

MICROVESICLE FORMULATIONS AND CONTACT ALLERGY -  
EXPERIMENTAL STUDIES IN *IN-VITRO*, MICE AND MAN

Ph.D. Thesis

by

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- II. Madsen, J.T., Vogel, S., Johansen, J.D. and Andersen, K.E. Encapsulating contact allergens in liposomes, ethosomes and polycaprolactone may affect their sensitizing properties. *Cutan Ocul Toxicol* 2011
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*Nomenclature*

POPC :1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine  
ROAT :Repeated open application test

## 1. PREFACE

The present studies are based on work carried out from 2007-2010 at the Department of Dermatology and Allergy Centre, Odense University Hospital, University of Southern Denmark in collaboration with the National Allergy Research Centre in Gentofte and the Department of Physics and Chemistry, University of Southern Denmark.

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*This Thesis is dedicated to my beautiful wife Milene, my lovely daughter Liva and my newborn son Magnus.*

Odense, August 2010

*Jakob Torp Madsen*

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## 2. Summary (English)

Attempts to improve formulation of topical products are a continuing process i.e. to increase cosmetic performance, enhance effects and protect ingredients from degradation. The development of micro and nano-vesicular systems has led to marketing of topical drugs and cosmetics using these technologies. Several papers have reported improved clinical efficacy by encapsulating pharmaceuticals in vesicular systems. Some vesicular systems may improve transdermal delivery of compounds compared to conventional vehicles. Few case reports have suggested that microvesicle formulations may affect allergenicity of topical products.

The aim of this thesis is to investigate the effect on the sensitizing and elicitation capacity of chemical allergens encapsulated in vesicular systems.

The first part examined how the encapsulation of isoeugenol, dinitro-chloro-benzene, and potassium dichromate in liposomes, ethosomes and polycaprolactone affects the sensitizing properties using the OECD and FDA approved skin sensitisation test method in mice: the Local Lymph Node Assay. Ethanolic liposome (Ethosome) formulation of lipophilic allergens increased the sensitising capacity and polycaprolactone protected against sensitisation compared to conventional vehicles. The formulation of the hydrophilic allergen, potassium dichromate, in all three drug delivery systems did not affect the sensitisation capacity. Further, the effect of vesicle size was studied and conflicting results were found.

The second part examined whether encapsulation of allergens in ethosomes affects the patch test reactivity and outcome of the Repeated Open Application Test (ROAT) compared to test with ethanol:water formulations. Pre-sensitized volunteer individuals were patch tested with a dilution series of isoeugenol and methyldibromoglutaronitrile formulated in ethosomes and ethanol:water. Both contact allergens encapsulated in ethosomes showed significantly enhanced patch test reactions compared to the allergen preparation in ethanol:water without ethosomes. No significant difference in the median lag time was recorded between preparations in the repeated open application test.

The third part examined the percutaneous absorption *in vitro* of dinitro-chloro-benzene and isoeugenol formulated in ethosomes and ethanol:water using Franz cells and human cadaver skin. We found no significant relationship between percutaneous skin absorption /penetration of the allergens and the sensitising properties of the test formulations.

*Conclusion*

Encapsulation of lipophilic contact allergens in lipid vesicles and nanospheres may affect the sensitising and elicitation capacity of the encapsulated allergen. Encapsulation of the hydrophilic allergen potassium dichromate did not alter the sensitizing capacity in the Local Lymph Node Assay. We did not find a correlation between the percutaneous skin absorption/penetration pattern and the sensitising capacity. The clinical implications of these results are so far uncertain. However, the cosmetic industry should consider the effect of encapsulation on a case by case basis because certain ingredients may become more allergenic when encapsulated. Dermatologists investigating patients with allergic reactions to consumer products using encapsulation technology should consider the risk of false negative results, if testing with ingredients in conventional patch test vehicles. Testing with encapsulated ingredients should be performed when possible.

### 3. Resumé (dansk)

Kosmetik og lægemiddelindustrien udvikler hele tiden nye formuleringstyper til lokal anvendelse med det formål at forbedre effekten, beskytte de aktive stoffer mod nedbrydning og ikke mindst øge den kosmetiske oplevelse. Udviklingen af mikro og nanovesikler i 1960'erne gjorde det muligt at indkapsle aktive ingredienser i produkterne for at beskytte dem mod nedbrydning, og for at øge penetrationen i huden med henblik på at øge effekten. Flere hudprodukter, der anvender denne teknologi, er markedsført. Enkelte kasuistiske meddelelser har antydnet, at disse nye formuleringsmetoder kan øge det indkapslede stofs allergifremkaldende egenskaber.

Formålet med denne ph.d afhandling er at undersøge, hvorvidt indkapsling af kendte allergener i sådanne vesikelformuleringer øger stoffernes sensibiliserings- og provokations egenskaber for udvikling af kontaktallergi.

Første del undersøger, hvordan indkapslingen af allergenerne isoeugenol, dinitro-chloro-benzen og kaliumdikromat indkapslet i liposomer, ethosomer og polycaprolacton påvirker sensibiliseringsfasen i en musemodel. Sensibiliserings testen "The Local Lymph Node Assay", der er valideret og godkendt af OECD og FDA, anvendes. Forsøgene viser, at ethosomer øger de lipofile allergeners (isoeugenol og dinitro-chloro-benzene) sensibiliseringsegenskaber, hvorimod polycaprolacton beskytter mod sensibilisering. Indkapslingen af kaliumdikromat i alle tre vesikelsystemer har ingen effekt på sensibiliseringen. Hvorvidt størrelsen af vesiklerne spiller en rolle er uklart, da der fremkommer modsat rettede resultater.

Anden del af afhandlingen viser resultaterne fra kliniske provokationsforsøg på præ-sensibiliserede, frivillige forsøgspersoner, der tidligere har fået påvist en positiv lappeprøve (epikutantest) over for isoeugenol eller metyldibromoglutaronitril. Epikutantest med allergenerne fremkalder en signifikant kraftigere eksem reaktion for ethosomformuleringens vedkommende sammenlignet med formuleringen uden brug af ethosomer. En "repeated open application test" viser ingen signifikant forskel mellem de to formuleringstyper.

Tredie del forsøger at påvise en sammenhæng mellem den påviste øgede sensibiliseringsgrad for ethosomformuleringerne og den perkutane absorption/penetration. Disse studier er udført på kadaver hud med brug af Franz celler. Der kan ikke påvises en sammenhæng mellem penetrationsdybden eller

den percutane absorption og sensibiliseringsgraden med allergenerne formuleret med og uden ethosomer. Tværtimod ser det ud til, at allergenets penetrationsdybde ikke spiller nogen rolle for sensibiliseringspotentialet.

#### *Konklusion*

Formuleringen af lipofile kontaktallergener i nogle vesikelsystemer kan øge allergenets sensibiliserende og provokerende egenskaber. Den kliniske betydning er ikke klarlagt, men kosmetik- og lægemiddelindustrien bør overveje risikoen for udvikling af kontaktallergi, når de udvikler nye produkter, der gør brug af denne teknologi. Når hudlæger undersøger for en kontaktallergi forårsaget af et forbrugerprodukt, der anvender denne indkapslingsteknologi, skal risikoen for et falsk negativt resultat nøje overvejes, hvis der udelukkende er testet med konventionelle vehikler. Hvis det er muligt, bør man teste med det anvendte vesikelsystem som vehikel.

## 4. Introduction

### 4.1. *Contact allergy*

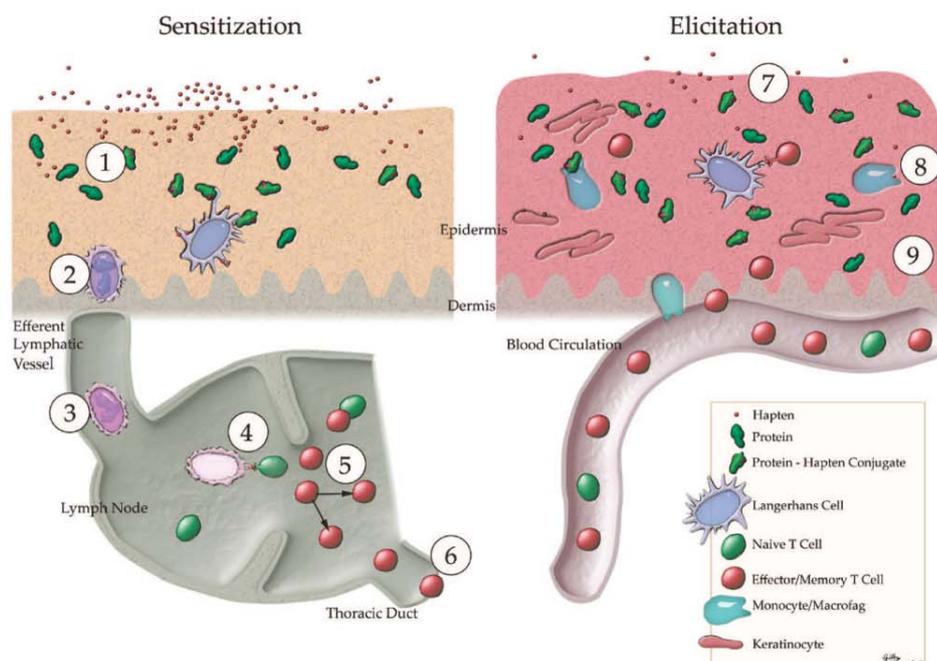
Allergic contact dermatitis is the clinical manifestation of contact allergy and can occur upon re-exposure to the allergen at the site of skin contact and results in eczema. Allergic contact dermatitis is a delayed type IV reaction and is responsible for only a minor part of the spectrum of contact dermatitis. The most common type of contact dermatitis is irritant contact dermatitis, a local inflammatory response in the skin that requires no prior sensitization. Irritant contact dermatitis results from direct chemical and physical irritant exposures to the skin - often due to wet work and detergents(1). Irritant and allergic contact dermatitis may result in sick leave and even change of job if the dermatitis is work related.

10% of the general population in Denmark were in 2006 sensitized to one or more chemicals from the environment (2). Cosmetic ingredients such as perfumes and preservatives often cause sensitization and allergic contact dermatitis and the labelling requirements given in the European Union Cosmetics Directive is of great help in tracing the causative allergenic ingredients (3). The most common causes of contact allergy are nickel followed by fragrances and preservatives (4).

Allergic contact dermatitis involves two phases. The induction (sensitization) phase and the effector (elicitation) phase. Key factors in developing contact allergy are the physicochemical properties of the allergen that allows it to penetrate stratum corneum into epidermis and its ability to react with proteins in epidermis making a hapten-protein complex capable of eliciting an immune response through contact with the Langerhans cells (5). The amount of allergen applied per skin surface area, the frequency of application and the vehicle used are other important factors in developing contact allergy (6-8). The mechanisms of both phases of contact allergy have been studied for more than seven decades and even though many factors are elucidated, others are not well understood, like how the effect of carrier vehicles for allergens affects skin absorption of the allergen and how this is related to the sensitising properties.

## 4.2. Sensitization

Skin sensitization is a T-cell mediated immune response. A hapten is a small molecule (below 1000 dalton) which reacts with proteins in the epidermis. During the sensitization process the hapten binds to skin components and the hapten-protein conjugate functions as an antigen, which is processed by the Langerhans cells (antigen presenting cells) and presented to and recognized by T-cells (Figure 1). The Langerhans cells migrate to the local lymph nodes where they present the antigen to the T-cells. If the hapten-protein complexes are formed *in* the Langerhans cell, which is the case for some lipophilic haptens, they will be presented on MHC class I molecules and presented to CD8<sup>+</sup> T cells. If the hapten-protein complexes are formed outside the Langerhans cell, they will be presented on MHC class II molecules to CD4<sup>+</sup> T cells. The exact role of these two routes is not elucidated in detail. T-



**Figure 1.**

Schematic presentation of the sensitization and elicitation phases of allergic contact allergy. (1) Hapten penetrates the epidermis and bind to a protein whereupon (2) the complex is being internalized by the Langerhans cell. (3) The Langerhans cell is activated and begins to migrate to the local lymph nodes where the hapten-protein complex is presented to naïve T-cells either on MHC-I or MHC-II molecules on the surface of the Langerhans cell (4). (5) proliferation of hapten specific T-cells are formed and (6) leave the lymph nodes into the circulation. Upon re-exposure to the hapten (7) a release of cytokines and chemokines are released and attract the hapten specific T-cells from the circulation and other non specific inflammatory cells to the area (8). (9) An inflammatory process begins resulting in dermatitis within 1-2 days depending on the dose and potency of the hapten. (illustration from Karlberg et al. (9))

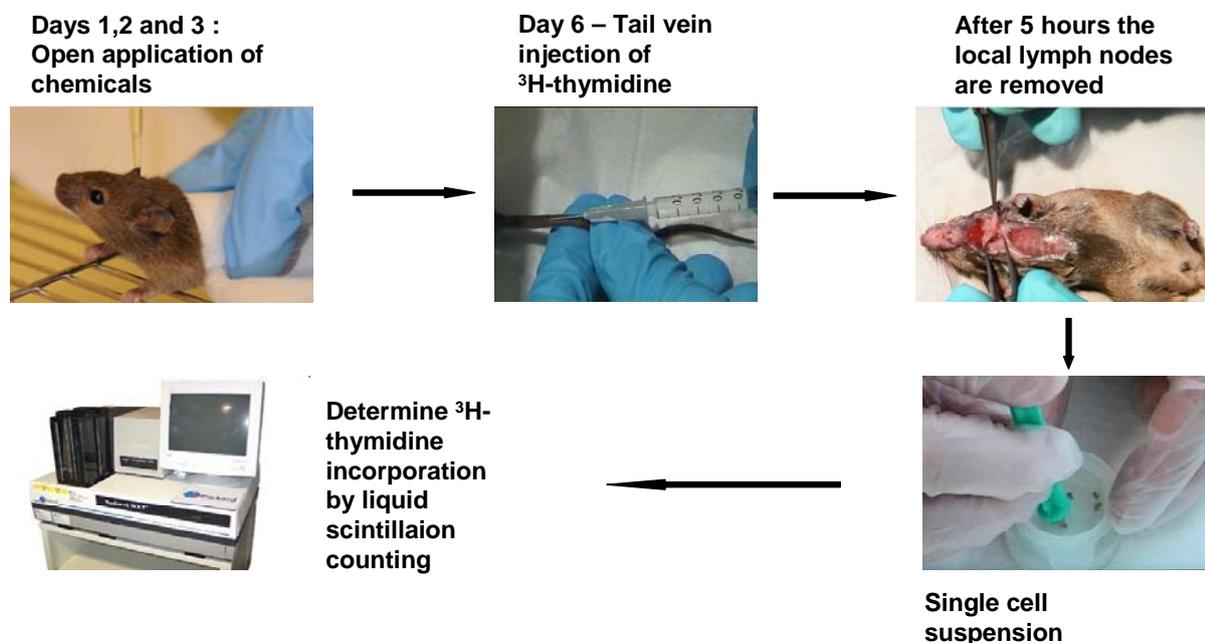
cells with receptors able to recognize the hapten-protein complex proliferates and circulate systemically throughout the body. These events constitute the induction, afferent or sensitization phase (10;11).

Hundreds of chemicals are today registered as contact sensitizers and new ones keep coming up as new chemicals are used in industry and in topical products. Predictive sensitization assays in animal and man are developed to detect sensitizers with the purpose to protect workers and consumers by regulating the presence of significant contact allergens in products on the market. Attempts are made to replace animal assays by *in-vitro* assays to reduce the need for animal experiments, since a ban within the European Union (EU) of allergy testing of cosmetics and toiletry ingredients is planned to be implemented in 2013 (10). Data on existing *in-vitro* assays show good correlation to animal predictive sensitization test methods, but only for moderate and strong sensitizers. These test methods are not yet approved to replace animal experiments, but they can be used to screen chemicals of interest before using animal test methods (9).

#### **4.2.1. The Local Lymph Node Assay (LLNA)**

Today the mouse Local Lymph Node Assay is the predictive sensitization test of choice (Figure 2). It has almost replaced the use of guinea pig test methods, with the guinea pig maximization test and the Buehler test as the most widely used. The Local Lymph Node Assay is less stressful for the animals, gives an objective outcome and reduces the number of animals compared to the guinea pig tests. The Local Lymph Node Assay only requires 4 groups of 4 mice to run a sensitization experiment compared to 15 animals in each group of guinea pigs. The Local Lymph Node Assay has a quantitative outcome (dose-response) allowing discriminating between four degrees of sensitization compared to the semi-quantitative guinea pig methods only dividing the allergens in weak or strong sensitizers. The Local Lymph Node Assay is internationally validated and results correlate well with human data, even though exceptions exist i.e. false positive results with some skin irritants i.e. sodium lauryl sulfate (11;12) and false negative results with some metals in certain vehicles (13). The vehicle is thus of major importance and may also affect the sensitizing capacity (14). It is important that the test chemical is soluble in the vehicle chosen and that the test chemical suspected being a sensitizer is in the same oxidative state as when the chemical is in contact with the skin. Some fragrances like

linalyl acetate listed on the product label of toiletries may oxidize when in contact with air and become more potent sensitizers. (15).



**Figure 2.**

The protocol of The Local Lymph Node Assay. At days 1, 2 and 3 25µl of test substance is applied on the dorsum of both ears. At day 6 the mice are injected with 250 µl phosphate buffered saline (PBS) containing 20 µCi [methyl]-<sup>3</sup>H-thymidine in the tail vein. Five hours later the mice are killed and the auricular lymph nodes removed and a single cell suspension is made. The lymph nodes of each group of animals are not pooled. The single cell suspension is washed with PBS and centrifuged twice. The DNA is precipitated with 5% trichloro acetic acid (TCA) overnight and then resuspended in 1ml TCA and transferred to scintillation vials and [methyl]-<sup>3</sup>H-thymidine is measured by β-scintillation.

### **4.3. Elicitation**

After proliferation and dissemination of specific T-cells the sensitized individual is capable of developing an allergic contact dermatitis following renewed skin contact with the hapten or a chemical cross reactive with the primary sensitizer. The hapten-protein complex is again presented to the circulating T-cells by the Langerhans cells. The activated T-cells trigger a cascade of biochemical and cellular processes leading to inflammation of the skin at the site of contact. A much lower concentration of allergens is needed in this process due to the higher amount of circulating memory T-cells compared to naïve T-cells required in the sensitizing phase. These events constitute as the

elicitation, effector or efferent phase. Contact allergy is thus regarded as a life long specific immunologic hypersensitivity (11).

#### **4.4. Test methods for diagnosing contact allergy**

##### **4.4.1. Diagnostic patch testing**

Patch testing (epicutaneous testing) is the standard method for diagnosing contact allergy in humans. Eczema patients are usually tested with a baseline series encompassing the most commonly occurring contact allergens in the population. By application of the allergen in an appropriate concentration and vehicle under occlusion for two days, the patch test provokes a miniature eczema reaction in case the individual is sensitized. The patch test allergens are usually applied on normal skin on the back in standardized chambers like Finn chambers® (Epitest Ltd Oy, Tuusula, Finland) or IQ-chambers© (Chemotechnique Diagnostics, Vellinge, Sweden), secured with tape. Ready-to-use test systems such as the Thin-layer Rapid Use Epicutaneous (TRUE) test® is available for a limited number of allergens and it is another possibility.

The contact allergens used in routine clinic are often formulated in petrolatum, but this vehicle is not optimal for every allergen and alternative vehicles can be used when needed. Solvents like water, ethanol, propylene glycol and acetone may be used as alternative vehicles increasing the skin penetration, but they also have drawbacks as i.e. propylene glycol being a sensitizer and irritant (16). Allergens formulated in different vehicles but in the same concentration may produce different strength of reactions (8;17). Usually the highest possible concentration that does not produce irritancy is used, so the number of false positive and false negative results are minimized (16). For rare allergens it is necessary to carefully select the patch test concentration to avoid both false positive and false negative reactions.

Readings are usually done on day 2-3 and on day 5-7 and both readings are important due to some early or late occurring reactions. Patch testing has been standardized by recommendation of the International Contact Dermatitis Research Group (ICDRG) (16). Patch test reactions are scored by a visual reading scale. For a positive patch test is required at least homogeneous redness and infiltration in the entire test area, scored as a 1+ reaction, if vesicles are also present the reaction is scored as a 2+ reaction, and if coalescing vesicles and spreading is present it is scored as a 3+ reaction. Irritant

responses are classified as IR and doubtful reactions as +?. A more detailed reading scale has been developed by Hindsen and Bruze (18) and later modified by Fischer et al (19) in order to recognize smaller differences in the allergic reactions primary for research purposes.

#### **4.4.2. Repeated open application test (ROAT)**

The ROAT is a supplementary provocative use test which can be used to confirm the presence of contact allergy if the patch test reaction is difficult to evaluate. The ROAT was standardized in 1986 regarding recommended test site on the body, for influence of skin region, area and application time (6;7). The advantage of the ROAT is that it mimics a real life exposure situation and is important in determining threshold values for sensitizers in risk assessment which may be more accurate compared to patch test thresholds. ROAT and patch test thresholds correlate very well (19-22). In this thesis the ROAT was used to investigate the effect of allergens formulated in different vehicles.

#### **4.5. *Skin penetration and absorption of chemicals – related to allergenicity***

In order for a contact allergen to get in contact with the cutaneous immune system it has to penetrate into the viable epidermis. Thus allergens should have appropriate physicochemical properties to cross the stratum corneum which normally is an effective skin barrier. A certain degree of lipophilicity ( $\log P$  around 2) is advantageous. Extremely lipophilic and hydrophilic molecules are poor skin penetrators (9;23). Formulating a chemical/allergen in different vehicles for topical administration may change the skin penetration profile (25-27) and the sensitizing and elicitation capacity of the allergen (8;14;24-26) but how these outcomes are related to penetration and absorption properties are not well elucidated. It is important to distinguish between skin penetration and absorption.

Percutaneous absorption corresponds to the transfer of a substance via the skin from the external environment to the systemic circulation. Penetration is a passive diffusion into the epidermis, dermis or cutaneous annexes (5). Formulating an allergen in different vehicles for topical administration may change the sensitizing and elicitation capacity of the allergen (8;16;29;30). Contact allergens are reactive compounds and interact with enzymes in the skin. This may change the availability to the Langerhans cells and thus affect sensitization. The strong allergen fluorescein isothiocyanate was found mainly to be retained in or adjacent to stratum corneum whereas a structurally similar non

sensitizing compound was found to be distributed more diffuse through the epidermis studied by two photon microscopy (27). The two compounds have similar lipophilicity and comparable molecular weights. The authors concluded that the highly reactive fluorescein isothiocyanate reacts with molecules in the skin thus obstructing its further transdermal transport.

#### **4.6. Common vehicle systems in cosmetic and topical drugs**

The development of new formulations for topical products is a continuing process. Encapsulation of product ingredients into different carrier molecules (like liposomes) may improve product efficiency, and it is a promising tool for dermal and transdermal delivery of drugs and cosmetic ingredients. The encapsulation technology has been used since the late 1960's and several topical products are marketed today claiming benefits from this technology. Bearing the enormous number of research papers dealing with encapsulation technologies in mind, surprisingly few pharmaceutical products have reached the market. This chapter focuses on the use of different types of encapsulating technologies in topical drugs and cosmetics and describes potential effects on product allergenicity.

One advantage of encapsulating a drug into liposomes is the possibility of delivering the drug directly to the site of action in the skin at a higher concentration and obtaining a reduced percutaneous absorption at the same time. The penetration pattern is determined by the composition of the liposome and the encapsulated compound. It is difficult to get approval from health service authorities of topical drugs using encapsulation technology because it is problematic for the manufacturer to prove the presence and stability of the microvesicles in the product. Some pharmaceutical products using microvesicle carriers are commercially available (Table 1). Examples are Pevaryl Lipogel® (econazole encapsulated in liposomes, Cilag Corps, Schaffhausen, Switzerland) and a local anaesthetic formulated in liposomes (LMX4™, Ferndale Pharmaceuticals Ltd, UK). Estrasorb™ is estradiol encapsulated in micelles in a nanoemulsion for transdermal drug delivery, reducing hot flares in menopause women (28). Several clinical trials have shown improved biological effects of products with microvesicle formulations compared to conventional formulations (for treatment of herpes simplex, psoriasis, acne, xerosis, atopic dermatitis, vitiligo, and superficial thrombophlebitis (29-36)). An example is 5-aminolevulinic acid formulated in 50 nm liposomes, which gives a more precise drug delivery that allows a 40% reduction in the amount of active ingredient when used to treat

acne with photodynamic therapy. The liposomes concentrate in the pilosebaceous units thereby reducing the side effects and open doors for new treatment modalities (37). Another example is topical administration of methotrexate (MTX) which is hydrophilic and present in dissociated form at physiological pH. Its capacity for passive diffusion is thus limited. By encapsulating MTX in liposomes clinical trials have shown better efficacy compared to placebo and marketed MTX-gel, probably due to increased bioavailability (38).

**Table 1.**

Commercially available drug delivery systems for the topical therapy of skin diseases and the transdermal application (39).  
<sup>a</sup>Medical device.

Active compound	Vehicle	Commercial product	Company	Indication
Econazole	Liposomes	Pevaryl Lipogel	Cilag, Switzerland	Dermatomycoses
Methoxycinnamates butyl methoxy-dibenzoylemethane	Liposomes	Daylong Actinica <sup>a</sup>	Spirig, Switzerland	Prophylaxis of actinic keratosis
Diclofenac	Liposomes	Diclac Lipogel	Hexal, Germany	Osteoarthritis
Tretinoin	Microsponges	Retin-A Micro	Ortho-Neutrogena, USA	Acne vulgaris
Fluorouracil	Microsponges	Carac	Sanofi Aventis, USA	Actinic keratosis

The carrier particles themselves are all considered safe for topical use, but the interaction between the carrier particle and the active ingredient may cause biological effects due to altered skin penetration, release profile or deposition of the active ingredient.

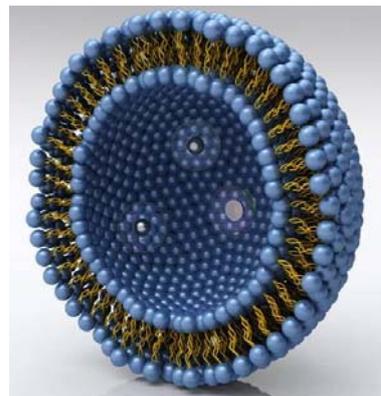
Lipid vesicles, solid lipid nanoparticles and polymeric nanoparticles are used in cosmetic formulations to increase bioavailability in stratum corneum and to protect light and oxygen sensitive cosmetic ingredients against degradation. Cosmetic ingredients like retinyl palmitate may cause physiological changes of the skin, but do not claim to treat skin diseases. Examples of encapsulated cosmetic ingredients are numerous e.g. coenzyme Q10, ascorbyl palmitate, tocopherol (vitamin E) and retinol (vitamin A) (44;45).

### 4.6.1. Liposomes

Liposomes are produced in sizes ranging from 25 nm to several micrometers. They consist of a single or multiple lipid double layer (unilamellar or multilamellar vesicles) (Figure 3). Liposomes are capable of carrying amphiphilic active ingredients either in the lipid layer or in the hydrophilic core. They are believed to protect the active ingredients from degradation. Liposomes tend to break down into its constituent components when in contact with the skin.

Therefore, liposomes at best can modulate drug transport to stratum corneum, but penetration will require more stable liposomes such as solid lipid nanoparticles (40). The concentration in the epidermis of active ingredients may increase up to five times administered in liposome formulations compared to more conventional vehicles (41). Liposome formulation in water can easily be incorporated in an aqueous cream for better cosmetic performance. Examples of active ingredients incorporated in liposomes in cosmetic industry are antioxidants, vitamin A derivatives and vitamin E.

**Figure 3.** Model of a unilamellar liposome consisting of one double lipid bilayer.



### 4.6.2. Transfersomes™

When adding different amounts of so called edge activator to the bilayer of classical liposomes eg. cholesterol or sodium cholate and a small concentration of ethanol these vesicles are called Transfersomes™ or Flexosomes™. The edge activators destabilise the membrane creating a more flexible structure and have been shown to penetrate in stratum corneum better compared to classical liposomes, thereby deliver their encapsulated ingredients deeper in the epidermis but not to the blood circulation (42). The mechanism of enhancement of skin penetration is not completely elucidated, but because of the flexibility of Transfersomes™, they are believed to squeeze between the corneocytes driven by an osmotic force due to the difference in water content of the relatively dehydrated epidermis compared to the viable dermis (43). No evidence supporting this theory have been published though. Because of that theory, Transfersomes™ should not be applied under occluded conditions which abolished the osmotic effect. Several drugs encapsulated in transfersomes have been tested in

animal experiments (eg. NSAID and local anaesthetics) showing increased dermal delivery and clinical effect compared to conventional formulations (42).

#### **4.6.3. Ethosomes**

Ethosomes (ethanolic liposomes) are made of phospholipids, a high content of ethanol (20-50%) and water. They deliver encapsulated drugs to the deep skin layers and the systemic circulation. Ethosomes have a much higher loading capacity of lipophilic drugs compared to classic liposomes. A clinical trial in humans has shown that treatment with ethosomal encapsulated acyclovir significantly improved a herpetic infection compared to the traditional Zovirax™ cream of the same concentration of active drug. Insulin loaded ethosomes has been found suitable for systemic transdermal delivery and the antibiotic bacitracin has likewise been encapsulated in ethosomes reaching the deep layers of the skin in animal experiments (44). Ethosomes may play a role in future transdermal drug delivery. Examples of cosmetics using ethosomes are Lipoduction™ and Noicellex™

#### **4.6.4. Niosomes**

Niosomes consists of non-ionic surfactant vesicles and are an alternative to liposomes. They can entrap both hydrophilic and hydrophobic chemicals, enhance the delivery to the skin and sustain the release of the drug. A phase I and II study in psoriasis patients concludes that methotrexate loaded niosomes are more efficacious than marketed methotrexate gel (38).

#### **4.6.5. Solid lipid nanoparticles**

Solid lipid nanoparticles have been developed in the 1990s and are produced by replacing the liquid lipid in an oil in water emulsion with a solid lipid (both at room and body temperature). Incorporation of pharmaceuticals and cosmetics in solid lipid nanoparticles is feasible and can easily be formulated in a cream (45). An advantage of solid lipid nanoparticles compared to conventional creams is an increase in skin hydration due to a better occlusive effect by solid lipid nanoparticles (46). Burst or sustained release of incorporated ingredients have been reported as well as increased percutaneous absorption compared to conventional formulations and is probably due to the unique composition of the solid lipid nanoparticles and incorporated ingredient. Examples of pharmaceuticals formulated in solid lipid nanoparticles are podophyllotoxin, antimycotics, non steroidal anti inflammatory drugs,

psoralen and topical glucocorticoids. No human studies with pharmaceuticals incorporated in solid lipid nanoparticles have been performed yet, but in 2008 more than 30 cosmetic products containing solid lipid nanoparticles were market (47). No side effects have been reported.

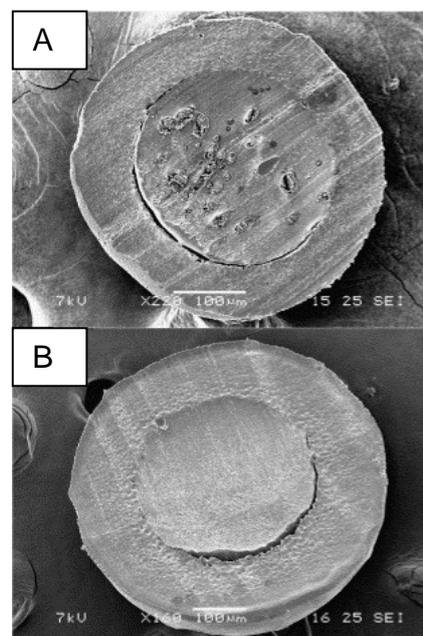
#### 4.6.6. Nanoemulsions

Nanoemulsions consist of two phases with droplets of 50-100nm in the external phase. Emulsifiers used to bind together oil and water in products such as hair conditioner and makeup remover yield a less oily mixture when they are broken down into nanoparticles. Nanoemulsions are used in both rinse-off and stay-on products. Different results are obtained on skin penetration and its correlation with droplet size. Nanoemulsions increase transdermal bioavailability of Vitamin E (48), but penetration of tetracaine from a nanoemulsion was not affected by droplet size on the skin within the range from 100-3500nm (49). Different emulsion components have been used and other authors have found increasing transdermal penetration with decreasing droplet size. There is so far no simple relationship between chemical, particle size and penetration, and each new emulsion carrying different active ingredients must be investigated separately to characterize skin penetration pattern. Estrasorb™ is an emulsion of estradiol nanoparticles and soybean oil which is on the market for treating hot flares of menopausal women (28).

#### 4.6.7. Nanospheres

Nanospheres are produced from different polymers e.g polycaprolactone, a biodegradable product widely used in cosmetic industry. When produced, the polymer wrap around itself, creating lipo- and hydrophilic spaces (Figure 4). Several drugs have been incorporated in nanospheres (50) as well as cosmetic ingredients (51). LÓreal has developed a nanocarrier system called Nanosome™ consisting of the biodegradable polymer polycaprolactone and other cosmetic companies have

**Figure 4.** Scanning electron microscopy of a nanosphere loaded with Paclitaxel (A) and unloaded (B). Picture from Wang et al. (54)



developed similar products from other polymeric particles. Polycaprolactone nanoparticles loaded with the lipophilic dye Nile Red showed enhanced penetration of the molecule into the stratum corneum layers (up to 60 $\mu$ m), compared to non-nano particle formulation (59;60). The distribution of another topically applied nanosphere-Nile Red formulations was studied by Sheihet et al in human cadaver skin using cryosectioning and fluorescence microscopy (52). Permeation analysis revealed that the nanospheres delivered nine times more Nile Red to the lower dermis than a control formulation using propylene glycol. Few papers have been published on the skin penetration/absorption behaviour and clinical effect of carrier molecules manufactured by cosmetic companies.

#### 4.6.8. Mechanism of penetration enhancement

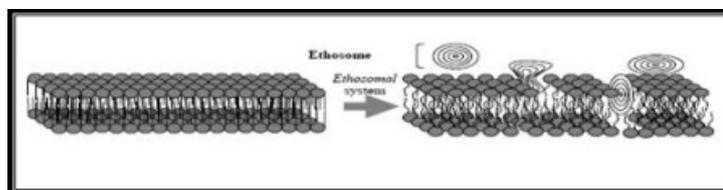
It is difficult to establish how vesicular drug delivery systems behave individually once applied to the skin and the exact mechanism is not known.

However different scenarios have been proposed. (a) Particle constituents may act as penetration enhancers after particle disruption on skin surface and

subsequently alter the skin lipid lamellae within the skin layer, (b) particles may serve as a depot of sustained release of dermally active compounds or (c) particles may serve as a rate limiting membrane barrier for the modulation of systemic absorption, hence providing a controlled transdermal delivery system(54). Another possibility is that intact vesicles penetrate beyond the superficial layers of the skin, but this is still a matter of discussion. Some theories state that intact Transfersomes™ and ethosomes may penetrate into the deeper layers of the skin and maybe even through the skin due to their elasticity (Figure 5). Several published studies have shown conflicting results and the theory is still very controversy (42;53).

**Figure 5.**

Suggested mechanism of transdermal penetration of ethosomes which is believed to be caused by the increased elasticity due to the ethanol content. The theory has been tested in several studies with conflicting results and is still very controversy (53).



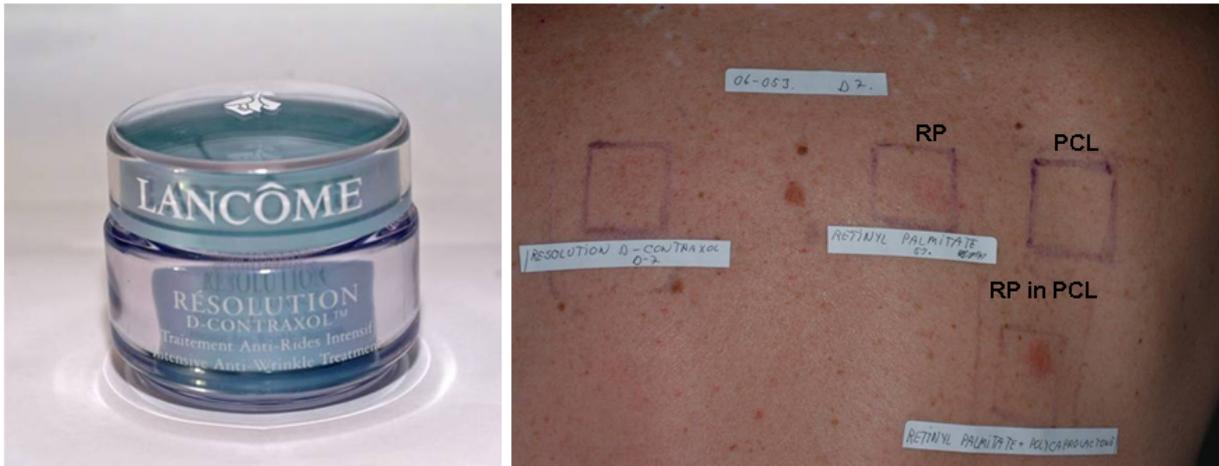
## 5. Aims of project

The project is based on the hypothesis that formulation of contact allergens in drug delivery systems using encapsulating technologies may affect the sensitizing and elicitation capacity of contact allergens. Few clinical reports have suggested that carrier molecules for ingredients in topical products have boosted the development of allergic contact dermatitis to the ingredient in question. Liposomes with encapsulated propyl gallate have been suggested to boost the development of contact allergy to propyl gallate in thirteen patients. However, patch tests with and without the liposomal formulation were not performed (55). Further, a case report described a woman developing severe allergic contact dermatitis from an anti wrinkle cream containing retinyl palmitate encapsulated in polycaprolactone (Figure 6) (51). Polycaprolactone is a polymeric drug delivery system capable of encapsulating lipophilic and hydrophilic agents. Retinyl palmitate is a rare contact allergen, and diagnostic patch tests revealed that the patient reacted more strongly to encapsulated retinyl palmitate compared to retinyl palmitate in petrolatum, even though the retinyl palmitate concentration was much lower when formulated in polycaprolactone compared to the petrolatum formulation.

The aim of this thesis is to investigate the effect of encapsulation of selected contact allergens in topical drug delivery systems with regard to sensitization and elicitation capacity of the allergens. Furthermore, laboratory experiments were performed to elucidate the relationship between percutaneous absorption of contact allergens and the sensitization properties of allergens formulated with and without encapsulation in ethosomes.

**Figure 6.**

Patch test results of retinyl palmitate (RP) in petrolatum (pet), encapsulated retinyl palmitate in polycaprolactone (PCL) and pure PCL. RP 5% in petrolatum showed a + reaction, PCL: negative and RP in PCL: ++. Note that RP in PCL is in a much lower concentration compared to RP in petrolatum (confidential information). Encapsulating RP in PCL increased the patch test reactions.



**5.1. Sensitization studies with three contact allergens encapsulated in three different carrier systems using the mouse Local Lymph Node Assay (paper I and II)**

Isoeugenol, dinitro-chloro-benzene and potassium dichromate were encapsulated in liposomes, ethosomes and in the microsphere “polycaprolactone”. These preparations were investigated for sensitizing properties in a controlled design. Further, the impact of the size of liposomes was studied.

**5.2. Elicitation studies in sensitized human volunteers with isoeugenol and methyldibromoglutaronitrile formulated in ethosomes (paper III)**

The encapsulated contact allergens in ethosomes were patch tested using a dilution series in volunteer patients in comparison with the same allergens in a control vehicle without ethosomes. Furthermore, a repeated open application test was performed in a subgroup of volunteers comparing the response to the contact allergens formulated with and without ethosomes.

**5.3. Skin penetration properties and release kinetics of the contact allergens encapsulated in ethosomes (paper IV)**

It is known that formulation of contact allergens in different vehicles may alter the sensitizing and elicitation capacity of contact allergens, but the relationship between sensitization response and percutaneous absorption/penetration is not clear. This study examined the percutaneous absorption and penetration of dinitro-chloro-benzene and isoeugenol formulated in ethosomes and ethanol:water using human cadaver skin mounted on Franz cells.

## 6. Methods

### 6.1. *Allergens*

The contact allergens and carrier systems used in the project were selected to fulfill certain criteria: The contact allergens had to be strong sensitizers due to limited encapsulating capacities of the carrier molecules and at the same time be common causes of allergic contact dermatitis so volunteer sensitized patients from the Department of Dermatology and Allergy Centre, Odense University Hospital, could be recruited for challenge studies. Further we wanted to test both lipophilic and hydrophilic contact allergens in order to see if the solubility played a role (and thereby the storage of the contact allergen in the vesicles) in the generation of contact allergy.

The carrier systems should be well defined and commonly used by the industry either on the market or in research phases.

Four strong contact sensitizers: potassium dichromate, isoeugenol, methyldibromo glutaronitrile and dinitrochlorobenzene were selected, also because quantitative methods for chemical analyses were available. The compounds were encapsulated in three selected drug delivery systems relevant for topical use were chosen: liposomes which are widely used in cosmetic and pharmaceutical research industry, ethosomes (ethanolic liposomes) because they contain ethanol allowing for a control solution of a lipophilic allergen in an ethanol:water mixture, making the lipids the only difference between the two solutions, and finally polycaprolactone, which is a polymeric particle used to encapsulate retinyl palmitate in an anti wrinkle crème (51).

The dermatotoxicologic risks from skin exposure to these carrier systems are considered low.

## 6.2. Preparation and validation of test solutions

### 6.2.1. Allergen loaded carrier systems

*Ethosomes* loaded with allergens were prepared as described by Touitou (56). Briefly, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), Avanti Polar Lipids (Alabaster, USA) was dissolved in 96% ethanol (pure or containing isoeugenol (Aldrich Denmark (CAS: 97-54-1)), dinitrochloro-benzene (CAS No 97-00-7, Sigma-Aldrich, Denmark)) or methyldibromogutaronitrile (Alfa-Aesar, Karlsruhe, Germany (CAS no 35691-65-7)) and MilliQ water (either pure or containing potassium dichromate (analytical grade, Alfa Aesar, London, UK, CAS: 7778-50-9)) was added slowly to a final concentration of 40% (v/v) ethanol under magnetic stirring (700 rpm). The final concentration of the allergens was measured by High-Performance Liquid Chromatography. The suspension was stirred for 5 minutes and then extruded 10 times through two polycarbonate filters with a pore size of 50, 100 or 200 nm using a Lipex® Extruder (Northern Lipids INC.). Empty *ethosomes* and an ethanol:water solution (4:6) of a corresponding concentration of allergen were used as control substances.

*Liposome* preparation was made by the thin film method. Briefly POPC and isoeugenol or dinitrochloro-benzene were dissolved in chloroform and methanol (2:1, v/v) in a 250-ml round-bottomed flask. The mixture was evaporated in a rotary evaporator above the transition temperature of the phospholipids and solvent traces were removed under vacuum. The thin film was hydrated with MilliQ water (pure or containing potassium dichromate) for 30 min. The vesicle suspension was extruded

**Figure 7.**  
Extrusion of liposomes through a nanopore filter.



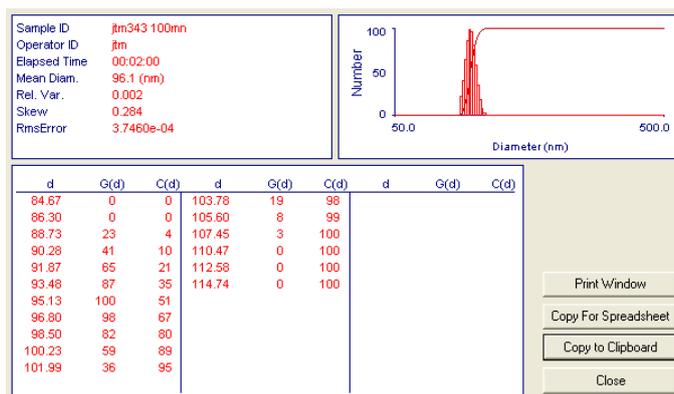
through a 50, 100 or 200nm polycarbonate filter 10 times using the Lipex® Extruder (Figure 7) and allergen concentration was determined by High-Performance Liquid Chromatography.

*Polycaprolactone* (CAS no: 2498-41-4, Aldrich, Denmark) was dissolved in acetone (pure or containing isoeugenol or dinitro-chloro-benzene) at 45°C and injected in MilliQ water containing 0,17g Pluronic F-68™ (CAS 9003-11-6, Aldrich, Denmark) (and potassium dichromate in certain cases) in a round bottomed flask under magnetic stirring (1200 rpm) at room temperature (Figure 8). Acetone and a large amount of the aqueous phase were eliminated under reduced pressure to a final volume of 5 ml. Allergen concentration was measured by High-Performance Liquid Chromatography. All formulations were kept at 5°C. A surfactant (Poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol), CAS nr: 9003+11-6, Aldrich, Denmark) was added to a final concentration of 1% (w/v) to liposome and polycaprolactone batches immediately before each Local Lymph Node Assay experiment to ensure sufficient contact with the skin. The concentrations of allergens were determined by High-Performance Liquid Chromatography to make sure the control solution matched the formulation containing polycaprolactone.

**Figure 8.** Manufacturing of polycaprolactone particles loaded with potassium dichromate.



**Figure 9.** Example of size measurement of liposomes loaded with dinitro-chloro-benzene extruded through a filter of 100 nm pore size. A Gaussian distribution is seen with a mean diameter of 96 nm.



### *Dynamic light scattering*

Hydrodynamic particle diameters and polydispersity index of ethosomes were determined by dynamic light scattering using a BI-200SM from Brookhaven Instruments. This incorporates a 632.8 nm HeNe laser operated at a fixed scattering angle of 90°. A sample of 10 µl was diluted in 1190µl 40% ethanol:MilliQ water mixture or pure MilliQ water dependent of the original vehicle. The measurements were conducted in triplicate; in a multimodal mode of 120 s. Figure 9 shows an example of the size distribution of extruded liposomes through a 100 nm pore size filter.

### **6.2.2. Encapsulation efficiency**

The encapsulation efficiency (EE%) of allergens formulated in polycaprolactone, ethosomes and liposomes was determined by ultracentrifugation as described by Heeremans et al. (57). Ethosomal, polycaprolactone and liposomal preparations containing dinitro-chloro-benzene, isoeugenol, methyl dibromoglutaronitrile or potassium dichromate were kept overnight at 5°C where after they were spun at 40.000 RPM for three hours in an Hitachi Sorvall Discovery 90SE ultracentrifuge with a swingout rotor from Sorvall (SW50.1). The supernatant was immediately removed and drug quantity was determined in both the sediment and the supernatant. Binding efficiency was calculated as follows:  $[(T-C)/T]*100$ , where T is the total amount of chemical detected in both the supernatant and sediment, and C is the amount of chemical detected only in the supernatant. The procedure was done in triplicates.

### **6.2.3. Quantification of isoeugenol, dinitro-chloro-benzene and potassium dichromate**

High-Performance Liquid Chromatography analysis was conducted on an ultimate 3000 series from DIONEX™ with a diode array detector. A DIONEX Acclaim® Surfactant column was used to separate isoeugenol and dinitro-chloro-benzene (Figure 10). Potassium dichromate was separated using a DIONEX Acclaim® 300 C18 column. The temperature of the column and the sample rack in the autosampler was set to 20°C. Mobile phase used for dinitro-chloro-benzene and isoeugenol: 75% methanol, 25% MilliQ water; isocratic elution for 30 min; and flow rate of 1 ml/min. The separations were monitored at 270nm. Mobile phase used for potassium dichromate consisted of 20% methanol

for 10 minutes followed by a linear gradient of 90% methanol performed over 5 min, followed by 40 min of 100% methanol to wash the column and 5 min of 20% methanol to equilibrate the column for the next run. Potassium dichromate was monitored at 260 nm. Pure reference compounds were used to make external calibration curves from which the concentrations of allergen were determined.

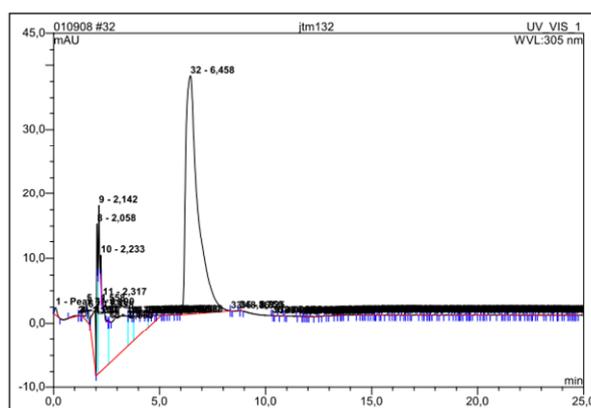
#### 6.2.4. Quantification of methyldibromoglutaronitrile

Methyldibromoglutaronitrile is not UV-active and content was measured by evaporative light scattering detection (Varian 385-LC) using a reversed phase C-5 column from Supelco©. Separation was achieved using a 0.8 ml/min flow rate with an isocratic mobile phase of 75% methanol and 25% MilliQ water Injection volume was 50µl and external calibration was done by pure methyldibromoglutaronitrile.

#### 6.2.5. Sensitization experiments

The Local Lymph Node Assay was performed according to standard procedure (58) with the lymph node cell proliferation determined for each animal and expressed as mean disintegrations per minute. Female CBA/Ca mice purchased from Harlan (Netherlands) 8 weeks of age were housed in cages with hepa-filtered airflow, under conventional conditions in light-, humidity- and temperature controlled rooms with food and water ad lib. Test substances were applied on the dorsum of both ears of each mouse for three consecutive days. On day five [methyl-<sup>3</sup>H]-thymidine was injected in the tail vein and after five hours the mice were sacrificed and the draining lymph nodes removed. A single cell suspension from each mouse was made and after two washing procedures with phosphate buffered saline, the DNA was precipitated with trichloroacetic acid for 18 hours and the thymidine incorporation was measured using β-scintillation counting. The standard vehicle in the Local Lymph Node Assay is acetone-olive oil (4:1) which dissolves polycaprolactone particles, ethosomes and liposomes. Therefore, we modified the Local Lymph Node Assay by using either water:ethanol (6:4

**Figure 10.** HPLC diagram of quantification of dinitro-chloro-benzene. Peak is seen at 6.458 minutes. Area under the curve is automatically generated for further calculation of concentration.



v/v) as control vehicle for ethosomes or water added 1% surfactant as control vehicle for liposome and polycaprolactone preparations loaded with hydrophilic allergens making the drug delivery system the only difference between batches. Lipophilic allergens were dissolved in ethanol:water (4:6, v/v), propylene glycol (analytical grade, CAS 57-55-6, Riedel-de Haën) or aceone:olive oil (acetone, analytical grade purchased from Aldrich, Denmark CAS 67-64-1 and olive oil purchased from Fluka, Denmark, CAS 8001-25-0) making the comparison with the drug delivery systems less comparable. The experiments were in accordance with Danish and European animal welfare regulations and were licensed by the Danish Animal Experimentation Inspectorate.

### **6.2.6. Test subjects**

The inclusion criteria were: age over 18 years, a previous positive patch test to methyldibromoglutaronitrile or isoeugenol within the last ten years at the Department of Dermatology, Odense University Hospital, University of Southern Denmark. Exclusion criteria were: active eczema on test sites, not being able to co-operate to the repeated open application test, pregnancy, and breast feeding.

48 persons with a previous positive patch test to isoeugenol and 89 persons with a previous positive patch test to methyldibromogutaronitrile were invited. The study was performed according to the Helsinki II declaration and approved by the local Ethics Committee (The Southern region of Denmark, S-20090022).

### **6.2.7. Patch test**

3 concentrations of methyldibromogutaronitrile and 2 concentrations of isoeugenol formulated in ethosomes and ethanol:water and blank controls were tested. The placement of the test concentrations and vehicles in both tests were randomized and blinded for the investigator and the subjects. After termination of the study the randomization code was broken. The study was performed according to the Helsinki II declaration and approved by the local Ethics Committee (The Southern region of Denmark, S-20090022). The patch tests were applied on IQ-chambers (Chemotechnique® Diagnostics, Sweden), occluded for two days and the reactions were read on D3. The reading scale developed by Fischer et al (19) was chosen in order to recognize smaller differences in the allergic

responses. The scale was as follows: 0 = no reaction; 1 = few papules with no erythema, no infiltration; 2 = faint erythema with no infiltration or papules; 3 = faint erythema with few papules and no homogeneous infiltration; 4 = erythema, homogeneous infiltration; 5 = erythema, infiltration and a few papules; 6 = erythema, infiltration and papules; 7 = erythema, infiltration, papules and a few vesicles; 8 = intensive erythema, infiltration and vesicles. The author performed all readings. All formulations were kept in darkness at 5°C and all preparations were made no more than 5 days prior to beginning of the patch testing and repeated open application test. Volunteers were instructed to keep the test material for the repeated open application test in the refrigerator. The concentrations of isoeugenol were: 0.0, 2.80, and 6.54 mg/ml and of methyldibromoglutaronitrile: 0.00, 0.10, 0.21, and 0.63mg/ml.

#### **6.2.8. Repeated open application test (ROAT)**

Repeated open application tests' were performed with one concentration of allergen formulated in ethosomes and ethanol:water. Two 3x3 cm areas on the volar aspect of both forearms were used. Twenty microlitres of test preparation were applied two (methyldibromoglutaronitrile) or three times (isoeugenol) daily using a micropipette (Acura 815, 20 µL, Buch & Holm A/S, Herlev, Denmark) with a fixed volume. Test subjects received 2 marked bottles, each mark referring to a test area. The solutions were spread on the area with the tip of the pipette and allowed to dry by evaporation. The subjects received written instructions and were instructed orally and manually in using the pipette. The dose of one application was 5.66 mg/ml isoeugenol or 0.10mg/ml methyldibromoglutaronitrile. When an area showed a positive reaction (verified by investigator), the subjects stopped application on that test area and continued on the other area. A reaction was defined as positive when 70% of the area had erythema, papules, or vesicles. Numbers of days until positive reactions occurred were counted. The author performed all readings. If no reaction developed within 4 weeks, application was stopped (except in one case: Here the repeated open application tests on one arm were positive after 18 days and on the other after 45 days). The concentration of isoeugenol was 5.66 mg/ml and of methyldibromoglutaronitrile: 0.10 mg/ml.

### 6.2.9. Skin penetration and absorption model

#### *Skin Membranes*

The human skin samples were obtained from the Department of Plastic and Reconstructive Surgery, Odense University Hospital. Skin was sampled from three women (26-37 years old) who underwent breast reconstruction. Skin samples were kept at  $-20^{\circ}\text{C}$  for periods not exceeding 12 months. The skin was allowed to thaw at room temperature 1 hour before being cleaned with distilled water.

Subcutaneous fat was removed. Skin thickness varied between 0.90 and 0.96 mm. Skin samples from individual donors were equally distributed between experimental groups.

#### *Skin penetration and absorption model*

Percutaneous penetration experiments were carried out using Franz diffusion cells with a permeation area of  $2.12\text{ cm}^2$  and a receptor volume between 15 and 19 ml (measured for each individual cell) as described by Nielsen et al (23). The system consists of two half-cells where the upper cell compartment represents the donor chamber and the lower the receptor chamber (Figure 11). The cells were kept at a constant temperature ( $32^{\circ}\text{C}$ ) in a water bath with individual magnetic stirring. Prior to experiments, the epidermal site was exposed to ambient laboratory conditions and the dermis was exposed to an aqueous solution of 0.9% NaCl and 5% bovine serum albumin containing 10% ethanol for 18 hours. Further, the barrier integrity was evaluated by capacitance measurements (Lutron DM-9023, Acer AB, Sweden) before the exposure to test substances, and cells with a capacitance above 110 nF were excluded.

**Figure 11.**

Franz cell chamber used with human cadaver full thickness skin (A). 106 $\mu\text{l}$  sample is applied on the donor side (B) and samples from receptor chamber were taken at selected time intervals (note the parafilm occlusion to prevent evaporation) (C). Epidermis is gently separated from dermis for individual allergen measurement (D).



During the experimental periods, donor and receptor chambers were covered with parafilm to avoid evaporation. The skin was exposed to 106 $\mu$ L test substance (50  $\mu$ L/cm<sup>2</sup>) and samples of 1 mL were taken at 2, 4, 6, 12, and 24 hours from the receptor chamber and replaced with 1 mL of fresh receptor fluid. At the end of experiment, remaining test compound in the donor chamber and on top of the skin was sampled using repeated washings with cotton swabs and 50% acetonitrile. Cotton swabs and skin samples were left for 72 hours to extract in acetonitrile before chemical analysis.

After termination of experiments, the epidermis was gently removed from the skin samples with a sharp knife, and both dermis and epidermis were transferred to individual vials containing 100% acetonitrile and left for extraction for 72 hours before measuring the amount of dinitro-chloro-benzene or isoeugenol.

The adherence of test compounds to glass in the receptor chamber, to proteins in the receptor fluid, and to the skin after extraction procedures was evaluated to assure complete recovery of penetrated test compounds.

The amount of dinitro-chloro-benzene applied in ethanol:water was 0.035 mg and 0.036 mg in ethosomes. The amount of isoeugenol applied in ethanol:water was 1.58 mg and 1.24 mg when applied in ethosomes.

#### **6.2.10. Release kinetics of allergens from ethosomes**

Dialysis membranes (Spectra-por 6, pore size: 10 000 Daltons, Spectrum Labs, purchased from Bie & Berntsen AS, Herlev, Denmark) were filled with 300 $\mu$ L test solution of dinitro-chloro-benzene or isoeugenol formulated in ethanol:water, 30, 60 or 90 mg/mL ethosomes and left in 75mL ethanol:water (4:6 v/v) covered with parafilm on a magnetic stirrer. Samples of 500 $\mu$ L were taken out at specific time intervals (Figure 12) and replaced with an equal amount of ethanol:water. Samples were analysed by high pressure liquid chromatography and expressed as % of the applied amount of allergen. The concentration of dinitro-chloro-benzene was 0.79 mg/mL in ethanol:water, 0.67 mg/mL in 30mg/ml ethosomes, 0.62 mg/mL in 60mg/mL ethosomes, and 0.63 mg/mL in 90 mg/mL ethosomes. The concentration of isoeugenol was 8.79 mg/mL in ethanol:water, 8.79 mg/mL in 30mg/mL ethosomes, 7.23 mg/mL in 60mg/mL ethosomes, and 7.63 mg/mL in 90 mg/mL ethosomes.

A T50% value was calculated in a similar way as the EC3% value of the Local Lymph Node Assay (59), now estimating the time needed for 50% of the allergen to diffuse through the dialysis membrane.

### 6.3. Statistical data analysis

Results are expressed as means  $\pm$ Standard deviation (SD) or standard error of mean (SEM). Statistically significant differences in the Local Lymph Node Assay experiments were determined using One Way ANOVA and Student-Newman-Keuls test for post hoc analysis. Differences in the patch test reactions and percutaneous absorption experiments were determined by two-way analysis of variance (two-way ANOVA) using applied dose and vehicle (ethosomes/ethanol:water) as factors. Repeated open application test experiments were analysed by Wilcoxon Signed Rank Test. Statistically significant differences of penetration over time of isoeugenol and dinitro-chloro-benzene and the release kinetics of allergens from ethosomes were determined using Two Way ANOVA. Mann Whitney test was used to test for different amount of allergen stored in epidermal and dermal compartments for ethanol:water and ethosome formulations.  $P < 0.05$  was chosen as minimal level of significance. The statistical software package: "Graphpad Prism 4" from GraphPad Software inc. San Diego, California, USA was used.

**Figure 12.** Dialysis of liposome formulation of potassium dichromate. The lower left picture shows the formulation before (right) and after (left) dialysis.



## 7. Experimental results and discussion

### 7.1. Sensitization studies (paper I & II)

#### 7.1.1. Results

Empty ethosomes, liposomes, polycaprolactone particles and the surfactant did not sensitize themselves in the Local Lymph Node Assay (Table 2, 3 and 4). Ethosomes, liposomes and polycaprolactone encapsulated with potassium dichromate showed no significant effect on the sensitizing capacity compared to potassium dichromate in ethanol:water or MilliQ water added 1% surfactant (Table 2). Dinitro-chloro-benzene and isoeugenol loaded polycaprolactone particles and dinitro-chloro-benzene in propylene glycol showed a significantly reduced sensitisation response compared to dinitro-chloro-benzene and isoeugenol in liposomes, acetone:olive oil and ethanol:water (Table 3 and 4). As opposed to the above results isoeugenol and dinitro-chloro-benzene loaded ethosomes showed a significant increased sensitizing capacity compared to formulations without ethosomes, and the dose of ethosomes was an additional factor as there was a linear dose-response relationship between concentration of ethosomes and the sensitization obtained, reaching a significant level at 60 mg/ml POPC (60). The size of dinitro-chloro-benzene loaded liposomes did not affect their sensitizing capacity but dinitro-chloro-benzene loaded ethosomes enhanced the allergenicity compared to a solution of dinitro-chloro-benzene, ethanol, water and POPC (larger vesicles) (Table 3). The effect of the surfactant on the sensitizing capacity is seen in Table 5. The surfactant is not a sensitizer but increases the sensitizing capacity of the potassium dichromate formulation by a factor of two when doubling the surfactant concentration. Table 6 shows the size of vesicles and the encapsulation efficiency. The encapsulation efficiency is low for potassium dichromate (0.7-16%) and in a higher range for dinitro-chloro-benzene and isoeugenol (77-98%).

**Table 2.**

Results of Local Lymph Node Assay experiments performed with potassium dichromate in different vehicles. §surfactant 1% added. Statistical differences were calculated by one way ANOVA and Student-Newman-Keuls test for post hoc analysis with  $P < 0.05$  as minimal level of significans. \*\*\* $P < 0.001$ .

<b>Potassium dichromate [Lipid], (n)</b>	<b>Allergen % (w/v)</b>	<b>Lymphocyte proliferation (mean DPM ± SD)</b>
<b>Ethanol:water (5)</b>	0.5	6933±3833
<b>Ethosomes (5)</b>	0.5	5049±1329
<b>Polycaprolactone (50mg/ml) (6)§</b>	0,0	428±181***
<b>Polycaprolactone (50mg/ml) (7)§</b>	0.5	1999±1184
<b>Polycaprolactone (5mg/ml) (6)§</b>	0.5	2231±2167
<b>Water (5)§</b>	0.5	2165±1018
<b>Liposomes (80mg/ml) (5)§</b>	0.0	1198±611***
<b>Liposomes (40mg/ml) (5)§</b>	0.5	4343±1377
<b>Liposomes (80mg/ml) (5)§</b>	0.5	4843±1339
<b>Water (4)§</b>	0.5	4987±3069

**Table 3.**

Results of Local Lymph Node Assay experiments performed with dinitro-chloro-benzene in different vehicles. §wetting agent 1% added. Statistical differences were calculated by one way ANOVA and Student-Newman-Keuls test for post hoc analysis with  $P < 0.05$  as minimal level of significans. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

Dinitro-chloro-benzene [Lipid], (n)	Allergen % (w/v)	Lymphocyte proliferation (mean DPM ± SD)	
Ethanol:water (5)#	0.03	1349±443*	
Ethosomes (60mg/ml) (5)#	0.03	2151±925**	
Ethosomes (60mg/ml) (5)#	0.00	387±108	
Ethanol:water (6)#	0.04	1017±290	***
Ethanol:water:POPC (60mg/ml) (6)#	0.04	3912±310	
Ethosomes (60mg/ml) (4)#	0.04	6007±944	*
Ethosomes (60mg/ml) (5)#	0.00	575±165	***
Polycaprolactone (60mg/ml) (5)§	0.05	1211±449	
Liposomes (60mg/ml)(5)§	0.05	7602±2658***	
Ethanol:water (5)	0.05	5349±2151***	
Acetone:olive oil (5)	0.05	5633±666***	
Polycaprolactone (60mg/ml) (5)§	0.00	778±234	
Liposomes (60mg/ml) (6)§ not extruded	0.04	1785±705	
Liposomes (60mg/ml) (6)§ 200nm	0.04	2106±391	
Liposomes (60mg/ml) (6)§ 100nm	0.04	2923±626	
Liposomes (60mg/ml) (6)§ 50nm	0.04	1806±514	

**Table 4.**

Results of Local Lymph Node Assay experiments performed with isoeugenol in different vehicles. n= number of mice. DPM=disintegrations per minute. §Surfactant 1% added. Statistical differences were calculated by one way ANOVA and Student-Newman-Keuls test for post hoc analysis with  $P<0.05$  as minimal level of significance. \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$ .

<b>Isoeugenol [Lipid], (n)</b>	<b>Dose % (w/v)</b>	<b>Lymphocyte proliferation (mean DPM <math>\pm</math> SD)</b>
Ethanol:water (7)	1.5	641 $\pm$ 349
Ethosomes (60mg/ml) (6)	1.5	2343 $\pm$ 533***
Ethosomes (60mg/ml) (6)	0.0	777 $\pm$ 420
Ethanol:water (5)	1.5	569 $\pm$ 289
Ethosomes (20mg/ml) (5)	1.5	850 $\pm$ 124
Ethosomes (40mg/ml) (5)	1.5	1053 $\pm$ 289
Ethosomes (60mg/ml) (5)	1.5	1359 $\pm$ 531*
Polycaprolactone (50mg/ml) (5)§	1.3	1100 $\pm$ 406
Liposomes (60mg/ml) (5)§	1.3	3868 $\pm$ 950***
Acetone:olive oil (5)	1.3	4491 $\pm$ 819***
Propylene glycol (5)	1.3	861 $\pm$ 346

**Table 5.**

Results of Local Lymph Node Assay experiments performed with potassium dichromate dissolved in water added a surfactant. Adding a surfactant agent to potassium dichromate dissolved in water increased the sensitizing capacity of the Local Lymph Node Assay. Lymph nodes were pooled for each group.

<b>Sample description</b>	<b>Potassium dichromate (% w/v)</b>	<b>Lymphocyte proliferation</b>
Surfactant 2% (n=4)	0.0	3419
Surfactant 1% (n=4)	0.5	7372
Surfactant 1% + (n=4)	1.0	16074
Surfactant 2% + (n=4)	0.5	15737

**Table 6.**

Size and encapsulation efficiencies of different drug delivery systems loaded with potassium dichromate, dinitro-chloro-benzene (DNCB) or isoeugenol. Results are mean $\pm$ SD.  $n=3$  in all experiments.

<b>Sample description</b>	<b>Allergen</b>	<b>Size<math>\pm</math>SD (nm)</b>	<b>Encapsulation efficiency % <math>\pm</math>SD</b>
Polycaprolactone	Potassium dichromate	313 $\pm$ 13	0.7 $\pm$ 0.3
Liposome	Potassium dichromate	91 $\pm$ 5	7 $\pm$ 2.1
Ethosome	Potassium dichromate	436 $\pm$ 9	16 $\pm$ 0.4
Polycaprolactone	DNCB	231 $\pm$ 20	83 $\pm$ 0.6
Liposome	DNCB	120 $\pm$ 17	92 $\pm$ 0.1
Ethosome	DNCB	245 $\pm$ 17	90 $\pm$ 0.3
Polycaprolactone	Isoeugenol	343 $\pm$ 21	84 $\pm$ 0.1
Liposome	Isoeugenol	155 $\pm$ 25	98 $\pm$ 0.1
Ethosome	Isoeugenol	396 $\pm$ 20	77 $\pm$ 0.3

### 7.1.2. Discussion

For the first time it is shown that contact allergens encapsulated in ethosomes can show enhanced sensitizing capacity compared to the same allergen concentration in solution. Encapsulated isoeugenol in ethosomes showed repeatedly, significantly increased sensitization in a modified Local Lymph Node Assay compared to isoeugenol in solution. Isoeugenol has previously been tested in the Local Lymph Node Assay in different vehicles and the EC<sub>3</sub> values obtained were 0.9 (dimethylsulfoxide), 1.5 (acetone:olive oil), 1.8 (ethanol:water 1:9), 2.5 (propylene glycol), and 4.9 (water/ethanol 1:1) (25). The dose of isoeugenol (1.1% w/v) in the present experiments was selected due to limited solubility of isoeugenol in the ethanol:water solution. Higher concentrations were not possible due to instability of the ethosome formulation with change in vesicle size and polydispersity index. The isoeugenol concentration is thus below the EC<sub>3</sub> values reported from other Local Lymph Node Assay experiments with isoeugenol using ethanol:water as vehicle. In accordance with this, isoeugenol did not sensitize in the ethanol:water solution, only in the ethosome formulation. Dinitro-chloro-benzene is a more potent allergen which permitted a concentration above its EC<sub>3</sub> value. Dinitro-chloro-benzene 0.03% (w/v) showed stronger sensitization in the ethanol:water solution compared to empty ethosomes

and the sensitization was further enhanced when formulated in ethosomes. However, the presence of POPC in the dinitro-chloro-benzene ethanol:water solution (0.04%) without extrusion of ethosomes also had an enhancing effect on sensitization compared the ethanol:water solution (Table 3).

The importance of vehicle effects on sensitisation and elicitation in contact allergy is well known from animal studies in both guinea pigs (61) (62) and mice (the Local Lymph Node Assay)(63)). No simple relationship between allergen, vehicle and sensitization has been found (14;63;64) suggesting that allergens should be tested in vehicles as close as possible to the vehicle used in the product. Our results show how encapsulation of allergens in three different drug delivery systems relevant for pharmaceutical and cosmetic industry may affect the sensitizing potency in the Local Lymph Node Assay. The results show that no simple relationship exist between the drug delivery system and the sensitising capacity in the Local Lymph Node Assay exist, since e.g. ethosomes loaded with isoeugenol, dinitro-chloro-benzene or potassium dichromate increase or do not change the sensitising capacity in the Local Lymph Node Assay compared to a solution of the allergens without the ethosomes. It may be the unique combination of the allergen and the drug delivery system which causes the change in the sensitising properties and therefore each system should be evaluated on a case by case basis for risk assessment. Polycaprolactone loaded with lipophilic allergens (dinitro-chloro-benzene and isoeugenol) showed reduced sensitisation in the Local Lymph Node Assay compared to acetone:olive oil and liposomes. This is in contrast to the suggestion in a case report (51). However, the Local Lymph Node Assay is a sensitisation experiment and the case report concerns elicitation. Further, we do not know the exact composition of the polycaprolactone in the cosmetic product. Octyl-methoxycinnamate, a UV filter used in sunscreens, penetrate significant less when encapsulate in PCL compared to non-encapsulated octyl-methoxycinnamate (65). Potassium dichromate encapsulated in polycaprolactone did not alter the sensitizing capacity in the Local Lymph Node Assay compared to Potassium dichromate in water. This is somehow expected since hydrophilic chemicals do not bind to the lipid membrane or to the polycaprolactone but rather stay in the aqueous phase as seen from the encapsulation efficiencies in Table 6. Therefore, the altered sensitisation capacity may not be caused by the lipid itself, but is more likely due to the *encapsulation* of the allergen in the lipid membrane. Isoeugenol formulated in acetone:olive oil was a significant stronger sensitizer compared to propylene glycol, which also is reported in the literature (14;66). We found approximately the same lymph node

proliferation as Ryan et al when testing potassium dichromate with a wetting enhancer (even though we used a polymer with a molecular weight of 4400 MW compared to Ryans' pluronic L92 (MW:3650))(15).

Liposomes and polycaprolactone are formulated in an aqueous solution which makes it impossible to compare the effect of polycaprolactone and liposomes alone, since lipophilic allergens must be formulated in an organic control solution which would dissolve the liposomes and polycaprolactone particles. We have added a surfactant to the lipophilic allergens formulated in liposomes and polycaprolactone and compared it to the allergens formulated in an organic solution (acetone:olive oil, ethanol:water or propylene glycol). This difference should be kept in mind when results are interpreted. Reducing the size of liposomes did not alter the sensitizing capacity in the Local Lymph Node Assay but reducing the size of ethosomes did increase the sensitizing capacity. Diverging results are found in the literature of the relation between the size of liposomes and bioavailability of the encapsulated compound, but this might depend on the exact composition of the liposomes (67;68).

## **Conclusion**

Formulating contact allergens in different microvesicular systems may alter their sensitizing properties. Ethosomes was able to enhance the sensitizing capacity of dinitro-chloro-benzene and isoeugenol and polycaprolactone protected the lipophilic allergens against sensitization. Diverging results where obtained on the size of vesicles. A case by case evaluation is recommended for the assessment of sensitising properties of product ingredients encapsulated in microvesicles.

## **7.2. *Elicitation studies with isoeugenol and methyldibromoglutaronitrile (paper III)***

### **7.2.1. Results**

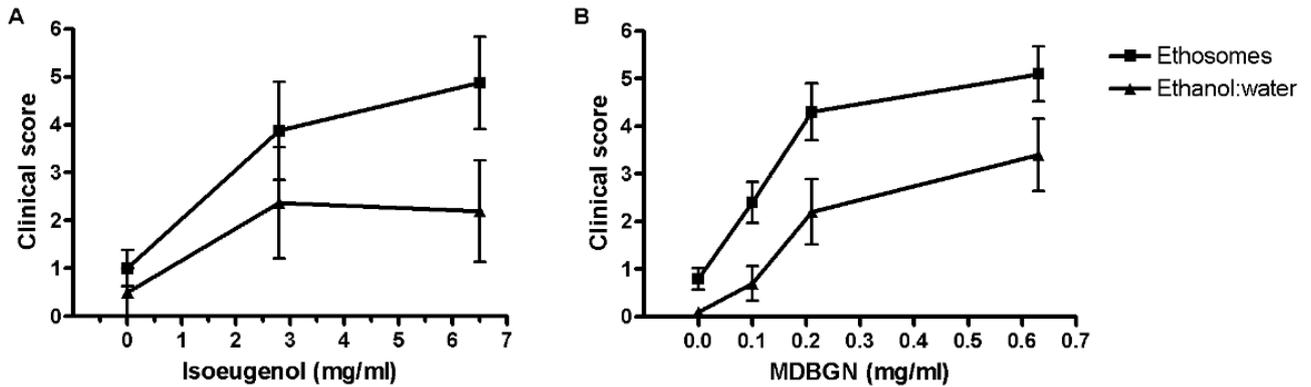
20 subjects participated in the methyldibromoglutaronitrile serial dilution patch test and eighteen in the Repeated Open Application Test . One subject had negative patch tests and 8 subjects a negative Repeated Open Application Test and they were removed from further analysis.

8 subjects participated in the isoeugenol serial dilution patch tests and the Repeated Open Application Test and all subjects had a positive patch test. Six subjects had a positive Repeated Open Application Test (one subject after 45 days) and two did not react during the exposure period.

Isoeugenol and methyldibromoglutaronitrile formulated in ethosomes significantly enhanced the patch test reactions compared to the same allergens in ethanol:water, making ethosomes the only difference (Figure 13 and 14). However, when POPC was added to ethanol:water – without extrusion of vesicles – there was no difference in response to isoeugenol in ethosomes (Figure 15). The Repeated Open Application Test did not show a significant difference for any of the allergens, but a trend towards a more rapid developing positive reaction was found for isoeugenol formulated in ethosomes compared to isoeugenol formulated in ethanol:water (Table 7).

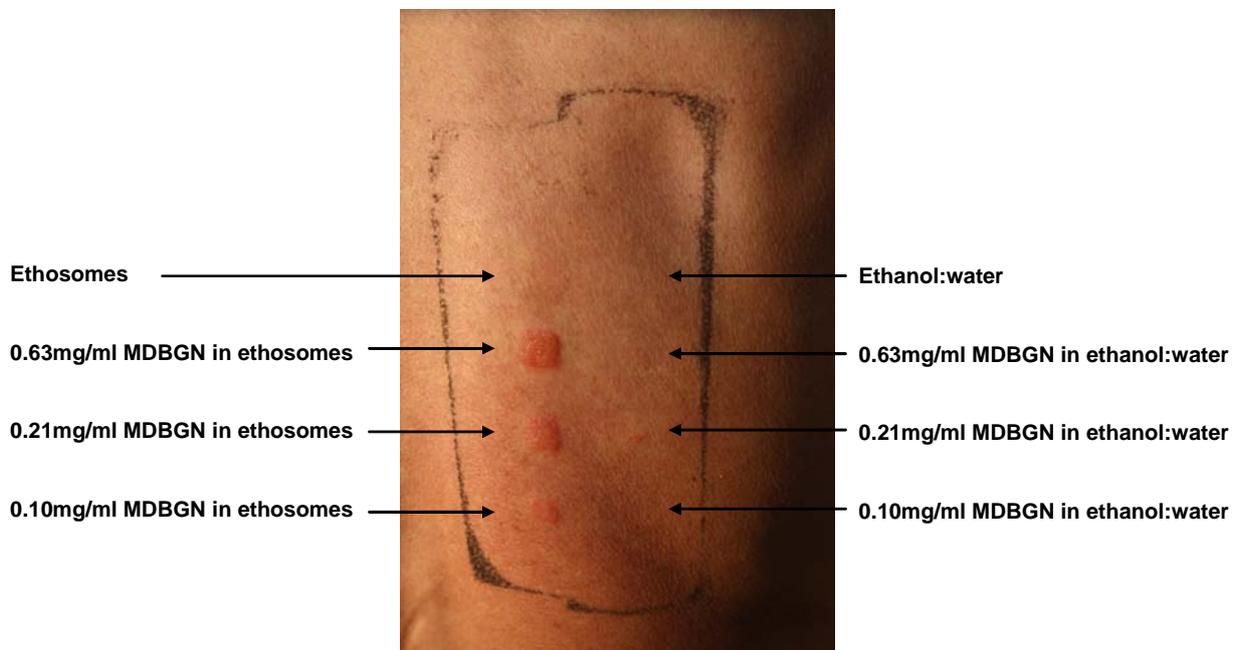
**Figure 13.**

Patch test results of methyldibromoglutaronitrile (MDBGN)(n=19) and isoeugenol (n=8) encapsulated in ethosomes (100mg/ml) compared to the same concentrations of allergen in ethanol:water. A significant increase in patch test response is seen for both allergens encapsulated in ethosomes (MDBGN,  $p<0.0001$  and isoeugenol  $p<0.05$ ). Increased allergen concentration also increased the elicitation response (MDBGN  $p<0.0001$  and isoeugenol  $p<0.007$ )( two-way ANOVA). Results are expressed as means  $\pm$  SEM.



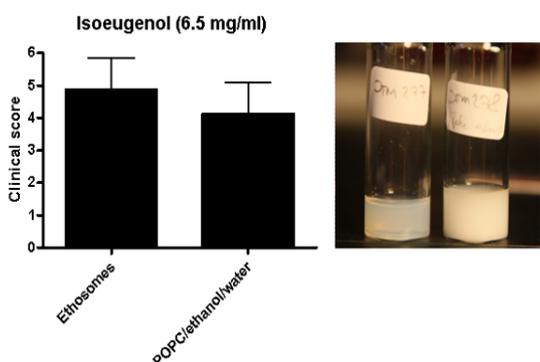
**Figure 14.**

Result of a serial dilution patch test in a sensitised volunteer with methyldibromoglutaronitrile (MDBGN) using IQ chambers and 15 $\mu$ l test substance formulated in ethosomes and ethanol:water.



**Figure 15.**

Patch test results with 6.5 mg/ml isoeugenol formulated in ethosomes (300nm) and in POPC:ethanol:water (n=8). No significant difference was observed (Wilcoxon signed rank test). Results are expressed as means  $\pm$  SEM. The picture shows the light scattering effect of small extruded vesicles of 300nm (left) versus non extruded vesicles (right).



**Table 7.**

Repeated open application test performed with isoeugenol (n=6) and methyldibromoglutaronitrile (MDBGN) (n=10) formulated in ethosomes and ethanol:water as vehicles. Results are presented as mean days  $\pm$  SEM to a positive reaction. No significant difference was observed (Wilcoxon Signed Rank Test) for both allergens even though a trend towards a faster developing reaction was seen by isoeugenol formulated in ethosomes compared to the ethanol:water formulation (p=0.31).

	Isoeugenol	
	Ethosomes	Ethanol:water
Days to positive ROAT $\pm$ SD	7.7 $\pm$ 2.4	15.3 $\pm$ 7.3
	Methyldibromoglutaronitrile	
	Ethosomes	Ethanol:water
	10.7 $\pm$ 2.3	10.1 $\pm$ 2.0

### Characterisation of ethosomes

Vesicle size measured before and after experiments remained stable in the test tubes for the duration of the experiment. All ethosomes were between  $333 \pm 13$  and  $463 \pm 13$  nm and polydispersity index ranged from  $0.06 \pm 0.04$  -  $0.22 \pm 0.03$ , and can be regarded as monodisperse. The encapsulation efficiency of isoeugenol to ethosomes was  $77.3 \pm 0.3\%$  and for methyldibromoglutaronitrile  $21.8 \pm 4.3\%$ .

### 7.2.2. Discussion

Using a protocol with precise dosing and characterization of test preparations, it is for the first time shown that lipophilic contact allergens encapsulated in ethosomes can enhance the patch test reactions in sensitized individuals compared to the same allergens in control solution of 40 % ethanol in water without lipid vesicles. Other vehicle effects on both sensitization and elicitation responses have previously been reported in experiments using the Local Lymph Node Assay, guinea pigs, and human volunteers as test subjects (14;26). However, the effect of new encapsulating vehicles has not been

studied before. No difference was seen when POPC was added to the ethanol:water solution compared to the ethosome formulation (Figure 15). A tentative explanation of the results is that spontaneous formation of vesicles occurs when POPC is mixed with water (or ethanol:water). However, the vesicles are not of homogeneous size and they are multilamellar compared to vesicles extruded through a filter of equal pore size which makes the vesicles more uniform and single lamellar. The light scattering effect of small extruded vesicles (300 nm) versus non extruded vesicles is clearly seen in Figure 15. Due to very high polydispersity indexes dynamic light scattering measurements were not applicable in the POPC:ethanol:water formulation.

Repeated Open Application Test performed with methyldibromoglutaronitrile and isoeugenol with and without ethosomes showed no significant difference in lag time until a positive response, even though a trend towards a more rapid developing reaction occurred with encapsulated isoeugenol compared to isoeugenol in ethanol:water. We have no explanation for this discrepancy between patch test results and Repeated Open Application Test, but occlusion may play a role. It has been reported that occlusion decreases penetration of compounds through the skin when encapsulated in Transfersomes™ (43) but since there is no clear documented relation between skin penetration and the sensitizing capacity of an allergen (5;69), altered penetration is probably not the key to the different findings in our results. Further experiments are needed to clarify this problem.

Increased patch test reactivity correlates with increased Repeated Open Application Test reactivity for some allergens as methyldibromoglutaronitrile and isoeugenol (19;70), but it is not always the case (71). Isoeugenol is less lipophilic and better retained inside the ethosomes compared to methyldibromoglutaronitrile as expressed by higher encapsulation efficiency (77% vs. 22%). Whether this difference accounts for the discrepancy between the Repeated Open Application Test and patch test reactions of methyldibromoglutaronitrile and isoeugenol encapsulated in ethosomes remains speculative, but obviously the low encapsulation efficiency of methyldibromoglutaronitrile is enough to produce significant changes in the test reactions if the encapsulation efficiency is an important parameter. A direct comparison is only valid for a single allergen when formulated in different vehicles and not between different allergens, since allergens with significantly different chemical structures and thereby physico-chemical properties (e.g.  $\log P$ ) will influence the vesicle properties

(e.g. stability, encapsulation efficiency and skin penetration) and subsequently complicate data discussion.

The clinical implications of these results are so far uncertain. However, the cosmetic industry should consider the effect of encapsulation on a case by case basis because certain ingredients may become more allergenic when encapsulated. Dermatologists investigating patients with allergic reactions to consumer products using encapsulation technology should consider the risk of false negative results, if testing with ingredients in conventional patch test vehicles. Testing with encapsulated ingredients should be performed when possible.

### **7.3. *Skin penetration properties and release kinetics (paper IV)***

#### **7.3.1. Results**

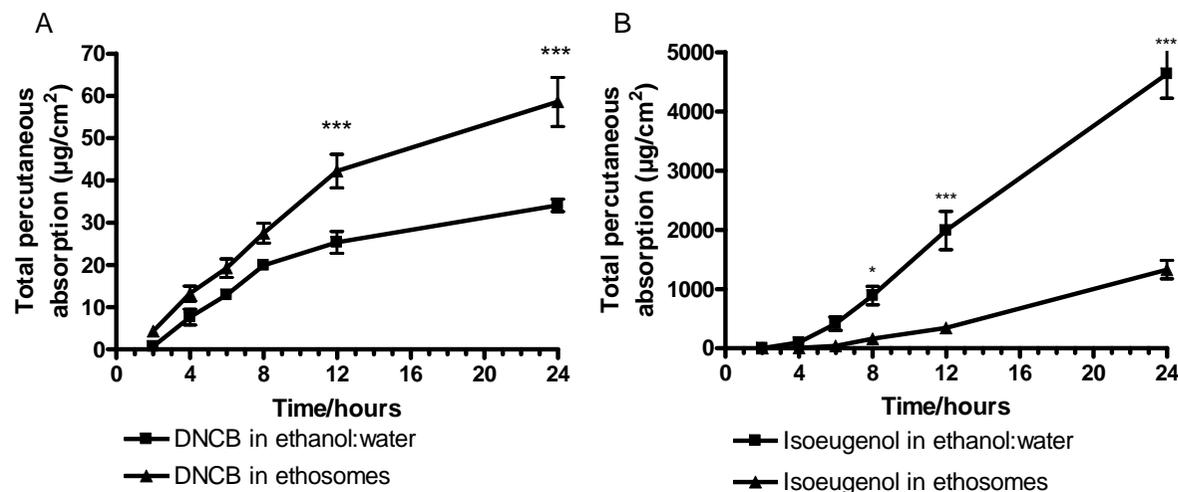
Ethosome formulation of dinitro-chloro-benzene significantly increased the percutaneous absorption of dinitro-chloro-benzene compared to an ethanol:water formulation of dinitro-chloro-benzene (Figure 16 and Table 8). In contrast, the percutaneous absorption of isoeugenol formulated in ethosomes was significantly reduced compared to an ethanol:water formulation of isoeugenol. dinitro-chloro-benzene formulated in ethosomes had a slightly (non significant) increased dermis deposition compared to the ethanol:water formulation, but no difference in epidermal deposition. On the contrary, the ethosome formulation significantly decreased the dermis deposition of isoeugenol and caused a more limited and non-significant increase in epidermal deposition of isoeugenol. The ethosome formulation caused a significantly increased relative skin deposition of isoeugenol, whereas the ethosomes had a more limited but opposite effect on the relative skin deposition of dinitro-chloro-benzene. A significantly increased lag-time was found for isoeugenol formulated in ethosomes compared to the ethanol:water formulation, whereas the lag-time of dinitro-chloro-benzene was not significantly affected by the ethosome formulation. The max flux as well as the permeability coefficient of isoeugenol was significantly lower, when isoeugenol was formulated with ethosomes compared to the ethanol:water formulation, whereas no difference was seen for the dinitro-chloro-benzene formulations. In summary all parameters showed an opposite trend for the two allergens in ethosomes and ethanol:water. This observation is a consequence of the decreased release rate when dinitro-chloro-benzene as well as isoeugenol was formulated in ethosomes (Figure 17 and Table 9). An interesting observation was that

the effect of ethosome formulation was evident at the lowest concentration of ethosomes applied for isoeugenol (30 mg/mL), whereas a three time's higher concentration of ethosomes was required to decrease the release rate significantly for dinitro-chloro-benzene. No measureable adherence of dinitro-chloro-benzene or isoeugenol to glass, protein binding, or remaining test compounds in skin samples following the extraction procedures were observed.

Size and encapsulation efficiencies show that ethosomes loaded with isoeugenol are slightly larger compared to dinitro-chloro-benzene loaded ethosomes (Table 10). Encapsulation efficiencies are of the same magnitude.

**Figure 16.**

(A) shows a significant increased percutaneous absorption after 12 hours when dinitro-chloro-benzene (DNCB) is formulated in ethosomes compared to an ethanol:water formulation and (B) shows a significant decreased percutaneous absorption after 8 hours when isoeugenol is formulated in ethosomes compared to an ethanol:water formulation. ( $n=8$ , \*\*  $P<0.01$ , \*\*\* $P<0.001$ , two-way ANOVA). Results are expressed as means  $\pm$  SEM.

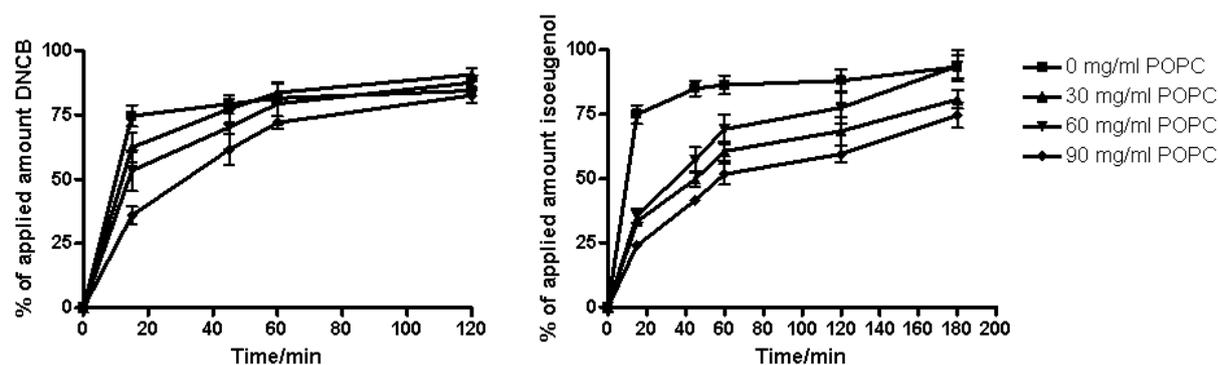

**Table 8.**

Fraction of dinitro-chloro-benzene (DNCB) and isoeugenol retained in dermis and epidermis after 24 hours treatment of DNCB and isoeugenol formulated in ethanol:water or ethosomes using Franz diffusion cells. Furthermore, the max flux, lag-time and  $K_p$  are shown. Data are expressed as  $\mu\text{g} \pm$  standard deviation. ( $n=8$ , \* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$ )

		Epidermis deposition ( $\mu\text{g}/\text{cm}^2$ )	Dermis deposition ( $\mu\text{g}/\text{cm}^2$ )	Dermis/Epidermis ratio	Total percutaneous absorption at 24h/ ( $\mu\text{g}/\text{cm}^2$ )	Total skin deposition in percent of total penetration	max flux ( $\mu\text{g}/\text{cm}^2 \cdot \text{h}$ )	Lag-time(h)	$K_p/(\mu\text{m}/\text{h})$
DNCB	Ethanol:water	$0.05 \pm 0.04$	$0.66 \pm 0.32$	$17.9 \pm 10.7$	$34 \pm 4$	$2.09 \pm 0.96$	$1.3 \pm 0.6$	$2.4 \pm 0.9$	$39 \pm 17$
	Ethosomes	$0.04 \pm 0.01$	$0.82 \pm 0.42$	$19.7 \pm 7.8$	$59 \pm 16^{***}$	$1.68 \pm 1.31$	$1.6 \pm 0.8$	$1.9 \pm 1.2$	$47 \pm 24$
Isoeugenol	Ethanol:water	$2.83 \pm 1.57$	$49 \pm 21$	$18.7 \pm 6.6$	$4635 \pm 1167$	$1.30 \pm 1.00$	$206 \pm 91$	$4.5 \pm 1.1$	$138 \pm 61$
	Ethosomes	$3.30 \pm 1.58$	$22 \pm 6^{**}$	$8.7 \pm 5.9^{**}$	$1327 \pm 443^{***}$	$2.05 \pm 0.55^*$	$69 \pm 21^*$	$6.8 \pm 1.4^{**}$	$59 \pm 18^*$

**Figure 17.**

Release time for dinitro-chloro-benzene (DNCB) and isoeugenol in an ethanol:water and in 3 concentrations of ethosomes evaluated by dialysis. Both allergens are released significantly slower when formulated in increasing ethosomes concentrations. Data represents mean±standard error of mean. (N=3,  $p < 0.0001$  for DNCB and  $p < 0.0025$  for isoeugenol, two-way ANOVA).



**Table 9.**

Dialysis experiments show an increased T50% value with increasing amount of ethosomes in the sample of dinitro-chloro-benzene and isoeugenol formulated in increasing concentrations of ethosomes. Data represents means ±standard deviations. (N=3,  $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ . One-Way ANOVA with Newmann-Keuls post hoc test).

T 50%	POPC (mg/ml)			
	0	30	60	90
Dinitro-chloro-benzene	10±1	14±1	21±15	33±9
Isoeugenol	10±1	32±6*	26±5**	44±8***

**Table 10.**

Overview of physical-chemical properties, size of ethosomes, skin penetration and release time formulated in ethosomes of isoeugenol and DNCB. \* indicates experimental values obtained using the software: US EPA. [2010]. Estimation Programs Interface Suite™ for Microsoft® Windows, v 4.00]. United States Environmental Protection Agency, Washington, DC, USA. # Data represents mean ± standard deviations (N=3).

	DNCB	Isoeugenol
Molecular weight	202.5	164.21
Water solubility	Insoluble	Slightly soluble
Log <i>P</i> (O:W)	2.17*	3.04*
Encapsulation efficiency in ethosomes %	90±0.3#	77±0.3#
Release time when formulated in ethosomes compared to ethanol:water	Increased	Increased
Skin penetration when formulated in ethosomes compared to ethanol:water	Increased	Decreased

### 7.3.2. Discussion

We found contradictory percutaneous absorption and penetration patterns when comparing dinitro-chloro-benzene and isoeugenol formulated in ethanol:water and ethosomes and hence penetration/absorption characteristics could not explain the increased sensitizing capacity of both allergens when formulated in ethosomes. A marked difference between dinitro-chloro-benzene and isoeugenol is the water solubility, the latter being much more water soluble compared to dinitro-chloro-benzene. Further, isoeugenol has higher  $\log P$  and lower encapsulation efficiency compared to dinitro-chloro-benzene but both allergens showed a sustained release when formulated in ethosomes (Table 10). Despite these differences both allergens increase their sensitizing potential when formulated in ethosomes, suggesting that the sustained release might be an important parameter of the observed differences in sensitizing capacity. All previously published studies investigating ethosome formulations and skin penetration show an increased penetration/absorption of the encapsulated compound. For the first time it is now shown that an ethosome formulation of a compound (isoeugenol) inhibited the percutaneous penetration compared to a control formulation without the vesicles. Andersen et al. showed in 1985 that chlorocresol formulated in propylene glycol had a lower sensitization capacity compared to an acetone:olive oil formulation. Both formulations had the same bioavailability of chlorocresol in the skin after 24 hours but the authors did not distinguish between skin deposition and did not measure skin absorption (62). In 1996 Heylings et al investigated vehicle effects of dinitro-chloro-benzene formulated in acetone and propylene glycol and skin absorption in the Local Lymph Node Assay (72). They found an increased sensitizing capacity which correlated with an increased flux from 2 hours and onwards when dinitro-chloro-benzene was formulated in acetone compared to propylene glycol, the latter having lowest EC3% value. After 24 hours the total skin absorption was similar for the two formulations (17). Further, the percentage of the applied dose absorbed through the skin at 4 hours was substantially greater when dinitro-chloro-benzene was administered in acetone (17). For both vehicles, similar amounts of dinitro-chloro-benzene were found on top of the skin at 4 hours, but markedly less had penetrated into or beyond the skin when propylene glycol was used as the vehicle, suggesting that increased absorption at 4 hours may be more important than absorption profile after 24 hours. We found comparable fluxes from 2 to 8 hours for dinitro-chloro-benzene formulated in ethosomes and ethanol:water. Beyond 8 hours only a slight increase in

flux was seen for the ethosome formulation. On the contrary we found a significant decreased flux and lag-time when isoeugenol was formulated in ethosomes compared to ethanol:water resulting in a lower  $K_p$ .

Pendlington et al studied the sensitizer hexyl cinnamic aldehyde (HCA) in four different vehicles (73) of which three previously had been tested in the Local Lymph Node Assay (25) in an attempt to study the epidermal/dermal disposition of the allergen. The authors did, however, not correlate the skin deposition of HCA in the three vehicles to the EC3% values of HCA in the different vehicles. When correlating the sensitizing potency of HCA in the three vehicles (in order of increasing potency: AOO, PG and ethanol) and skin disposition of HCA, a consistent correlation was found between low EC3% value and high flux (0-6 hours) and high cumulative skin absorption, but not between low EC3% value and HCA deposition in stratum corneum, epidermis and dermis. This is largely consistent with Heylings findings that the flux is important but not with our findings.

In conclusion, there is no simple relationship between bioavailability, skin absorption and sensitizing capacity of contactallergens in different formulations. It appears that the first hours of skin penetration is decisive for sensitization development, In this study we focussed on 24 hours data for the skin deposition. It would be interesting to study allergen skin deposition from 0-8 hours. Ethosome formulations may affect allergen concentration deeper in the epidermis or dermis within this spectrum of time. New visualization techniques like confocal and two-photon microscopy allow real-time non invasive measurements of the penetration of fluorescent allergens in the different skin departments over time (27) and would be a suitable method for such studies. The time points of interest regarding penetration behaviour of allergens may be the first hours after topical application.

It has been stated that skin penetration/absorption of allergens is of only minor importance, foran extremely strong sensitizer like trimellitic anhydride with a  $\log P$  value of -2.5, because it would be considered too hydrophilic to penetrate readily (69). Vehicle effects have been studied extensively using the mouse Local Lymph Node Assay. No cases have been reported where a compound classified as a weak sensitizer in one vehicle was classified as a strong sensitizer in another vehicle (14;25;66). It has been suggested that the enhanced lymph node cell proliferative responses induced by dintro-

chloro-benzene when applied in sodium lauryl sulphate may be due to increased numbers of dendritic cells reaching the lymph nodes (74). Further, it has been postulated that the vehicle in which dinitro-chloro-benzene is delivered to the skin may influence cutaneous metabolism secondary to, or independent of, altered absorption kinetics (72). Presumably similar mechanisms could explain the consistent higher sensitising capacity found in the Local Lymph Node Assay when a lipophilic allergen is formulated in ethosomes compared to ethanol:water solution. The mechanisms of allergic contact allergy are complex and perhaps it is the unique combination of allergen and vehicle that determines the sensitizing and elicitation properties and not just the skin penetration/absorption characteristics of the allergen alone.

Formulating dinitro-chloro-benzene and isoeugenol in ethosomes increased the release time of the allergens from the dialysis bag (Figure 17). It took more than 1 h before the released amount of allergen from the ethosome formulation reached the amount of ethanol:water formulation. The speed of release of allergen from the formulation is perhaps more important than the speed of penetration when comparing sensitization properties in different vehicles. However, the exact mechanism of how a vehicle influences the sensitizing properties remains uncertain. The present study on two different allergens suggests that skin penetration properties on a wider scale (not just amount but also kinetics) are important parameters in relation to understanding the allergenicity of chemicals in various vehicles.

## **8. General discussion – Dermatitis related to exposure to products containing micro vesicles.**

The thesis contributes to the risk assessment of modern vehicle systems used in cosmetic and pharmaceutical products concerning contact allergy. Micro and nano vesicular dermal delivery systems as well as other carrier molecules have not been subject to a rigorous risk assessment concerning the effect on contact allergy before. Only two case reports have raised the possibility of increased allergenicity by incorporation of a contact allergen in vesicular dermal drug delivery systems.. However, patch tests with and without the liposomal formulation of propyl gallate were not performed (55), and no comparable vehicle was used in the case report of retinyl palmitate incorporated in polycaprolactone micro particles (petrolatum vs. water containing polycaprolactone particles) (51), so proof is absent.

It is important to emphasize that the selected allergens are only model allergens. It is unlikely that potassium dichromate encapsulated in liposomes should find its way into the market. On the other hand, the fragrance ingredient isoeugenol could very well be encapsulated in liposomes along with the preservative methyldibromoglutaronitrile added an active antiwrinkle compound like a vitamin A derivative.

### *Sensitization and elicitation*

For the first time a systematic controlled study characterizing the effect of encapsulating allergens in topical drug delivery systems with reference to the sensitizing and elicitation capacity of contact allergy has been performed. Four different contact allergens with different physico-chemical and sensitizing properties were investigated. Increased sensitization response was found in the Local Lymph Node Assay when dinitro-chloro-benzene and isoeugenol were encapsulated in ethosomes compared to formulations without ethosomes (75). Challenge experiments in sensitized volunteer patients using ethosomes loaded with methyldibromoglutaronitrile or isoeugenol showed enhanced patch test response compared to challenge tests with the same allergens in an ethanol:water (4:6)

formulation making the lipid vesicles the only difference between formulations (76). More classical vehicle effects on both sensitization and elicitation responses have previously been reported in experiments using the Local Lymph Node Assay and human volunteers as test subjects (14;26). However, the effect of new encapsulating vehicles on product allergenicity has not been studied systematically until now. An important conclusion is that not all micro particle delivery systems increase the sensitising capacity in the Local Lymph Node Assay. Polycaprolactone showed reduced sensitization when the lipophilic allergens (dinitro-chloro-benzene and isoeugenol) were encapsulated and no effect when the hydrophilic potassium dichromate was formulated in polycaprolactone micro particles. We cannot make general conclusions based on present results, but a trend is that formulation of hydrophilic allergens in the tested dermal delivery systems does not change the sensitizing capacity. No patch tests were performed with a hydrophilic allergen. Hydrophilic allergens do not bind to the lipid membrane but lipophilic allergens do, as seen from the encapsulation efficiencies in Table 6. Therefore, encapsulation efficiency seems to be an important parameter in risk assessment of sensitization.

Interpretation of the sensitising capacity of polycaprolactone and liposomes with encapsulated lipophilic allergens should be done with caution; because the compared vehicle is organic solution vs. the micro particles in water added 1% surfactant. An experiment comparing different concentrations of polycaprolactone and liposomes with the same amount of lipophilic allergen would give more accurate and detailed information of the impact of the delivery systems on the sensitization capacity. This would be an interesting study in the future. However, since the only difference between liposomes and ethosomes is ethanol, it would not be over interpretation to conclude, that liposomes also enhance the sensitizing and probably the elicitation capacity as well.

#### *Nanoparticles - Does size matters?*

The strict definition of nanotechnology deals with structures of the size of 100 nm or smaller. Unfortunately, conflicting definitions of nanotechnology and blurry distinctions between different scientific fields have complicated the area (77). In the cosmetic and pharmaceutical industry many products may carry the name 'nanotechnology', even if it is not nanotechnology in the original meaning of the word. It is the change in physical, chemical and biological properties when downsizing

particles that are of importance. Different size limits have been proposed for the term nanotechnology; from <100nm to < 1000nm(77;78). The EU Parliament and the Council of Ministers have accepted the definitions of <100nm used in the Opinion on nanotechnology and cosmetics from the Scientific Committee on Consumer Products. When considering the importance of vesicle size on dermal penetration and bioavailability of the encapsulated substance conflicting results exist.

Major parts of the particles in these experiments exceeded the 100 nm size limit for nanoparticles. However, particles larger than 100nm also may show size specific properties e.g. liposomes of 120nm penetrate human skin in greater extend compared to liposomes of 810nm (68). The results presented in Table 3 shows diverging results on the sensitizing capacity when reducing the sizes of vesicles. Liposomes of different sizes loaded with dinitro-chloro-benzene did not reveal a change in sensitizing capacity but smaller ethosomes did increase the sensitizing capacity compared to larger one. On the other hand, ethosomes of different sizes loaded with isoeugenol did not change the strength of patch test reaction in sensitized human volunteers. These conflicting results make it hard to conclude whether or not size is an important parameter of these encapsulating technologies when speaking about sensitization and elicitation. If particle size matters, it is probably of minor importance or it could be dependent on the combination of the specific particle and allergen.

A limited number of studies have tried to elucidate a possible correlation between sensitization and percutaneous absorption/penetration of contact allergens. Previous studies have shown that vehicle induced effects on the sensitizing capacity could be related to changes in the skin absorption or penetration of allergens (79-81). However, no clear correlation of dermal bioavailability, percutaneous absorption/penetration and sensitization has been found even though it seems reasonable that increased dermal penetration should result in increased sensitization due to better bioavailability of the allergens to the Langerhans cells. Ethosomes are in general believed to increase the bioavailability in the skin. The present results showed that even though the ethosome formulation of isoeugenol and dinitro-chloro-benzene increased the sensitizing capacity compared to control formulations, it could not be correlated to a change in percutaneous penetration or absorption pattern, since the two allergens showed opposite penetration behaviour. These findings correlate very well with Andersen's results of chlorocresol, which found no relationship between sensitization and bioavailability (62). Heylings

conclude that an increased flux might be responsible for increased sensitization, but also here our studies showed opposite results from the two allergens (72).

An interesting finding was that ethosome formulations also are capable of decreasing the skin deposition and percutaneous absorption, which has not been reported in the literature before.

## 9. Conclusion

The dermatotoxicologic risks from skin exposure to products using these carrier systems are considered low. No general rules can be concluded from the experiments presented and risk assessment should be done on a case by case basis. Given the limited information available it is important that dermatologists are aware of the use of encapsulation technology in products causing contact dermatitis, because encapsulation of product ingredients may affect allergenicity in some cases. It may be difficult to discover whether a product contains microvesicles if it is not mentioned on the label. Words like “nanosphere”, “liposome” and “encapsulated” can be looked for, but often not in the label but rather in the marketing folder. The website “[www.nanotechproject.org](http://www.nanotechproject.org)” registers consumer products using nanotechnology and different carrier technologies based on information from the manufacturers or other sources. The list is far from complete but can be helpful. Dermatologists investigating patients with allergic reactions to consumer products using encapsulation technologies should consider the risk of false negative results, if testing with ingredients in conventional patch test vehicles. It is important to collaborate with the manufacturer. Sometimes they can provide the dermatologist with samples of encapsulated compounds for patch testing (51). If these new formulation systems really pose a risk for consumers regarding development of allergic skin reactions from use of topical products containing this technology, is so far not documented but experimental data shows that it is possible. Dermatologists are incited to look for dermatitis patients with possible allergic skin reactions from topical products using nano- or microvesicle technologies, and be aware of the importance of patch test vehicle.

## **10. Future perspectives**

The increased use of vesicle systems in topical skin products requires a continuous search for possible dermatotoxicological side effects, e.g. enhanced skin penetration and development of cutaneous allergy to product ingredients encapsulated. The thesis focuses on few types of encapsulating vehicles. Other test designs are possible for investigating interaction between encapsulation technologies in animal and man. More dose response studies are needed. Further, new vehicle systems should be tested when they enter the market. It is important that independent researchers in the field of contact allergy continue to investigate vehicle effects. Also clinical dermatologist should look for new possible vehicle effects when they do patch testing.

It seems obvious that the degree of penetration of an allergen is an important parameter in the sensitizing and elicitation phases of contact allergy. Much work needs to be done here, since the few studies that exist show conflicting results. In order to investigate the penetration factor of sensitization, two-photon microscopy and confocal microscopy could be useful methods, since they allow for a non-invasive measurement of fluorescent contact allergens like rhodamine B isothiocyanate, which makes it possible to follow the allergen over time when it penetrates the skin. These techniques also make it possible to show exactly what happens to the vesicular carrier systems and might answer following important questions: For how long time are the vesicles intact? Do they break down into its constituents on the skin or does this happen deeper in the skin? Does the size of the vesicles play a role in the depth of penetration of the active compound? All these important questions may inspire for additional research projects.

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## **12. Papers I-V**

## INVESTIGATIVE REPORT

# Ethosome Formulations of Known Contact Allergens can Increase their Sensitizing Capacity

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Vesicular systems, such as liposomes and ethosomes, are used in cosmetic and pharmaceutical products to encapsulate ingredients to protect ingredients from degradation, to increase bioavailability, and to improve cosmetic performance. Some reports have suggested that formulation of cosmetic ingredients in vesicular carrier systems may increase their contact allergy elicitation potential in humans. However, no sensitization studies have been published. We formulated two model contact allergens (isoeugenol and dinitrochlorobenzene) in ethosomes and investigated the sensitization response using a modified local lymph node assay (LLNA). The results were compared with those for the same allergens in similar concentrations and vehicles without ethosomes. Both allergens encapsulated in 200–300 nm ethosomes showed increased sensitizing potency in the murine assay compared with the allergens in solution without ethosomes. Empty ethosomes were non-sensitizing according to LLNA. The clinical implications are so far uncertain, but increased allergenicity from ethosome-encapsulated topical product ingredients cannot be excluded. **Key words:** skin sensitization; contact dermatitis; liposomes; ethosomes; local lymph node assay.

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Liposomes and ethosomes are used in cosmetic products to increase delivery of certain ingredients to the skin with the purpose of enhancing an alleged effect and/or to protect the ingredients from degradation. Increased biological effects of topical drugs formulated in different kinds of liposomes have been reported; for example, acyclovir encapsulated in ethosomes demonstrated improved clinical efficacy in herpes simplex treatment compared with conventional formulation (1), and methotrexate encapsulated in niosomes showed increased clinical efficacy compared with placebo (2). Other promising clinical results have been obtained with liposome-en-

capsulated drugs in the treatment of acne, xerosis, atopic dermatitis, vitiligo, and superficial thrombophlebitis, and demonstrate the possibilities for liposome formulations in dermatology (1, 3–9). Whether encapsulation of chemicals in liposomes and other vesicular systems affects the allergenicity of product ingredients is not documented. Few clinical reports raise this question. Propyl gallate incorporated in liposomes has been suggested to boost the allergic potential in 13 patients. However, patch tests with and without the liposomal formulation were not performed (10). Furthermore, a case report described a woman developing severe allergic contact dermatitis from an anti-wrinkle cream containing retinyl palmitate encapsulated in polycaprolactone (PCL) (11). PCL is a polymeric carrier system capable of encapsulating lipophilic and hydrophilic agents. Retinyl palmitate is a rare contact allergen, and diagnostic patch tests have revealed that the patient reacted more strongly to encapsulated retinyl palmitate than to retinyl palmitate in petrolatum, even though the retinyl palmitate concentration was much lower in PCL compared with the petrolatum preparation. The size of the PCL particles was larger than 100 nm (11).

Liposomes are spherical vesicles with membranes consisting of one (unilamellar) or more (oligolamellar, multilamellar) bilayers of polar lipids, e.g. phosphatidylcholine (POPC). Liposomes are able to encapsulate hydrophilic molecules in the aqueous core and incorporate lipophilic molecules in the lipid bilayer (Fig. 1). The skin penetration properties of liposomes depend on modifications in size and composition of the vesicles, e.g. by adding different chemicals into the bilayer, such as cholesterol, surfactants and ethanol (12). Vesicles consisting of pure lipids are often referred to as “liposomes”, whereas they are called flexosomes or transfersomes when surfactants and/or cholesterol are added in the bilayer, and ethosomes when ethanol is added. Formulating certain chemicals in ethosomes may increase skin penetration compared with transfersomes, while liposomes are believed not to penetrate the stratum corneum (13–15). How these vesicles behave once applied to the skin is not known, but different scenarios have been proposed. The vesicles can act as drug carriers controlling release of the encapsulated agent, provide a localized depot on the

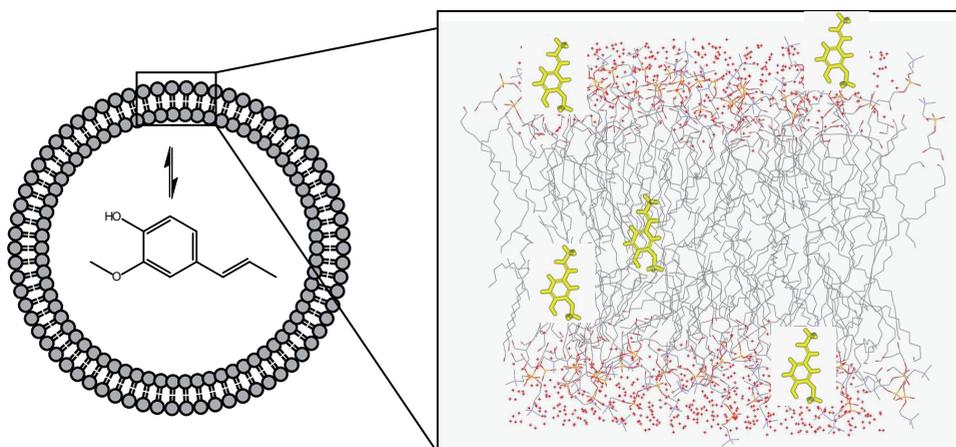


Fig. 1. Model of isoeugenol (yellow) encapsulated in an ethosome. *Left-hand figure*: schematic representation of a unilamellar liposome. *Right-hand figure*: enlarged view from a coarse-grain computer simulation of phosphatidylcholine (POPC). The membrane patch represents a  $45 \times 50$  Å area from the published coarse grain simulation data. The temperature was set to 20°C. Since the solution contains 40% ethanol, an equilibrium of the lipophilic allergen isoeugenol is established between the POPC membrane and the inner and outer fluid.

skin, or provide delivery to the skin appendages (hair and follicles and sweat glands). Some liposomes may possess more of the above-mentioned characteristics, depending on the constituents of the liposomes and the encapsulated compound (16).

The level and degree of sensitization in experimental contact allergy depends on the potency of the allergen and the induction dose. Furthermore, the vehicle is of major importance, both in the sensitization and the elicitation phase, as documented previously (17, 18). No simple correlation exists between the skin absorption of the allergen and the degree of sensitization and elicitation (17).

The present study is based on the hypothesis that formulation of contact allergens in drug delivery systems may affect the sensitizing potential. Ethosomes were selected as the carrier system because they contain ethanol, thus allowing research into lipophilic allergens in water/ethanol mixtures with and without the phospholipids. According to previous studies with ethosomes loaded with lipophilic compounds, the lipophilic compound is located both on/in the lipid bilayer as well as in the core (19).

A modified murine local lymph node assay (LLNA) was chosen as the sensitization test. The skin sensitization response is determined by measuring the cell proliferation in the draining lymph nodes as a function of concentration after topical application of test compounds. Two potent model allergens (isoeugenol and dinitrochlorobenzene (DNCB)) were selected to test our hypothesis, as only limited amounts of allergen can be associated with the ethosomes.

## METHODS

### *Sensitization experiments*

The standard LLNA assay was modified by use of water-ethanol (6:4) as a vehicle for comparison between encapsulated and dissolved allergen, making the ethosomes the only difference between these two test materials. The lymph node cell proliferation was determined for each animal and expressed as mean disintegrations per minute (dpm) (20). Female CBA/Ca mice purchased from Harlan (Horst, The Netherlands), 8 weeks

old, were housed in cages with HEPA-filtered airflow under conventional conditions in light-, humidity- and temperature-controlled rooms with *ad lib* food and water. The animals were allowed to acclimatize for one week prior to the study. The experiments were carried out in accordance with Danish and European animal welfare regulations and were licensed by the Danish Animal Experimentation Inspectorate.

### *Ethosome preparation*

Ethosomes with isoeugenol (CAS No. 97-54-1) (Aldrich, Brøndby, Denmark) or dinitrochlorobenzene (DNCB) (CAS No. 97-00-7) (Sigma-Aldrich, Denmark) were prepared as described by Touitou (19). Briefly, POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) (Avanti Polar Lipids, Alabaster, USA) was dissolved in 96% ethanol containing isoeugenol or DNCB, and MilliQ water was added slowly to a final concentration of 40% (v/v) ethanol under magnetic stirring (700 rpm) at 30°C. The suspension was stirred for 5 min and then extruded 10 times through two polycarbonate filters with a pore size of 200 nm using a Lipex® Extruder (Northern Lipids Inc., Burnaby, Canada).

To study the effect of ethosome formulation the following control solutions were prepared: dinitrochlorobenzene (DNCB) or isoeugenol was dissolved in ethanol, whereafter MilliQ water was added to a final concentration of 40% (v/v) ethanol. Furthermore, another experiment was performed with DNCB in ethanol/water (4:6) solution with POPC added to investigate the effect of the lipid without subsequent extrusion of ethosomes.

The concentration of isoeugenol and DNCB in experimental solutions was determined by high-performance liquid chromatography (HPLC). Allergen concentration was measured by HPLC to ensure the allergen concentration matched the ethosomal formulation (see figures).

A further experiment was performed to investigate varying amounts of POPC in order to study the impact of ethosomal concentration by adding 20, 40 and 60 mg POPC to the same volume of isoeugenol-ethanol solution. The ethosome preparation was compared with a 4:6 ethanol/water solution made from the same batch of isoeugenol in ethanol. The concentration of isoeugenol was not measured by HPLC in the POPC dose-response experiment. The formulations were kept in the darkness at 5°C and all preparations were made on the same day or the day before the LLNA experiment.

### *Characterization of ethosomes*

Hydrodynamic particle diameters and polydispersity index (PI) of ethosomes, which describes the size distribution of the

particles, were determined by dynamic light scattering (DLS) using a BI-200SM instrument from Brookhaven Instruments (Holtville, USA). This incorporates a 632.8 nm HeNe laser operated at a fixed scattering angle of 90°. A 20 µl volume of ethosome-solution was diluted in 1.5 ml ethanol-MilliQ water (40%). The measurements were conducted in triplicate in a multimodal mode of 180 s. The size of ethosomes was measured on the day of preparation and directly after the experiment.

#### Encapsulation efficiency

The encapsulation efficiency (EE%) of isoeugenol and DNCB by ethosomal vesicles was determined by ultracentrifugation, as described by Heeremans et al. (21), and later used on ethosomal systems by Tuitou et al. (19). Ethosomal preparations containing DNCB or isoeugenol were kept overnight at 5°C, whereafter they were spun at 40,000 rpm for 3 h in a Hitachi Sorvall Discovery 90SE ultracentrifuge with a swing-out rotor from Sorvall (Breda, The Netherlands) (SW50.1). The supernatant was removed immediately and drug quantity was determined in both the sediment and the supernatant. Binding efficiency was calculated as follows:  $[(T-C)/T]*100$ , where T is the total amount of chemical detected in both the supernatant and sediment, and C is the amount of chemical detected only in the supernatant. The procedure was performed in triplicate.

#### Quantification of isoeugenol and DNCB in ethosomes

HPLC analysis was conducted on an Ultimate 3000 series from Dionex® (Hvidovre, Denmark) with a diode array detector. A Dionex® RP-18 Acclaim 300 C18 reversed phase column was used. The temperature of the column and the sample rack in the autosampler was set to 20°C. Mobile phase: 75% methanol, 25% MilliQ water; isocratic elution for 30 min; and flow rate of 1 ml/min. The separations were monitored at 270 nm. The injection volume was 10 µl. Pure reference compounds were used to make external calibration curves from which the concentrations of DNCB and isoeugenol were determined.

#### Statistical data analysis

Results are expressed as means ± standard error of mean (SEM). Statistically significant differences in the isoeugenol and DNCB experiments were determined using one-way analysis of variance (ANOVA) and Student-Newman-Keuls test for *post hoc*

analysis with  $p < 0.05$  as a minimal level of significance. We used the statistical software package: Graphpad Prism 4 from GraphPad Software Inc (San Diego, CA, USA).

## RESULTS

The LLNA experiments showed a significantly increased sensitization from isoeugenol-loaded ethosomes compared with isoeugenol dissolved in ethanol/water (Fig. 2A). Isoeugenol concentration in all formulations was 1.1% w/v. The experiment was repeated twice with equivalent results. A significantly increased sensitizing capacity was also found for ethosomes loaded with DNCB (0.03% w/v) compared with DNCB in the aqueous-ethanol solution and empty ethosomes (Fig. 2B). The dose of ethosomes was another important factor as there was a linear dose-response relationship between concentration of ethosomes and the sensitization obtained, reaching a significant level at 60 mg/ml POPC (Fig. 3). The formation of ethosomes had a significant enhancing effect on sensitization with DNCB compared with DNCB in the ethanol/water/POPC solution without extrusion (Fig. 4).

Vesicle size measured before and after experiments remained stable for the duration of the experiment. All ethosomes were between  $210 \pm 8$  and  $317 \pm 30$  nm and polydispersity index ranged from 0.09 to 0.20 can be regarded as monodispersed. All batches showed an increase in PI of approximately 0.05 over the three experimental days. The encapsulation efficiency of isoeugenol into ethosomes was  $24 \pm 6\%$  and into DNCB  $18 \pm 1\%$ .

## DISCUSSION

These results indicate that contact allergens encapsulated in ethosomes can show enhanced sensitizing capacity compared with the same allergen concentra-

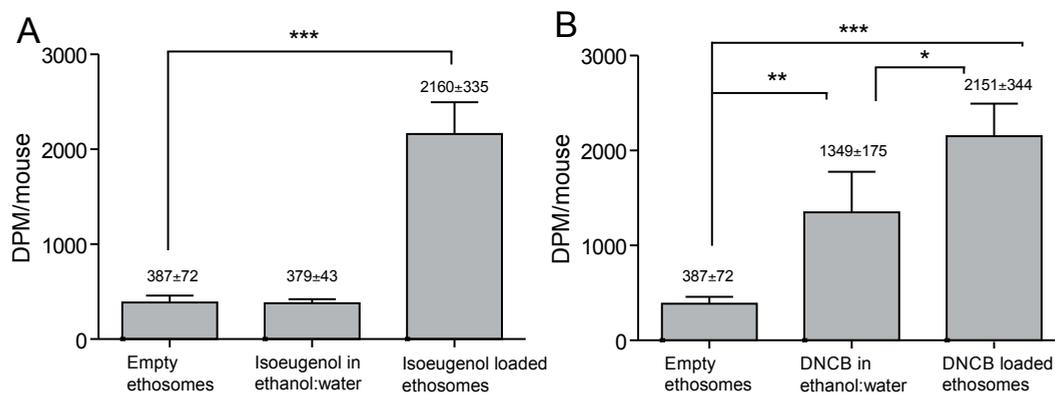


Fig. 2. Encapsulation of isoeugenol and dinitrochlorobenzene (DNCB) in ethosomes increases their sensitizing capacity. (A) Isoeugenol (1.1% w/v) loaded ethosomes (60 mg/ml) significantly increase the sensitizing capacity in the local lymph node assay compared with empty ethosomes and isoeugenol dissolved in ethanol/water (4:6).  $*p < 0.05$  ( $n = 6$  in each group). (B) DNCB (0.03% w/v) loaded ethosomes (60 mg/ml) significantly increase the sensitizing capacity compared with DNCB in an ethanol/water solution ( $*p < 0.05$ ) and compared with empty ethosomes ( $***p < 0.001$ ). DNCB in ethanol/water (4:6) significantly increases the sensitizing capacity compared with empty ethosomes ( $***p < 0.01$ ). Results are presented as mean ± standard error of the mean of disintegrations per minute (dpm) per mouse ( $n = 6$  in each group).

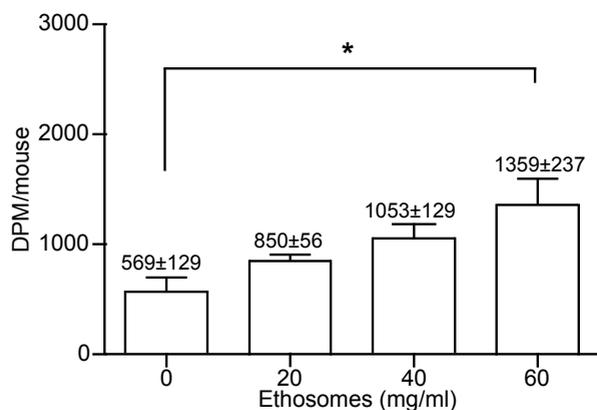


Fig. 3. Encapsulated isoeugenol in ethosomes reaches a significant sensitizing potency by increasing the amount of phosphatidylcholine (POPC) to 60 mg/ml ethosomes compared with the control vehicle (0 mg/ml POPC) in the local lymph node assay (LLNA). \* $p < 0.05$  ( $n = 5$  in each group) (Mean  $\pm$  standard error of the mean).

tion in solution. Encapsulated isoeugenol in ethosomes showed repeatedly, significantly increased sensitization in a modified LLNA compared with isoeugenol in solution. Isoeugenol has previously been tested in the LLNA in different vehicles. The EC<sub>3</sub> values (estimated concentration reduced to produce a stimulation index of 3) obtained were 0.9 (dimethylsulphoxide), 1.5 (acetone/olive oil), 1.8 (water/ethanol 1:9), 2.5 (propylene glycol), and 4.9 (water/ethanol 1:1) (22). The dose of isoeugenol (1.1%) in the present experiments was selected due to limited solubility of isoeugenol in the ethanol/water solution. Higher concentrations were not possible due to instability of the ethosome formulation with change in vesicle size and PI. The isoeugenol concentration is thus below the EC<sub>3</sub> values reported from other LLNA experiments with isoeugenol using ethanol/water as vehicle. In accordance with this,

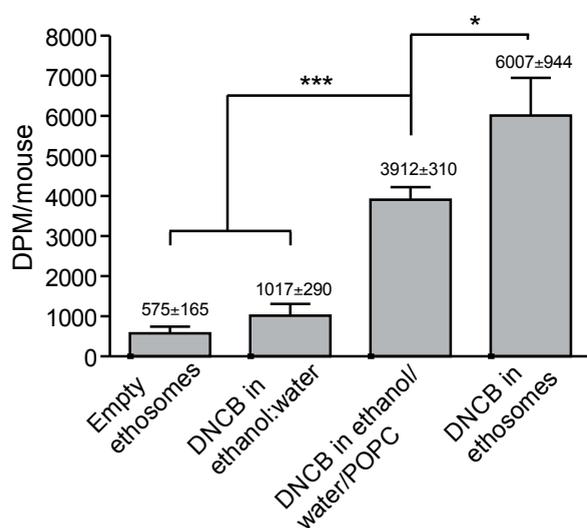


Fig. 4. Encapsulated dinitrochlorobenzene (DNCB) (0.04% w/v) in ethosomes increases the sensitizing capacity significantly (\* $p < 0.05$ , \*\*\* $p < 0.001$ ) compared with DNCB in ethanol/water/phosphatidylcholine (POPC) solution. ( $n = 4-6$  in each group) (Mean  $\pm$  standard error of the mean).

isoeugenol did not sensitize in the solution, only in the ethosome formulation. DNCB is a more potent allergen, which permitted a concentration above its EC<sub>3</sub> value. DNCB 0.03% (w/v) showed stronger sensitization in the aqueous-ethanolic solution compared with empty ethosomes and the sensitization was further enhanced when formulated in ethosomes. However, the presence of POPC in the DNCB ethanol/water solution (0.04%) without extrusion of ethosomes also had an enhancing effect on sensitization (Fig. 4) compared with the ethanol/water solution.

The vehicle effect on the sensitizing capacity differs between allergens, but the exact mechanism is unclear (18). Skin penetration appears not to be the major factor in the guinea pig maximization test (17) and the relationship between the percutaneous absorption and the extent to which sensitization is induced is still unclear in the LLNA, even though the rate of skin penetration appears to be important (23).

Skin penetration properties of vesicular systems depend on physicochemical characteristics of the vesicles, and chemicals in vesicular systems may use varying pathways through the epidermis (24). In order for a contact allergen to sensitize an individual, close contact with dendritic cells is necessary, as would be expected to occur in damaged and eczematous skin, while penetration is less pronounced through normal skin. Hair follicles may represent a shunt that allows efficient and fast penetration through the skin barrier for encapsulated compounds (25-27). It has been suggested that encapsulation of possible allergens protects against sensitization (28), but this was not the case in the present experiments.

The term encapsulation or entrapment is often used in the literature, although true encapsulation probably occurs very little in these vesicular formulation systems, since they to some extent are dynamic systems that aim to obtain equilibrium between encapsulated and non-encapsulated compound (21). Therefore, the ethosome formulation contains encapsulated and non-encapsulated compound. Furthermore, there appears to be a synergistic effect on enhancement of drug penetration through the skin between non-entrapped and entrapped drug compared with entrapped drug alone (29). Heeremans et al. (21) stated that the term encapsulation or entrapment should be interpreted as binding or association of the chemical to the lipids.

The size of the vesicle carriers may also be important, since decreasing liposomal size may increase the concentration of encapsulated substance in the skin (12). However, this was not studied here due to difficulties in producing stable ethosomes in different sizes. The conclusion of the present study is that formulation of chemicals in vesicular carrier systems can enhance the sensitizing capacity. This may be of particular importance for weaker allergens. Further research is

needed to clarify the clinical implications for topical drugs and cosmetics.

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*The authors declare no conflict of interest.*

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ORIGINAL ARTICLE

# Encapsulating contact allergens in liposomes, ethosomes, and polycaprolactone may affect their sensitizing properties

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## Abstract

Attempts to improve formulation of topical products are a continuing process and the development of micro- and nanovesicular systems as well as polymeric microparticles has led to marketing of topical drugs and cosmetics using these technologies. Encapsulation of some well-known contact allergens in ethanolic liposomes have been reported to enhance allergenicity compared with the allergens in similar vehicles without liposomes. The present report includes data on more sensitization studies using the mouse local lymph node assay with three contact allergens encapsulated in different dermal drug-delivery systems: liposomes, ethosomes, and polycaprolactone particles. The results show that the drug-delivery systems are not sensitizers in themselves. Encapsulating the hydrophilic contact allergen potassium dichromate in all three drug-delivery systems did not affect the sensitizing capacity of potassium dichromate compared with control solutions. However, encapsulating the lipophilic contact allergen dinitrochlorobenzene (DNCB) in polycaprolactone reduced the sensitizing capacity to  $1211 \pm 449$  compared with liposomes ( $7602 \pm 2658$ ) and in acetone:olive oil (4:1) ( $5633 \pm 666$ ). The same trend was observed for encapsulating isoeugenol in polycaprolactone ( $1100 \pm 406$ ) compared with a formulation in acetone:olive oil ( $4491 \pm 819$ ) and in liposomes ( $3668 \pm 950$ ). Further, the size of DNCB-loaded liposomes did not affect the sensitizing properties. These results suggest that modern dermal drug-delivery systems may in some cases magnify or decrease the sensitizing capacity of the encapsulated contact allergen.

**Keywords:** Encapsulation, liposomes, sensitization, local lymph node assay, polycaprolactone, ethosomes, allergen

## Introduction

Micro- and nanosized vesicles and different kinds of polymeric microparticles are used in topical products to encapsulate pharmaceutical and cosmetic ingredients in order to improve their efficiency. Different lipid-based vesicular systems were developed with different properties depending on composition, for example, by adding ethanol or different surfactants into the bilayer (1,2). Vesicles consisting of pure lipids are often referred to as liposomes, whereas they are called ethosomes when ethanol is added, and flexosomes or transfersomes when surfactants and/or cholesterol are added in the bilayer, but this terminology is far from consistent. Changing the length of the lipid molecules and different coatings for the vesicles also changes their

properties. Owing to the destabilizing effect of ethanol on lipid bilayers, it was thought that high concentrations of ethanol were destructive to liposomal structures. However, the existence of vesicles as well as the ethosome structure was demonstrated by several techniques including proton-decoupled nuclear magnetic resonance, transmission electron microscopy, and scanning electron microscopy, and the vesicles show a unimodal size distribution (3). Polymeric particles like polycaprolactone and solid-lipid nanoparticles are also capable of encapsulating chemical compounds for topical delivery to the skin and such products are on the market (4,5).

Increased treatment effects of topical products with encapsulated drugs have been reported in a clinical

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trial for herpes simplex, psoriasis, acne, xerosis, atopic dermatitis, vitiligo, and superficial thrombophlebitis (6–13). The expected benefits of such formulations include improved bioavailability, protection of ingredients from oxidization and photodegradation, and in some cases reduced skin irritancy (6). Further, a precise drug delivery to target cells may allow for a reduction of the concentration of an active ingredient as reported for 5-aminolevulinic acid (5-ALA) formulated in 50 nm liposomes for photodynamic therapy for treatment of acne. The liposomes concentrate in the pilosebaceous units thereby reducing the side effects, which open doors for new treatment modalities (14).

Encapsulation of contact allergens may also affect allergenicity as suggested in case reports (15,16). A woman developed severe allergic contact dermatitis from an anti-wrinkle cream containing retinyl palmitate encapsulated in polycaprolactone. Retinyl palmitate is a rare contact allergen, and diagnostic patch tests revealed that the patient reacted more strongly to encapsulated retinyl palmitate compared with retinyl palmitate in petrolatum, even though the retinyl palmitate concentration was much lower when formulated in polycaprolactone compared with the petrolatum formulation (15). Polycaprolactone nanoparticles loaded with the lipophilic dye Nile Red have shown enhanced penetration of the molecule into the stratum corneum layers (up to 60  $\mu\text{m}$ ), compared with non-nanoparticle formulation (17).

Experimental studies using the mouse local lymph node assay (LLNA) for sensitization experiments showed that encapsulation of dinitrochlorobenzene (DNCB) and isoeugenol in ethosomes enhanced the sensitizing capacity compared with an ethanol:water (4:6 v/v) formulation (18). Volunteer patients showed in a clinical study that isoeugenol and methyl dibromoglutaronitrile encapsulated in ethosomes significantly enhanced the patch test reactions in sensitized volunteers (19). The present report describes further LLNA studies with more allergens and other drug-delivery systems. This is important due to the lack of knowledge of sensitization properties for these new encapsulating technologies used increasingly in cosmetics and pharmaceutical products (20). Results from previously published experiments are included in the result figures (18).

## Materials and methods

Two clinically important common contact allergens (the hydrophilic potassium dichromate and the lipophilic isoeugenol) were selected as model allergens due to their documented sensitizing properties, and in order to test a lipophilic and a hydrophilic allergen. Further, DNCB was included as an extreme experimental sensitizer because it limited how much allergen can be encapsulated in the drug-delivery systems.

## Preparation of test solutions

### *Ethosomes*

Ethosomes with encapsulated potassium dichromate were prepared as described by Touitou et al. (3). In brief, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) (Avanti Polar Lipids, Alabaster, USA) was dissolved in 96% ethanol and Milli-Q water containing potassium dichromate (analytical grade; Alfa Aesar, London, UK, CAS 7778-50-9) was added slowly to a final concentration of 40% (v/v) ethanol under magnetic stirring (700 rpm). The final concentration of the allergen was measured by high-performance liquid chromatography (HPLC). In case of empty ethosomes for control measurements, water without potassium dichromate was added to the POPC/ethanol solution. The suspension was stirred for 5 min and then extruded 10 times through two polycarbonate filters with a pore size of 50, 100, or 200 nm using a Lipex<sup>®</sup> Extruder (Northern Lipids Inc.). Empty ethosomes and an ethanol:water solution (4:6 v/v) of a corresponding concentration of allergen were used as control transport vehicles.

The isoeugenol (Aldrich, Denmark; CAS 97-54-1)- or DNCB (CAS 97-00-7; Sigma-Aldrich, Denmark)-loaded ethosomes were manufactured using the same techniques with slight modifications. POPC was dissolved in 96% ethanol containing isoeugenol or DNCB and Milli-Q water was added slowly to a final concentration of 40% (v/v) ethanol under magnetic stirring (700 rpm). In case of empty ethosomes for control measurements, ethanol without dissolved allergen was used.

### *Liposomes*

Liposomes loaded with DNCB or isoeugenol were made by the thin film method (21). In brief, POPC and isoeugenol or DNCB were dissolved in chloroform and methanol (2:1 v/v) in a 250-mL round-bottomed flask. The mixture was evaporated in a rotary evaporator above the transition temperature of the phospholipids to remove solvents under vacuum and producing a thin film in the flask consisting of POPC. The thin film was hydrated with Milli-Q water for 30 min. The vesicle suspension was extruded through a 50, 100, or 200 nm polycarbonate filter 10 times using the Lipex<sup>®</sup> Extruder to make a homogeneous size distribution of the vesicles. A non-extruded control solution containing larger vesicles of different sizes was also made for comparison. In manufacturing potassium dichromate-loaded liposomes, the POPC was dissolved in chloroform and methanol (2:1 v/v), solvents were evaporated under reduced pressure and afterward hydrated with Milli-Q water containing potassium dichromate, and the solution was extruded through a filter as described above. Allergen concentration was determined by HPLC.

### *Polycaprolactone microparticles*

Polycaprolactone particles loaded with DNCB or isoeugenol were manufactured based on the solvent displacement process (22). In brief, polycaprolactone (CAS

2498-41-4; Aldrich, Denmark) and a lipophilic allergen (DNCB or isoeugenol) were dissolved in 125 mL acetone at 45°C, and this organic phase was injected into 125 mL Milli-Q water containing 0.17 g Pluronic F-68™ (CAS 9003-11-6; Aldrich, Denmark) in a round-bottomed flask under magnetic stirring (1200 rpm) at room temperature. Acetone and the aqueous phase were reduced to 5 mL under reduced pressure. Allergen concentration was measured by HPLC. All formulations were kept at 5°C. In case of empty polycaprolactone particles, no allergen was dissolved in the organic phase, but otherwise the same procedures were followed.

In case of potassium dichromate-loaded polycaprolactone particles, the polymer was dissolved in the organic phase and injected into an aqueous phase containing potassium dichromate and Pluronic F-68™. Otherwise, the same procedures as described above were followed.

To ensure sufficient contact with the skin in the LLNA, a surfactant (polyethylene glycol–polypropylene glycol, CAS 9003-11-6; Aldrich, Denmark) was added to liposomes and polycaprolactone particle batches to a final concentration of 1% (v/v) immediately before each LLNA experiment.

To document vesicle size, stability, and encapsulation efficiency (EE), the following methods were used.

**Dynamic light scattering** Hydrodynamic particle diameters and polydispersity index (PI) of ethosomes were determined by dynamic light scattering (DLS) using a BI-200SM from Brookhaven Instruments. This incorporates a 632.8 nm HeNe laser operated at a fixed scattering angle of 90°. A sample of 10 µL was diluted in 1190 µL 40% ethanol:Milli-Q water mixture or pure Milli-Q water dependent of the original vehicle. The authors are aware that the dilution may change the microstructure of the vesicles, but the dilution was necessary to allow for sufficient amount of light to pass the test solution. The measurements were conducted in triplicate, in a multimodal mode of 120 sec. The size of particles was measured before and after the LLNA experiment.

**Encapsulation efficiency** The EE% of allergens formulated in polycaprolactone, ethosomes, and liposomes was determined by ultracentrifugation as described by Heeremans et al. (23). Ethosomal, polycaprolactone, and liposomal preparations containing DNCB, isoeugenol, or potassium dichromate were kept 12 h at 5°C and thereafter spun at 80.640 g for 3 h in an Hitachi Sorvall Discovery 90SE ultracentrifuge with a swing-out rotor from Sorvall (SW50.1). The supernatant was immediately removed and drug quantity was determined in both the sediment and the supernatant. Binding efficiency was calculated as follows:  $[(T-C)/T] \times 100$ , where  $T$  is the total amount of chemical detected in both the supernatant and sediment, and  $C$  is the amount of chemical detected only in the supernatant. The procedure was done in triplicates.

**Quantification of isoeugenol, DNCB, and potassium dichromate** HPLC analysis was conducted on an ultimate 3000 series from DIONEX™ with a diode array detector. A DIONEX Acclaim® Surfactant column was used to separate isoeugenol and DNCB. Potassium dichromate was separated using a DIONEX Acclaim® 300 C18 column. The temperature of the column and the sample rack in the thermostated autosampler was set to 20°C. Mobile phase used for DNCB and isoeugenol: 75% methanol, 25% Milli-Q water; isocratic elution for 30 min; and flow rate of 1 mL/min. The separations were monitored at 270 nm. Mobile phase used for potassium dichromate consisted of 20% methanol for 10 min followed by a linear gradient of 90% methanol performed over 5 min, followed by 40 min of 100% methanol to wash the column and 5 min of 20% methanol to equilibrate the column for the next run. Potassium dichromate was monitored at 260 nm. Pure reference compounds were used to make external calibration curves from which the concentrations of allergen were determined.

**Sensitization experiments** The LLNA was performed according to standard procedure (24) with the exception that the lymph node cell proliferation was determined for each animal and expressed as mean disintegrations per minute (dpm). The scintillation count data for each group were analyzed statistically. No EC3 values were calculated. Female CBA/Ca mice purchased from Harlan (the Netherlands), 8 weeks of age, were housed in cages with hepa-filtered airflow under conventional conditions in light-, humidity-, and temperature-controlled rooms with food and water *ad libitum*. Test substances were applied on the dorsum of both ears of each mouse for three consecutive days. On Day 5, [methyl-<sup>3</sup>H]-thymidine was injected in the tail vein and after 5 h the mice were sacrificed and the draining lymph nodes were removed. A single cell suspension from each mouse was made and after two washing procedures with phosphate-buffered saline, the DNA was precipitated with trichloroacetic acid for 18 h and the thymidine incorporation was measured using β-scintillation counting. The standard vehicle in the LLNA is acetone:olive oil (4:1 v/v), which dissolves polycaprolactone, ethosomes, and liposomes. Therefore, we modified the LLNA by using either water:ethanol (6:4 v/v) as control vehicle for ethosomes or water-added 1% surfactant as control vehicle for liposome and polycaprolactone batches loaded with hydrophilic allergens making the drug-delivery system the only difference between batches. Lipophilic allergens were dissolved in ethanol:water (4:6 v/v), propylene glycol (PG, analytical grade, CAS 57-55-6; Riedel-de Haën, Seelze, Germany) or acetone:olive oil (acetone, analytical grade purchased from Aldrich, Denmark, CAS 67-64-1 and olive oil purchased from Fluka, Denmark, CAS 8001-25-0) making the comparison with the drug-delivery systems less comparable. The experiments were in accordance with Danish and European animal welfare regulations and were licensed by the Danish Animal Experimentation Inspectorate.

### Statistical data analysis

Results are expressed as mean  $\pm$  standard deviation. Statistically significant differences in the experiments were determined using one-way ANOVA and Student-Newman-Keuls test for post hoc analysis with  $P < 0.05$  as a minimal level of significance.

### Results

Empty ethosomes, liposomes, polycaprolactone particles, and the surfactants were not sensitizers themselves in the LLNA (Figures 1–3 and Table 1).

Ethosomes, liposomes, and polycaprolactone vesicles encapsulated with potassium dichromate showed no significant effect on the sensitizing capacity compared with potassium dichromate in ethanol:water or Milli-Q water with 1% surfactant added (Figure 1).

The lipophilic contact allergens DNCB encapsulated in polycaprolactone reduced the sensitizing capacity to  $1211 \pm 449$  dpm compared with DNCB encapsulated in liposomes ( $7602 \pm 2658$  dpm) and DNCB formulated in acetone:olive oil ( $5633 \pm 666$  dpm) (Figure 2). The same trend was observed when encapsulating isoeugenol in polycaprolactone ( $1100 \pm 406$  dpm) compared with isoeugenol in acetone:olive oil ( $4491 \pm 819$ ) and in liposomes ( $3668 \pm 950$ ) (Figure 3). In contrast, isoeugenol ( $2343 \pm 533$  dpm vs  $641 \pm 349$  dpm) and DNCB ( $2151 \pm 925$  dpm vs.  $1349 \pm 443$  dpm) in ethosomes showed a

significant increased sensitizing capacity compared with formulations without ethosomes (Figures 2 and 3) (18). It is noteworthy from Figures 2 and 3 that liposomes gave higher sensitization responses than PG and polycaprolactone particles.

The size of DNCB-loaded liposomes (50, 100, 200 nm, and non-extruded) did not affect their sensitizing capacity (Figure 2).

The surfactant was not a sensitizer itself but the sensitizing capacity of potassium dichromate increased by a factor of 2 (from 7372 to 15737 dpm) when doubling the surfactant concentration (Table 1).

The size of the particles ranged from 65 to 2466 nm in case of liposomes, 245 to 436 nm for the ethosomes, and 231 to 343 nm for the polycaprolactone particles (Table 2). The sizes of the particles were stable during the study and the PI was below 0.17, which can be regarded as monodisperse (except for the non-extruded liposomes). Every DLS measurement resulted in a unimodal size distribution curve. Encapsulation efficiencies of the hydrophilic potassium dichromate range from 0.7% in polycaprolactone to 16% in ethosomes as shown in Table 2. The lipophilic allergens (DNCB and isoeugenol) showed increased encapsulation efficiencies compared with the hydrophilic potassium dichromate, with liposomes being the best to retain the allergens (92% for DNCB and 98% for isoeugenol) compared with polycaprolactone (83% for DNCB and 84%

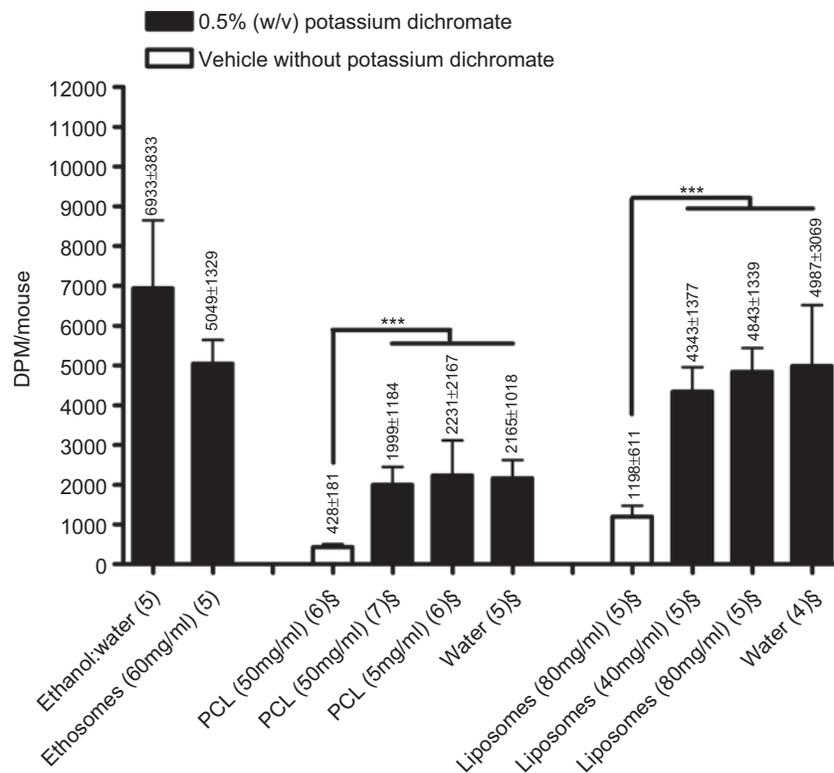


Figure 1. Results of local lymph node assay experiments performed with potassium dichromate in different vehicles. Number of mice is given in parenthesis. DPM = disintegrations per minute. PCL = polycaprolactone. §Surfactant 1% added. Statistical differences were calculated by one-way ANOVA and Student-Newman-Keuls test for post hoc analysis with  $P < 0.05$  as minimal level of significance. \*\*\* $P < 0.001$ .

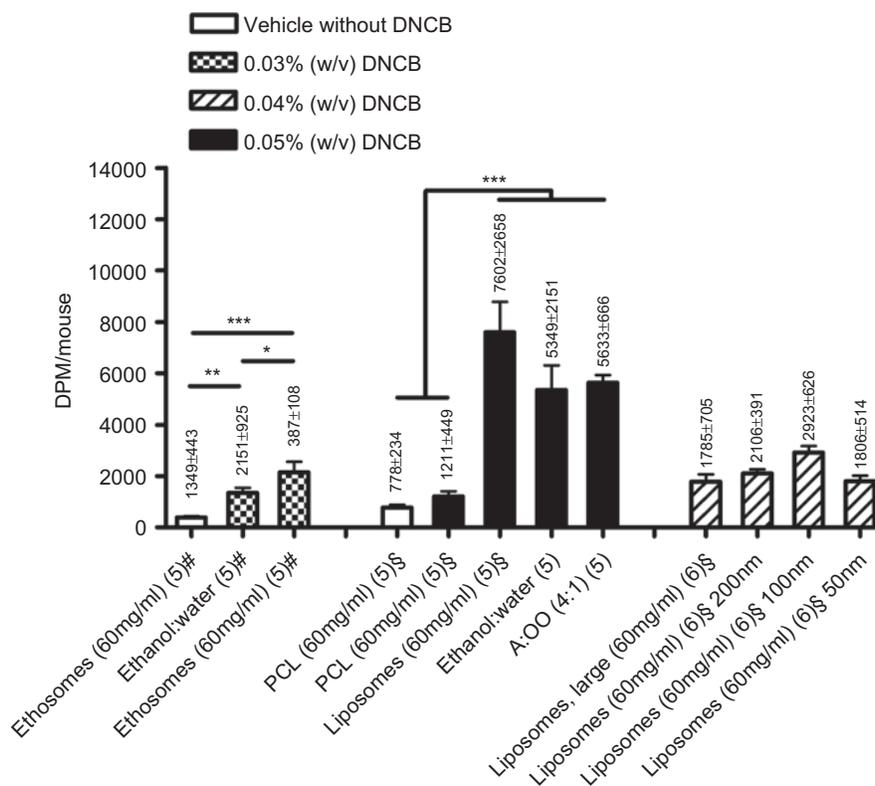


Figure 2. Results of local lymph node assay experiments performed with dinitrochlorobenzene (DNCB) in different vehicles. Number of mice is given in parenthesis. DPM = disintegrations per minute. PCL = polycaprolactone. A:OO = acetone:olive oil. §Surfactant 1% added. #Previously published data (18). Statistical differences were calculated by one-way ANOVA and Student-Newman-Keuls test for post hoc analysis with  $P < 0.05$  as minimal level of significance. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

for isoeugenol) and ethosomes (90% for DNCB and 77% for isoeugenol).

The size of liposomes, ethosomes, and polycaprolactone was stable during the time of experiment (3 days). The size of liposomes and polycaprolactone particles was stable for at least 22 days after adding 1% surfactant to the formulations (data not shown). Adding the surfactant to the polycaprolactone or the liposomal formulation did not produce a bi- or multimodal size distribution compared with the liposomal and polycaprolactone formulation alone.

## Discussion

The importance of vehicle effects on sensitization and elicitation in contact allergy is well-known from animal studies in both guinea pigs (25) and mice (26). There is no simple relationship between type of contact allergen, type of vehicle, and sensitization properties (26–28). These results show that encapsulation of contact allergens in three different drug-delivery systems relevant for the pharmaceutical and cosmetic industry may affect the sensitizing potency in the LLNA. The results confirm that there is no simple relationship between the drug-delivery system and the sensitizing potency in the LLNA. The different encapsulation efficiencies of the allergens in the three drug-delivery systems may partly explain the change in sensitizing capacity. For instance, potassium

dichromate showed very low encapsulation efficiencies in liposomes, ethosomes, and polycaprolactone, and no change in sensitizing capacity was seen when potassium dichromate was encapsulated in the three different drug-delivery systems. This is somehow expected since hydrophilic chemicals do not bind to the lipid membrane or to the polycaprolactone but rather stay in the aqueous phase as seen from the encapsulation efficiencies in Table 2. Therefore, the altered sensitization capacity may not be caused by the lipid itself but is more likely due to the encapsulation of the allergen in the lipid membrane. High EE may be an important parameter for effect on sensitization properties. The direction of the effect may depend on the specific combination of chemical and drug-delivery system, that is, DNCB and isoeugenol both showed high encapsulation efficiencies and a decreased sensitization capacity when encapsulated in polycaprolactone, and increased sensitization when encapsulated in ethosomes. The mechanism of action is not elucidated. When vesicle formulations or polymeric microparticles dry down on skin, their microstructure may change and therefore the characterization of their structure are only valid before applying them on the skin. To the authors' knowledge, no publication state that intact vesicles exist when they get in contact with the skin. Polycaprolactone loaded with lipophilic allergens (DNCB and isoeugenol) showed reduced sensitization in the LLNA compared with acetone:olive oil and liposomes. This is in contrast

to the suggestion in a case report (15). However, the LLNA is a sensitization experiment and the case report concerns elicitation. Further, we do not know the exact composition of the polycaprolactone in the cosmetic product. Octyl methoxycinnamate (OMC), a UV filter used in sunscreens, penetrates significantly less when encapsulated in polycaprolactone compared with non-encapsulated OMC (29) but other studies showed the opposite (17). Isoeugenol formulated in acetone:olive oil was a significantly stronger sensitizer compared with PG, which is also reported in the literature (28,30). We found approximately the same lymph node proliferation as Ryan et al. (31) when testing potassium dichromate with a surfactant (even though we used a polymer with

Table 1. Adding a surfactant to potassium dichromate dissolved in water increases the sensitizing capacity in the local lymph node assay.

Sample description	Potassium dichromate (%w/v)	Lymphocyte proliferation (mean DPM)
Surfactant 2% ( <i>n</i> =4)	0.0	855
Surfactant 1% ( <i>n</i> =4)	0.5	1843
Surfactant 1% + ( <i>n</i> =4)	1.0	4019
Surfactant 2% + ( <i>n</i> =4)	0.5	3934

Lymph nodes were pooled for each group and each result is expressed as disintegrations per minute (DPM) per mouse.

a molecular weight of 4400 MW compared with Ryan et al.'s pluronic L92 (MW 3650).

Liposomes and polycaprolactone are formulated in an aqueous solution, which makes it impossible to compare the effect of polycaprolactone and liposomes alone, since lipophilic allergens formulated in an organic solution would dissolve the liposomes and polycaprolactone particles. We added a surfactant to the liposome and polycaprolactone formulations with lipophilic allergen and compared sensitization response to the allergens dissolved in organic solutions (acetone:olive oil, ethanol:water, or PG). These minor variations in formulations should be kept in mind when results are interpreted. Changing the size of liposomes did not affect the sensitizing capacity in the LLNA. Diverging results are reported on the relationship between the size of liposomes and bioavailability of the encapsulated compound, and this will also be affected by the composition of the liposomes (1,32).

The surfactant may be able to coalesce into vesicles spontaneously, but this was not found in our experiments.

## Conclusion

Formulating contact allergens in different microvesicles and polymeric microparticles may affect their sensitizing properties. Ethosomes were able to enhance the

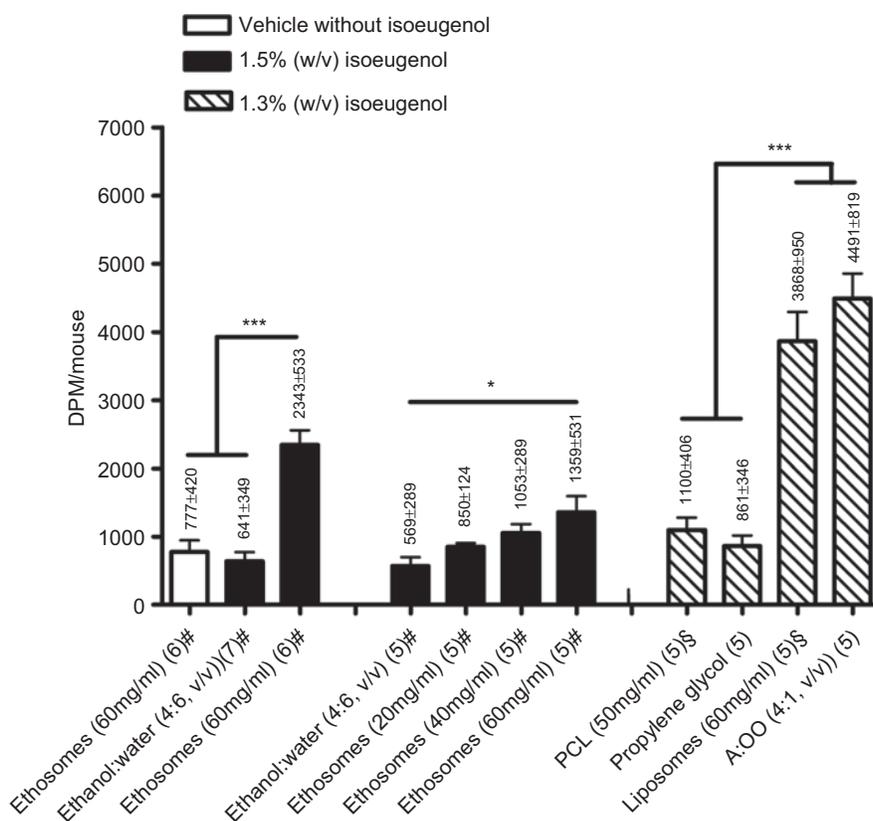


Figure 3. Results of local lymph node assay experiments performed with isoeugenol in different vehicles. Number of mice is given in parenthesis. DPM=disintegrations per minute. PCL=polycaprolactone. A:OO=acetone:olive oil. §Surfactant 1% added. #Previously published data (18). Statistical differences were calculated by one-way ANOVA and Student-Newman-Keuls test for post hoc analysis with  $P < 0.05$  as minimal level of significance. \* $P < 0.05$ , \*\*\* $P < 0.001$ .

Table 2. Size, polydispersity index (PI), and encapsulation efficiencies of different drug-delivery systems loaded with potassium dichromate, dinitrochlorobenzene (DNCB), or isoeugenol.

Sample description	Allergen	Size $\pm$ SD (nm)	PI	Encapsulation efficiency% $\pm$ SD
Polycaprolactone	Potassium dichromate	313 $\pm$ 13	0.12 $\pm$ 0.06	0.7 $\pm$ 0.3
Liposome	Potassium dichromate	91 $\pm$ 5	0.06 $\pm$ 0.01	7 $\pm$ 2.1
Ethosome	Potassium dichromate	436 $\pm$ 9	0.17 $\pm$ 0.02	16 $\pm$ 0.4
Polycaprolactone	DNCB	231 $\pm$ 20	0.02 $\pm$ 0.02	83 $\pm$ 0.6
Liposome	DNCB	65 $\pm$ 1	0.06 $\pm$ 0.04	
Liposome	DNCB	99 $\pm$ 1	0.05 $\pm$ 0.01	
Liposome	DNCB	184 $\pm$ 2	0.04 $\pm$ 0.00	92 $\pm$ 0.1
Liposome	DNCB	2466 $\pm$ 832	0.34 $\pm$ 0.03	
Ethosome	DNCB	245 $\pm$ 17	0.09 $\pm$ 0.03	90 $\pm$ 0.3
Polycaprolactone	Isoeugenol	343 $\pm$ 21	0.04 $\pm$ 0.01	84 $\pm$ 0.1
Liposome	Isoeugenol	155 $\pm$ 25	0.09 $\pm$ 0.04	98 $\pm$ 0.1
Ethosome	Isoeugenol	396 $\pm$ 20	0.17 $\pm$ 0.10	77 $\pm$ 0.3

Results are expressed as mean  $\pm$  SD.  $n=3$  in all experiments.

sensitizing capacity of DNCB and isoeugenol and polycaprolactone protected the lipophilic allergens against sensitization.

## Declaration of interest

All authors have no conflicts of interest.

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# Ethosome formulation of contact allergens may enhance patch test reactions in patients\*

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**Background:** Ethosomes and liposomes are ultra-small vesicles capable of encapsulating drugs and cosmetic ingredients for topical use, thereby potentially increasing bioavailability and clinical efficacy. So far, few reports have suggested that formulation of cosmetic ingredients in vesicular carrier systems may increase the allergenicity potential.

**Objectives:** To investigate the effect of ethosome formulation of isoeugenol and methyldibromo glutaronitrile on the elicitation response under patch test conditions and by repeated open applications.

**Patients/Materials/Methods:** A total of 27 volunteer patients with a previous positive patch test reaction to either isoeugenol or methyldibromo glutaronitrile were included in the study. In all patients, a serial dilution patch test was performed with the allergen in question formulated in ethosomes and in an ethanol/water solution. In addition, a repeated open application test (ROAT) was performed in a subset of 16 patients, and lag time until a positive response was recorded.

**Results:** Both contact allergens encapsulated in ethosomes showed significantly enhanced patch test reactions as compared with the allergen preparation in ethanol/water without ethosomes. No significant difference in the median lag time was recorded between preparations in the ROAT.

**Conclusions:** Encapsulating potential contact allergens in ethosomes may increase the challenge response as compared with the same concentrations in an ethanol/water base without ethosomes.

**Key words:** contact dermatitis; encapsulation; ethosome; liposome; patch test; repeated open application test. © John Wiley & Sons A/S, 2010.

Conflicts of interest: The authors have no conflicts of interest.

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Ethosomes (ethanolic liposomes) and other carrier systems for delivery of drugs and cosmetic ingredients to and through the skin have been of much interest, and show potential for use in clinical applications. Increased biological effects of encapsulated drugs have been reported in clinical trials for herpes simplex, psoriasis, acne, xerosis, atopic dermatitis, vitiligo, and superficial thrombophlebitis (1–7).

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Suggested explanations for these intriguing effects of encapsulated compounds as compared with conventional formulations include improved bioavailability, protection of encapsulated ingredients from degradation and photo-oxidation, and reduced irritancy.

We have recently shown that the sensitizing capacity in the local lymph node assay (LLNA) can be enhanced, as compared with conventional formulations, by encapsulating dinitrochlorobenzene and isoeugenol in ethosomes (8). The effect of

encapsulation on the challenge phase in sensitized humans has not been investigated, although a few clinical reports have raised this issue. Propyl galate incorporated in liposomes was suggested to boost the allergic potential in 13 patients; however, patch tests with and without the liposomal formulation were not performed (9). Furthermore, a case report described a woman developing severe allergic contact dermatitis from an 'anti-wrinkle' cream containing retinyl palmitate encapsulated in polycaprolactone (10).

The present study is based on the hypothesis that formulation of contact allergens in vesicular drug delivery systems may enhance the patch reaction as compared with allergen formulations in conventional vehicles. Ethosomes were selected as carriers because they contain ethanol, allowing experiments with lipophilic allergens in water/ethanol mixtures with and without the phospholipids, and because they have previously been shown to enhance the sensitizing capacity of allergens in the LLNA (8). Isoeugenol and methylidibromo glutaronitrile (MDBGN) were selected as model allergens, and formulated in ethosomes and ethanol/water for performance of patch tests and repeated open application test (ROATs) on human volunteers with a previous positive patch test reaction to the allergens.

## Materials and Methods

### Test subjects

The inclusion criteria were: age over 18 years, and a previous positive patch test reaction to MDBGN or isoeugenol within the last 10 years at the Department of Dermatology, Odense University Hospital, University of Southern Denmark. Exclusion criteria were: active eczema on test sites, not being able to cooperate for the ROAT, pregnancy, and breast-feeding.

Forty-eight persons with a previous positive patch test reaction to isoeugenol and 89 persons with a previous positive patch test reaction to MDBGN were invited to participate.

### Study design

Three concentrations of MDBGN and two concentrations of isoeugenol formulated in ethosomes and ethanol/water and blank controls were tested. ROATs were performed with one concentration of allergen formulated in ethosomes and ethanol/water.

The placement of the test concentrations and vehicles in both tests were randomized and blinded for the investigator and the subjects. After termination of the study, the randomization code was broken. The study was performed according to the

Helsinki II declaration, and approved by the local ethics committee (Southern Region of Denmark, S-20090022).

### Patch test

The patch tests were applied on IQ-chambers (Chemotechnique® Diagnostics, Vellinge, Sweden), and occluded for 2 days; the reactions were read on D3. The reading scale developed by Fischer et al. (11) was chosen, in order to detect smaller differences in the allergic responses. The scale was as follows: 0 = no reaction; 1 = few papules with no erythema and no infiltration; 2 = faint erythema with no infiltration or papules; 3 = faint erythema with few papules and no homogeneous infiltration; 4 = erythema with homogeneous infiltration; 5 = erythema with infiltration and a few papules; 6 = erythema with infiltration and papules; 7 = erythema with infiltration, papules, and a few vesicles; and 8 = intensive erythema with infiltration and vesicles. J.T.M. performed all readings.

### ROAT

Two 3 × 3 cm areas on the volar aspects of both forearms were used. Twenty microlitres of test preparation were applied two (MDBGN) or three (isoeugenol) times daily, with a micropipette (Acura 815, 20 µl; Buch & Holm A/S, Herlev, Denmark) with a fixed volume. Test subjects received two marked bottles, each mark referring to a test area. The solutions were spread on the area with the tip of the pipette, and allowed to dry by evaporation. The subjects received written instructions, and were instructed orally and manually in using the pipette. The dose of one application was 5.66 mg/ml isoeugenol or 0.10 mg/ml MDBGN. When an area showed a positive reaction (verified by the investigator), the subjects stopped application on that test area and continued on the other area. A reaction was defined as positive when 70% of the area had erythema, papules, or vesicles. Numbers of days until positive reactions occurred were counted. J.T.M. performed the readings. If no ROAT reaction developed within 4 weeks, application was stopped (except in one case: here, the ROAT result on one arm was positive after 18 days, and that on the other after 45 days).

**Ethosome preparation.** Ethosomes with isoeugenol (CAS no. 97-54-1; Aldrich, Brøndby, Denmark) or MDBGN (CAS no. 35691-65-7; Alfa-Aesar, Karlsruhe, Germany) were prepared as described by Touitou (12). Briefly, 100 mg/ml 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) (Avanti Polar Lipids, Alabaster, AL, USA) was dissolved in 96% ethanol containing isoeugenol

or MDBGN, and MilliQ water was added slowly to a final concentration of 40% (v/v) ethanol in water. The suspension was stirred for 5 min and then extruded 10 times through two polycarbonate filters with a pore size of 100 nm, using a Lipex® Extruder (Northern Lipids, Burnaby, Canada).

The effect of the ethosomes was compared with the following control formulations. MDBGN or isoeugenol was dissolved in ethanol, and MilliQ water was then added to a final concentration of 40% (vol/vol) ethanol in water. An extra experiment was performed with isoeugenol in an ethanol/water (4:6) solution with POPC (100 mg/ml) added to investigate the effect of the lipid without subsequent extrusion of ethosomes.

The concentrations of isoeugenol and MDBGN were determined by high-performance liquid chromatography (HPLC), to ensure that the allergen concentration of the control solution matched the allergen concentrations in the ethosomal formulations. All formulations were kept in darkness at 5°C, and all preparations were made no more than 5 days prior to beginning the patch testing and ROAT. Volunteers were instructed to keep the test material for the ROAT in the refrigerator.

The final concentrations of isoeugenol were: 0.0, 2.80 and 6.54 mg/ml for the patch test, and 5.66 mg/ml for the ROAT. The final concentrations of MDBGN were 0.00, 0.10, 0.21 and 0.63 mg/ml for the patch test experiment, and 0.10 mg/ml for the ROAT.

*Characterization of ethosomes.* Hydrodynamic particle diameters and the polydispersity index (PI) of ethosomes, which describes the size distribution of the particles, were determined by dynamic light scattering (DLS), using a BI-200SM instrument from Brookhaven Instruments (Holtsville, NY, USA). This incorporates a 632.8-nm HeNe laser operated at a fixed scattering angle of 90°. Twenty microlitres of ethosome solution was diluted in 1.5 ml of ethanol/MilliQ water (40%). The measurements were conducted in triplicate in a multimodal mode of 180 seconds.

*Encapsulation efficiency.* The efficiency of encapsulation (EE%) of isoeugenol and MDBGN by ethosomal vesicles was determined by ultracentrifugation, as described by Heeremans et al. (13) and later used on ethosomal systems by Tuitou et al. (12). Ethosomal preparations containing MDBGN or isoeugenol were kept overnight at 5°C, after which they were spun at 143 360 g rpm for 3 hrs in a Hitachi Sorvall Discovery 90SE ultracentrifuge with a swingout rotor (SW50.1; Sorvall, Breda, The Netherlands). The supernatant was immediately removed, and drug quantity was determined in both the sediment and the supernatant.

Binding efficiency was calculated as follows:  $[(T - C)/T] \times 100$ , where  $T$  is the total amount of chemical detected in both the supernatant and sediment, and  $C$  is the amount of chemical detected only in the supernatant. The procedure was performed in triplicate.

*Quantification of isoeugenol and MDBGN in ethosomes.* HPLC analysis of isoeugenol was conducted on an Ultimate 3000 series from DIONEX™ (Hvidovre, Denmark) with a diode array detector. A DIONEX® Acclaim® Surfactant column was used for separation of isoeugenol. The temperature of the column and the sample rack in the autosampler was set to 20°C. The conditions were as follows: mobile phase, 75% methanol/25% MilliQ water; isocratic elution for 30 min; and flow rate, 1 ml/min. The separations were monitored at 270 nm. The injection volume was 10 µl. Pure reference compounds were used to make external calibration curves, from which the concentrations of isoeugenol were determined. MDBGN is not UV-active, and content was measured by evaporative light scattering detection (Varian 385-LC, Analytical Instruments AS, Værløse, Denmark), using a reversed phase C-5 column from Supelco® (Aldrich, Brøndby, Denmark). Separation was achieved using a 0.8 ml/min flow rate with an isocratic mobile phase of 75% methanol and 25% MilliQ water. The injection volume was 50 µl, and external calibration was performed with pure MDBGN.

*Statistical data analysis.* Results are expressed as means ± standard error of the mean. Differences in the patch test reactions were determined by two-way analysis of variance (ANOVA), with applied dose and vehicle (ethosomes and ethanol/water) as factors. ROAT experiments were analysed with the Wilcoxon signed rank test. The GRAPHPAD PRISM 4 from GraphPad Software (San Diego, California, USA) was used.

## Results

Twenty subjects participated in the MDBGN patch test and 18 in the ROAT study. One subject had a negative patch test result and 8 subjects a negative ROAT result, and were excluded from further analysis.

Eight subjects participated in the isoeugenol patch test and ROAT, and all subjects had a positive patch test reaction. Six subjects had a positive ROAT reaction (one subject after 45 days), and two did not react during the exposure period.

Isoeugenol and MDBGN formulated in ethosomes gave significantly enhanced patch test reactions as compared with the same allergens in ethanol/water (Figs. 1 and 2). However, when POPC

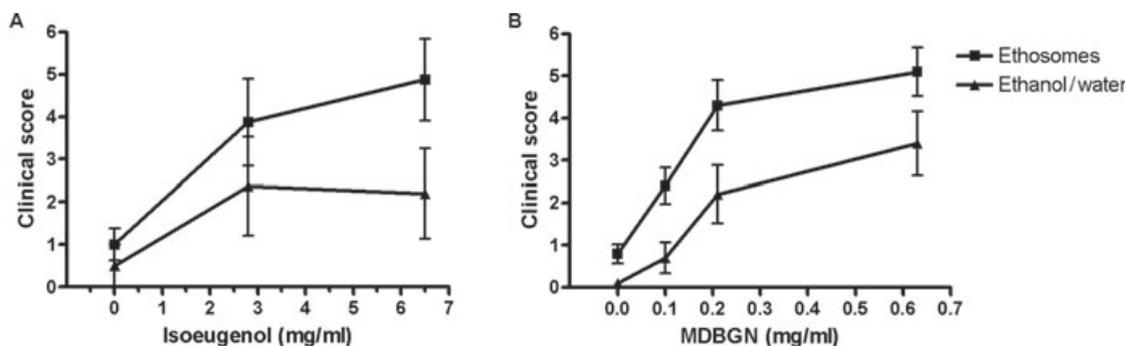


Fig. 1. Patch test results for methyldibromo glutaronitrile (MDBGN) ( $n = 19$ ) and isoeugenol ( $n = 8$ ) encapsulated in ethosomes (100 mg/ml) as compared with the same concentrations of allergen in ethanol/water. Significant increases in patch test reactions are seen for both allergens encapsulated in ethosomes (MDBGN,  $P < 0.0001$ ; isoeugenol,  $P < 0.05$ ). An increased allergen concentration also increased the elicitation response (MDBGN,  $P < 0.0001$ ; isoeugenol,  $P < 0.007$ ) (two-way ANOVA). Results are expressed as means  $\pm$  standard error of the mean.

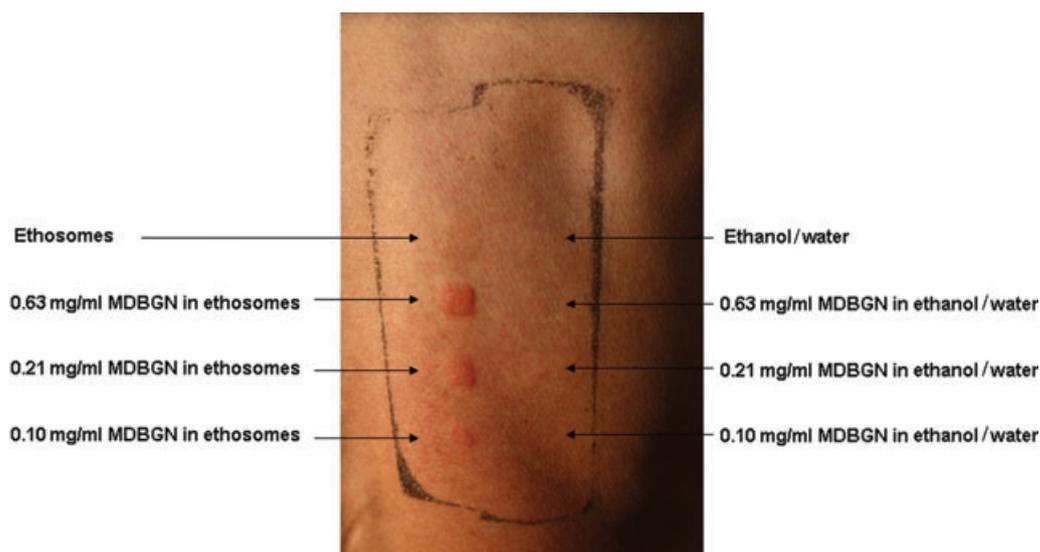


Fig. 2. Result of a serial dilution patch test in a sensitized volunteer with methyldibromo glutaronitrile (MDBGN), using IQ-chambers and 15  $\mu$ l of test substance formulated in ethosomes and ethanol/water.

was added to ethanol/water – without extrusion of vesicles – there was no difference in response to isoeugenol in ethosomes (Fig. 3). The ROAT did not show a significant difference for any of the allergens, but a trend towards a more rapidly developing positive reaction was found for isoeugenol formulated in ethosomes as compared with isoeugenol formulated in ethanol/water (Table 1).

#### Characterization of ethosomes

Vesicle size measured before and after experiments remained stable in the test tubes for the duration of the experiment. All ethosomes were between  $333 \pm 13$  and  $463 \pm 13$  nm, and the PI ranged from  $0.06 \pm 0.04$  to  $0.22 \pm 0.03$ . The PI values can be regarded as monodisperse. The EE% of isoeugenol in ethosomes was  $77.3\% \pm 0.3\%$ , and for MDBGN it was  $21.8\% \pm 4.3\%$ .

#### Discussion

Using a protocol with precise dosing and characterization of test preparations, we have, for the first time, shown that lipophilic contact allergens encapsulated in ethosomes can enhance patch test reactions in sensitized individuals as compared with the same allergens in a control solution of 40% ethanol in water without lipid vesicles. Other vehicle effects on both sensitization and elicitation responses have previously been reported in experiments using the LLNA, guinea pigs and human volunteers (14, 15). However, the effect of new encapsulating vehicles has not been studied before.

No difference was seen when POPC was added to the ethanol/water solution as compared with the ethosome formulation (Fig. 3). A tentative explanation of the results is that spontaneous formation of vesicles occurs when POPC is mixed with water (or ethanol/water). However, the vesicles

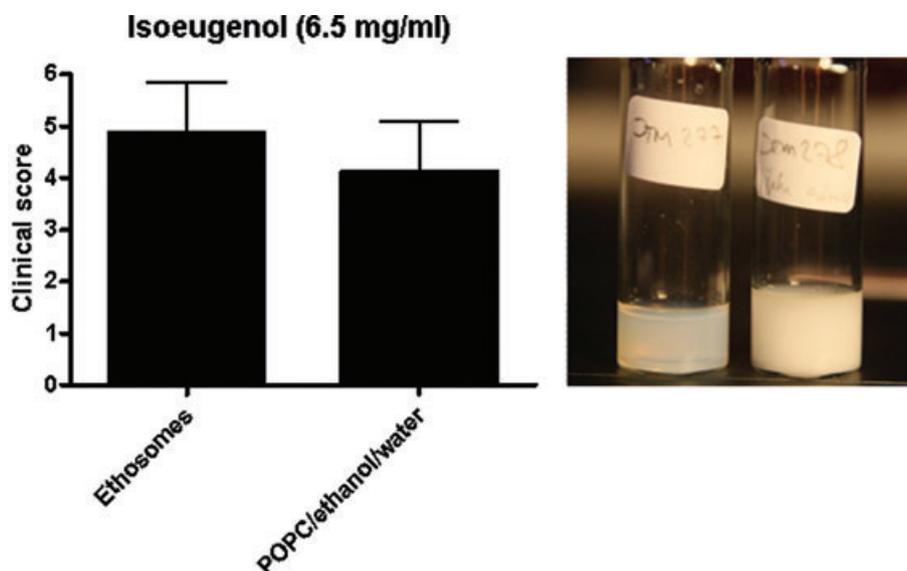


Fig. 3. Patch test results with 6.5 mg/ml isoeugenol formulated in ethosomes (400 nm) and in 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC)/ethanol/water ( $n = 8$ ). No significant difference was observed (Wilcoxon signed rank test). Results are expressed as means  $\pm$  standard error of the mean. The picture shows the light scattering effect of small extruded vesicles of 300 nm (left) versus non-extruded vesicles (right).

Table 1. The repeated open application test (ROAT) performed with isoeugenol ( $n = 6$ ) and methyl dibromo glutaronitrile (MDBGN) ( $n = 10$ ) formulated in ethosomes and ethanol/water as vehicles

	Days to positive ROAT $\pm$ SEM
Isoeugenol	
Ethosomes	7.7 $\pm$ 2.4
Ethanol:water	15.3 $\pm$ 7.3
MDBGN	
Ethosomes	10.7 $\pm$ 2.3
Ethanol:water	10.1 $\pm$ 2.0

Results are presented as mean days  $\pm$  standard error of the mean (SEM) to a positive reaction. No significant difference was observed (Wilcoxon signed rank test) for either allergen, even though a trend towards a faster developing reaction was seen for isoeugenol formulated in ethosomes as compared with the ethanol/water formulation ( $P = 0.31$ ).

are not homogeneous in size, and they are multilamellar, whereas vesicles extruded through a filter of equal pore size are more uniform and unilamellar. The light scattering effect of small extruded vesicles (300 nm) versus non-extruded vesicles is clearly seen in Fig. 3. Owing to very high PIs, DLS measurements were not applicable for the POPC/ethanol:water formulation.

The ROAT performed with MDBGN and isoeugenol with and without ethosomes showed no significant difference in lag time until a positive response, even though a trend towards a more rapidly developing reaction occurred with encapsulated isoeugenol as compared with isoeugenol in ethanol/water. We have no explanation for this discrepancy between patch test results and the ROAT, but occlusion may play a role. It has been

reported that occlusion decreases the penetration of compounds through the skin when they are encapsulated in Transfersomes<sup>®</sup> (16), but as there is no clearly documented relationship between skin penetration and the sensitizing capacity of an allergen (17, 18), altered penetration is probably not the key to the discrepancies in our results. Further experiments are needed to clarify this issue.

Increased patch test reactivity correlates with increased ROAT reactivity for some allergens, such as MDBGN and isoeugenol (11, 19), but this is not always the case (20). Isoeugenol is less lipophilic and is better retained inside the ethosomes than MDBGN, as expressed by higher EE% (77% versus 22%). Whether this difference accounts for the discrepancy between the ROAT and patch test reactions of MDBGN and isoeugenol encapsulated in ethosomes remains speculative, but obviously the low encapsulation efficiency of MDBGN is enough to produce significant changes in the test reactions if the encapsulation efficiency is an important parameter. A direct comparison is only valid for a single allergen when formulated in different vehicles, and not between different allergens, as allergens with significantly different chemical structures, and therefore physicochemical properties (e.g. log  $P$ ), will influence the vesicle properties (e.g. stability, encapsulation efficiency, and skin penetration) and subsequently complicate discussions on the findings.

The clinical implications of these results are, so far, uncertain. However, the cosmetic industry should consider the effect of encapsulation on a case by case basis, because certain ingredients

may become more allergenic when encapsulated. Dermatologists using encapsulation technology to investigate patients with allergic reactions to consumer products should consider the risk of false-negative results, if testing with ingredients in conventional patch test vehicles. Testing with encapsulated ingredients should be performed when possible.

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RESEARCH ARTICLE

# Percutaneous penetration characteristics and release kinetics of contact allergens encapsulated in ethosomes

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## Abstract

**Background:** Formulation of the contact allergens dinitrochlorobenzene and isoeugenol in ethanolic liposomes (ethosomes) increases their sensitizing properties in the local lymph node assay compared with an ethanol–water formulation of the allergens. Likewise, isoeugenol and methyl dibromo–glutaronitrile formulated in ethosomes enhanced the patch test reactions in sensitized human volunteers. The relationship between the percutaneous penetration/absorption and sensitization/elicitation phases of contact allergy is not well elucidated.

**Objective:** The aim of this study was to investigate whether the observed increased sensitizing and elicitation properties following the formulation of selected contact allergens in ethosomes could be explained by a change in release kinetics of the allergens and their pattern of percutaneous penetration and absorption as well as allergen deposition in epidermis and dermis.

**Methods:** Release kinetics were studied using dialysis bags, and samples were taken at selected time points until equilibrium was reached. Percutaneous absorption and penetration were studied using human skin on Franz cells, and receptor fluid samples were taken at selected time points. Experiments were terminated after 24 hours, and deposition of allergen in epidermis and dermis was measured. Maximum flux and lag time were calculated.

**Results:** Ethosome formulation decreased the release of both allergens compared with the ethanol–water formulation. Ethosome formulation of dinitrochlorobenzene increased its percutaneous penetration but reduced the percutaneous penetration of isoeugenol compared with control formulations. Likewise, all other calculated parameters showed an opposite trend for the 2 allergens in ethosomes and ethanol–water.

**Conclusions:** The present study demonstrates that identical ethosomes affect the percutaneous penetration characteristics of 2 allergens differently. Thus, our results indicate that each combination of an allergen and a vehicle needs to be evaluated separately. The exact mechanistic relationship between percutaneous penetration, release kinetics, and allergenicity of chemicals in various vehicles remains to be clarified.

**Keywords:** *Percutaneous absorption; ethosomes; liposomes; allergen; sensitization; Franz cell*

## Introduction

In order for a contact allergen to get in contact with the cutaneous immune system, it has to penetrate the viable epidermis. Thus, allergens should have

appropriate physicochemical properties to cross the stratum corneum, which normally is an effective skin barrier. A certain degree of lipophilicity ( $\log P$  [logarithm of the ratio of the concentrations of the unionized solute in solvents] around 2) is advantageous.

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Extremely lipophilic and hydrophilic molecules are poor skin penetrators (1,2). Formulating a chemical/allergen in different vehicles for topical administration may change the skin penetration profile (2–4) and the sensitizing and elicitation capacity of the allergen (5–9), but how these outcomes are related to penetration and absorption properties is not well elucidated.

We have previously shown that formulation of contact allergens (dinitrochlorobenzene [DNCB] and isoeugenol) in ethanolic liposomes (ethosomes) increased the sensitizing properties in the local lymph node assay (LLNA) and that isoeugenol and methyl dibromo-glutaronitrile formulated in ethosomes enhanced the patch test reactions in 27 sensitized human volunteers (2,10,11). Several reports have shown that encapsulating lipophilic and cationic compounds in ethosomes increases their skin penetration and bioavailability in stratum corneum compared with formulations without ethosomes (12). The aim of this study was to investigate whether the observation of increased sensitizing and elicitation properties following formulation of selected contact allergens in ethosomes could be explained by a change in release kinetics and penetration pattern. We used dialysis and Franz diffusion cells and compared release kinetics and penetration profiles of 2 ethosome-encapsulated contact allergens with the same contact allergens formulated in ethanol–water making the lipids (ethosomes) the only difference.

## Methods

### *Ethosome preparation*

Ethosomes with isoeugenol (Sigma-Aldrich Denmark A/S, Copenhagen, Denmark; Chemical Abstract Service [CAS] No. 97-54-1) or DNCB (Sigma-Aldrich Denmark A/S; CAS No. 97-00-7) were prepared as described by Touitou et al. (13). Briefly, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC; Avanti Polar Lipids, Alabaster, AL, USA) was dissolved in 96% ethanol containing isoeugenol or DNCB, and Milli-Q water (Millipore Corp., Billerica, MA, USA) was added slowly to a final concentration of 40% (percent volume in volume [v/v]) ethanol. The suspension extruded 10 times through 2 polycarbonate filters with a pore size of 200 nm using a Lipex Extruder (Northern Lipids Inc. Burnaby, BC, Canada). The concentration of isoeugenol and DNCB in experimental solutions was determined by high-performance liquid chromatography (HPLC). The DNCB or isoeugenol was dissolved in ethanol after Milli-Q water was added to a final concentration of 40% (v/v) ethanol. The ethosome

preparation was compared with a 4:6 ethanol-to-water solution containing isoeugenol or DNCB.

### *Characterization of ethosomes*

The hydrodynamic particle diameters and polydispersity index (PI) of ethosomes, which describes the size distribution of the particles, were determined by dynamic light scattering (DLS) using a BI-200SM instrument from Brookhaven Instruments (Holtville, NY, USA). This incorporates a 632.8-nm helium–neon (HeNe) laser operating at a fixed scattering angle of 90°. Twenty-microliter ethosome solution was diluted in 1.5 mL of ethanol–Milli-Q water (4,6). The measurements were conducted in triplicate in a multimodal mode of 180 seconds. The sizes of the ethosomes were measured on the day of preparation and immediately after the experiments.

### *Encapsulation efficiency*

The encapsulation efficiency (EE%) of isoeugenol and DNCB by ethosomal vesicles was determined by ultracentrifugation as described by Heeremans et al. (14) and later used on ethosomal systems by Touitou et al. (13). Ethosomal preparations containing DNCB or isoeugenol were kept for 12 hours at 5°C and thereafter spun at 40,000 revolutions per minute (RPM) for 3 hours in a Hitachi Sorvall Discovery 90SE ultracentrifuge (Tokyo, Japan) with a swing-out rotor from Sorvall (SW50.1; Thermo Fisher Scientific, Asheville, NC, USA). The supernatant was immediately removed and the allergen quantity was determined in both the sediment and the supernatant. Binding efficiency was calculated as follows:

$$[(T - C) / T] \times 100$$

where T is the total amount of chemical detected in both the supernatant and sediment, and C is the amount of chemical detected only in the supernatant. The procedure was done in triplicate.

### *Quantification of isoeugenol and dinitrochlorobenzene in ethosomes*

High-performance liquid chromatography analysis was conducted on an ultimate 3000 series from Dionex Corporation (Sunnyvale, CA, USA) with a diode array detector. A Dionex RP-18 Acclaim 300 C18 reversed-phase column was used. The temperature of the column and the sample rack in the autosampler was set to 20°C. The autosampler mobile phase included 75% methanol and

25% Milli-Q water; isocratic elution for 30 minutes; and a flow rate of 1 mL/min. The separations were monitored at 270 nm. The injection volume was 10  $\mu$ L. Pure reference compounds were used to make external calibration curves from which the concentrations of DNCB and isoeugenol were determined.

### *Skin membranes*

The human skin samples were obtained from the Department of Plastic and Reconstructive Surgery, Odense University Hospital. Skin was sampled from 3 women (26–37 years old) who underwent breast reconstruction. Skin samples were kept at  $-20^{\circ}\text{C}$  for periods not exceeding 12 months. The skin was allowed to thaw at room temperature 1 hour before being cleaned with distilled water. Subcutaneous fat was removed. Skin thickness varied between 0.90 and 0.96 mm. Skin samples from individual donors were equally distributed between experimental groups.

### *Skin penetration and absorption model*

Percutaneous penetration experiments were carried out using Franz diffusion cells with a permeation area of 2.12  $\text{cm}^2$  and a receptor volume between 15 and 19 mL (measured for each individual cell) as described by Nielsen et al. (2). The system consists of 2 half-cells where the upper cell compartment represents the donor chamber and the lower, the receptor chamber. The cells were kept at a constant temperature ( $32^{\circ}\text{C}$ ) in a water bath with individual magnetic stirring. Prior to experiments, the epidermal site was exposed to ambient laboratory conditions and the dermis was exposed to an aqueous solution of 0.9% sodium chloride (NaCl) and 5% bovine serum albumin (BSA)-containing 10% ethanol for 18 hours. Further, the barrier integrity was evaluated by capacitance measurements (Lutron DM-9023, Acer AB, Bromma, Sweden) before the exposure to test substances, and cells with a capacitance above 110 nF were excluded.

During the experimental periods, donor and receptor chambers were covered with parafilm to avoid evaporation. The skin was exposed to 106  $\mu$ L of test substance (50  $\mu$ L/ $\text{cm}^2$ ) and samples of 1 mL were taken at 2, 4, 6, 12, and 24 hours from the receptor chamber and replaced with 1 mL of fresh receptor fluid. At the end of the experiment, remaining test compound in the donor chamber and on top of the skin was sampled using repeated washings with cotton swabs and 50% acetonitrile. Cotton swabs and skin samples were left for 72 hours to extract in acetonitrile before chemical analysis.

After termination of the experiments, the epidermis was gently removed from the skin samples with a sharp knife, and both dermis and epidermis were transferred to individual vials containing 100% acetonitrile and left for extraction for 27 hours before measuring the amount of DNCB or isoeugenol.

The adherence of test compounds to glass in the receptor chamber, to proteins in the receptor fluid, and to the skin after extraction procedures was evaluated to ensure complete recovery of penetrated test compounds.

The amount of DNCB applied in ethanol–water was 0.035 mg and 0.036 mg in ethosomes. The amount of isoeugenol applied in ethanol–water was 1.58 mg and 1.24 mg when applied in ethosomes.

### *Release kinetics*

Dialysis membranes (Spectra-por 6, pore size: 10,000 daltons, Spectrum Labs, purchased from Bie & Berntsen AS, Herlev, Denmark) were filled with 300  $\mu$ L of test solution of DNCB or isoeugenol formulated in ethanol–water, 30, 60 or 90 mg/mL ethosomes, and left in 75 mL of ethanol–water (4:6 v/v) on a magnetic stirrer. Samples of 500  $\mu$ L were taken out at specific time intervals (Figure 2) and replaced with an equal amount of ethanol–water. Samples were analyzed by HPLC and expressed as the percentage of the applied amount of allergen. The concentration of DNCB was 0.79 mg/mL in ethanol–water, 0.67 mg/mL in 30-mg/mL ethosomes, 0.62 mg/mL in 60-mg/mL ethosomes, and 0.63 mg/mL in 90-mg/mL ethosomes. The concentration of isoeugenol was 8.79 mg/mL in ethanol–water, 8.79 mg/mL in 30-mg/mL ethosomes, 7.23 mg/mL in 60-mg/mL ethosomes, and 7.63 mg/mL in 90-mg/mL ethosomes.

A *T50% value* was calculated in a way similar to the EC3 value (concentration of test chemical required to provoke a 3-fold increase in lymph node cell proliferation) of the LLNA (15), with the *T50%* value being an estimate of the time needed for 50% of the allergen to diffuse through the dialysis membrane.

### *Statistical data analysis*

Results are expressed as mean  $\pm$  standard error of the mean (SEM) or standard deviation (SD). Statistically significant differences in penetration over time of isoeugenol and DNCB and the release kinetics were determined using 2-way analysis of variance (ANOVA). The Mann-Whitney test was used to test for different amounts of allergen stored in epidermal and dermal compartments for ethanol–water and

ethosomal formulations. A p-value < .05 was chosen as the minimal level of significance. Differences of the T50% values were determined by 1-way ANOVA. We used the statistical software package GraphPad Prism 4 from GraphPad Software Inc. (La Jolla, CA, USA).

## Results

Ethosome formulation of DNCB significantly increased the percutaneous absorption of DNCB compared with an ethanol-water formulation of DNCB (Figure 1, Table 1). In contrast, the percutaneous absorption of isoeugenol formulated in ethosomes was significantly reduced compared with an ethanol-water formulation of isoeugenol (Figure 1, Table 1). The DNCB formulated in ethosomes had a slight (nonsignificant) increase in dermis deposition compared with the ethanol-water formulation, but no difference in epidermal deposition (Table 1). On the contrary, the ethosome formulation significantly decreased the dermis

deposition of isoeugenol and caused a more limited and nonsignificant increase in epidermal deposition of isoeugenol. The ethosome formulation caused a significant increase in the relative skin deposition of isoeugenol, whereas the ethosomes had a more limited but opposite effect on the relative skin deposition of DNCB (Table 1). A significantly increased lag time was found for isoeugenol formulated in ethosomes compared with the ethanol-water formulation, whereas the lag time of DNCB was not significantly affected by the ethosome formulation. The maximum flux as well as the permeability coefficient of isoeugenol was significantly lower, when isoeugenol was formulated with ethosomes compared with the ethanol-water formulation, whereas no difference was seen for the DNCB formulations. In summary, all parameters showed an opposite trend for the 2 allergens in ethosomes and ethanol-water. No measurable adherence of DNCB or isoeugenol to glass, protein binding, or remaining test compounds in skin samples following the extraction procedures were observed.

**Table 1.** Fraction of dinitrochlorobenzene and isoeugenol retained in epidermis and dermis after 24 hours treatment of dinitrochlorobenzene and isoeugenol formulated in ethosomes and ethanol-water.

		Epidermis deposition ( $\mu\text{g}/\text{cm}^2$ )	Dermis deposition ( $\mu\text{g}/\text{cm}^2$ )	Dermis/epidermis ratio	Total percutaneous absorption at 24h ( $\mu\text{g}/\text{cm}^2$ )	Total skin deposition in percent of total penetration	Maximum flux ( $\mu\text{g}/\text{cm}^2\cdot\text{h}$ )	Lag time (h)	$K_p$ ( $\mu\text{m}/\text{h}$ )
DNCB	Ethanol-water	0.05 $\pm$ 0.04	0.66 $\pm$ 0.32	17.9 $\pm$ 10.7	34 $\pm$ 4	2.09 $\pm$ 0.96	1.3 $\pm$ 0.6	2.4 $\pm$ 0.9	39 $\pm$ 17
	Ethosomes	0.04 $\pm$ 0.01	0.82 $\pm$ 0.42	19.7 $\pm$ 7.8	59 $\pm$ 16***	1.68 $\pm$ 1.31	1.6 $\pm$ 0.8	1.9 $\pm$ 1.2	47 $\pm$ 24
Isoeugenol	Ethanol-water	2.83 $\pm$ 1.57	49 $\pm$ 21	18.7 $\pm$ 6.6	4,635 $\pm$ 1,167	1.30 $\pm$ 1.00	206 $\pm$ 91	4.5 $\pm$ 1.1	138 $\pm$ 61
	Ethosomes	3.30 $\pm$ 1.58	22 $\pm$ 6**	8.7 $\pm$ 5.9**	1,327 $\pm$ 443***	2.05 $\pm$ 0.55*	69 $\pm$ 21*	6.8 $\pm$ 1.4**	59 $\pm$ 18*

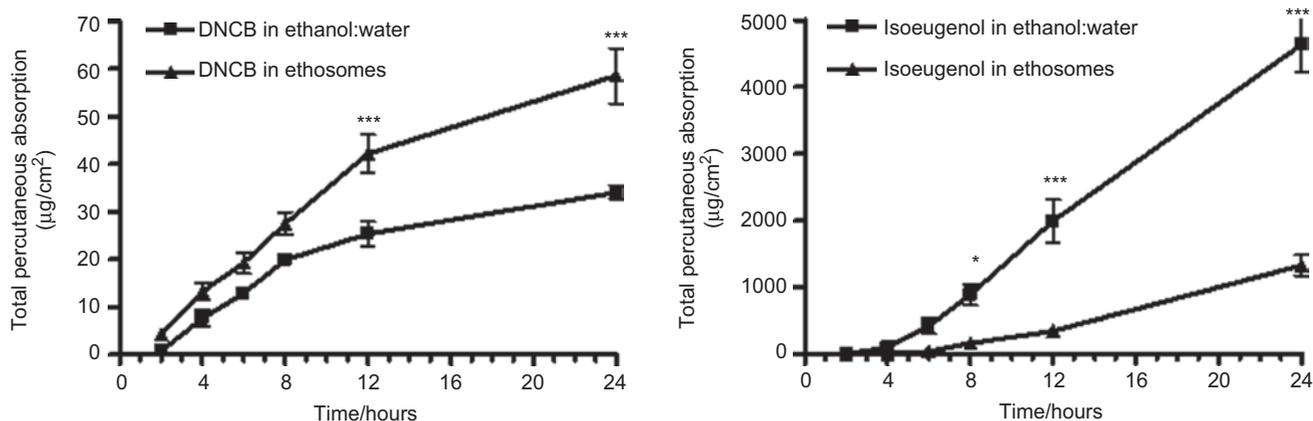
Data are expressed as  $\mu\text{g} \pm$  standard deviation ( $n=8$ ).

\*p < .05.

\*\*p < .01.

\*\*\*p < .001.

DNCB = dinitrochlorobenzene;  $K_p$  = permeability coefficient.



**Figure 1.** The graph on the left shows a significantly increased percutaneous absorption after 12 hours when dinitrochlorobenzene (DNCB) is formulated in ethosomes compared with an ethanol-water formulation, and the graph on the right shows a significantly decreased percutaneous absorption after 8 hours when isoeugenol is formulated in ethosomes compared with an ethanol-water formulation ( $n=8$ , \*\*p < .01, \*\*\*p < .001, 2-way analysis of variance). Results are expressed as mean  $\pm$  standard error of the mean.

The dialysis experiment showed increasing T50% values with increasing amounts of ethosomes in the sample (Table 2). This observation is a consequence of the decreased release rate when DNCB as well as isoeugenol was formulated in ethosomes (Figure 2). An interesting observation was that the effect of ethosome formulation was evident at the lowest concentration of ethosomes applied for isoeugenol (30 mg/mL), whereas a 3-times higher concentration of ethosomes was required to decrease the release rate significantly for DNCB (Table 2).

Size and encapsulation efficiencies show that ethosomes loaded with isoeugenol are slightly larger than DNCB-loaded ethosomes (Table 3). Encapsulation efficiencies are of the same magnitude.

## Discussion

We found contradictory percutaneous absorption and penetration patterns when comparing DNCB

**Table 2.** Dialysis experiments show increasing T50% values with increasing amount of ethosomes in the sample.

T50%	POPC (mg/mL)			
	0	30	60	90
DNCB	10 ± 1	14 ± 1	21 ± 15	33 ± 9
Isoeugenol	10 ± 1	32 ± 6*	26 ± 5**	44 ± 8***

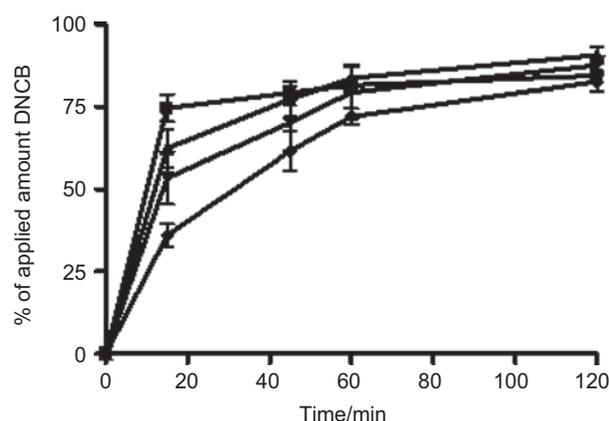
Data represent mean ± standard deviation;  $N=3$ ; one-way analysis of variance with Newman-Keuls post hoc test.

\* $p < .05$ .

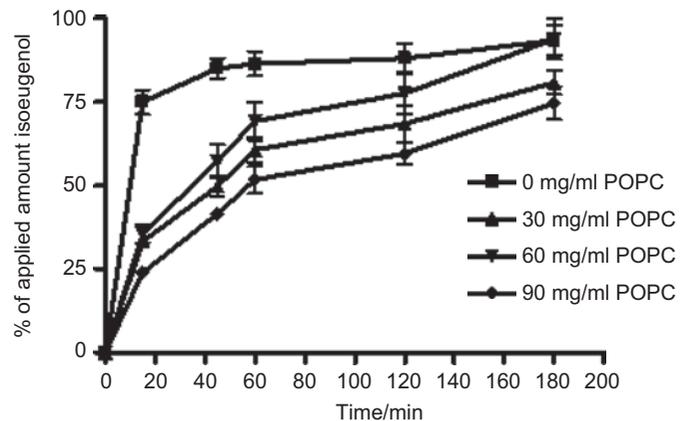
\*\* $p < .01$ .

\*\*\* $p < .001$ .

DNCB = dinitrochlorobenzene; POPC = 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; T50% = estimation of the time needed for 50% of the allergen to diffuse through the dialysis membrane.



and isoeugenol formulated in ethanol-water and ethosomes and hence penetration/absorption characteristics could not explain the increased sensitizing capacity of both allergens when formulated in ethosomes. A marked difference between DNCB and isoeugenol is in the water solubility, with isoeugenol being much more water-soluble than DNCB. Further, isoeugenol has higher logP and lower encapsulation efficiency than DNCB, but both allergens showed a sustained release when formulated in ethosomes (Table 3). Despite these differences, both allergens increase their sensitizing potential when formulated in ethosomes, suggesting that the sustained release might be an important parameter of the observed differences in sensitizing capacity. All previously published studies investigating ethosome formulations and skin penetration show an increased penetration/absorption of the encapsulated compound. We have shown for the first time that an ethosome formulation of a compound (isoeugenol) inhibited the percutaneous penetration compared with a control formulation without the vesicles. Andersen et al. showed in 1985 that chlorocresol formulated in propylene glycol had a lower sensitization capacity compared with an acetone-olive oil formulation. Both formulations had the same bioavailability of chlorocresol in the skin after 24 hours, but the authors did not distinguish between skin deposition and did not measure skin absorption (16). In 1996, Heylings et al. investigated the vehicle effects of DNCB formulated in acetone and propylene glycol and skin absorption in the LLNA (17). They found an increased sensitizing capacity, which correlated with an increased flux from 2 hours and onwards when DNCB was formulated in acetone compared with propylene glycol, the latter having the lowest EC3 value. After 24 hours, the total skin absorptions



**Figure 2.** The release time for dinitrochlorobenzene (DNCB) and isoeugenol in an ethanol-water formulation and in 3 concentrations of ethosomes evaluated by dialysis. Both allergens are released significantly slower when formulated in increasing concentrations of ethosomes. Data represents mean ± standard error of the mean ( $n=3$ ,  $p < .0001$  for DNCB and  $p < .0025$  for isoeugenol, 2-way analysis of variance). POPC = 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine.

**Table 3.** Overview of physical-chemical properties, size of ethosomes, skin penetration formulated in ethosomes, and release time formulated in ethosomes of isoeugenol and dinitrochlorobenzene.

	DNCB	Isoeugenol
Molecular weight	202.5	164.21
Water solubility	Insoluble	Slightly soluble
LogP (O:W)	2.17 <sup>a</sup>	3.04 <sup>a</sup>
Encapsulation efficiency in ethosomes (%)	90 ± 0.3 <sup>b</sup>	77 ± 0.3 <sup>b</sup>
Release time when formulated in ethosomes compared with ethanol-water	Increased	Increased
Skin penetration when formulated in ethosomes compared with ethanol-water	Increased	Decreased
Size of ethosomes (nm)	245 ± 17 <sup>b</sup>	396 ± 20 <sup>b</sup>

<sup>a</sup>Indicates experimental values obtained using the following software: US EPA. Estimation Programs Interface Suite for Microsoft Windows, version 4.00. Washington, DC: United States Environmental Protection Agency, 2010.

<sup>b</sup>Data represent mean ± standard deviation ( $N=3$ ).

DNCB = dinitrochlorobenzene; LogP = logarithm of the ratio of the concentrations of the un-ionized solute in solvents.

O:W = Octanol-Water Partition Coefficient

were similar for the 2 formulations (17). Further, the percentage of the applied dose absorbed through the skin at 4 hours was substantially greater when DNCB was administered in acetone (17). For both vehicles, similar amounts of DNCB were found on top of the skin at 4 hours, but markedly less had penetrated into or beyond the skin when propylene glycol was used as the vehicle, suggesting that increased absorption at 4 hours may be more important than the absorption profile after 24 hours. We found comparable fluxes from 2 to 8 hours for DNCB formulated in ethosomes and ethanol-water. Beyond 8 hours, only a slight increase in flux was seen for the ethosome formulation. On the contrary, we found a significant decrease in flux and lag time when isoeugenol was formulated in ethosomes compared with ethanol-water, resulting in a lower permeability coefficient ( $k_p$ ).

Pendlington et al. studied the sensitizer hexyl cinnamic aldehyde (HCA) in 4 different vehicles (18), of which 3 previously had been tested using the LLNA (7) in an attempt to study the epidermal/dermal disposition of the allergen. The authors did not, however, correlate the skin deposition of HCA in the 3 vehicles to the EC3 values of HCA in the different vehicles. When correlating the sensitizing potency of HCA in the 3 vehicles (in order of increasing potency: acetone-olive oil, propylene glycol, and ethanol) and skin disposition of HCA, a consistent correlation was found between low EC3 value and high flux (0–6 hours) and high cumulative skin absorption, but not between low EC3 value and HCA deposition in stratum corneum, epidermis, and dermis. This is largely consistent with Heylings et al.'s finding that the flux is important, but not with our findings.

In conclusion, there is no simple relationship between bioavailability, skin absorption, and sensitizing capacity of contact allergens in different formulations. It appears that the first hours of skin penetration are decisive for sensitization development. In this study

we focused on 24 hours' data for the skin deposition. It would be interesting to study allergen skin deposition from 0 to 8 hours. Ethosome formulations may affect allergen concentration deeper in the epidermis or dermis within this spectrum of time. New visualization techniques such as confocal and 2-photon microscopy allow real-time noninvasive measurements of the penetration of fluorescent allergens in the different skin departments over time (19) and would be a suitable method for such studies. The time points of interest regarding penetration behavior of allergens may be the first hours after topical application.

It has been stated that skin penetration/absorption of allergens is of only minor importance, for an extremely strong sensitizer such as trimellitic anhydride, with a logP value of  $-2.5$ , because it would be considered too hydrophilic to penetrate readily (20). Vehicle effects have been studied extensively using the mouse LLNA. No cases have been reported where a compound classified as a weak sensitizer in one vehicle was classified as a strong sensitizer in another vehicle (6,7,21). It has been suggested that the enhanced lymph node cell proliferative responses induced by DNCB when applied in sodium lauryl sulfate may be due to increased numbers of dendritic cells reaching the lymph nodes (22). Further, it has been postulated that the vehicle in which DNCB is delivered to the skin may influence cutaneous metabolism secondary to, or independent of, altered absorption kinetics (17). Presumably similar mechanisms could explain the consistently higher sensitizing capacity found in the LLNA when a lipophilic allergen is formulated in ethosomes compared with ethanol-water solution. The mechanisms of allergic contact allergy are complex, and perhaps it is the unique combination of allergen and vehicle that determines the sensitizing and elicitation properties and not just the skin penetration/absorption characteristics of the allergen alone.

Formulating DNCB and isoeugenol in ethosomes increased the release time of the allergens from the dialysis bag (Figure 2). It took more than 1 hour before the released amount of allergen from the ethosome formulation reached the amount of ethanol–water formulation. The speed of release of allergen from the formulation is perhaps more important than the speed of penetration when comparing sensitization properties in different vehicles. However, the exact mechanism of how a vehicle influences the sensitizing properties remains uncertain. The present study on 2 different allergens suggests that skin penetration properties on a wider scale (not just amount, but also kinetics) are important parameters in relation to understanding the allergenicity of chemicals in various vehicles.

## Declaration of interest

The authors report no declarations of interest.

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# Microvesicle Formulations Used in Topical Drugs and Cosmetics Affect Product Efficiency, Performance and Allergenicity

Jakob Torp Madsen and Klaus Ejner Andersen

Attempts to improve the formulations of topical products are continuing processes (ie, to increase cosmetic performance, enhance effects, and protect ingredients from degradation). The development of micro- and nanovesicular systems has led to the marketing of topical drugs and cosmetics that use these technologies. Several articles have reported improved clinical efficacy by the encapsulation of pharmaceuticals in vesicular systems, and the numbers of publications and patents are rising. Some vesicular systems may deliver the drug deeper in the skin as compared to conventional vehicles, or even make transdermal delivery more efficient for a number of drugs. Vesicular systems may also allow a more precise drug delivery to the site of action (ie, the hair follicles) and thereby minimize the applied drug concentration, reducing potential side effects. On the other hand, this may increase the risk of other side effects. Few case reports have suggested that microvesicle formulations may affect the allergenicity of topical products. This article gives an overview of the current knowledge about the topical use of microvesicular systems and the dermatoallergologic aspects.

THE DEVELOPMENT of new formulations for topical products is a continuing process. The encapsulation of product ingredients into different carrier molecules (such as liposomes) may improve product efficiency and is a promising tool for dermal and transdermal delivery of drugs and cosmetic ingredients. The encapsulation technology has been used since the late 1960s, and several topical products marketed today claim benefits from this technology. This review focuses on the use of different types of encapsulating technologies in topical drugs and cosmetics and describes their possible effects on product allergenicity.

## Encapsulation Technology Used in Topical Drugs and Cosmetics

One advantage of encapsulating a drug into liposomes is the possibility of delivering the drug directly to the site of action in the skin at a higher concentration and obtaining a decreased percutaneous absorption at the same time. The penetration pattern is determined by the composition of the

liposome and the encapsulated compound. It is difficult to get approval from health service authorities for topical drugs using encapsulation technology because it is problematic for the manufacturer to prove the presence and stability of the microvesicles in the product. Some pharmaceutical products using microvesicle carriers are commercially available; examples are Pevaryl Lipogel (Cilag Corp., Schaffhausen, Switzerland), which is econazole encapsulated in liposomes, and a local anesthetic formulated in liposomes (LMX4, Ferndale Pharmaceuticals Ltd, UK). Estrasorb (Graceway Pharmaceuticals, Exton, PA) is estradiol encapsulated in micelles in a nanoemulsion for transdermal drug delivery; it is used for reducing hot flares in menopausal women.<sup>1</sup>

Several clinical trials have shown improved biologic effects of products with microvesicle formulations as compared to products with conventional formulations (for treatment of herpes simplex, psoriasis, acne, xerosis, atopic dermatitis, vitiligo, and superficial thrombophlebitis<sup>2-5</sup>). An example is 5-aminolevulinic acid formulated in 50 nm liposomes, which gives a more precise drug delivery that allows a 40% reduction in the amount of active ingredient when used to treat acne with photodynamic therapy. The liposomes concentrate in the pilosebaceous units, thereby reducing side effects and opening doors for new treatment modalities.<sup>6</sup> Another example is the topical administration of methotrexate (MTX), which is hydrophilic and present in dissociated form at physiologic

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hydrogen ion concentration (pH); its capacity for passive diffusion is thus limited. Clinical trials have shown better efficacy of MTX encapsulated in liposomes when compared to placebo and marketed MTX gel, probably owing to increased bioavailability.<sup>2</sup>

The carrier particles themselves are all considered safe for topical use, but the interaction between the carrier particle and the active ingredient may cause biologic effects due to altered skin penetration, release profile, and deposition of the active ingredients.

Lipid vesicles, solid lipid nanoparticles, and polymeric nanoparticles are used in cosmetic formulations to increase bioavailability in stratum corneum and to protect light- and oxygen-sensitive cosmetic ingredients against degradation.<sup>7</sup> Cosmetic ingredients such as retinyl palmitate may cause physiologic changes of the skin but are not claimed to treat skin diseases. Examples of encapsulated cosmetic ingredients are numerous (eg, coenzyme Q<sub>10</sub>, ascorbyl palmitate, tocopherol [vitamin E], and retinol [vitamin A]).<sup>8,9</sup> The types of nanoparticles and micro-particles discussed below are components of marketed cosmetics.

### Liposomes

Liposomes are produced in sizes ranging from 25 nm to several micrometers. They consist of a single or multiple lipid double layer (unilamellar or multilamellar vesicles). Liposomes are capable of carrying amphiphilic active ingredients either in the lipid layer or in the hydrophilic core. They are believed to protect the active ingredients from degradation. Liposomes tend to break down into their constituent components when in contact with the skin. Therefore, liposomes at best can modulate drug transport to stratum corneum, but penetration will require more-stable carriers such as solid lipid nanoparticles or Transfersomes.<sup>10</sup> The concentration of active ingredients in the epidermis may be up to five times greater with liposome formulations than with formulations that use more-conventional vehicles.<sup>11</sup> Liposome formulation in water can easily be incorporated in an aqueous cream for better cosmetic performance. In the cosmetic industry, examples of active ingredients incorporated in liposomes are antioxidants, vitamin A derivatives, and vitamin E.

### Transfersomes

Vesicles produced by adding different amounts of so-called edge activators to the bilayer of classic liposomes (eg,

cholesterol or sodium cholate and a small concentration of ethanol) are called Transfersomes and Flexosomes. The edge activators destabilize the membrane, creating a more flexible structure, and have been shown to penetrate in stratum corneum better as compared to classic liposomes, thereby delivering their encapsulated ingredients deeper in the epidermis but not to the blood circulation.<sup>12</sup> The mechanism of enhancement of skin penetration has not been completely elucidated, but because of their flexibility, Transfersomes are believed to squeeze between the corneocytes, driven by an osmotic force due to the difference in water content of the relatively dehydrated epidermis and the viable dermis.<sup>13</sup> Because of that theory, Transfersomes should not be applied under occluded conditions, which abolishes the osmotic effect. Several drugs encapsulated in Transfersomes (eg, nonsteroidal antiinflammatory drugs [NSAIDs] and local anesthetics) have been tested in animal experiments and showed increased dermal delivery and clinical effect when compared to conventional formulations.<sup>12</sup>

### Ethosomes

Ethosomes are nanocarriers made of phospholipids, ethanol at a high concentration (20–50%), and water. They can deliver drugs to the deep skin layers and the systemic circulation. Ethosomes have a much higher loading capacity of lipophilic drugs as compared to classic liposomes. A clinical trial showed that treatment with ethosomal encapsulated acyclovir significantly improved a herpetic infection when compared to treatment with the traditional Zovirax (GlaxoSmithKline, Brentford, UK) cream with the same concentration of active drug. Insulin-loaded ethosomes have been found to be suitable for systemic transdermal delivery, and the antibiotic bacitracin has likewise been encapsulated in ethosomes and reaches the deep layers of the skin in animal experiments. Ethosomes may play a role in future transdermal drug delivery.<sup>14</sup> Examples of cosmetics using ethosomes are Lipoduction (Osmotics, New York, NY) and Noicellex (Novel Therapeutic Technologies, Inc., Wilmington, DE).

### Niosomes

Niosomes consist of non-ionic surfactant vesicles and are an alternative to liposomes. They can entrap both hydrophilic and hydrophobic chemicals, enhance delivery to the skin, and sustain the release of the drug. A phase I and II study in psoriasis patients concluded that MTX-

loaded niosomes are more efficacious than marketed MTX gel.<sup>2</sup>

### Solid Lipid Nanoparticles

Solid lipid nanoparticles (SLNs) were developed in the 1990s and are produced by replacing the liquid lipid in an oil-in-water emulsion with a lipid that is solid at both room and body temperature. The incorporation of pharmaceuticals and cosmetics in SLNs is feasible and can easily be formulated in a cream.<sup>15</sup> An advantage of SLNs compared to conventional creams is an increase in skin hydration owing to a better occlusive effect by SLNs.<sup>16</sup> Burst or sustained release of incorporated ingredients has been reported, as well as increased percutaneous absorption as compared to conventional formulations; this is probably due to the unique composition of the SLN and incorporated ingredient. Examples of pharmaceuticals formulated in SLNs are podophyllotoxin, antimycotics, NSAIDs, psoralen, and topical glucocorticoids. No human studies with pharmaceuticals incorporated in SLNs have been performed yet, but more than 30 cosmetic products containing SLNs were marketed in 2008.<sup>17</sup> No side effects have been reported.

### Nanoemulsions

Nanoemulsions consist of two phases, with droplets of 50 to 100 nm in the external phase. Emulsifiers that are used to bind together oil and water in products such as hair conditioners and makeup removers yield a less oily mixture when they are broken down into nanoparticles. Nanoemulsions are used in both rinse-off and stay-on products. Opposing results are obtained on the relation between emulsion droplet size and the depth of dermal penetration of the active ingredients. Nanoemulsions increase the transdermal bioavailability of vitamin E,<sup>18</sup> but penetration of tetracaine from nanoemulsion is not affected by a droplet size of 100 to 3,500 nm on the skin.<sup>19</sup> Different emulsion components have been used, and other authors have found increasing transdermal penetration with decreasing droplet size. There is so far no simple relationship between chemical, particle size, and penetration, and each new emulsion carrying different active ingredients must be investigated separately to ascertain its skin penetration pattern.

### Nanospheres

Nanospheres are produced from different polymers (eg, polycaprolactone, a biodegradable product widely used in

the cosmetic industry). When produced, the polymer wraps around itself, creating lipo- and hydrophilic spaces. Several drugs have been incorporated in nanospheres,<sup>20</sup> as have cosmetic ingredients.<sup>21</sup> L'Óreal (Paris, France) has developed a nanocarrier system called Nanosome, which consists of the biodegradable polymer polycaprolactone; other cosmetic companies have developed similar products. Polycaprolactone nanoparticles loaded with the lipophilic dyeing agent Nile red showed enhanced penetration of the molecule into the stratum corneum layers (up to 60  $\mu\text{m}$ ) as compared to non-nanoparticle formulations.<sup>22,23</sup> The distribution of another topically applied nanosphere Nile red formulation was studied in human cadaver skin with cryosectioning and fluorescence microscopy by Sheihet and colleagues.<sup>24</sup> Permeation analysis revealed that the nanospheres delivered nine times more Nile red to the lower dermis than a control formulation using propylene glycol did. Few articles have been published about penetration and absorption in skin and the clinical effect of carrier molecules manufactured by cosmetic companies.

### Dermatitis Related to Exposure to Products Containing Microvesicles

Few case reports have suggested that microvesicles in topical products may have been involved in the development of allergic contact dermatitis. Propyl gallate incorporated in liposomes has been suggested to boost the allergic potential of propyl gallate in 13 patients; however, patch tests with and without the liposomal formulation were not performed.<sup>25</sup> Another case report described a woman who developed severe allergic contact dermatitis from an antiwrinkle cream containing retinyl palmitate encapsulated in polycaprolactone.<sup>21</sup> Polycaprolactone is a polymeric drug delivery system capable of encapsulating lipo- and hydrophilic agents. Retinyl palmitate is a rare contact allergen, and diagnostic patch tests revealed that the patient reacted more strongly to encapsulated retinyl palmitate than to retinyl palmitate in petrolatum, even though the retinyl palmitate concentration was much lower when formulated in polycaprolactone when compared to the petrolatum formulation (Fig 1).

### Enhanced Allergenicity of Compounds Encapsulated in Microvesicle Formulations

Increased sensitization response was found by local lymph node assay (LLNA) when dinitrochlorobenzene and isoeugenol encapsulated in ethosomes were compared to



**Figure 1.** Patch-test results of retinyl palmitate in petrolatum, encapsulated retinyl palmitate in polycaprolactone, and pure polycaprolactone. Retinyl palmitate 5% in petrolatum yielded a + reaction; polycaprolactone, a negative reaction; and retinyl palmitate in polycaprolactone, a ++ reaction. Note that retinyl palmitate in polycaprolactone is at a lower concentration than retinyl palmitate in petrolatum. Encapsulating retinyl palmitate in polycaprolactone increased the strength of the patch-test reactions. (PCL = polycaprolactone; pet = in petrolatum; RP = retinyl palmitate.)

formulations without ethosomes.<sup>26</sup> Controlled patch-test experiments in selected sensitized volunteer patients and using ethosomes loaded with methyldibromoglutaronitrile or isoeugenol showed enhanced patch-test responses in comparison to patch tests with the same allergens in ethanol and water (4:6) formulations, making the vesicles the only difference.<sup>27</sup> Vehicle effects on both sensitization and elicitation responses in experiments using LLNA and human volunteers as test subjects were previously reported.<sup>28,29</sup> However, the effect of new encapsulating vehicles on product allergenicity is rarely studied. The particles in these experiments exceeded the 100 nm size limit for nanoparticles. However, particles larger than 100 nm also may show size-specific properties; for example, liposomes of 120 nm penetrate human skin to a greater extent than do liposomes of 810 nm.<sup>30</sup> So far, no reports have suggested that vesicles of nanosize range increase the skin penetration of encapsulated compounds as compared to similar formulations without the nano-sized vesicles.

## Conclusion

The dermatotoxicologic risk from skin exposure to microvesicle carrier systems is considered to be low. No general rules can be determined from the reported experiments, and risk assessment should be done on a case-by-case basis. Given the limited information available, it is important that dermatologists be aware of the use of encapsulation technology in products that cause contact dermatitis as

encapsulation of product ingredients may affect allergenicity in some cases. Whether a product contains microvesicles may be difficult to ascertain if it is not mentioned on the label. Words such as “nanosphere,” “liposome,” and “encapsulated” can be looked for, but often these words appear not on the label but rather in the marketing folder. Based on information from the manufacturers or other sources, the Web site <[www.nanotechproject.org](http://www.nanotechproject.org)> lists consumer products that use nanotechnology and different carrier technologies. The list is far from complete but can be helpful. Dermatologists investigating patients with allergic reactions to consumer products that use encapsulation technology should consider the risk of false-negative results when testing with ingredients in conventional patch-test vehicles. It is important to collaborate with the manufacturer; manufacturers can sometimes provide dermatologists with samples of encapsulated compounds for patch testing.<sup>21</sup> Whether these new formulation systems really pose a risk for consumers in regard to allergic skin reactions from the use of topical products using this technology is not documented so far, but experimental data show that such a risk is possible. Dermatologists are urged to look for dermatitis patients with possible allergic skin reactions from topical products using nano- or microvesicle technology and to be aware of the importance of patch-test vehicles.

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