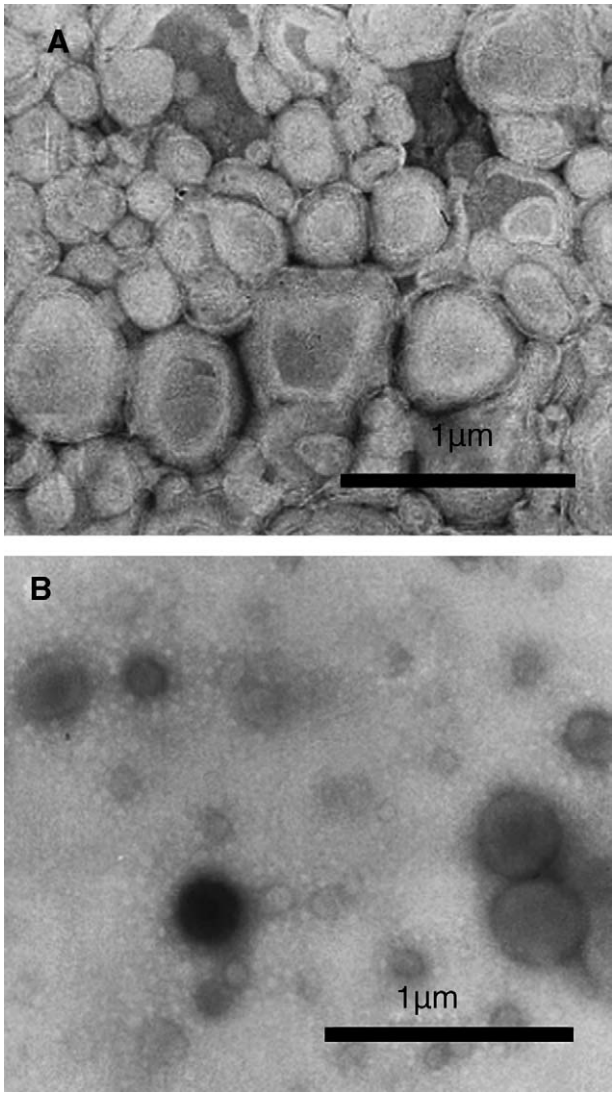

Fig. 2. Negative stain electron micrographs of tretinoin-loaded vesicles prepared with OrNs10: (A) MLVs; (B) UVs.

On the contrary, as can be seen from Table 2, the TRA non-saturated formulations always showed smaller sizes.

The large diameter of all vesicles saturated in TRA is the consequence of the presence in the bilayers of highly hydrophilic amphiphiles (i.e. non-ionic surfactant and charger inducers, DCP

Table 1
Incorporation efficiency (*E*%) and mean diameter of vesicular formulations saturated with tretinoin

Composition	*E*%		Average size (nm ± S.D.)	
	MLV	UV	MLV	UV
P90/SA	71.80 ± 5.1	93.03 ± 1.7	598 ± 67	297 ± 74
Br30/SA	71.92 ± 3.3	94.96 ± 0.5	655 ± 58	278 ± 61
OrNS10/SA	87.77 ± 1.4	96.56 ± 0.3	756 ± 65	312 ± 27
OrCG110/SA	97.17 ± 1.3	99.50 ± 0.9	923 ± 49	366 ± 46
P90/DCP	97.32 ± 1.8	75.98 ± 2.5	536 ± 49	293 ± 53
Br30/DCP	93.85 ± 1.0	93.00 ± 0.8	598 ± 31	225 ± 29
OrNS10/DCP	96.57 ± 1.1	95.35 ± 0.5	658 ± 44	292 ± 52
OrCG110/DCP	98.47 ± 0.8	97.38 ± 1.3	714 ± 47	328 ± 27

Table 2
Mean size of vesicular formulations not saturated with tretinoin

Composition	Average size (nm ± S.D.)	
	MLV	UV
P90/SA	298 ± 56	137 ± 41
Br30/SA	345 ± 38	163 ± 29
OrNS10/SA	356 ± 47	213 ± 36
OrCG110/SA	393 ± 53	229 ± 49
P90/DCP	219 ± 59	99 ± 39
Br30/DCP	236 ± 68	87 ± 32
OrNS10/DCP	264 ± 77	96 ± 29
OrCG110/DCP	313 ± 58	107 ± 32

or SA) together with an excess of tretinoin. The hydrophilic amphiphiles increase the vesicle surface energy, which causes vesicle enlargement (Fang et al., 2001; Carafa et al., 1998). For the same reason vesicle formulations containing SA always showed larger sizes than those containing DCP. Thus, in the case of niosomes, a correlation between amphiphile hydrophile lipophile balance (HLB) and vesicle size can be observed. In fact, niosomes made from Br30 (HLB = 9.7) showed a smaller mean size than those prepared with OrNS10 (HLB = 11) whose niosomes in turn were smaller than vesicles made from OrCG110 (HLB = 16). This behaviour can be explained because the higher the surfactant hydrophilicy, the higher the water uptake into the bilayer. Therefore, vesicle size increases as well as the amount of TRA, which can be incorporated into the vesicular bilayers. In fact, the highest TRA incorporation efficiency (96–99%) was observed for the largest vesicles made with the most hydrophilic surfactant OrCG110 (HLB = 16). This improved incorporation of the lipophilic TRA is due to the increased capacity of the lipophile environment in the surfactant bilayer, which is capable of incorporating this amphipatic molecule to a higher extent. Surfactants like Br30, OrNS10 and OrCG110 have high HLB and small CPP (critical packing parameter) values, and are likely to form micelles in aqueous solution. They are able to form vesicles only in the presence of a large amount of cholesterol, which allows them to achieve CPP values and molecular geometry suitable for vesicle formation (Israelachvili, 1985). In this case the hydrophobic tretinoin molecule ($\log K_{o/w} = 6.3$), as well as cholesterol, can improve the CPP for the bilayer formation (Uchegbu and Vyas, 1998; Paulsson and Edsman, 2001). Therefore, the higher the surfactant HLB, the higher the amount of lipophilic drug, which can be intercalated into the niosomal bilayers. Therefore, TRA incorporation efficiency (E%) decreased with the decrease of the surfactant HLB as reported by Shahiwala and Misra (2002) regarding nimesulide. On the other hand, formulations unsaturated in tretinoin always showed a very high entrapment efficiency (98–100%), which was independent from the bilayer composition.

Table 1 shows that also TRA saturated formulations demonstrated high incorporation capability, ranging from 72% (Br30/SA MLVs) to 99% (OrCG110/SA UVs). Positively charged unilamellar vesicles always showed higher entrapment efficiency than the MLV counterparts, as previously observed for similar TRA-loaded positively charged liposomes (Sinico et al., 2005). This result seems to confirm the hypothesis that a higher energy is required for vesicle formation when SA is present in the lipid phase. As can be seen in Table 1, P90/DCP sonicated liposomes showed the lowest incorporation efficiency (75.98%) in comparison with UV niosomes and P90/SA liposomes. Similar results were obtained in a previous work also with hydrogenated soy phosphatidylcholine, where a reduction of E% was found from 96.75% (MLVs) to 78.53% (UVs) (Sinico et al., 2005). This outcome could be due to several negative factors. In particular, there is a lower entrapment capability of the single layered membrane because it is prepared with the more bulky double chain surfactant soy phosphatidylcholine. This is particularly true when a negative charger inducer is associated to the partially ionised trans-retinoic acid. In fact, the same does not happen when UVs are prepared with the positive charge inducer SA, which can electrostatically link the ionised TRA. On the other hand, in the case of niosomes prepared with more hydrophilic surfactants, a higher incorporation of the drug into the bilayer is achieved because, as reported above, TRA contributes to a higher stability of the niosomal bilayer.

3.2. In vitro skin permeation study

In order to evaluate the influence of drug content and vesicle composition, structure, and surface charge on the TRA accumulation in and diffusion through the skin, we carried out in vitro permeation studies using newborn pig skin and vertical Franz diffusion cells. During this study, we compared the permeation data obtained from niosomal tretinoin with those obtained from a liposomal formulation of the drug. The permeation study was carried out using both tretinoin saturated and non-saturated vesicular formulations, which were all diluted in order to obtain the same drug concentration, that is 0.2 mg/ml. We diluted vesicular formulations with the assumption that dilution did not lead to TRA release because of the high hydrophobicity and low water solubility of this drug. Experiments in which leakage of tretinoin from the diluted vesicular suspension was studied by dialysis did prove this assumption as no appreciable amount of TRA was released from all the studied formulations. Moreover, diluted formulations were checked for TRA crystals using light microscopy, which did not show any drug loss from the vesicles. As a control, we chose a commercial topical formulation of tretinoin (Retin-A®, 0.25 mg/ml) because it was reported that liposomes with less TRA concentration than commercial formulations had shown the same efficacy (Schäfer-Korting et al., 1994).

The in vitro permeation study was carried out through the whole skin in occlusive conditions that are known to improve drug accumulation into the skin layers, while non-occlusive conditions are more suitable to improve the flux (Van Kuijk-Meuwissen et al., 1998; Sinico et al., 2005). The mean amount of TRA permeated per unit of surface area from vesicles or control formulations was determined during 9 h experiments. Fig. 3 shows permeation profiles (cumulative amounts of TRA permeated versus time) of tretinoin through the skin obtained from TRA saturated MLV and UV niosomes in comparison with the commercial formulation used as a control. On the other hand,

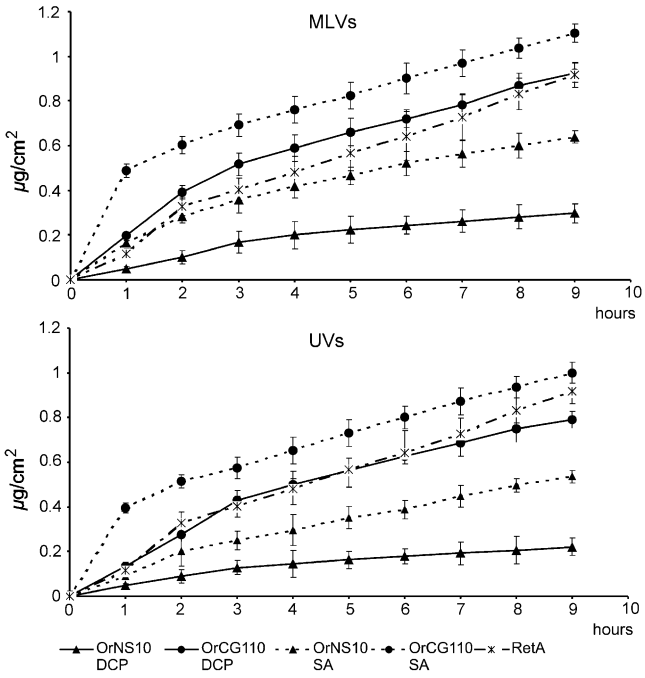

Fig. 3. In vitro diffusion of niosomal TRA through newborn pig skin (9 h).

tretinoin permeation from the non-saturated formulations was negligible, never reaching 0.1% of the applied dose.

As can be seen in Fig. 3, permeation curves do not show a classic profile with a steady state phase. Tretinoin flux is higher in the initial period of the permeation experiments (0–3 h). Afterwards, for all vesicular formulations, TRA permeation rate was lower and almost constant in all sampling times. Results seem to indicate that TRA release and skin permeation occurred rapidly especially when the drug was delivered from positively charged vesicles. In fact, as shown in Fig. 3, after only 1 h from the beginning of the experiments both SA-containing MLVs and UVs delivered a TRA amount that was at least two-fold higher than that permeated from the negatively charged vesicles. As already observed for TRA-loaded liposomes, for each composition the permeation profile was very similar for both multi- or unilamellar vesicle dispersions. Therefore, results obtained during this work seem to confirm that vesicle size and lamellarity do not affect TRA delivery through the pig skin (Sinico et al., 2005). These results also suggest that vesicles do not penetrate intact through the skin (Du Plessis et al., 1994).

Results of this study are reported in Table 3, which shows the cumulative amounts of tretinoin delivered in the receiver compartment at 1 and 9 h after the beginning of the experiments carried out with TRA saturated formulations. It is evident that there are some differences in the total amount of TRA permeated. In the first analysis, the permeated dose of liposomal and niosomal TRA was dependent on the main bilayer component. In fact, these values increased according to this order: OrNS10 < Br30 < P90 < OrCG110 vesicles both in MLV and UV formulations. MLVs always gave a higher TRA delivery than UVs, although no stastistically relevant differences could be observed. In addition, positively charged vesicles always gave a higher permeation rate than the negatively charged ones. The only exceptions were OrCG110 formulations, which always showed the highest permeation rate independently from the vesicle charge. It is well known that the skin may act as a negatively charged membrane and it has generally been reported that the presence of a charge on the vesicle surface affects the drug diffu-

Table 3
Results of in vitro permeation study from vesicular formulations saturated with tretinoin and control

Component		TRA delivered (μg/cm^2 ± S.D.)		TRA skin accumulated (μg/cm^2 ± S.D.)	LAC
		1 h	9 h	9 h	9 h
P90/SA	MLV	0.499 ± 0.024	0.888 ± 0.059	13.56 ± 1.33	5.3
	UV	0.464 ± 0.018	0.799 ± 0.038	23.47 ± 2.55	12.9
Br30/SA	MLV	0.464 ± 0.021	0.764 ± 0.028	39.26 ± 8.54	26.8
	UV	0.339 ± 0.016	0.606 ± 0.032	46.72 ± 8.03	39.4
OrNS10/SA	MLV	0.165 ± 0.011	0.638 ± 0.028	37.22 ± 4.33	23.5
	UV	0.088 ± 0.005	0.536 ± 0.027	44.37 ± 8.84	39.7
OrCG110/SA	MLV	0.488 ± 0.031	1.103 ± 0.040	16.81 ± 2.42	3.9
	UV	0.396 ± 0.022	0.999 ± 0.047	21.87 ± 4.98	8.8
P90/DCP	MLV	0.098 ± 0.010	0.555 ± 0.023	30.45 ± 7.58	26.6
	UV	0.080 ± 0.006	0.452 ± 0.021	32.67 ± 8.98	34.8
Br30/DCP	MLV	0.079 ± 0.009	0.419 ± 0.020	52.26 ± 8.33	53.3
	UV	0.042 ± 0.006	0.346 ± 0.013	79.47 ± 8.44	111.3
OrNS10/DCP	MLV	0.049 ± 0.007	0.298 ± 0.011	32.68 ± 7.42	54.1
	UV	0.047 ± 0.009	0.221 ± 0.040	52.50 ± 4.79	127.7
OrCG110/DCP	MLV	0.047 ± 0.012	0.926 ± 0.043	12.81 ± 2.90	5.6
	UV	0.135 ± 0.015	0.791 ± 0.035	23.48 ± 6.21	9.8
RetA		0.114 ± 0.023	0.917 ± 0.057	12.03 ± 0.41	4.2

Amount of TRA delivered through the skin at 1 h and at the end of the experiments (9 h); amount of tretinoin accumulated into the skin at the end of the experiments; and Locally Accumulation Efficiency (LAC) value: TRA accumulated into SC/TRA delivered through the skin ratio.

sion through the skin (Katahira et al., 1999; Ogiso et al., 2001). Negatively charged vesicles generally give a higher flux than their positively charged counterparts, which in turn can improve drug accumulation in the superficial skin strata. However, in the present investigation positively charged vesicles provided a permeation rate higher than negatively charged liposomes and niosomes. In order to verify if the higher tretinoin permeation obtained from the SA containing vesicles could have been due to skin damage caused by the cationic surfactant, we also carried out experiments by pre-treating the pig skin with an SA solution (1 mg/ml) for 8 h. Successively, RetA or TRA loaded neutral vesicular formulations were applied. However, results of this study revealed that the pre-treatment with SA always strongly decreased the TRA permeation rate when compared to formulations applied to the non-treated skin (data not shown). Therefore, the enhanced TRA diffusion through the pig skin observed here with the SA-containing formulations could be explained as the consequence of ion pair formation between the partially ionized retinoic acid ($pK_a = 7.85$; pH = 7) and SA counter ions thus promoting tretinoin delivery through the skin (Trotta et al., 2003; Sinico et al., 2005).

3.3. In vitro TRA regional distribution on the skin

The amounts of TRA accumulated in the whole pig skin are reported in Table 3. As can be seen, vesicular formulations enhanced TRA accumulation in newborn pig skin to a factor of 1–6.6 over RetA. Moreover, the accumulation of TRA provided by OrNS10 and Br30 DCP-containing vesicles was found statistically higher ($P < 0.05$) than that resulting from OrCG110 vesicles and the control.

Results showed that the accumulation of TRA provided by vesicular formulations was related both to vesicle structure and composition. In fact, UVs always showed to be a better carrier than MLVs for the delivery of TRA locally to the skin. This result could be due to the different rate of vesicle flocculation on the skin surface according to their size. After their topical application, vesicles settle down on the skin surface close to the outermost corneocyte layer. In contact with the skin the smaller UVs are less stable than larger MLVs. They disintegrate faster on the skin surface and a close contact between skin lipids and vesicle constituents is obtained. In these conditions, material exchange between vesicles and intercellular lipids may occur, allowing the diffusion of free molecules as well as small membranal fragments into the SC. MLVs are larger than UVs and are formed with several concentric lipid bilayers. Therefore, they settle down on the skin surface faster and stay on the SC surface forming a thick lipid layer that increases the diffusion pathway of tretinoin (Verma et al., 2003).

The highest accumulation values were obtained from the negatively charged vesicles, while the highest TRA permeation was obtained from positive vesicles. This contradictory result can be explained because of the physicochemical properties of TRA. In fact, as reported above vesicles form a lipid film on the skin, which can retain both endogen and liposome entrapped water causing an improvement of SC intra- and intercellular hydration. Therefore, the compact structure of the SC is opened and the barrier permeability is increased. All these factors cause the retention of tretinoin in the horny layer. However, they cannot improve TRA diffusion through the inner more hydrophilic skin layers because of the lipophilicity of the drug, which shows higher affinity for the SC lipid matrix. Only when TRA was incorporated into positively charged liposomes a slightly improved diffusion through the skin was obtained. This behaviour can be explained as a consequence of a better diffusion pattern of the more hydrophile ionized TRA electrostatically linked to the counter ion stearylamine.

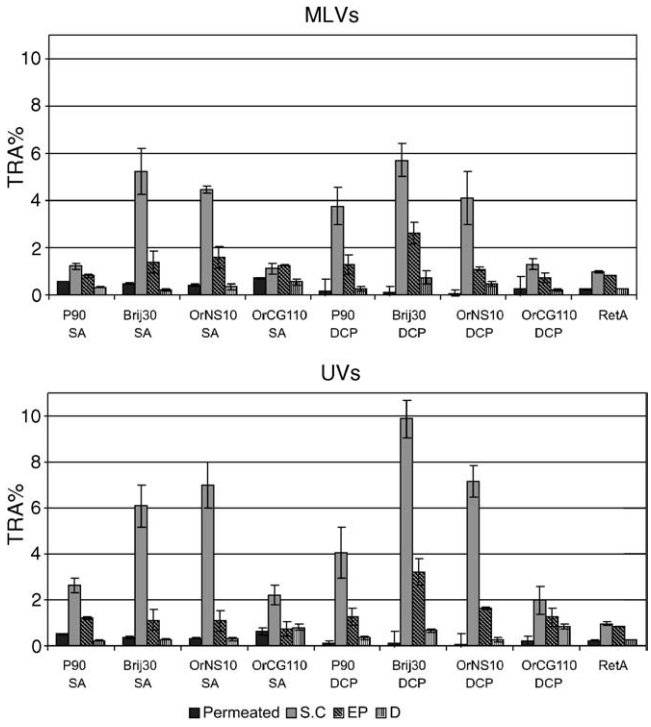

Fig. 4. Cumulative amount of tretinoin permeated through pig skin after 9 h and retained into the stratum corneum (SC), epidermis (E) and dermis (D) layers from the vesicular formulations, MLVs and UVs, saturated with tretinoin.

In particular, niosomes made with Br30 and OrNS10 always gave the best performances as potential carriers for the treatment of skin pathologies.

Fig. 4 shows the percentage of TRA delivered and accumulated in the stratum corneum (SC), in the epidermis (EP) and in the dermis (D) for all the studied vesicular formulations and controls at the end of the experiments. As can be seen the highest accumulation of tretinoin was found in the SC, except for P90 and OrCG110 positively charged MLVs, which on the other hand had shown the highest TRA transdermal permeation.

A useful dimensionless parameter that allows the evaluation and comparison of vesicular formulations as topical carriers for TRA is the locally accumulation efficiency (LAC). LAC values can easily be obtained from the TRA accumulated on SC/TRA delivered ratio. LAC values obtained from the several formulations are listed in Table 3. The LAC value of niosomes was higher by a factor of 1.3–30.4 ($P < 0.05$) than that of the commercially available RetA (LAC = 4.2), suggesting that the main effect of these systems was to accumulate the drug in the skin. The only exception was OrCG110/SA multilamellar niosomes

whose LAC value (3.9) was somewhat lower than that of RetA. It is interesting to point out that this formulation had the highest transdermal permeation as compared to the other vesicular preparations. Comparison of LAC values also shows that positive niosomes made from Br30 and OrNS10 are better carriers for tretinoin than the corresponding P90 liposomes. In fact, LAC values of these niosomes are 4.5–7.5-fold higher than those of positively charged liposomes. However, best LAC values were always obtained with negatively charged vesicles, both liposomes and niosomes.

The comparison of LAC values suggests that the accumulation of the tretinoin in the skin is affected by vesicle charge, vesicle structure and size, vesicle composition and therefore amphiphile physico-chemical properties (T_c, HLB, polar head). In particular it is promoted by small, negatively charged vesicles and it is strongly affected by the vesicle composition. In fact, niosomes made from Br30 (HLB = 9.7) and OrNS10 (HLB = 11) showed the highest LAC values.

Some interesting results were obtained for alkyl polyglucoside vesicles. In fact, while OrNS10 (HLB = 11) vesicles gave the highest accumulation values and a low permeation rate, OrCG110 (HLB = 16) niosomes showed higher fluxes and very low LAC values. Results obtained during this study give evidence that interactions between skin and vesicles mainly depend on physico-chemical properties of the major component of the vesicular bilayer. As discussed by other authors (Junginger et al., 1991), the polar moiety of the amphiphile plays the main role with respect to interaction with skin lipids. OrCG110 with its very strong hydrophilic head group (n = 1–5) is not able to penetrate significantly into the SC. However, when penetration occurs, its highly polar head can strongly perturb the SC intercellular lipid bilayer giving rise to a facilitated pathway for the drug that can reach the dermis and receiver compartment more easily and in a greater amount.

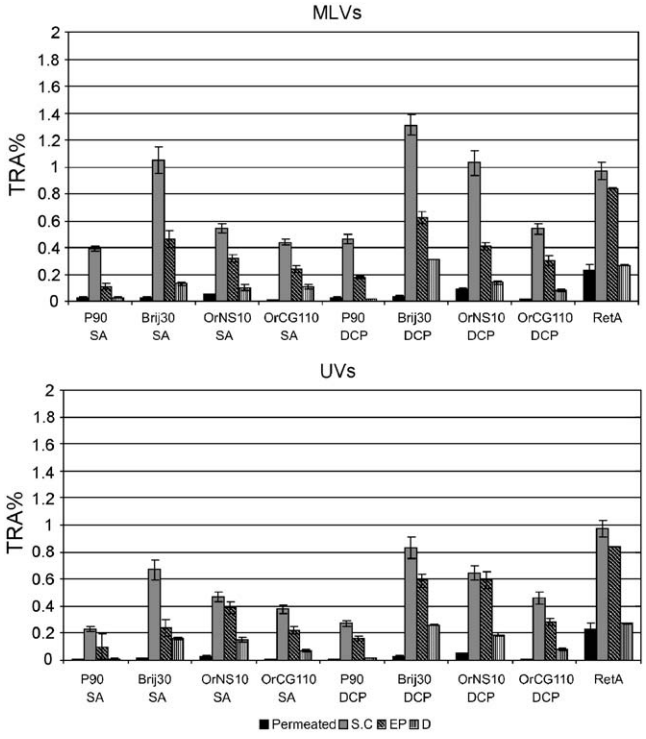

Fig. 5. Cumulative amount of tretinoin permeated through pig skin after 9 h and retained in the stratum corneum (SC), epidermis (E) and dermis (D) layers from the vesicular formulations, MLVs and UVs, not saturated with tretinoin.

Fig. 5 shows that when the same amount of vesicle-incorporated TRA (2 mg/ml) is applied to the pig skin, but using liposomes or niosomes not saturated with the drug, results in drug permeation and retention are similar or even lower than those obtained with the control. Moreover, Fig. 5 shows that also in these formulations the highest amount of tretinoin is retained in the SC. Therefore, comparison of results obtained from the two sets of formulations shows that the same amount of vesicle-incorporated drug gives a 10-fold lower accumulation in the skin when vesicles are unsaturated with tretinoin.

Finally, results also show that niosomes made from Oramix NS10 or Brij30 are better carriers than liposomes for the cutaneous delivery of tretinoin.

4. Conclusions

In vitro permeation results of this work have shown that composition of niosomes is very important for improving cutaneous or transdermal delivery of a lipophilic drug such as tretinoin. In fact, very hydrophilic surfactants (i.e. OrCG110, HLB = 16) may improve diffusion of TRA through the pig skin. On the other hand, Brij® 30 or Oramix NS 10 (HLB 9 and 11, respectively) niosomes have shown to be able to greatly enhance drug cutaneous retention, especially if compared to the commercial formulation RetinA® and P90 liposomes. Therefore, APG surfactants seem to be an interesting class of amphiphiles for niosome formation as, depending on their structure, they are able to improve either transdermal or cutaneous delivery of niosomal tretinoin.

However, these results are strongly affected by the thermodynamic activity of the drug. Vesicular formulations, which are saturated with TRA have shown to highly promote drug accumulation in the pig skin, while the same does not occur when the vesicular bilayer is not saturated with the drug.

Therefore, these results show an interesting advantage of vesicular systems: once drug saturated vesicular formulations are prepared, they can be diluted to reach the desired drug concentration without loosing the saturation of the vesicular bilayer and therefore maintaining the same thermodynamic activity of the drug and the same driving force for skin permeation.

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