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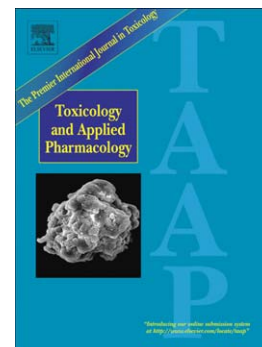
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A study of the enhanced sensitizing capacity of a contact allergen in lipid vesicle formulations

Carl Simonsson^{a*}, Jakob Torp Madsen^b, Annette Graneli^c, Klaus E. Andersen^b, Ann-Therese Karlberg^a, Charlotte A. Jonsson^{a#}, Marica B. Ericson^{c#}

^aDepartment of Chemistry, University of Gothenburg, SE-412 96, Gothenburg, Sweden

^bDepartment of Dermatology, Odense University Hospital, University of Southern Denmark, DK-5000, Odense, Denmark

^cDepartment of Physics, University of Gothenburg, SE-412 96, Gothenburg, Sweden

*Corresponding author. Tel: +46 31786 9110; fax: +46 786 3840. E-mail address: carl.simonsson@chem.gu.se

#Joint responsibility for the study

Abstract

The growing focus on nanotechnology and the increased use of nano-sized structures, e.g. vesicles, in topical formulations has led to safety concerns. We have investigated the sensitizing capacity and penetration properties of a fluorescent model compound, rhodamine B isothiocyanate (RBITC), when administered in micro- and nano-scale vesicle formulations. The sensitizing capacity of RBITC was studied using the murine local lymph node assay (LLNA) and the skin penetration properties were compared using diffusion cells in combination with two-photon microscopy (TPM).

The lymph node cell proliferation, an indicator of a compounds sensitizing capacity, increased when RBITC was applied in lipid vesicles as compared to an ethanol:water (Et:W) solution. Micro-scale vesicles showed a slightly higher cell proliferative response compared to nano-scale vesicles. TPM imaging revealed that the vesicle formulations improved the skin penetration of RBITC compared to the Et:W solution. A strong fluorescent region in the stratum corneum and upper epidermis implies elevated association of RBITC to these skin layers when formulated in lipid vesicles.

In conclusion, the results indicate that there could be an elevated risk of sensitization when haptens are delivered in vehicles containing lipid vesicles. Although the size of the vesicles seems to be of minor importance, further studies are needed before a more generalized conclusion can be drawn. It is likely that the enhanced sensitizing capacity is a consequence of the improved penetration and increased formation of hapten-protein complexes in epidermis when RBITC is delivered in ethosomal formulations.

Keywords:

Lipid vesicles, nano, ethosomes, contact allergy, two-photon microscopy, skin penetration

Abbreviations:

Allergic contact dermatitis (ACD); dinitrochlorobenzene (DNCB); ethanol:water (Et:W); local lymph node assay (LLNA); 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC); rhodamine B isothiocyanate (RBITC); two-photon microscopy (TPM).

Introduction

Allergic contact dermatitis (ACD) is a T-cell mediated skin disease triggered by low molecular weight chemicals called haptens (Karlberg et al., 2008b). An estimate of 15-20% of the population in the Western world are sensitized to one or more chemicals (Thyssen et al., 2007). The risk of being sensitized depends on several different factors. For example, there is a well documented correlation between sensitizing capacity and hapten reactivity towards nucleophilic amino acids in proteins (Landsteiner and Jacobs, 1935; Gerberick et al., 2007; Karlberg et al., 2008a). Other aspects that may influence the risk of being sensitized are exposure and the physical state of the skin barrier (Allenby and Basketter, 1993; Basketter et al., 2006). Also, the choice of vehicle has been shown to affect the sensitizing capacity of compounds (Kligman, 1966; Basketter et al., 2001; Jowsey et al., 2008). Thus, the development of new topical and transdermal delivery systems must take the possibility of skin sensitizing effect of active compounds into consideration. For example, the growing focus on nanotechnology and use of nano-sized particles and lipid vesicles (particles and vesicles having dimensions less than 100 nm) in pharmaceutical and cosmetic formulations, has raised questions about whether the sensitizing capacity of potential allergens could be increased in these type of formulations.

Lipid vesicles, e.g. liposomes, are colloidal particles composed of bilayers of amphiphatic molecules forming spherical shaped lipid bodies with aqueous cores. The use of lipid vesicles in cutaneous formulations was initiated in the beginning of 1980 (Mezei and Gulasekharam, 1980). Since then there has been increasing use of vesicles in cosmetic- and pharmaceutical skin products. One proposed benefit of vesicle formulations is their ability to improve the penetration of compounds into the skin, while limiting the systemic uptake (Mezei and Gulasekharam, 1980; Masini et al., 1993; Bernard et al., 1995; Fresta and Puglisi, 1997; Kim et al., 1997; Ferreira et al., 2004; Sinico et al., 2008). Increased transdermal delivery using vesicles has also been reported (El Maghraby et al., 1999; Guo et al., 2000; Touitou et al., 2000; Dubey et al., 2007).

Skin sensitizing effects related to vesicle formulations have so far been scarcely studied. Recently Madsen et al (Madsen et al., 2010b) demonstrated that formulations containing ethosomes, i.e. lipid vesicles prepared with ethanol in the aqueous phase (Touitou et al., 2000), enhanced the sensitizing capacity of two contact allergens, dinitrochlorobenzene (DNCB) and isoeugenol, in the murine local lymph node assay (LLNA). Furthermore, isoeugenol and methyldibromo-glutaronitrile in ethosomal vehicles likewise increased the elicited patch test reactions in sensitized volunteers (Madsen et al., 2010a). Thus, there seems to be an elevated risk of allergenic reactions when haptens are delivered in vehicles containing lipid vesicles; however, the reason for the observed effect has not been elucidated.

We have recently used two-photon microscopy (TPM) as a novel approach to study processes related to contact (Samuelsson et al., 2009; Simonsson et al., 2011). TPM is a non-invasive imaging technique, which can be used for three dimensional visualisation of the cellular localisation and distribution of chromophores in highly light scattering and light absorbing tissue (Denk et al., 1990), such as human skin. For example, TPM has been applied to study the skin barrier and its modulation by penetration enhancers (Bender et al., 2008), as well as for diagnostic purposes (Paoli et al., 2008). Compared to confocal microscopy, TPM offers improved imaging depth in biological tissue due to superior penetration of the near infrared excitation light. Another advantage is the inherently confined excitation volume which improves sensitivity, i.e. no pinhole is needed in front of the detector, and reduces out of focus photobleaching and phototoxic effects.

The aim of this work was to investigate the sensitizing capacity of haptens when delivered in vehicles containing nano- or micro-scale lipid vesicles, i.e. ethosomes, and to study the role of skin permeation and penetration on the sensitizing capacity by employing a fluorescent model compound, rhodamine B isothiocyanate (RBITC) (Fig. 1A). The sensitizing capacity of RBITC in nano- and micro-scale ethosomal formulations, and in an ethanol-water (Et:W) solution was investigated using a modified murine LLNA. TPM was utilized to visualise the penetration of RBITC from the different vehicles in *ex vivo* human skin samples and trans-epidermal fluxes were measured using diffusion chambers.

Material and methods

Preparation of test formulations

The lipid vesicles used in this study were prepared from 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC, Fig. 1B) 5-6 % w/v (Avanti Polar Lipids, Alabaster, Al) in 40 % v/v ethanol in water. Nano-scale ethosomal formulations (here referred to as nano-scale ethosomes) were prepared using an extrusion technique (Hope et al., 1985). Briefly, POPC and RBITC (Sigma Aldrich Chemie GmbH, Steinheim, Germany) were dissolved in ethanol and water was injected whilst maintaining continuous stirring at 700 rpm. The solution was subsequently extruded through Nucleopore Track-Etch polycarbonate membranes (Whatman, Springfield Mill, UK), pore size 800 nm, 100 nm, and 50 nm at room temperature, using an Avanti mini-extruder (Avanti Polar Lipids), to obtain ethosomes <100 nm. Non-extruded micro-scale ethosomal formulations (here referred to as micro-scale ethosomes) were prepared by the thin film hydration method. Briefly a film of POPC was hydrated with a hydroethanolic solution (40 % v/v ethanol) of RBITC by spinning (approx. 120 rpm) 1 h in a round bottom flask on a rotary evaporator model R-210 (Buchi Labortechnik AG, Flawil, Switzerland) at room temperature. RBITC was also formulated in a 40% v/v Et:W control solution. Two different concentrations of RBITC were used, i.e. a final concentration of approx. 5 % w/v for the LLNA experiments and approx. 0.5 % w/v for the *ex vivo* penetration and permeation experiments.

Quantitative analysis of RBITC

The concentrations of RBITC in the formulations and in the acceptor liquid in the *ex vivo* permeation experiments were analyzed using a fluorescence spectrophotometer (Spectra Max M2, Molecular Devices, Sunnyvale, Ca). The excitation wavelength was 544 nm and the emission wavelength 590 nm. Vesicle formulations were disrupted by incubation in 0.1 % w/w Triton X (Sigma Aldrich Chemie GmbH) in phosphate buffer (pH 7.4) prior to analysis.

Quantitative analysis of POPC

The concentration of POPC in the vesicle formulation before and after extrusion was analysed using an ammonium ferrothiocyanate assay previously described by Stewart (Stewart, 1980). Briefly, ammonium ferrothiocyanate was prepared by mixing 4.1 g ferric chloride hexahydrate (Sigma Aldrich Chemie) and 4.6 g ammonium thiocyanate (Sigma Aldrich

Chemie) in 100 mL water. Extruded and non extruded ethosomal formulations were diluted 1:1000 with chloroform (Sigma Aldrich Chemie), vortexed, mixed 3:2 with ammonium ferrothiocyanate and incubated for 1 h on a orbital shaker model Swip (Edmund Buhler GmbH, Hechingen, Germany) at room temperature. The POPC concentration was determined by analysing the absorbance of the organic phase at λ 490 nm.

Size distribution

The hydrodynamic diameter and polydispersity of the nano-scale ethosomes were determined by dynamic light scattering (DLS) (Zetasizer Nano ZS, Malvern Instruments, Worcesterchire, UK). The instrument incorporates a He-Ne 633 nm laser and a non-invasive back-scatter detection system. Measurements were performed in disposable capillary cells at 22 °C. Data acquisition and processing were controlled via the integrated Malvern Zetasizer computer program Dispersion Technology Software. The ethosomes were prepared and analysed by DLS just before they were used in either the LLNA or the *ex vivo* skin experiments. Also, a stability study of extruded ethosomes over time, up to 10 days, at room temperature was performed.

Encapsulation efficiency

The encapsulation efficiency of RBITC in the nano-scale ethosomes was determined by size-exclusion gel chromatography (Fry et al., 1978). Briefly, Sephadex G50 was incubated in water at 90°C for 1 h, loaded in 2 mL disposable plastic syringes and packed by centrifugation (2000 rpm). The gel was pre-loaded with empty vesicle formulations and spun at 2000 rpm, before RBITC ethosomal formulations (approx. 0.5 or 5 % w/v), diluted 1:10 with water, were applied and separated by centrifugation (2000 rpm). The encapsulation efficiency was calculated by dividing the concentration of RBITC in the eluate from the column with the concentration in the same formulation before separation.

Modified LLNA with non-pooled lymph nodes

The sensitizing capacity of RBITC was investigated using a modified murine LLNA (OECD, 2002; Christensson et al., 2008; Niklasson et al., 2009), i.e. by comparing the cell proliferation in non-pooled lymph nodes induced by a fixed concentration of RBITC in either nano- or micro-scale ethosomes, or in Et:W solution. Female CBA/Ca mice, 8-12 weeks old, purchased from Scanbur AB (Sollentuna, Sweden) were housed in hepa-filtered airflow cages in light-, humidity- and temperature controlled rooms. The animal experiments were approved

by the local ethics committee. Briefly, mice were exposed to RBITC (approx. 5 % w/v) in either nano-scale ethosomes, micro-scale ethosomes, or in Et:W solution, topically applied on the ears, for three consecutive days. A sham treated control group was concurrently exposed to empty ethosomes. All formulations were freshly prepared on the day of the application. Two days after the last topical application, the mice were injected with 20 μ Ci [methyl-3H] thymidine (Amersham Biosciences, UK) in PBS (Sigma-Aldrich) and sacrificed 5 h later. Draining auricular local lymph nodes were excised and single cell suspensions from individual mice were prepared and analysed by measuring the [methyl-3H]thymidine incorporation by β -scintillation counting (Beckman LS 6000TA Instruments). The results are expressed as mean dpm/mouse lymph nodes.

Skin samples

Ex vivo skin penetration and permeation experiments were performed using human skin samples obtained as leftovers from breast reduction surgery at the Sahlgrenska University Hospital, Gothenburg, Sweden. The skin was stored at -70 °C and used within 3 months of the operation. Percutaneous absorption experiments were performed using skin samples from two different donors, and the skin penetration experiments using skin samples from 3 different donors.

Percutaneous absorption

Ex vivo skin permeation experiments were performed according to COLIPA guidelines (COLIPA, 1997) using heat separated human epidermis and vertical diffusion chambers (Laboratory Glass Apparatus Inc, Berkley, CA). The receiver volume of the diffusion chambers were 4 mL and a permeation area approx. 1 cm². The skin was equilibrated with the acceptor liquid, PBS, stirred with a bar magnet at 32 °C, for 30 minutes before RBITC (approx. 0.5 % w/v) in nano-scale ethosomes or in Et:W solutions (200 μ l) were added to the donor compartment. Passive diffusion was maintained for 24 h at 32 °C under continuous stirring and sampling with a flow rate of 1.5 mL/h. The acceptor liquid flow was driven by a Minipuls 2 peristaltic pump (Gilson Inc, Middleton, WI) and acceptor liquid was collected using an autosampler Model 203 (Gilson). The concentration of RBITC in the acceptor was analyzed with a Spectra Max M2 fluorescence spectrophotometer (Molecular Devices).

Two-photon microscopy investigations

Full thickness human skin was incubated with RBITC in either nano- or micro-scale ethosomal formulations, or Et:W solution in vertical diffusion chambers (Laboratory Glass Apparatus Inc) during 24 h at 32 °C. The skin was then rinsed with PBS before mounted in imaging chambers for subsequent imaging by TPM. Non-invasive imaging of the skin samples was performed with a Radiance 2100MP Rainbow laser scanning multiphoton microscope system (Bio-Rad, Hemel Hempstead, UK; company purchased by Carl Zeiss) coupled to an Olympus IX71 inverted microscope (Olympus, Hamburg, Germany). Two photon excitation was obtained using a femtosecond Titanium:Sapphire laser (Tsunami, Spectra-Physics, Mountain View, CA), pumped at 7.5 W by a 10 W Millennia X's laser. The output wavelength was tuned to 800 nm, the pulse length was approx. 80 fs and the pulse repetition rate approx. 80 MHz. An Achroplan NIR 40x/0.8 W lens (Carl Zeiss MicroImaging GmbH Jena, Germany) was used. Detection was performed in descanned mode with a fully opened pinhole and a signal enhancing lens to achieve maximum sensitivity. The filter set-up for detection was a 650 nm dichroic mirror and a 570 nm long pass filter. Image acquisition was controlled via the integrated computer software LaserSharp 2000. Image stacks showing the distribution of RBITC in the skin were generated by stepwise focusing the microscope through the samples while raster scanning the fluorescence in the xy-plane. The distance between each focal plane was 2-4 μm and the xy-pixel size 0.59 $\times$ 0.59 μm . The scan area was 302 $\times$ 302 μm and the scan rate 166 lines per second. Images stacks were processed, analysed and displayed using the computer software Image J (National Institute of Health, Bethesda, MD).

Statistical Analysis

Statistical analysis was performed using the Mann-Whitney U-test in the statistical toolbox of Matlab (Mathworks Inc, Natick, MA).

Results

Characterization of vesicle systems

The concentration of RBITC in the formulations used in the LLNA and the *ex vivo* skin penetration experiments was analysed by fluorescence spectrometry. The concentration of RBITC in the vehicles compared in LLNA 1 (Fig 2A) was 5.8 ± 0.1 % w/v (Et:W) and 5.5 ± 0.1 % w/v (nano ethosomes). The concentrations of RBITC in the vehicles compared in LLNA 2 (Fig 2B) was 5.2 ± 0.2 % w/v (nano-scale ethosomes) and 5.0 ± 0.8 % w/v (micro-scale ethosomes). The concentration of RBITC in vehicles compared in the *ex vivo* skin penetration experiments was 0.46 ± 0.02 % w/v (nano-scale ethosomes), 0.44 ± 0.02 % w/v (micro-scale vesicles) and 0.46 ± 0.01 % w/v (Et:W). Analysis of the lipid composition before and after extrusion showed that approx. 80 % POPC remained in the formulation after the extrusion of the vesicles, i.e. 6.1 ± 0.4 % w/v before and 4.8 ± 0.2 % w/v after extrusion.

The size distribution of the nano-scale ethosomes was analysed by DLS. The average size was 76 ± 17 nm ($n = 14$) and the polydispersity index 0.14 ± 0.06 . The ethosomes were prepared and the size distribution analysed immediately prior to the LLNA and *ex vivo* skin experiments. In a stability study it was demonstrated that the average nano-scale ethosome size after 4 days at room temperature was 110 ± 10 nm, and after 10 days 170 ± 16 nm.

The encapsulation efficiency of RBITC in the nano-scale ethosomes used in the LLNA experiments (approx. 5 % w/v) was 17 ± 9 % ($n = 7$). At the lower concentration of RBITC (approx. 0.5 % w/v), used in the *ex vivo* permeation and penetration experiments the encapsulation efficiency of the ethosomes was higher, i.e. 62 ± 4 % ($n = 4$).

It was not possible to obtain an accurate size distribution of RBITC in non-extruded ethosomes with DLS due to their size and high polydispersity. Instead, the distribution of the spontaneously formed vesicles was imaged using confocal fluorescence microscopy which showed that large lipid vesicles of sizes ranging from a few μm up to approx. 20 μm were formed (data not shown).

Modified LLNA with non-pooled lymph nodes

The relative sensitizing capacity of RBITC in the investigated vehicles was analysed using the modified murine LLNA with non-pooled lymph nodes. RBITC in lipid vesicles induced lymph node cell proliferation increased by almost a factor of five, compared to the group of

mice treated with RBITC in Et:W solution ($p < 0.001$) as well as to the negative control, i.e. empty ethosomes, ($p < 0.001$) (Fig. 2A). As the cell proliferation is an indicator of a compounds sensitizing capacity, this result implies elevated sensitizing capacity for RBITC when delivered in ethosome containing vehicles. There was also a small increase in the cell proliferation in the group exposed to RBITC in Et:W solution compared to the negative control ($p < 0.05$).

Comparing the cell proliferative response in the local lymph nodes following topical application of RBITC in two structurally different vesicle systems, i.e. micro-scale and nano-scale ethosomal formulations, revealed a slight increase in the cell proliferation when micro-scale ethosomes were used ($p < 0.05$). Both vesicle formulations induced a significant increase in cell proliferation compared to the negative control (empty ethosomes) by at least a factor of four ($p < 0.001$) (Fig. 2B).

Ex vivo percutaneous absorption

The percutaneous absorption of RBITC in the nano-scale ethosome formulation and in the Et:W solution was measured using diffusion chambers ($n = 4$) and is presented in Fig. 3. The result revealed no significant difference in the transepidermal flux between the two vehicles. The cumulative amount absorbed after 24 h was 1.18 ± 0.95 nmol from the Et:W solution and 1.29 ± 0.61 nmol from the ethosomes.

Visualization of skin penetration

Fig. 4 shows three dimensional reconstructions generated from TPM measurements of skin samples after 24 h topical application of RBITC in either nano-scale ethosome formulations (A and B), micro-scale ethosome (C and D) formulations, or in Et:W solution (E and F). All vehicles resulted in a significant swelling of the corneocytes in the SC. The TPM images show that the two vesicle formulations, i.e. nano- and micro-scale ethosomes increased the penetration of RBITC into the skin compared to the Et:W solution. Fluorescence intensity with depth profiles, constructed from the TPM image stacks (Fig. 5) shows a higher fluorescence signal, i.e. higher concentration of RBITC, deeper into the tissue when using either of the vesicle-containing vehicles compared to the Et:W solution. The signal was significantly higher ($p < 0.001$) from a depth of 32 to 88 μm after topical application of RBITC in nano- and micro-scale ethosomes compared to the Et:W solution. Furthermore, a noticeable elevated fluorescence of RBITC in the SC could be noted when administered using

the vesicle formulations (Fig.4 A - D). No difference between the two vesicle formulations could be detected.

Discussion

The growing focus on nanotechnology and the increased use of nano-sized structures, e.g. vesicles, in topical formulations has led to concerns regarding the safety of nano-sized materials. Potential dermatotoxicological side effects, e.g. skin sensitizing effects in these kinds of formulations needs to be investigated. However, studies focusing on the skin sensitizing effects of compounds in nano-sized vesicle systems used in dermatological formulations have to our knowledge not been published. We have here investigated the sensitizing capacity and the skin penetration of a fluorescent model hapten, RBITC, when administered in formulations containing lipid vesicles, i.e. ethosomes.

The sensitizing capacity of RBITC in nano-scale ethosomes and in an Et:W solution was compared using a modified murine LLNA. We also compared the sensitizing capacity of RBITC in nano-scale ethosomes (< 100 nm) and non-extruded micro-scale ethosomes, to investigate the effect of the micro-structure of the lipid vesicles on the sensitizing capacity. The results showed that both vesicle formulations increased the sensitizing capacity of RBITC compared to the Et:W solution. This corroborates previous findings by Madsen et al (Madsen et al., 2010b), which recently showed that formulations containing ethosomes increased the sensitizing capacity of two known contact allergens, DNCB, a strong experimental sensitizer and isoeugenol a fragrance compound and a moderate sensitizer, compared to an Et:W solution. However, we could not find any evidence that nano-scale vesicles increases the sensitizing capacity compared to micro-scale vesicle formulations. Instead, the micro-scale formulation was found to be slightly more sensitizing compared to the nano-scale ethosomes. One possible explanation for this effect might be that a fraction of the phospholipids are lost in the extruder membrane during the extrusion process when producing the nano-scale formulation. Analysis of the phospholipid content in the formulation before and after extrusion using an ammonium ferrothiocyanate assay showed that approx. 80 % of the lipid fraction remained after the extrusion, an effect not accounted for in the study. As Madsen et al (Madsen et al., 2010b) have previously demonstrated that the sensitizing capacity of isoeugenol is related to the concentrations of POPC, this is a likely explanation. Altogether the results indicate that the presence of lipid vesicles affects the sensitizing capacity of RBITC, but that the size of the vesicles seems to be of minor importance.

It was hypothesised that the enhanced sensitizing capacity could be related to an increase in the bioavailability of the hapten when applied in the vesicle formulations. However

subsequent skin permeation experiments, using the standard COLILPA method, did not show any variations in the trans-epidermal flux of RBITC, from the ethosomal formulation compared to the Et:W solution. Thus variations in the skin permeability could reasonably not explain the differences between the vehicles seen in the LLNA. Permeation studies using diffusion cells do not necessarily give a good measure of the local bioavailability of the hapten. TPM has previously been shown to be a useful method to study relative concentrations of topical applied compounds in the skin (Yu et al., 2001; Bender et al., 2008; Samuelsson et al., 2009). Further studies using TPM were therefore conducted, in order to evaluate the relative concentration of RBITC in the epidermis following application in the vesicle formulations and in the Et:W solution. Contrary to the permeation study, non-invasive visualization of hapten exposed human skin by TPM revealed a different epidermal distribution of RBITC following topical application in the ethosomal formulations compared to the Et:W solution. Higher concentration of RBITC could be detected deeper into the skin when either nano- or micro-scale ethosomal formulations were used. Intensity profiles constructed from TPM z-stacks showed a significant increase in the concentration of RBITC from a depth of 32 μm following topical application with the ethosomal formulations. Despite a small difference in sensitizing capacity in the LLNA, it was not possible to detect any variations in the penetration profile of RBITC between the nano- and micro scale vesicle systems.

Altogether, the results indicate that the enhanced sensitizing capacity could be, at least partly, explained by an improved delivery of the hapten into the epidermis from the vesicle formulation. This is in agreement with previous studies reporting that vehicle induced changes in the skin permeation and penetration properties of haptens can affect the sensitizing capacity. (Fullerton et al., 1988; Dearman et al., 1996; Heylings et al., 1996; Robinson and Cruze, 1996). However there is not always a clear correlation (Brunner et al.; Roberts and Aptula, 2008) and alternative or complementary reasons for vehicle induced effects have been proposed, e.g. vehicle induced adjuvant effects (Grabbe and Schwarz, 1998; Sato et al., 1998; Kimber et al., 2000; Basketter et al., 2001).

We have earlier observed that depending on the reactivity and sensitizing capacity of a compound, the skin penetration and epidermal distribution will be affected (Samuelsson et al., 2009). Similar to FITC in our earlier study, RBITC shows a distribution profile indicating formation of hapten-protein complexes in upper epidermis. Interestingly, in the present study, the strong fluorescent region in the SC and upper epidermis was more profound after topical

application of RBITC in the ethosome containing vehicles compared to a hydroethanolic solution. As the formation of hapten-protein complexes is a key step in skin sensitization, the reaction between the RBITC and macromolecules in these skin layers might be of importance for causing the elevated sensitizing capacity of compounds when delivered using lipid vesicles.

It is interesting to note that neither the biological response (sensitizing capacity) nor the skin penetration of RBITC increased using nano-scale vesicles compared to micro-scale. The result is in accordance with other studies investigating skin penetration effects using vesicle formulations (Michel et al., 1992; El Maghraby et al., 1999; Šentjurc et al., 1999; Sinico et al., 2005; Mura et al., 2007). Assuming uptake of intact vesicles, skin penetration should be a function of vesicle size (El Maghraby et al., 1999). Consequently it seems unlikely that the sensitizing and penetration enhancing effect induced by the vesicle formulations are related to the uptake of intact vesicles. Instead, it is more likely that the lipid vesicles are disrupted on the skin surface and that the penetration of RBITC proceeds independently of the vesicles. It has been proposed that the lipids can act as penetration enhancers by affecting the packing of the intercellular lipid lamellae or that vesicles increase the vehicle-SC drug partitioning by adsorbing to and either fuse or transfer encapsulated compounds into SC (Elsayed et al., 2007), but there are contradictory studies (Verma et al 2003). Thus, it is still a matter of debate as to whether intact vesicles can penetrate the skin barrier or not.

In conclusion, the sensitizing capacity of RBITC increased following topical application in nano- and micro-scale ethosomal formulations compared to an Et:W solution, according to results obtained using a modified LLNA. These results in combination with data previously published by Madsen et al (Madsen et al., 2010a; Madsen et al., 2010b) indicate that there could be an elevated risk of sensitization when potential haptens are present in topical formulations containing lipid vesicles. Although, the size of the vesicles seems to be of minor importance, further studies are needed before any more generalized conclusion can be drawn. Subsequent, non-invasive visualization of hapten exposed human epidermis by TPM, revealed that the skin penetration of RBITC increased significantly from the ethosomal formulations compared to the Et:W solution. Together, the results suggest that the observed variations in the sensitizing capacity could be, at least partly, a consequence of differences in the local bioavailability of the hapten.

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Figure Legends

Fig. 1. Chemical structures of (A) rhodamine B isothiocyanate (RBITC) and (B) 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC).

Fig. 2. Relative sensitizing capacity of RBITC in a nano-scale ethosome formulation compared to an Et:W solution (A) and nano-scale compared to a micro-scale ethosomal formulation (B) analyzed by measuring the cell proliferation in non-pooled draining auricular lymph nodes, expressed as disintegrations per minute (DPM).

Fig. 3. Permeation of RBITC through heat separated human epidermis in vertical diffusion cells. The data presented in the graph are calculated average of 4 independent experiments using skin from 2 different donors. The error bars indicate the standard deviations from the average values. The cumulative amount absorbed after 24 h was 1.18 ± 0.95 nmol from the Et:W solution (o) and 1.29 ± 0.61 from the ethosomes (x).

Fig. 4. TPM images of human skin samples after 24 h topical application of RBITC *ex vivo*, in either nano-scale ethosome (A and B) or micro-scale ethosome formulations (C and D) or in Et:W solution (E and F). Three dimensional constructs (A, C and E) and optical cross sections (B, D, F) are displayed. The x,y-image plane corresponds to an area of 302×302 μm . The z-depth (A, C, E) is 96 μm .

Fig. 5. Graphs presenting the fluorescence intensity obtained as function of depth from TPM imaging of human skin after 24 h topical application of RBITC in either nano-scale ethosome (solid line) or in micro-scale ethosome formulations (dotted line) or in Et:W solution (dashed line). The intensity profiles showing the distribution of RBITC after topical application in nano-scale ethosome formulations and in Et:W solution are calculated from 19 TPM z-stacks of samples from 6 independent diffusion cell experiments with skin from 3 different donors. The intensity profile of RBITC after topical application in micro-scale ethosome formulations is calculated from 12 TPM z-stacks of samples from 4 independent diffusion cell experiments with skin from 2 different donors.

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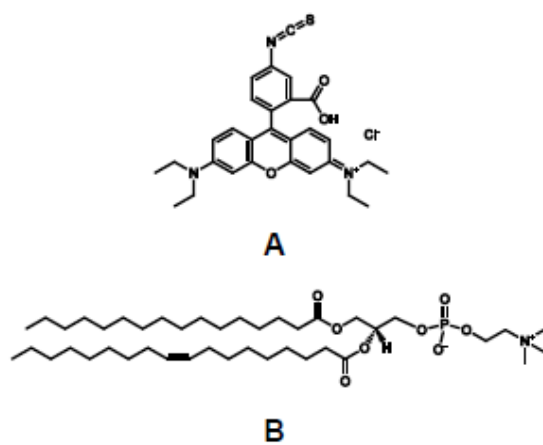


Figure 1

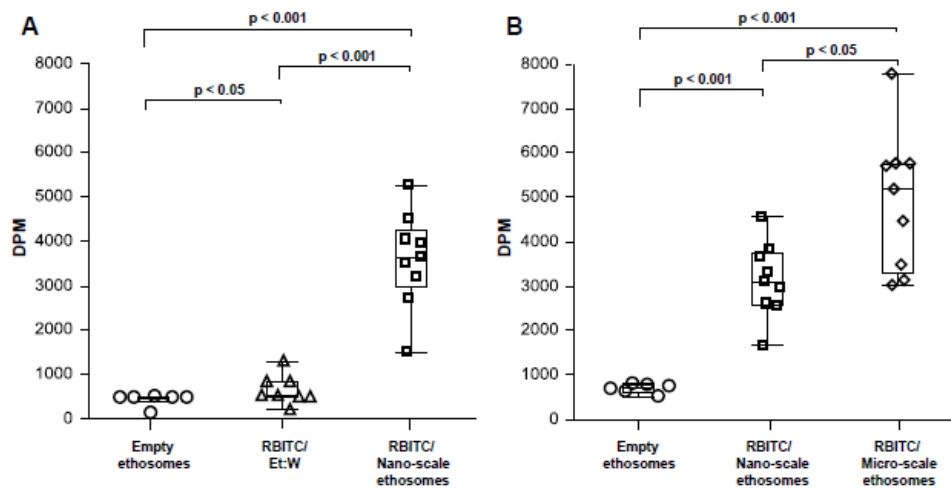


Figure 2

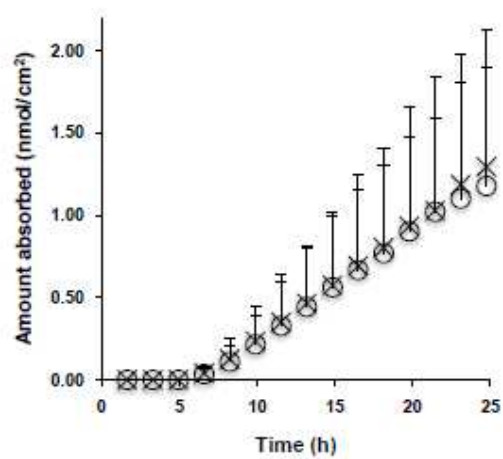


Figure 3

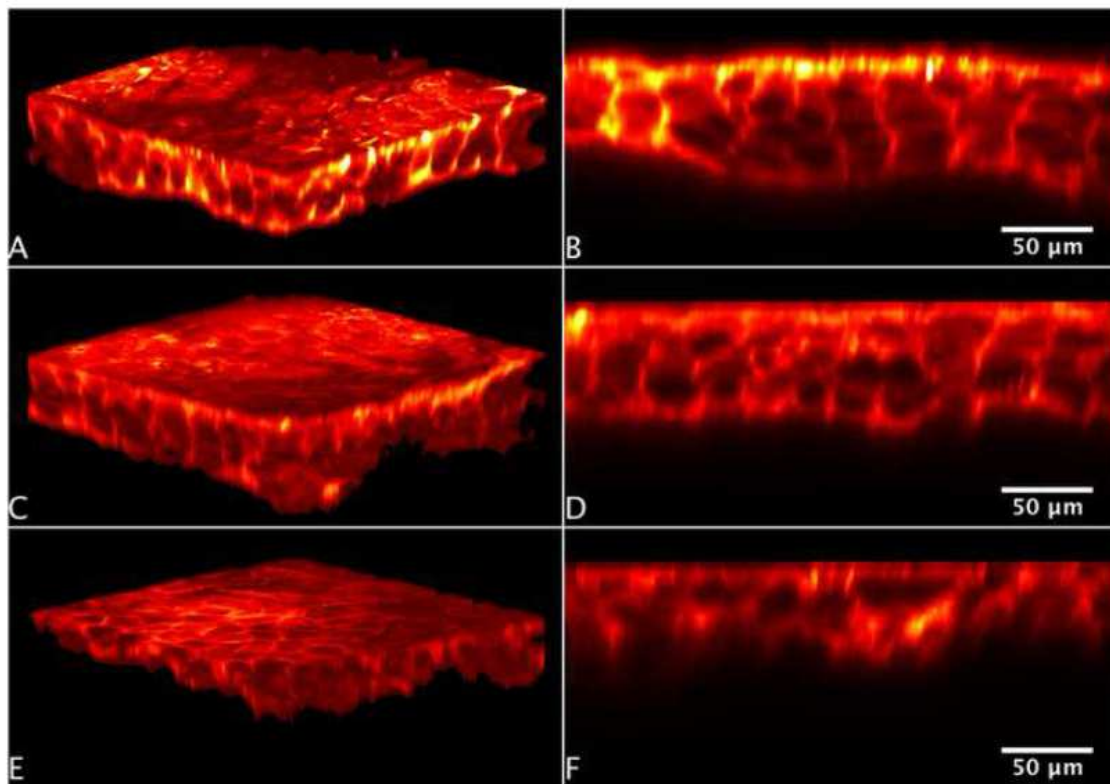


Figure 4

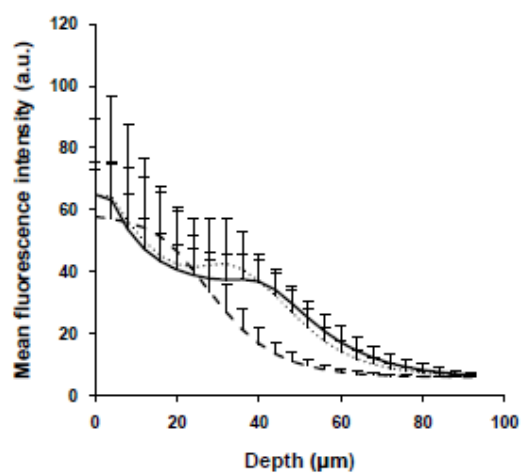


Figure 5

Graphical Abstract

