Research Article

***In Vitro* Cutaneous Application of ISCOMs on Human Skin Enhances Delivery of Hydrophobic Model Compounds Through the Stratum Corneum**

Henriette Baun Madsen,¹ Peter Ifversen,² Flemming Madsen,² Birger Brodin,¹
Ingrid Hausser,³ and Hanne Mørck Nielsen^{1,4}

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Abstract. This study aimed to investigate the effect of a novel kind of immune-stimulating complexes (ISCOMs) on human skin penetration of model compounds *in vitro* to evaluate their potential as a delivery system, ultimately for transcutaneous vaccination. Special focus was on elucidating the mechanisms of penetration. Preparation of ISCOMs was done by dialysis and subsequent purification in a sucrose density gradient. The penetration pathways of acridine-labeled ISCOMs were visualized using confocal laser scanning microscopy (CLSM). Transmission electron microscopy (TEM) was used to evaluate the ultrastructural changes in the skin after application of the ISCOMs with or without hydration. Transcutaneous permeation of the model compound, methyl nicotinate, was evaluated in diffusion cells. The prepared ISCOMs were 42–52 nm in diameter as evaluated by dynamic light scattering with zeta potentials of –33 to –26.1 mV. TEM investigations verified the presence of ISCOM structures. Penetration of acridine into skin was greatly increased by incorporation into ISCOMs as visualized by CLSM. Permeation of methyl nicotinate was enhanced in the presence of ISCOMs. Ultrastructural changes of the intercellular space in the stratum corneum after exposure of ISCOMs were observed on micrographs, especially for hydrated skin. In conclusion, cutaneous application of ISCOMs leads to increased penetration of hydrophobic model compounds through human stratum corneum and thus shows potential as a transcutaneous delivery system. The increased penetration seems to be reflected by a change in the intercellular space between the corneocytes, and the effect is most likely caused by the components of the ISCOMs rather than intact ISCOMs.

KEY WORDS: CLSM; penetration; Posintro™; TEM; vaccine.

INTRODUCTION

Immune-stimulating complexes (ISCOMs) constitute a well-known type of adjuvant delivery system. Upon injection or mucosal administration in both animals and humans, ISCOMs effectively deliver antigens with following induction of efficient immune responses (1,2). ISCOMs appear as 40–60 nm spherical cage-like structures to which the antigen can be associated. The size of ISCOMs is thus comparable to most viruses, which in itself increases their uptake by antigen-presenting cells (APC). ISCOMs are generally composed of the immunological adjuvant saponin, cholesterol, and

phospholipids organized to form a negatively charged nanoparticle to which the lipophilic or positively charged sites of the relevant antigen can be associated. In addition, positively charged ISCOMs (PLUSCOMs) described in the literature also indicate effective immune responses after subcutaneous injection (3,4).

An interesting route of administration, as an alternative to injection of vaccines, is transcutaneous administration. Immunization through the skin is attractive because it is noninvasive, pain free, and nontraumatic for the patient, and it does not require trained staff for proper administration. Furthermore, APC (Langerhans cells) present in the epidermis can be targeted by transcutaneous vaccination (5,6). However, the targeting of vaccines to Langerhans cells is strongly dependent on the application of a delivery system, which can facilitate the penetration through stratum corneum and present the antigen to APC in the epidermis. Furthermore, it is beneficial if the delivery system itself acts as an adjuvant for activation of an effective immune response against the delivered antigen.

A new generation of ISCOMs, the Posintro™ nanoparticles (7), is considered to function as a delivery vehicle for delivery of antigens across the stratum corneum, in addition to their well-known adjuvanticity. This new generation of ISCOMs provides the possibility to control the surface charge

¹ Department of Pharmaceutics and Analytical Chemistry, Faculty of Pharmaceutical Sciences, University of Copenhagen, Universitetsparken 2, 2100, Copenhagen, Denmark.

² Coloplast A/S, Bakkegårdsvej 406A, 3050, Humlebæk, Denmark.

³ Universitäts-Hautklinik, Vossstrasse 2, 69115, Heidelberg, Germany.

⁴ To whom correspondence should be addressed. (e-mail: hmn@farma.ku.dk)

ABBREVIATIONS: APC, Antigen-presenting cells; CLSM, Confocal laser scanning microscopy; DLS, Dynamic light scattering; ISCOMs, Immune-stimulating complexes; LDE, Laser Doppler electrophoresis; TEM, Transmission electron microscopy; UC, Ultracentrifugation.

by adding a positively charged cholesterol derivative (DC-cholesterol) to the nanoparticulate structure. Adding positive charges to the nanoparticles will not only increase the surface association of negatively charged antigen sites (4) but hypothetically also increase the interaction with negatively charged lipids and cell surfaces, thereby increasing uptake by APC. Effective immune responses against tetanus toxoid have been observed *in vivo* in rabbits and mice after transcutaneous vaccination with Posintro™ nanoparticles with associated tetanus toxoid antigen (Schjødt *et al.*, presentation, Second International MVADS 2006, “Efficient transcutaneous immunization using ISCOM-based formulations”). Furthermore, activation and migration of Langerhans cells after application of Posintro™ were observed *in vivo* in hairless rats upon cutaneous exposure to the formulation (8). The mechanism of Posintro™ interaction with and penetration into the skin after cutaneous application and the following activation of the immune response are, however, still unknown.

The Posintro™ nanoparticles as such or components hereof are believed to serve as a penetration enhancer for transcutaneous delivery of the antigen. The transcutaneous penetration pathway of either fluorescent drugs or various nanoparticles has previously been identified using confocal laser scanning microscopy (CLSM) (9–12). Supplementary to this, transmission electron microscopy (TEM) provides a higher resolution and has previously been used to evaluate the mechanism of penetration of elastic vesicles into both human and mice skin *in vitro* and to study interactions between vesicles and skin (11,13). The results of those studies showed the location of intact elastic vesicles in the upper three layers of the stratum corneum and that the intercellular lipid layer was disturbed by the application of elastic vesicles. TEM has also been used to demonstrate that metallic nanoparticles with a size of 10 nm were able to penetrate the skin (14) and to study changes in ultrastructure in the skin after drug delivery by iontophoresis (15,16).

The objectives of the current study were to investigate the effect of Posintro™ nanoparticles on human skin penetration of model compounds and to elucidate the mechanism by which this takes place. The results can be used to evaluate their potential as a transcutaneous delivery system.

MATERIALS AND METHODS

Materials

Quillaja saponaria A (Quil A) is a saponin derivative and was obtained from Brenntag Biosector, Denmark, and mega-10 (decanoyl-*N*-methylglucamide) was from Bachem, Germany. Cholesterol (>98%), dioleoyl phosphatidylethanolamine (>99%), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC, >99%) were obtained from Avanti Polar Lipids, AL, USA. Methyl nicotinate, DC-cholesterol (~95%), sucrose (>99.5%), 4'-6-diamidino-2-phenylindole (DAPI), and agarose were purchased from Sigma-Aldrich, Denmark. Acridine (2,8-bis(dimethylamino)-10-dodecyl-acridinium bromide) was obtained from ACROS, Belgium. Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin,

500 ng/ml amphotericin B, and 10% (v/v) fetal calf serum were all purchased from BioWhittaker, Cambrex. Fluorescent mounting medium was purchased from DakoCytomation, Denmark. Tissue-TEK OCT was purchased from Sakura Finetek, Denmark. Phosphate-buffered saline (PBS) pH 7.4 was prepared from distilled, deionized water (Milli-Q Water system, Millipore, Axeb, Denmark). Three percent (w/v) glutaraldehyde, glycid ether 100 (Epon), 1% (w/v) aqueous osmium tetroxide, methylene blue, and ethanol were all purchased from Roth GmbH, Germany, while copper grids and formvar solution were from Plano, Germany. Uranyl acetate was obtained from SERVA, Germany, and propylene oxide and cacodylate buffer pH 7.4 were from Polysciences Europe, Germany, and lead citrate was from Merck, Germany.

Preparation and Characterization of Posintro™ Nanoparticles

Posintro™ nanoparticles were prepared by the standard dialysis method first described by Höglund *et al.* in 1989 (17). The particles used for the CLSM studies were slightly modified, as the fluorophore (acridine) was incorporated into the nanoparticles, which were subsequently named acridine-Posintro™. Briefly, cholesterol, DC-cholesterol, and POPC (and acridine) were dissolved in 20% (w/v) mega-10 in Milli-Q water. The saponin, Quil A, was dissolved in Milli-Q water, and appropriate amounts of the components were mixed and stirred for 2 h at 37°C. The theoretical weight ratio before dialysis between Quil A/POPC/total cholesterol was 5:1:1, and the total lipid concentration was 0.2% (w/v). The amount of DC-cholesterol was either 25% of the total cholesterol or 50% of the total cholesterol. Where nothing else is mentioned, the amount of DC-cholesterol is 25% of the total cholesterol. The mixture was dialyzed (Slide-A-Lyzer® cassette, 10,000 MW cutoff, Thermo Scientific, Denmark) against PBS for 48 h at 26°C and 48 h at 5°C with buffer changes every 24 h in order to remove the detergent.

Acridine-Posintro™ particles were ultracentrifuged (UC) in a continuous sucrose gradient prepared by the freeze-thaw method described previously (18). Briefly, 4.5 ml 25% (w/v) sucrose solution was placed in an ultraclear centrifuge tube (Beckman Coulter, CA, USA) and frozen at -20°C. The day before centrifugation, the tube was placed at 4°C for slow thawing. Acridine-Posintro™ suspension of 0.7 ml was placed on top of the sucrose gradient and centrifuged for 4 h at 15°C and 50,000 rpm (112,000×g). The visible band was collected by gradient harvesting (Auto-Densi Flow®, MO, USA) and dialyzed against PBS at room temperature for 48 h with two buffer changes in order to remove the sucrose. Size distribution was measured by dynamic light scattering (DLS), and zeta potential was measured by laser Doppler electrophoresis (LDE; Zetasizer Nano ZS, Malvern, UK) in small volume cuvettes before and after UC. TEM images were recorded of the prepared Posintro™ and acridine-Posintro™ by negative staining. Briefly, Posintro™ was placed on a formvar-coated copper grid and negatively stained using a filtered aqueous solution of 1% (w/v) uranyl acetate.

Skin Penetration Model

Fresh human abdominal skin was obtained after cosmetic surgery from Frederiksborg Klinikken, Denmark (approved

by the local ethical committee, no. H-Ø-2001-1-39G) and processed the same day. Subcutaneous fat was removed, and skin biopsies with a diameter of 8 mm were punched out.

The skin penetration model (Fig. 1) was prepared by pouring heated liquid agar into the bottom of cell culture inserts (Becton Dickinson Biosciences, Denmark). The inserts were transferred to deep six-well plates (Becton Dickinson Biosciences, Denmark) and incubated at 4°C for 1 h. The skin biopsies were placed in the hardened agar with the stratum corneum facing upwards, and 11 ml of supplemented DMEM medium was added to each well. The skin biopsies were cultured and maintained at 37°C and 5% CO₂ in a humidified atmosphere.

Confocal Laser Scanning Microscopy

CLSM was used to visualize the penetration of acridine-Posintro™ nanoparticles by localization of the fluorescent label (acridine) in human skin. Samples of 20 µl were applied on the surface of the skin biopsies, which were subsequently incubated as described above for 24, 48, or 72 h. The applied samples were composed of 0.05 mg/ml acridine in 5% (w/v) mega-10 in PBS and acridine-Posintro™ nanoparticles in PBS, corresponding to 0.05 mg/ml acridine. After 24, 48, or 72 h of exposure, skin biopsies were removed from the penetration model setup, washed in PBS, and quick-frozen in Tissue-Tek. Subsequently, the frozen samples were cut in cryo-dermatome (Leica CM1100, Germany) into 4-µm-thin sections, which were incubated at room temperature for 30 min with DAPI nuclear stain (1 µg/ml in PBS). The sections were mounted in fluorescent mounting medium and visualized on a CLSM (Leica TCS SPE, Germany). The excitation wavelength was 405 nm for DAPI and 488 nm for acridine. The emission was visualized in the ranges 410–490 and 500–535 nm for DAPI and acridine, respectively. Each treatment was repeated three times, and representative images obtained with the same settings were recorded.

Transmission Electron Microscopy

The potential of the Posintro™ nanoparticles to penetrate into the stratum corneum upon cutaneous application was examined using TEM. Samples of 20 µl Posintro™ were applied to human skin *in vitro*, either in the absence or presence of a cross-linked polyvinyl-pyrrolidone-based hydrogel containing 65% water (Coloplast A/S, Denmark, patent no. WO/2004/031253) covering the skin surface. The hydrogel

was applied in order to study the effect of hydration. After 24 and 72 h of incubation under the conditions described above, skin biopsies exposed to Posintro™ with or without hydration were cut in half and divided into seven small skin pieces of approximately 1 mm³. The small skin pieces were fixed in 3% (v/v) glutaraldehyde in Milli-Q water overnight at 4°C, followed by fixation in 1% (w/v) osmium tetroxide in 0.1 M cacodylate buffer pH 7.4 for 1 h at room temperature. After fixation, the small pieces were dehydrated in a series of graded ethanol solutions (50%, 70%, 80%, 96%, and 100% (v/v)) and in propylene oxide with gradually increasing amounts of Epon. The tissue samples were embedded in Epon overnight at 60°C for polymerization. Semithin sections (1 µm) were cut and stained with methylene blue for visualization with light microscopy to verify the integrity of the skin. Several ultrathin (approximately 90 nm) sections were cut on a microtome (Ultracut E, Reichert-Jung, Austria), collected on formvar-coated grids and counterstained with uranyl acetate and lead citrate. The sections were examined in a Philips TEM (EM400, Eindhoven, The Netherlands). Each treatment was repeated three times, and representative micrographs were collected.

In Vitro Permeation Study in Franz Diffusion Cells

Human abdominal or mammary skin obtained from cosmetic surgery (same approval as described previously) was frozen at –20°C immediately after receipt and removal of the subcutaneous fat. Prior to the experiment, the skin was thawed and hydrated overnight in PBS at 5°C. The skin was then placed in a 60°C water bath for 1 min, after which the epidermis was carefully separated from the dermis and placed on top of a cellulose membrane in Franz diffusion cells. The donor solution consisted of 10 mg/ml of the model substance, methyl nicotinate, in the absence or presence of Posintro™ or the different components of the nanoparticles in similar concentrations as theoretically present in the intact nanoparticles. Methyl-nicotinate-containing donor solutions comprised: PBS, 10% (w/v) Tween 80 in PBS, Posintro™ with 25% (w/w) DC-cholesterol in PBS, Posintro™ with 50% (w/w) DC-cholesterol in PBS, Quil A in PBS, cholesterol in PBS with 10% (w/v) Tween 80, DC-cholesterol in PBS with 10% (w/v) Tween 80, POPC in PBS with 10% (w/v) Tween 80, and a mixture of the above-mentioned components in PBS with 10% (w/v) Tween 80.

A test volume of 200 µl was applied to a skin diffusion area of 0.2 cm², and the 3 ml stirred receptor phase (PBS) was

Fig. 1. The skin penetration model. Skin biopsies were placed in agar in cell inlets. The inlets were placed in supplemented DMEM medium and incubated at 37°C and 5% CO₂

kept at 37°C, maintaining a skin surface temperature of 32°C. Three-hundred-microliter samples were withdrawn from the receptor compartment after 1/2, 1, 3, 5, 8, 12, and 24 h and immediately replaced by 300 µl PBS. For each test solution, eight replicates were performed on skin from eight different skin donors. The samples were diluted and analyzed within the linear range (3–150 µg/ml) of the standard curve using a monochromatic plate reader at 263 nm.

Data Analysis

Cumulative amounts (Q) versus time (t) plots were used to determine steady-state flux (J_{ss} ; Eq. 1) and calculate the apparent permeability (P_{app} ; Eq. 2) for methyl nicotinate in the time range 0–24 h.

$$J_{ss} = \frac{dQ}{dt} \times \frac{1}{A} \quad (1)$$

$$P_{app} = \frac{J_{ss}}{\Delta C} \quad (2)$$

A is the area of diffusion, and ΔC is the concentration gradient of methyl nicotinate between the donor and the receptor compartment. Sink condition was maintained at all times throughout the experiment; thus, ΔC approximately equals the concentration in the donor compartment. All statistical analyses were carried out using a paired t test with a 95% significance level. Calculated P values indicate the significance. P_{app} is stated as mean $\pm$ SEM of the eight repetitions.

RESULTS

Preparation and Characterization of Posintro™ Formulations

In order to visualize the nanoparticle penetration pathway in the skin, Posintro™ and acridine-Posintro™ were prepared in parallel by the dialysis method. UC of an acridine-Posintro™ suspension in a sucrose gradient gave rise to a clear fluorescent band in the gradient that was distinct from the band of free acridine (Fig. 2), which strongly indicates that acridine was incorporated in the particulate structure. The presence of spherical, cage-like structures with sizes of 40–60 nm was confirmed by TEM for both the Posintro™ and the acridine-Posintro™ (Fig. 3, black arrow). Quil A micelles (Fig. 3, white arrow) were also observed in the ISCOM preparations as described in the literature (19,20).

All particle batches were characterized by DLS size measurement as Z average of four measurements, and zeta potential was measured by LDE as an average of ten measurements before and after UC; the results are shown in Table I. For both types of nanoparticles, size ranges corresponded to the expected structures with a size of 40–60 nm. The mean size of acridine-Posintro™ decreased slightly from 47.4 ± 0.2 to 41.7 ± 0.3 nm upon UC, and the polydispersity index (PdI) also decreased from 0.112 to 0.078 after UC, indicating narrow and homogeneous size distributions in both preparations. The mean size of the Posintro™ was 52.3 ± 2.7 nm, and the PdI was 0.102. The reproducibility

Fig. 2. UC gradients of acridine-Posintro™ (left) and acridine (right). The presence of acridine-Posintro™ nanoparticles in the lower band in the left tube was confirmed by size measurement by DLS

of the preparation method was confirmed by low interbatch variability as evaluated by DLS size measurements and location of visible UC bands in the sucrose gradient in different batches (data not shown).

No Effect on Skin Macromorphology

Light microscopy images were recorded after exposure to the nanoparticles in order to evaluate the potential damaging effects of treatment with Posintro™ nanoparticles on the skin. The skin appeared to be intact and unaffected by the application of Posintro™ nanoparticles and PBS for up to 72 h (Fig. 4). Generally, the epidermis retained its morphological characteristics with an intact stratum corneum and showed no sign of parakeratosis. Furthermore, the junction zone looked intact. It was concluded that neither the Posintro™ nanoparticles nor the treatment affects the skin significantly, as judged by visual inspection of the micrographs.

Enhanced Penetration of Acridine into Epidermis

The visualization of acridine-Posintro™ nanoparticle penetration pathway and depth was carried out using CLSM. When acridine was incorporated into Posintro™ nanoparticles, penetration of acridine into the epidermis was detected. Acridine was only detected in the stratum corneum after 24 h of application (Fig. 5a), whereas permeation through the stratum corneum and into the stratum granulosum was observed after 48 h of application (Fig. 5b). After 72 h of

a – Posintro™ **b – Acridine-Posintro™**
Fig. 3. TEM images of Posintro™ **a** and acridine-Posintro™ **b** nanoparticles before UC negatively stained with 1% (w/v) uranyl acetate. Size bar 100 nm. Black arrow: Posintro™ nanoparticle, white arrow: Quil A micelle

exposure, acridine fluorescence could be detected throughout the whole epidermis, but not beyond the stratum basale (Fig. 5c). Acridine deposition was evident in the intercorneocyte space, as visualized in the horizontal cross section (Fig. 5d) and in the magnification in Fig. 5e. As a control, free acridine in 5% (w/v) mega-10 in PBS did not seem to permeate through the stratum corneum and into the epidermis when administered for up to 72 h, since fluorescence is not observed beyond the stratum corneum (Fig. 5f–h). Mega-10 was added in order to increase solubility of acridine in water, and although mega-10 would be expected potentially to enhance penetration, we did not observe any penetration of acridine in the mega-10 solution into the epidermis.

Thus, only when acridine was incorporated into Posintro™ nanoparticles was it detectable in the epidermis. This was confirmed by the cross-sectional penetration profiles derived from the CLSM images (Fig. 6). Free acridine only penetrated about 10 μm into the stratum corneum, whereas acridine incorporated into Posintro™ was observed in larger amounts in depths of up to 70 μm in the epidermis, where the target cells for immune stimulation, the Langerhans cells, are located.

The penetration pathway of acridine-Posintro™ could clearly be visualized with the CLSM method. These confocal microscopy studies show that Posintro™ nanoparticles were able to enhance the penetration of a small very hydrophobic molecule (acridine, MW 515 Da) into the epidermis.

Change in Intercellular Space Between the Corneocytes

In order to further elucidate the penetration mechanism of the Posintro™ nanoparticles as indicated from the results of the CLSM studies, TEM studies were conducted to

evaluate the ultrastructure of the skin after exposure to Posintro™. Human skin without any pretreatment clearly showed the expected corneocyte structures, which were flattened, dead, and fully keratinized cells that did not contain any organelles (Fig. 7a). The lipid barrier, located between the corneocytes, was washed out during the sample preparation procedure encompassing several steps of organic solvent washing and dehydration of the specimen. Therefore, these spaces appear mostly empty. Below the stratum corneum, the stratum granulosum is visualized (Fig. 7a–d). As a control sample, skin exposed to PBS was imaged (Fig. 7b), which confirmed that the stratum corneum was not affected by treatment with PBS, as it appears similar to the nontreated skin in Fig. 7a

After application of Posintro™ nanoparticles without (Fig. 7c) or with (Fig. 7d) the presence of a hydrogel (hydration) for 24 h, spherical-like domains were observed but only rarely found in the intercorneocyte space (Fig. 7d, black arrow) and primarily observed after hydration. With hydration of the skin, the spherical-like domains were more pronounced, however, mainly in the deeper layers of the stratum corneum (Fig. 7d). After 72 h of exposure to the Posintro™ nanoparticles without (Fig. 8b) and with (Fig. 8c, d) hydration, more spherical-like domains were observed compared to application for 24 h, and the distribution of the domains was more homogeneous. Thus, in contrast to the 24-h samples, the spherical-like domains were also observed in the Posintro™-exposed skin without hydration (Fig. 8b). Also, after 72 h of PBS exposure, the stratum corneum (Fig. 8a) still did not seem noticeably affected by the treatment. It should be noticed that the spherical-like domains contain dark electron-dense spots, as observed in the magnification in Fig. 8d. The electron-dense spots presumably consist of aggregated lipid

Table I. Posintro™ Nanoparticle Size and Charge Before and After UC

	Size ^a before UC (nm)	Size ^a after UC (nm)	Zeta potential ^b before UC (mV)	Zeta potential ^b after UC (mV)
Posintro™	52.3 $\pm$ 2.7	–	–32.9 $\pm$ 12.7	–
Acridine-Posintro™	47.4 $\pm$ 0.2	41.7 $\pm$ 0.3	–29.6 $\pm$ 11.8	–26.1 $\pm$ 7.37

^a Stated as Z average of four measurements (mean $\pm$ SD) measured by DLS

^b Stated as average of ten measurements (mean $\pm$ SD) measured by LDE

Fig. 4. Light microscopy images of the skin after application of PBS (top) and Posintro™ (bottom) for 72 h in the skin penetration model. Size bar 50 µm

material originating either from the nanoparticles and/or remnants of the intercorneocyte lipids.

These observations clearly indicate that the Posintro™ nanoparticles cause a change in the intercellular space between the corneocytes in the form of increased spherical-like domains between the corneocytes.

Increase in Permeability of Methyl Nicotinate Across Human Skin

To investigate whether the changes observed by the microscopic techniques are specific for the nanoparticulate structure or can be affiliated to some of the constituents in the Posintro™ nanoparticles, *in vitro* permeability studies were performed. Methyl nicotinate permeability increased significantly ($P < 0.05$) in the presence of the Posintro™ nanoparticles compared to methyl nicotinate administered in PBS alone (Fig. 9a). The permeated amount of methyl nicotinate corresponded to 22% of the total applied amount in PBS and 44% of the total applied amount with Posintro™ nanoparticles containing 25% DC-cholesterol. There was no significant difference on the methyl nicotinate permeability in the presence of Posintro™ with 25% DC-cholesterol as compared to Posintro™ 50% DC-cholesterol.

Individual components as well as a mixture of all the components were also tested, but since most of the single components in the Posintro™ nanoparticles are not soluble in

PBS, 10% (w/v) Tween 80 was added to increase solubility. The presence of 10% (w/v) Tween 80 in PBS did not decrease the integrity of the tissue since no significant difference in P_{app} for methyl nicotinate in PBS and in PBS with 10% (w/v) Tween 80 (Fig. 9b) was detected. The presence of DC-cholesterol ($P = 0.024$), POPC ($P = 0.001$), and Quil A ($P = 0.008$) mediated a significant increase in P_{app} of methyl nicotinate compared to control studies, whereas cholesterol did not mediate an increase in permeability. When comparing the effect of the different components, Quil A appeared to have a higher permeability-enhancing effect than the other components, and this effect was significantly different from cholesterol ($P = 0.001$) and DC-cholesterol ($P = 0.014$) but not significantly different from POPC. A simple mixture of the components also indicated a significant increase in the permeability of methyl nicotinate compared to methyl nicotinate in 10% (w/v) Tween 80 ($P = 0.003$). The permeability of methyl nicotinate in the mixture was not significantly different from the P_{app} in Posintro™ nanoparticles with 25% DC-cholesterol.

These results show that the Posintro™ nanoparticles increase the permeability of methyl nicotinate, a small lipophilic molecule (Mw 137 Da, log P 3.1) across human skin. The increased permeability seems to be caused by the components of the Posintro™ nanoparticles equally to the nanoparticulate structure, yet the effects of the individual components seem not to be additive.

DISCUSSION

The objective of this study was to investigate the effect of ISCOM nanoparticles on the penetration of model compounds into and across human skin *in vitro* and to investigate the mechanism by which this might occur.

Penetration of Posintro™ Nanoparticles

In the present study, the penetration depth and the penetration pathway of acridine-Posintro™ in human skin was visualized (Figs. 5 and 6). Permeation of acridine through the stratum corneum was observed following *in vitro* exposure of acridine-Posintro™ to the surface of human skin (Fig. 5). The penetration occurred primarily via the intercellular pathway between the corneocytes, which ties in with the fact that acridine is a very hydrophobic molecule that can be incorporated into the lipid-based nanoparticles and therefore would be expected to distribute to the lipid-rich intercellular space (21,22). The same penetration profile was also observed after incorporation of another hydrophobic fluorophore, DiD-PE, into the Posintro™ nanoparticles. DiD-PE was observed to penetrate via the intercellular pathway through the stratum corneum into epidermis after 48 h of application (data not shown). Application of acridine in PBS with 10% mega-10 showed only minor penetration of the fluorescent label into the stratum corneum, indicating that the Posintro™ nanoparticles or their components increased the extent of acridine permeation. To elucidate whether the penetration of acridine was dependant on the incorporation into Posintro™ nanoparticles or merely caused by coadministration, the control of choice would be to coadministrate free acridine with Posintro™ nanoparticles. However, dissolution of acridine in aqueous buffer requires addition of detergents,

Fig. 5. Confocal micrographs of the penetration of acridine in human skin. Twenty microliters of 0.05 mg/ml acridine-Posintro™ in PBS or 0.05 mg/ml acridine in 5% (w/v) mega-10 was applied for 24, 48, or 72 h in the skin penetration model under incubation at 37°C and 5% CO₂. The biopsies were quick-frozen and cut into 4-μm thin sections, and the sections were incubated for 30 min with DAPI nuclear color. The CLSM images were recorded with a ×63 oil objective. *Green:* acridine, *blue:* DAPI. *Size bars* **a–c** 25 μm, **d** 50 μm, **e** 10 μm, and **f–h** 25 μm. *White cross-sectional lines* represent the penetration profiles depicted in Fig. 6

which would interrupt the cage-like structures of the nanoparticles and thereby change the nanoparticle characteristics.

The CLSM technique can produce valuable indications on skin penetration of various kinds of particles and the mechanism of penetration. Dubey *et al.* previously demonstrated, using CLSM, that rhodamine red loaded in elastic liposomes was able to penetrate much deeper than the probe loaded in conventional rigid liposomes (23). Furthermore,

CLSM was used to show that Quantum dots penetrate through the most superficial stratum corneum layers and are located near hair follicles (9,24). Despite the obvious advantages of the CLSM technique, a disadvantage of the method is that penetration of the fluorophore only is observed and that merely hypothetical conclusions regarding the penetration depth and pathway of the particles as such can be made. Data from the CLSM studies indicated that

Fig. 6. Cross-sectional penetration profiles of acridine in human skin after 48 h of application of acridine-Posintro™ (blue line) or acridine (red line). The profiles are based on the cross-sectional lines in Fig. 5b, g

Posintro™ nanoparticles increased the permeation of acridine through the stratum corneum. However, it could not be determined from these experiments whether Posintro™ nanoparticles penetrate as intact or disintegrated particles or whether they merely cause an increase in the permeability of the released acridine.

Mechanism of Penetration

To further elucidate the mechanism of penetration of the Posintro™ nanoparticles, TEM micrographs were recorded to evaluate the effect of cutaneous treatment with Posintro™ nanoparticles on human skin and possibly to localize intact nanoparticles in the skin. Spherical-like domains were clearly observed in the intercorneocyte space after exposure to Posintro™ nanoparticles (Fig. 8b) and not observed in the control samples. Similar expanded intercellular spaces in the stratum corneum have also been observed with topical application of various aliphatic and aromatic hydrocarbons from jet fuel to porcine skin *in vivo* (25) and after selective lipid extraction from the skin (26). A possible explanation for presence of the spherical-like domains is agglomeration of nanoparticles or particle remnants in clusters between the corneocytes increasing the intercorneocyte spacing locally. The increased spacing between the corneocytes caused by the Posintro™ nanoparticles could enhance the penetration of the associated or coadministered molecule via the intercellular route. The spherical-like domains might in reality also be remnants of lamellar bodies budded off from the stratum granulosum, but as the spherical-like domains are much more pronounced in the Posintro™-exposed skin compared to the control, the nanoparticles seem to have an influence on the occurrence of these domains. The occurrence of the spherical-like domains in the skin was greatly increased in hydrated

Fig. 7. Transmission electron micrographs of untreated human stratum corneum **a** and stratum corneum after 24 h of *in vitro* exposure to PBS **b**, Posintro™ **c**, or Posintro™ + hydration **d**. The specimens were fixed in 3% (v/v) glutaraldehyde and 1% (w/v) osmium tetroxide followed by dehydration in a gradient of graded ethanol solutions and in propylene oxide with gradually increasing amounts of Epon. Ultrathin sections were collected on formvar-coated grids and counterstained with uranyl acetate and lead citrate. SG stratum granulosum, SC stratum corneum; black arrows indicate examples of the spherical-like domains. Size bar 1 μ m

Fig. 8. Transmission electron micrographs of human stratum corneum after 72 h of *in vitro* exposure to PBS **a**, Posintro™ **b**, or Posintro™ + hydration **c**, **d**. The specimens were fixed in 3% (v/v) glutaraldehyde and 1% (w/v) osmium tetroxide followed by dehydration in a gradient of graded ethanol solutions and in propylene oxide with gradually increasing amounts of Epon. Ultrathin sections were collected on formvar-coated grids and counterstained with uranyl acetate and lead citrate. SG stratum granulosum, SC stratum corneum; black arrows indicate examples of the spherical-like domains; black dotted arrow indicates the electron-dense spots. Size bar 1 μm

tissue. The spherical-like domains were observed after 24 h of exposure (Fig. 7) on hydrated skin, but not until 72 h in the absence of hydration. The CLSM studies were performed in the absence of hydration, and thus no penetration of acridine-Posintro™ was observed after 24 h (Fig. 5a), whereas penetration was evident after 72 h of application (Fig. 5c). These CLSM results correlate with the occurrence of the spherical-like domains observed with TEM.

The evidence of penetration enhancement of a hydrophobic model compound and the observed structural alterations in the skin caused by the presence of Posintro™ nanoparticles may be explained by either a partial disintegration of the particles or a collapse of the Posintro™ nanoparticulate structure upon interaction with the skin. This interaction could possibly increase the spacing between the corneocytes and make the stratum corneum barrier more permeable to the drugs coadministered or encapsulated.

Penetration of Intact Nanoparticles?

The observed spherical-like domains contained dark electron-dense spots (Fig. 8d). The electron-dense spots presumably consist of lipid-aggregated material originating either from the nanoparticles or the intercorneocyte lipids.

Similar electron-dense spots in the lipid areas in the stratum corneum have also previously been observed by van den Bergh *et al.* after treatment with elastic vesicles and fixation with osmium tetroxide (11). In that study, the spots were concluded to originate from the vesicle material.

Lamellar stacks similar to the contents of lamellar bodies in the stratum granulosum were observed by van den Bergh *et al.* after application of elastic vesicles onto human and mouse skin (11,13). In the same studies, disorganized intercellular lipids were also observed. The lamellar stacks were found in the upper and lower part of the stratum corneum, and they were either perpendicular to the bilayers of the skin or randomly distributed. The lamellar stacks were suggested to resemble stacks of flattened elastic vesicles, and these occur similarly to the observed spherical-like domains in the present study, except that in the present study they occur as empty spaces after osmium tetroxide postfixation. In the studies of van den Bergh *et al.*, a mixture of osmium and ruthenium tetroxide was used as a fixation agent. This procedure improves the visualization of lipids between the corneocytes and thus detects the perpendicular bilayers of lipids and disorganized intercellular lipids. A major limitation of ruthenium tetroxide, however, is its high reactivity, which causes severe overall damage of the tissue, and a poor penetration

Fig. 9. Permeability of methyl nicotinate applied with Posintro™ nanoparticles or components of Posintro™ nanoparticles on human skin. The samples with cholesterol, DC-cholesterol, POPC, and the mixture of components are dissolved in PBS with 10% (v/v) Tween 80, whereas Posintro™ nanoparticles and Quil A are in PBS. $N=8$, P_{app} is stated as mean $\pm$ SEM of eight repetitions. All statistical analyses were carried out using a paired t test with a 95% significance level; asterisks indicate significance

into the stratum corneum and living tissue and thus a poor contrast in TEM imaging (27). Therefore, osmium tetroxide was chosen as a fixation agent in the present study in order to achieve a better contrast throughout the skin sample. The focus was thus on detection of the effects of the cutaneous treatment rather than localization of the nanoparticle. Osmium tetroxide is normally used as a postfixation agent of lipids in tissue, but due to the lack of double-bond-containing lipids in the intercorneocyte space, osmium tetroxide cannot fixate the lipid matrix between the corneocytes (28,29). Thus, confirmation of the presence of Posintro™ nanoparticles in the skin was carried out using ruthenium tetroxide as a fixation agent instead of osmium tetroxide. Electron-dense inclusions similar to the Posintro™ structure were observed between the corneocytes in the upper layers of the stratum corneum after 24 h of application on hydrated skin, but only in very small amounts (data not shown). Since

such inclusions were not detected in the control, this could be an indication of the penetration of a few intact nanoparticles into the upper layers of the stratum corneum.

The present studies clearly indicate that the Posintro™ nanoparticles have an influence on skin penetration, but no final conclusion can be drawn as to whether Posintro™ nanoparticles penetrate as intact particles. This should be considered along with the ongoing discussions on whether different types of nanoparticles are able to penetrate the skin in their intact form. Schreier and Bouwstra *et al.* have reviewed the penetration of vesicles, liposomes, and niosomes (21,30,31) and suggested that penetration of intact vesicles in general is unlikely to occur at all. However, Cevc *et al.* (32) stated that a combination of flexible vesicles (transfersomes) and nonocclusive application will enable vesicles to penetrate through the stratum corneum under the influence of a “hydration force”.

Posintro™ Nanoparticles or Their Components Act as Enhancers

In order to evaluate the effect of the nanoparticle structure, traditional permeation cell studies were performed. The human skin permeability of a less hydrophobic model compound, methyl nicotinate, was significantly increased upon coadministration with Posintro™ nanoparticles *in vitro* (Fig. 9a). Methyl nicotinate was considered a relatively good model substance as it is reasonably soluble in buffer and easily detectable and permeated untreated human skin in measurable amounts. Since it was subsequently found that all the individual components in the Posintro™ nanoparticle, except cholesterol, were able to increase methyl nicotinate permeability when added alone (Fig. 9b), the enhanced permeability might thus be an effect of the mixture of components rather than being mediated by the nanoparticulate structure itself. In particular, the detergent Quil A has a significant effect on the permeability of methyl nicotinate, corresponding to that observed by Recchia *et al.* where a semisynthetic *Quillaja* saponin increased the delivery of antibiotics across mucosal membranes (33). Also, detergents in general are known to act as penetration enhancers (reviewed in (34)). *In vitro* studies on human skin have indicated that both fatty acids and surfactants are able to increase the penetration of naloxone, which in size and lipophilicity is similar to methyl nicotinate (35). The proposed mechanism of enhancement involved was alterations of the normal skin structure along with an increased solubility of the drug in the skin lipids, which are both generally known mechanisms of various chemical enhancers (34,36). Similar to this, the observed enhancer effect of Posintro™ could simply be due to alterations in the skin structure as a result of detergent and lipid exposure.

Posintro™ nanoparticles are cage-like structures with less overall negative surface charge than traditional ISCOMs due to DC-cholesterol being a constituent. This would in itself increase the interaction with negatively charged antigens and biological membranes compared to the traditional ISCOMs (4). In the present study, it was, however, found that the amount of positive DC-cholesterol in the ISCOMs did not significantly enhance the permeation of methyl nicotinate

(Fig. 9a) and probably did not alter the interaction with the corneocytes in the stratum corneum. These results are thus not conclusive regarding the effect of variations in charge of the Posintro™ nanoparticles, and current research focuses on further evaluating the interaction of Posintro™ nanoparticles with the skin.

CONCLUSIONS

These studies show that the Posintro™ nanoparticles or components hereof interact with the stratum corneum and enhance the penetration of a small lipophilic fluorescent probe (acridine) into the epidermis and the transcutaneous permeation of a small rather lipophilic molecule (methyl nicotinate). Mechanistically, this seems to be the result of a change in the intercellular space between the corneocytes, enabling increased penetration by the intercellular pathway. The enhancing effect might be caused by the components of the Posintro™ nanoparticles rather than the nanoparticulate structure itself, since disintegration of the particles upon application cannot be excluded. Overall, the results indicate that ISCOMs are promising candidates for transcutaneous delivery systems.

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