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Research Paper

Tocopheryl acetate disposition in porcine and human skin when administered using lipid nanocarriers

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Abstract

Objectives Assessing the delivery of a drug into the skin when it has been formulated within a nanocarrier is a complex process that does not conform to the conventions of traditional semi-solid formulations. The aim of this study was to gain a fundamental understanding of drug disposition in both human and porcine skin when applied using a lipidic nanocarrier.
Methods A model system was generated by loading tocopheryl acetate into a well-characterised solid lipid nanoparticle and formulating this system as a traditional aqueous hyaluronic acid gel. Franz diffusion cells fitted with a silicone or nylon membrane were used to assess drug and particle transport independently whilst human and pig skin were employed to determine skin delivery.
Key findings The tocopheryl acetate, when loaded into the solid lipid nanoparticles, did not release from the particle. However, 1.65 ± 0.90% of an infinite dose of tocopheryl acetate penetrated into the stratum corneum of pig skin when delivered using a nanoparticle-containing gel.
Conclusions These results suggest that hydration of the stratum corneum in pig skin could lead to the opening of hydrophilic pores big enough for 50 nm-sized particles to pass into the superficial layers of the skin, a phenomenon that was not repeated in human skin.
Keywords human skin; nanoparticles; percutaneous absorption; pig skin; tocopheryl acetate; vitamin E

Introduction

The transport of a pharmaceutical compounds into the skin from a topical formulation involves two major steps: (1) release of the active agent from the formulation and (2) permeation of the released active compound into the barrier.[1] These processes are dictated by the physiochemical properties of the penetrant and the nature of the delivery vehicle after application to the skin.[2] Lipidic drugs (logP > 3) are especially difficult to deliver into the skin as it is problematic to load these agents into non-irritant topical formulation vehicles and the outermost layer of the skin, the stratum corneum (SC), restricts their passage into underlying tissue.[3]

In order to overcome the low solubility of highly lipophilic compounds in traditional cosmetically acceptable vehicles several new formulation approaches have been developed. The incorporation of therapeutic agents within lipid nanoparticles is one such novel approach.[4] Solid lipid nanoparticles (SLN) have previously been shown to enhance drug skin penetration[5] and employing an SLN carrier to administer an agent topically has been reported to improve drug loading, cosmetic formulation acceptability, skin moisturisation and patient compliance.[6]

When delivering a therapeutic agent to the skin using an SLN suspended in a simple gel base, the drug can exist within the formulation in one of several different forms. It can be encapsulated inside the particle, adsorbed to the surface of the particle or free in solution. In most cases the drug is probably presented to the skin in all of these forms, but very few previous studies have investigated how the drug and transport properties influence delivery.

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As it is believed that nanoparticles with a diameter greater than10 nm do not penetrate the SC, a drug that is loaded into most topically applied nanocarriers must first be released before it can be delivered into the skin.[7] However, if the particles release the entire drug load prior to application, the nanoparticle has no utility in the formulation, therefore particle and drug properties must be selected to enable rapid replenishment of a small 'sink' of free drug to facilitate efficient delivery. This dynamic process of drug release and permeation increases the complexity of the methods required to assess these systems. In addition, the results of drug delivery studies using topically applied nanosized systems must be interpreted with caution, as delivery is a multistage process only partly described by the conventional theory of skin delivery of drugs.

Franz cell diffusion testing is the most commonly employed method for assessing topical drug delivery. This method is very flexible and can be used to determine both formulation drug release and barrier permeation. However, the Franz cell methodology is heavily influenced by the choice of membrane and the selection of receiver fluid, hence the standardisation of these parameters is critical to allow accurate interpretation of the resultant data.[8] Ideally, the donor and receiver fluids selected for Franz cell studies should not alter the membrane or influence the permeation of the applied agent, i.e. violate sink conditions. Highly lipophilic compounds display low solubility in receiver fluids that do not modify the test membrane (i.e. non-alcoholic solvents) and therefore limited previous in-vitro assessment of these agents has been performed. In the assessment of a topical formulation containing nanoparticles the selection of the Franz cell receiver fluid is even more important because if the chosen solvent penetrates the membrane freely and enters the donor chamber it can solubilise the nanoparticles and release the drug. This can be particularly problematic if the barrier is porous in nature. However, judicious design of the Franz cell test system does allow determination of both drug and particle transport processes.

The aim of this study was to gain a fundamental understanding of drug disposition in both human and porcine skin when the active agent is applied using a lipidic nanocarrier. A model system was generated by loading tocopheryl acetate (TA) into a well characterised SLN and formulating this system as traditional aqueous hyaluronic acid gel. It was anticipated that drug release from the SLN into the hydrophilic suspending media would be minimal and hence particle and drug permeation properties could be isolated. The nanoparticle system was compared to two positive controls: pure TA oil and TA dispersed in silicone oil, which allowed unhindered drug permeation. Franz diffusion cells were fitted with a silicone membrane, to assess drug transport, and a nylon membrane, to assess particle transport. A lipid receiver fluid was employed in the studies to ensure sink conditions were maintained and to remove the possibility of back diffusion of the receiver fluid into the donor compartment. Full thickness human and pig skin were employed to assess how the drug and nanoparticle transport properties influenced TA delivery into the skin.

Materials and Methods

Materials

Labrafac WL 1349 (medium chain triglycerides) was provided by Gattefossé S.A. (France). Lipoid S75-3 (soybean lecithin 69%) was donated by Lipoid GmbH (Germany). Solutol HS 15 (mixture of free polyethylene glycol (PEG) 660 and polyethylene glycol 660 hydroxystearate) was provided by BASF (Univar, UK) D-α Tocopheryl acetate (TA) with 96.0–102.0% purity was provided by Roche (UK). NaCl, ethanol and methanol (high performance liquid chromatography (HPLC) grade) were purchased from VWR (UK). Hyaluronic acid (HA) powder (with molecular weight of 500 000 Da), Miglyol 810 (caprylic-capric triglycerides) were gifts from MedPharm (UK) and SBlack (UK), respectively. Folixane D4 silicone membrane was from Novatech (France) and the nylon membrane with pore size of 0.20 μm was purchased from Fisher Scientific (UK). Volpo 20 (polyethoxy, (n = 20) oleyl ether) and dimethicone (Dow Corning 200 Silicone fluid, 350) were obtained from BASF and SBlack (UK), respectively. Tween 80 (polysorbate 80) was supplied by Fisher Scientific (UK), PEG 400 and gamma-cyclodextrin by Sigma Aldrich (UK).

Methods

Formulation preparation

Lipid nanoparticles were prepared by a phase-inversion process described previously by Heurtault *et al*.[9] Briefly, TA oil (4% w/w) was dissolved in the triglyceride oil (16.5% w/w), which was then mixed with lecithin (1.75% w/w) and a non-ionic surfactant (16.25% w/w) and finally with the aqueous phase (NaCl in water, at 61.5% w/w). The dispersion was heated under magnetic stirring to the temperature above the phase inversion temperature of the system (approximately 75°C). The mixture was cooled down to 60°C and then heated to 80°C at a rate of approximately 4°C/min. This process was repeated three times. TA-loaded nanoparticles were obtained by adding cold water (2–3°C) to the emulsion at the end of the process to induce particle precipitation. The TA loaded nanoparticles were purified by ultracentrifugation (L7 ultra-centrifuge, Beckman, USA) at 40 000 rev/min for 1 h at a temperature of 20°C. The purified solution was used in the topical formulations. The TA nanoparticle gel was prepared by adding HA powder (2% w/w) to TA purified nanoparticles (98% w/w) and allowing the system to gel for 24 h at 24°C. The 5.7% (w/w) (maximum miscibility) TA (saturated) in non-volatile silicone oil was prepared by direct mixing using a magnet bar inside a jar, covered with aluminium foil, placed on a Variomag stirrer plate at room temperature. The four final formulations therefore consisted of two with TA nanoparticles (purified TA nanoparticles, and purified TA nanoparticles in HA gel) plus two controls that did not contain nanoparticles (100% TA and the saturated TA in oil).

Tocopheryl acetate miscibility and drug stability

Miscibility studies were conducted by introducing an excess amount of TA into a vial of solvent, which was capped and sealed with Parafilm, placed on a magnetic plate (Stuart, UK) and mixed with a magnetic stirrer bar for 24 h at room temperature. The compositions were assessed visually.

TA miscibility in simple solvents like Miglyol oil 810, mineral oil, PEG 400 and glycerol and in solvents with the addition of surface active agents was also assessed.[10,11] A range of concentrations of Volpo 20 with critical micelle concentration (CMC) value ≤ 25 μM and Tween 80 (CMC = 0.012–0.006 μM) in water/ethanol was investigated with a view to using surface active agents in a non-irritant and non-occluded form.[12,13] The chemical stability of TA in the chosen receiver fluid in the presence of the membrane for the permeation studies (i.e. silicone membrane, nylon membrane, full thickness pig skin and human skin) was investigated over 72 h by incubating a known amount of TA with Miglyol and the membrane in a water bath at 37°C. Samples of the TA solution were withdrawn and assessed by HPLC every 24 h.

In-vitro permeation

Individually calibrated static Franz diffusion cells were used to assess the permeation of TA across two synthetic membranes (nylon and silicone). The membranes were sealed between the two chambers using parafilm. The average area available for permeation was 2.2 ± 0.1 cm² and the average volume of the receiver compartment was 9.8 ± 0.5 ml. The most appropriate receiver fluid was selected using data from solubility/chemical stability studies and this was filled into the receiver compartment of the cells, a stir bar added and the fluid stirred constantly throughout the experiment. The cells were submerged in a water bath on a magnetic stirrer plate (Varimag, Telesystem HP15, Daytona, USA) at 37ºC. The cells were allowed to equilibrate in the water bath for 1 h and on initiation of the experiment an infinite dose (1 ml) of TA oil, TA saturated silicone fluid, TA-loaded nanoparticles or TA–SLN-loaded HA gel (2%) was applied onto the apical surface of the selected membrane. Samples were taken from the arm of the receiver chamber at predetermined intervals and replaced by fresh thermostatically regulated receiver solution. No samples were below the limit of detection for the HPLC assay or exceeded 10% of the total TA saturated solubility in the receiver solution, i.e. sink conditions were maintained during the study. Where appropriate, the steady-state flux of TA in the permeation profiles was taken as the most linear region of the time against cumulative drug concentration plot after the lag time and prior to dose depletion (at least eight points were considered and $r^2 \geq 0.999$ unless otherwise stated).

Skin permeation

Human skin was collected with consent from abdominoplasty surgery with approval from the Research Ethics Committee of Hertfordshire University and stored at –20°C. Prior to use it was defrosted at room temperature. The subcutaneous fat was carefully removed from the skin using forceps and a scalpel. Following removal of the fat, individual sections of skin (of adequate size to fit in the Franz diffusion cells) were cut using a cork borer and washed using distilled water. An identical procedure was used to prepare pig cheek skin sourced from a local abattoir.

Full thickness pig and human skin were sealed between the donor and receiver chamber of the Franz-type diffusion cells (using an identical method to the artificial membranes) and placed in a water bath at 37°C. The average exposed skin surface area available for permeation was 0.7 ± 0.0 cm² and the average receiver compartment volume was 2.3 ± 0.0 ml. The cells were allowed to equilibrate in the water bath for 1 h prior to experiment initiation and cells were checked for leaking by inversion. An equivalent finite dose (4.2 mg/cm²) of TA oil, TA saturated silicone fluid and TA lipid nanoparticle loaded HA gel (2% w/w) was applied on the apical surface of the skin using a pre-calibrated positive displacement pipette (Gilson, USA). After 24 h a single sample was taken from the receiver fluid (no TA detected in any of the samples and so not reported) and a mass balance recovery from the SC, epidermis and dermis was performed. The cells were checked for leakage by inversion at the end of the experiments. The Franz cells were dismantled and both receiver and donor compartments washed with ethanol. The washings were made up to a known volume and assayed by HPLC. The skin was cleaned with cotton buds and mounted on a firm base. Tape stripping was performed using Ultra Clear Scotch tape (Sellotape, UK). Each piece of tape was placed on the whole area, a defined pressure was applied (using a 300 mg weight) for 10 s and then the tape was peeled quickly (<1 s) from the test site with forceps. The first two strips were considered as containing residual formulation, hence they were combined with the cotton buds, soaked in ethanol for 24 h, and the solution was filtered and assayed by HPLC. The 22 subsequent strips removed from the skin were collected and defined as containing the TA that had permeated into the SC. TA was extracted from the tapes by adding ethanol, shaking for 6 h, filtering and assaying by HPLC (prior method validation showed a 104.9 ± 12.4% TA recovery, data not shown). After removal of the SC the underlying viable epidermis was carefully peeled away from the dermis with a scalpel and the remaining dermis was manually cut into small pieces, again using a scalpel, and was then dissolved in 5 ml ethanol. The samples from the surface of the skin, the SC, the viable epidermis and the dermis were soaked in ethanol separately and sonicated for 60 min (Decon F5300b, UK), then shaken overnight at room temperature whilst being protected from the light by covering with aluminium foil. The samples were filtered through a 0.2 ml syringe filter (which was shown previously not to bind to TA) and were centrifuged for 10 min at 11 000 rev/min (VWR, UK) prior to HPLC analysis.

HPLC analysis

The HPLC analysis of TA was performed using a Perkin Elmer system consisting of a 200 LC pump, a 200 LC autosampler, and a UV detector. Separation was achieved using a Kromasil C18 column (250 × 4.6 mm, pore size: 100Å, surface area: 340 m/g, carbon load 19.0%) at ambient temperature. The mobile phase consisted of a degassed and filtered 50:50 mixture of ethanol/methanol which was pumped through the column at a rate of 1 ml/min. The sample injection volume was 50 μl and TA was detected at 284 nm over a 15-min run time. The TA peak eluted at 8.30 ± 0.11 min (n = 108) and the solvent front approximately 4 min before. This reverse-phase HPLC method was shown to be fit for purpose with 2 μg/ml sensitivity for the quantification of TA. The method precision was good, with

an intra-day and inter-day variation of < 2%. The calibration plots showed excellent linearity ($r^2 > 0.999$) throughout the study with an accuracy of > 99%. Although endogenous RRR α-tocopherol is known to be present in human skin, the HPLC method employed in this work was capable of separating the natural form from the TA. As a consequence, in this work any endogenous α-tocopherol did not interfere with the analytical results.

Statistical analysis

All data were presented as mean ± standard deviation and statistical analysis of data was performed using the computer program SigmaStat (Version 3.5, Virginia, USA). All data were checked in terms of normality (Kolmogorov–Smirnov test) and homogeneity of variances (Levene's test) prior to analysis. When equal variance and normality were found, a parametric one-way repeated measurements analysis of variance (ANOVA) test was used. For non-Gaussian distribution, a non-parametric test (Mann–Whitney) was applied. Post-hoc Tukey analysis was used for all pairwise comparisons to enable comparison of the means of the measurements within the data set. The significance level was set at 0.05.

Results

Nanoparticles characterisation

The purified nanoparticles generated by the phase inversion method displayed a mean effective volume diameter of 50.83 ± 1.75 nm ($n = 3$) and contained 57.7 ± 4.9% of the original 110 mg of TA added. The recovery of TA from the purification method was found to be 90.4 ± 2.2% ($n = 3$). On storage, the mean effective volume diameters of the loaded particles increased by 5.1 ± 0.75% at 4°C, 5.0 ± 0.94% at 25°C and 1.81 ± 0.68% at 40°C.

Tocopheryl acetate miscibility and stability

TA miscibility was visually determined in two lipophilic solvents, Miglyol oil and mineral oil, and these were compared to a range of more polar vehicles with and without the addition of surfactants (Table 1). The TA was miscible in the Miglyol oil and mineral oil, but demonstrated poor solubility in PEG 400 and in water. Tween 80 (10% w/w) was the most effective surfactant of those employed as it increased the TA water miscibility to ≤ 0.07 mg/ml. Increasing the amount of Volpo 20 from 6 to 10% did not improve TA miscibility. Adding 20% (w/w) of the organic solvent (ethanol) to the TA systems containing Volpo 20 solution or PEG 400 improved TA miscibility significantly from ≤ 0.006 to ≤ 0.01 mg/ml and from ≤ 0.6 to ≤ 4.2 mg/ml, respectively. TA, when dissolved in Miglyol oil and incubated with nylon, regenerated cellulose and the mammalian membranes was found not to significantly degrade ($P \leq 0.05$) during a 72-h period (data not shown).

Artificial membrane permeation

TA, when presented as a pure oil, permeated freely through both nylon (1372.86 ± 241.13 μg/cm^2 per h) and silicone membranes (26 891.31 ± 904.23 μg/cm^2 per h). The nylon barrier permeation rate was significantly lower compared to the silicone (Mann–Whitney test, $P \leq 0.05$; Table 2) but in both these membranes steady state fluxes were generated rapidly and maintained for 24 h (profiles not shown). Saturating the TA in silicone oil (5.7% w/w) significantly reduced the TA permeation rate through both synthetic membranes (190.98 ± 67.15 μg/cm^2 per h and 836.15 ± 137.94 μg/cm^2 per h in nylon and silicone, respectively), compared to the pure TA oil, but steady state permeation was still achieved rapidly and maintained throughout the study. When the purified TA-loaded nanoparticles, either suspended in water or a HA gel, were presented to either the nylon or silicone membranes, no detectable TA permeation was observed. The lack of TA drug permeation through silicone was indicative of there being no free TA in the nanoparticle suspensions, i.e. no drug release in the presented vehicles, and a lack of permeation through the porous nylon membrane was indicative of no nanoparticle transport into the Miglyol receiver fluid.

Table 1 The miscibility/solubility of tocopheryl acetate (TA) in a range of solvents suitable for in-vitro topical permeability experiments

Solvent	TA solubility (mg/ml)
Miglyol oil 810	Freely miscible
Mineral oil	Freely miscible
PEG 400	Negligible
Water	Negligible
80/20 (w/w) PEG 400 in ethanol	≤ 4.2
10% (w/w) Tween 80 in water	≤ 0.07
10% (w/w) Solutol HS 15 in water	≤ 0.007
6% (w/w) Volpo 20 in water	≤ 0.006
6% (w/w) Volpo 20 in (20/80) ethanol/water	≤ 0.01
10% (w/w) Volpo 20 in water solution	≤ 0.005
10% (w/w) Volpo 20 in (20/80) ethanol/water	≤ 0.007
Gamma cyclodextrin	Negligible
Glycerin	Negligible

PEG 400, polyethylene glycol 400; Volpo 20, polyethoxyoleyl ether; Tween, polysorbate 80; Solutol HS 15, a mixture of free polyethylene glycol (PEG) 660 and PEG 660 hydroxystearate.

Pig skin and human skin penetration

Utilising tape-stripping to assess the TA penetration into the SC of pig skin showed that 0.51 ± 0.13%, 1.32 ± 0.57% and 1.65 ± 0.90% of the total TA applied was delivered in the upper layer of the skin using TA oil, TA saturated in silicone oil and TA-loaded nanoparticle gel respectively (Figure 1). There was a significant difference (one-way ANOVA, $P < 0.05$) in the TA penetration of the different formulations. More specifically, a comparison of means using the Tukey HSD test, showed that the penetration of TA into the pig skin SC, when applied from silicone fluid, was statistically higher compared to pure TA oil ($P < 0.05$). Furthermore, there was no statistical difference in the TA penetration of the SC when comparing the performance of the TA-loaded nanoparticle gel and TA in silicone solution. The amount of TA that passed through the pig skin SC and into the epidermis/dermis was significantly higher (Tukey HSD test, $P < 0.05$) at 3.12 ± 1.18% for the pure TA oil formulation compared with the other two test systems. TA in the silicone fluid and the

Table 2 Permeation of tocopheryl acetate through artificial membranes using different vehicles

Membrane	Formulation	Flux (mg/cm^2 per h)	Lag time (h)	Cumulative amount in 24 h (mg/cm²)
Nylon	TA oil	1.4 ± 0.2	0.74 ± 0.44	31.7 ± 5.6
	TA in silicone	0.2 ± 0	1.81 ± 1.38	4.6 ± 1.6
	NP in HA gel	No permeation	–	–
	NP in water	No permeation	–	–
Silicone	TA oil	26.9 ± 0.9	1.40 ± 0.93	58.6 ± 19.1
	TA in silicone	0.8 ± 0.1	3.25 ± 0.88	20.0 ± 3.3
	NP in HA gel	No permeation	–	–
	NP in water	No permeation	–	–

Figures shown for tocopheryl acetate (TA) oil, TA in silicone, TA loaded nanoparticles in hyaluronic acid (HA) gel and TA loaded nanoparticles (NP) suspended in water. Mean of n = 6 ± SD.

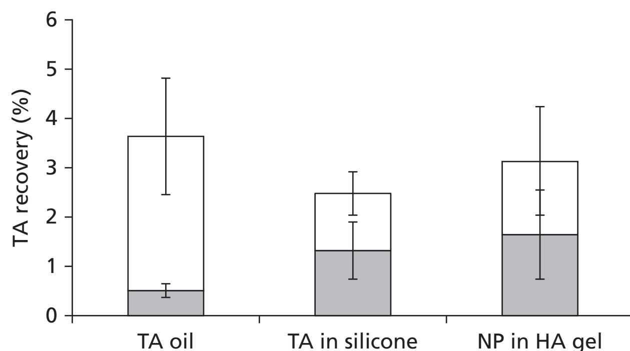

Figure 1 Tocopheryl acetate recovery after application to full thickness pig skin using three different vehicles. Figures shown for tocopheryl acetate (TA) oil, TA in silicone solution, TA loaded nanoparticles in a hyaluronic acid gel (NP in HA gel). Total TA recovery from the skin is expressed as percentage of dose applied, divided as amount extracted from stratum corneum (grey column) and amount extracted from epidermis/dermis (white column). Data represent mean of n = 6 ± SD

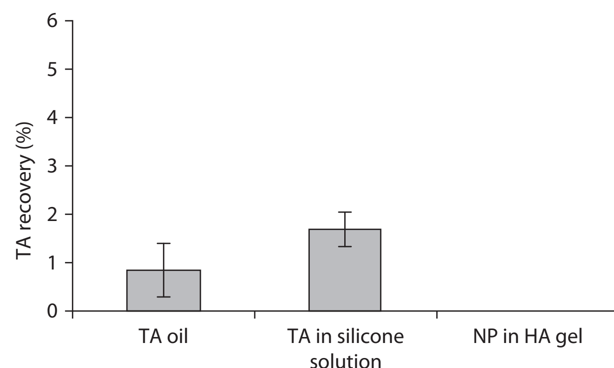

Figure 2 Tocopheryl acetate recovery after application to full thickness human skin using three different vehicles. Figures shown for tocopheryl acetate (TA) oil, TA in silicone solution, hyaluronic acid (HA) gel loaded with TA loaded nanoparticles (NP in HA gel). Total TA recovery from the skin is expressed as percentage of dose applied and displayed as amount extracted from stratum corneum only (grey column). Data represent mean of n = 6 ± SD

nanoparticle gel delivered 1.16 ± 0.43% and 1.48 ± 1.10% of the applied TA dose into the lower layers of the skin, respectively.

When full thickness human skin was employed as a barrier, 0.84 ± 0.56% of the applied TA was detected in the SC when administered as the pure oil and 1.70 ± 0.36% when administered using TA-saturated silicone oil (Figure 2). TA was not detected in the SC when the TA-loaded nanoparticle gel was applied to the human skin. TA was not detected beyond the SC when applied to human skin. The amount of TA recovered from the human or pig skin when the pure TA oil and silicone solution were used was not statistically different ($P > 0.05$, Tukey HSD test).

Discussion

The major driving force for a drug in solution to move passively across a membrane such as the skin is its concentration gradient. However, the passive movement is driven down this concentration gradient by the thermodynamic activity of the agent in the formulation, i.e. the degree of drug saturation in the vehicle.[14] As such, when an active compound is saturated in an application vehicle, an equivalent membrane permeation rate is attained irrespective of the vehicle composition if the membrane remains unaltered by the formulation. However, the influence of drug thermodynamic activity is negligible if there is a low concentration of the drug in the vehicle. A lack of drug solubility means the available drug will deplete almost instantly and the concentration gradient will rapidly be reduced to a negligible level.[1]

Depletion of a drug in a topical formulation vehicle can be slowed and sometimes prevented if a secondary reservoir can act to replenish the depleted drug. The high surface area displayed by nanoparticles make them ideally suited to act as a drug reservoir in a topical formulation. If the dissolution of the drug from the nanoparticle into the vehicle is rapid, barrier penetration of the permeate remains the rate-limiting step, i.e. drug release from the topical formulation has been optimised. However, the true potential of a nanoparticle to aid topical delivery has not been realised yet, as assessing the delivery of a drug into the skin when it has been formulated within a nanocarrier is a complex multistage process. Simply

assessing the rate or extent of drug delivery from such a system over 24 h is inappropriate if researchers wish to evaluate the ability of a topically applied nanocarrier to improve delivery.[15] To assess the applicability of nanosystems to resolve topical drug delivery problems the nanoparticle delivery process must be deconvoluted. One approach that can be used to achieve this is to use different in-vitro models to compare the nanoparticle drug release, nanoparticle membrane penetration and drug penetration independently, such that their effects on superficial skin delivery can be understood.

In the current study the highly hydrophobic model drug TA was loaded into a 50 nm SLN. It has previously been shown that the TA has no detectable release from this particle when suspended in water.[16] This was not surprising as the apparent solubility of TA in the SLN suspending medium was so low it was undetectable ($\leq$ 0.0001 mg/ml). The inability of TA to leave the SLN nanoparticle simplified the in-vitro permeation models that were employed in this work. In the absence of drug release from the particles, any drug deemed to have permeated into or through the synthetic test barriers from the nanocarrier systems must relate to either nanoparticle transport or a loss of nanoparticle integrity during delivery. Losing nanoparticle integrity during the release studies and not during the equivalent permeation experiments is indicative of inappropriate solvent 'back-diffusion' from the Franz cell receiver fluid, through the synthetic membrane, into the formulation. Careful Franz cell receiver fluid selection is therefore critical to allow accurate result interpretation.

Hydrophobic drug solubility can be improved in Franz cell receiver fluids by adding organic solvents or surface active agents to an inert liquid. For example, Mahamongkol[17] added ethanol when assessing the permeation of five TA formulations through human cadaver skin and Bronaugh[18] used 6% Volpo 20 in water to determine hydrophobic agent barrier penetration.[17,19] However, both these approaches failed to significantly enhance the TA solubility in a manner that would allow permeation through the synthetic membranes to occur under sink conditions in this work. The level of ethanol was capped at 20% as it is known to alter membrane thickness and induce solvent back-diffusion at higher levels.[8,20] The only way to solve the TA solubility problem appeared to be the use of an oil-based receiver fluid. Miglyol or mineral oil could have been selected as the receiver fluid as they both demonstrate adequate miscibility with the TA, but Miglyol demonstrates poor miscibility with the applied nanoparticle formulations and is less viscous than mineral oil and therefore easier to sample without introduction of air bubbles.

Miglyol oil has not been used as a receiver fluid in previous studies and hence the barrier properties of both the synthetic and biological membranes when immersed in this oil have not been assessed. However, it was apparent from visual observation that the barriers remained intact during the time course of all the permeation studies; no unusual thickening of the barrier or back-diffusion of the receiver fluid into the donor cells was observed. In addition, it should be noted that the Miglyol oil was primarily in contact with the underside of the dermis when the both the human and pig skin was employed as the barrier and the partition of the hydrophobic oil into the dermis was anticipated to be much less extensive compared to its uptake into the skin through the SC when applied on the skin surface. Hence, because of the logical perception that penetration of the oil-based receiver into skin was limited and the fact that all the experiments were designed to be of a comparative nature, Miglyol oil was selected as the receiver fluid in all subsequent work with TA. The hydration of long- and medium-chain triglyceride oils has been shown to influence compound solubility and this could be significant in a study where the active compound solubility is low, but as TA was highly miscible in the oil no special control methods were taken to ensure constant hydration of the oils in this work.[21]

The complexity of the human skin makes mechanistic permeation studies very difficult to undertake. Hence, barriers produced from synthetic materials are popular tools to investigate the multiple sequential processes involved in topical delivery. The choice of membrane is often dependent on the objectives of the study. For example, when using a confluent barrier such as silicone, mass transport of a compound dissolved in solution, down its concentration gradient, through the barrier and into the receiver fluid, can be determined. In contrast, the rate at which a topical formulation presents a drug or particle to the surface of a barrier can be determined by mass transport across a porous membrane. Nylon was selected in this study to determine the transport rate of the SLN particles as it is known to be highly inert, resistant to swelling by a wide range of solvents and have a pore size through which the nanoparticle can readily pass.[22]

The permeation of TA from four test preparations was assessed in this study; in two formulations where 100% of the TA was in solution (TA oil and TA silicone oil) and in two where the TA was immobilised in a nanocarrier (purified nanoparticle suspension, HA nanoparticle gel). The pure TA oil and TA silicone solutions were used to assess the ability of the test systems to measure free drug permeation and it was not surprising to discover that the TA oil had the highest flux through the synthetic membranes. The competition between the transport of silicone and TA through the membrane, in a similar manner to previously reported for lidocaine and prilocaine oils,[23] explains the reduction in the TA permeability from the silicone oil compared to the pure TA oil. The fact that TA appeared to permeate the silicone membrane more rapidly than the nylon was probably a consequence of the different hydrophobicity of membranes. TA oil is extremely hydrophobic and therefore the hydrated barrier of nylon, despite being porous, would pose a significantly more resistant barrier to TA transport than the silicone membrane. The resistance of porous membranes to permeation has previously been reported.[8] This comparison of the synthetic membranes was not considered to be of primary importance to the conclusions of this study as the two membranes were used for two different purposes: the silicone to assess free drug transport and the nylon to assess particle-loaded drug transport. The inability of the SLN formulations to deliver any drug through both the silicone and nylon membranes was an unexpected result. Whilst the inability of the TA to release from the SLN particle presumably prevented drug transport across the silicone membrane, the SLN particle would be expected to diffuse across the nylon

membrane intact. If the SLN did permeate the silicone membrane they would carry the drug and hence be detected by the HPLC assay, but this was not the case. To understand the reason for this, the properties of the SLN need to be considered. The lipid nanoparticles used in this work were produced by a phase inversion manufacturing method and their structure has previously been reported to be a hybrid between polymeric nanocapsules and liposomes.[9,23] Solutol, an ethoxylated surfactant containing a polyethylene glycol end-group, surrounds the SLN and this produces a hydrophilic surface, coating the oily core of these lipid nanoparticles, which stabilises them in water.[24,25] The size of the nanoparticles, i.e. 50 nm, means that their movement should be driven by Brownian motion.[26] However, the hydrophilic nature of the SLN surface may prevent free diffusion of the nanoparticle into the Miglyol receiver fluid. The effects observed in the experiment are undoubtedly dependent on the receiver fluid employed, but Miglyol is a much better model for SC nanoparticle interaction compared to traditionally employed receiver fluids as Miglyol has a solubility parameter of ca. 9.32 $(cal/cm^3)^{1/2}$ and in the porcine skin of ca. 10 $(cal/cm^3)^{1/2}$.[27]

When utilising TA, a free radical scavenging agent, to track the particle transport in human skin, the form of the agent being used must be considered, as tocopherol is found innately in human and animal tissue.[28] TA is most commonly used in topical formulations due to its greater chemical stability, and for this reason it too was used in this study. The HPLC method employed in this study was capable of separating naturally occurring tocopherol and TA, hence accurate detection of TA in the skin was assured.[29] No innate tocopherol was detected in any of the skin samples assessed, which is not surprising as the chemical instability of natural tocopherol means that it would probably have degraded prior to the skin being utilised in these studies.

Skin from several mammalian species, including humans, has been suggested as a suitable model for in-vitro adsorption testing.[30] Pig skin has been shown to have similar histological and physiological properties to human skin and has been suggested as a good model for human skin permeability testing.[31] However, for nanoparticle formulations a number of differences in the permeability of skins from different animals have been observed. For example, a study using guinea pig skin concluded faster transport for smaller nanoparticles in hairy skin but not in hairless skin.[32] Our results showed extensive absorption of TA when delivered using nanoparticles in pig skin, but no TA detected in the SC of human skin using the same formulations. The pig SC has previously been reported to be 1.6 times thicker and possess larger hair follicles that are 1.8 times more numerous compared to human skin.[10] In addition, it has been shown that nanoparticles with a size of approximately 300 nm penetrate deep into the hair follicles.[33] Although the lipid nanoparticles produced here were monodispersed, with mean effective diameters of about 50 nm, it is unusual to deposit such a great percentage in the SC considering that these particles had a very hydrophilic surface. It is also unlikely that the shunt route could support such extensive permeation into the SC and not deeper layers, as the hair follicles, for example, span the whole epidermis.[34,35]

The more extensive transport of TA into pig skin compared to human skin when the pure TA oil and the TA-loaded silicone oil were employed as test systems appears to support previous observations that human skin is significantly less permeable to compounds than porcine skin.[10,36] However, the passage of the highly hydrophobic TA beyond the SC and into the epidermis of the pig skin is more surprising and this is more suggestive of a structural modification induced by formulation application. One hypothesis that could be employed to explain the differences observed in TA permeation is that the porcine skin is undergoing a type of structural modification that is not apparent in human skin when it is hydrated for prolonged time periods. Visualising the SC lipid layers *in vitro* by transmission electron microscopy demonstrates that prolonged exposure of skin to water (24 h) causes disruption of lipidic lamellae in both pig skin *in vitro* and human skin *in vivo*. However, for pig skin, the disruption was similar to that caused by a surfactant.[37] It has also previously been suggested that prolonged hydration mechanically disrupts animal skin but not human skin.[38] This difference may be related to their different lipidic structure. Intercellular lipid structures in the SC are responsible for the barrier function of mammalian skin.[39] Analysing the lipids extracted from human and pig skin have revealed that human skin is unique with regard to its high content of long chain ceramides.[40] It has also been found that the presence of long-chain ceremide is of great importance for the formation of the lateral lipid packing as well as the long range lamellar ordering in the SC and consequently its barrier permeability properties.[41] Stable nanoparticles are ideal to probe such differences and the data generated in this study indicates that they may provide excellent discrimination in permeation pathways for different skin types. Further studies using a range of nanoparticle types and sizes would be beneficial to investigate both the size and the properties of permeation pathways that may be present in porcine skin after exposure to water for prolonged periods of time. Such information is critical to aid the interpretation of pre-clinical data for novel topical formulations.

Conclusions

Assessing drug release from nanoparticle formulations is a complex process that is dependent on both drug release from nanoparticles and the transport of nanoparticles from the formulation towards the barrier. In this work, a novel lipid receiver fluid combined with porous and non-porous synthetic membranes was employed to assess the permeation of TA from a nanoparticle formulation. Particle diffusion was hindered by the lipid receiver fluid employed in the Franz cells. However, as this lipidic receiver fluid displayed similar properties to the SC, the results from this study suggest that nanoparticles with a hydrophilic surface have limited contact with hydrophobic membranes. The contact of pig skin with water for 24 h appeared to create a new shunt route for the nanoparticles to permeate the SC. As human skin did not produce the same effects, the reliability of pig skin for assessment of nanoparticle-facilitated drug delivery and toxicity needs to be reconsidered.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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References

1. Scheuplen RJ, Blank IH. Permeability of skin. *Physiol Rev* 1971; 51: 702.
2. Williams AC. *Transdermal and topical drug delivery*, 2nd edn. London: Pharmaceutical Press.
3. Elias PM. Epidermal lipids, membranes, and keratinization. *Int J Dermatol* 1981; 20: 1–19.
4. Muller RH *et al*. Solid lipid nanoparticles – a novel carrier system for cosmetics and pharmaceutics. 1. Properties, production and scaling up. *Pharmazeutische Industr* 1997; 59: 423–427.
5. Maia CS *et al*. Solid lipid nanoparticles as drug carriers for topical glucocorticoids. *Int J Pharm* 2000; 196: 165–167.
6. Dingler A *et al*. SLN (solid lipid nanoparticles) as new solid carrier of active ingredients in cosmetics. *Eur J Pharm Sci* 1996; 4: 148–155.
7. Stracke F *et al*. Multiphoton microscopy for the investigation of dermal penetration of nanoparticle-borne drugs. *J Invest Dermatol* 2006; 126: 2224–2233.
8. Reid ML *et al*. An investigation into solvent–membrane interactions when assessing drug release from organic vehicles using regenerated cellulose membranes. *J Pharm Pharmacol* 2006; 60: 1–10.
9. Heurtault B *et al*. A novel phase inversion-based process for the preparation of lipid nanocarriers. *Pharm Res* 2002; 19: 875.
10. Bronaugh RL *et al*. Methods for in vitro percutaneous absorption studies II. Animal models for human skin. *Toxicol Appl Pharmacol* 1982; 62: 481–488.
11. Lee AR, Tojo K. Evaluation for intrinsic skin permeation of unstable compounds. *Chem Pharm Bull* 1996; 44: 1621–1623.
12. Chou DK *et al*. Effects of Tween 20 (R) and Tween 80 (R) on the stability of albutropin during agitation. *J Pharm Sci* 2005; 94: 1368–1381.
13. Dai WG *et al*. Combination of Pluronic/Vitamin E TPGS as a potential inhibitor of drug precipitation. *Int J Pharm* 2005; 355: 31–37.
14. Higuchi T. Physical chemical analysis of percutaneous absorption process from creams and ointments. *J Soc Cosmetic Chem* 1960; 11: 85–97.
15. Jenning V *et al*. Vitamin A-loaded solid lipid nanoparticles for topical use drug release properties. *J Control Release* 2000; 66: 115–126.
16. Zhao Y *et al*. A dynamic topical hydrofluoroalkane foam to induce nanoparticle modification and drug release in situ. *Eur J Pharm Biopharm* 2009; 72: 521–528.
17. Mahamongkol H *et al*. Permeation study of five formulations of alpha-tocopherol acetate through human cadaver skin. *J Cosmet Sci* 2005; 56: 91–103.
18. Bronaugh RL, Stewart RF. Methods for *in vitro* percutaneous-absorption Studies. 3. Hydrophobic compounds. *J Pharm Sci* 1984; 73: 1255–1258.
19. Bronaugh RL *et al*. Determination of percutaneous absorption by *in vitro* techniques. In: Bronaugh RL, Maibach H.I, eds. *Percataneous absorption drugs-cosmetics mechanism*. New York: Marcel Dekker, 1989: 229–235.
20. Dias M *et al*. ATR-FTIR spectroscopic investigations on the effect of solvents on the permeation of benzoic acid and salicylic acid through silicone membranes. *Int J Pharm* 2001; 216: 51–59.
21. Land LM *et al*. The influence of water content of triglyceride oils on the solubility of steroids. *Pharm Res* 2005; 22: 784–788.
22. Haigh JM, Smith EW. The selection and use of natural and synthetic membranes for in-vitro diffusion experiments. *Eur J Pharm Sci* 1994; 2: 311–330.
23. Fiala S *et al*. An investigation into the influence of binary drug solutions upon diffusion and partition processes in model membranes. *J Pharmacy Pharm Sci* 2008; 60: 1615–1623.
24. Moddaresi M *et al*. Vitamin E nanoparticles for dermal application. *J Pharm Pharmacol* 2006; 58: A13–A14.
25. Heurtault B *et al*. Interfacial stability of lipid nanocapsules. *Colloids and Surfaces B: Biointerfaces* 2001; 30: 225–235.
26. Teeguarden JG *et al*. Particokinetics *in vitro*: dosimetry considerations for *in vitro* nanoparticle toxicology assessments. *Toxicol Sci* 2007; 95: 300–312.
27. Liron Z, Cohen S. Percutaneous absorption of alkanoic acids 2. application of regular solution theory. *J Pharm Sci* 1984; 73: 538–542.
28. Packer L, Valacchi G. Antioxidants and the response of skin to oxidative stress: vitamin E as a key indicator. *Skin Pharmacol Applied Skin Physiol* 2002; 15: 282–290.
29. Zhao Y. Engineering novel dynamic hydrofluoroalkane foams to improve topical drug delivery. UK, London: King's College, 2009 (dissertation).
30. OECD 427. OECD Guideline for testing of chemicals – Draft Guideline 427. Skin absorption: *In vivo* method. Paris: Organization for Economic Cooperation and Development, 2007.
31. Dick IP, Scott RC. Pig ear skin as an in-vitro model for human skin permeability. *J Pharm Pharmacol* 1994; 44: 640–645.
32. Shim J *et al*. Transdermal delivery of minoxidil with block copolymer nanoparticles. *J Control Release* 2004; 97: 477–484.
33. Lademann J *et al*. Hair follicles; a long term reservoir for drug delivery. *Skin Pharmacol Physio* 2006; 19: 232–236.
34. Ogiso T *et al*. Mechanism for enhancement effect of lipid disperse system on percutaneous absorption. *J Pharm Sci* 1996; 85: 57–64.
35. Munster U *et al*. RU 58841-myristate-prodrug development for topical treatment of acne and androgenetic alopecia. *Pharmazie* 2005; 60: 8–12.
36. Middelkoop E *et al*. Porcine wound models for skin substitution and burn treatment. *Biomaterials* 2004; 25: 1559–1567.
37. Warner RR *et al*. Water disrupts stratum corneum lipid lamellae: damage is similar to surfactants. *J Invest Dermatol* 1999; 113: 960–96.
38. Bond JR, Barry BW. Limitation of hairless mouse skin as a model for in vitro permeation studies through human skin: hydration damage. *J Invest Dermatol* 1998; 90: 486–489.
39. Blank IH. Factors which influence the water content of stratum corneum. *J Invest Dermatol* 1952; 18: 433–440.
40. Bouwstra JA *et al*. Role of ceramide 1 in the molecular organization of the stratum corneum lipids. *J Lipid Res* 1999; 39: 186–196.
41. Forslind B. A domain mosaic model of the skin barrier. *Acta Derm Venereol (Stockh)* 1994; 74: 1–6.