

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

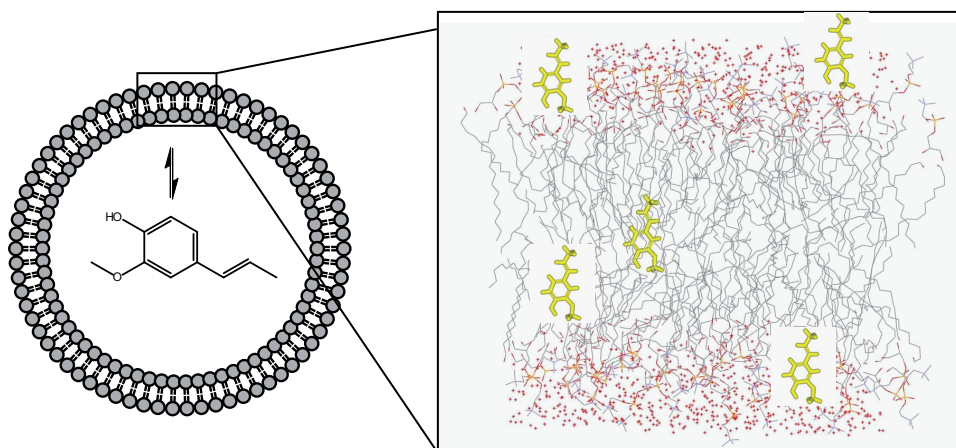


Fig. 1. Model of isoeugenol (yellow) encapsulated in an ethosome. *Left-hand figure*: schematic representation of a unilamellar liposome. *Right-hand figure*: enlarged view from a coarse-grain computer simulation of phosphatidylcholine (POPC). The membrane patch represents a 45×50 Å area from the published 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 ± 8 and 317 ± 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-

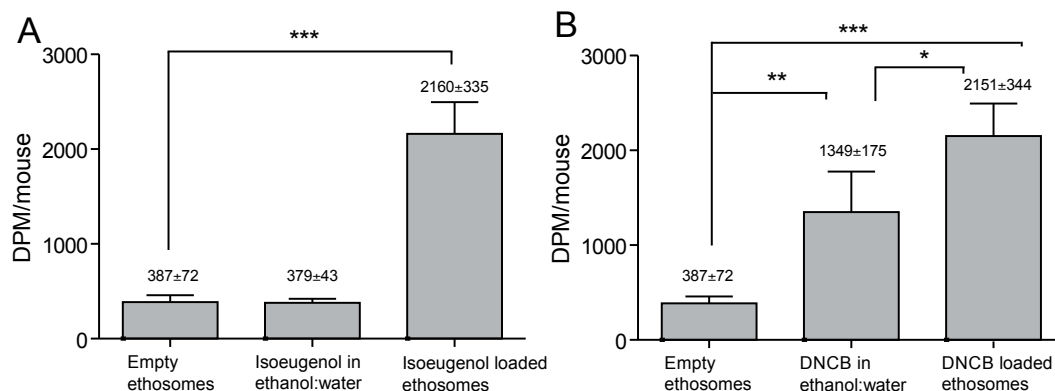


Fig. 2. Encapsulation of isoeugenol and dinitrochlorobenzene (DNCB) in ethosomes increases their sensitizing capacity. (A) Isoeugenol (1.1% w/v) loaded ethosomes (60 mg/ml) significantly increase the sensitizing capacity in the local lymph node assay compared with empty ethosomes and isoeugenol dissolved 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).

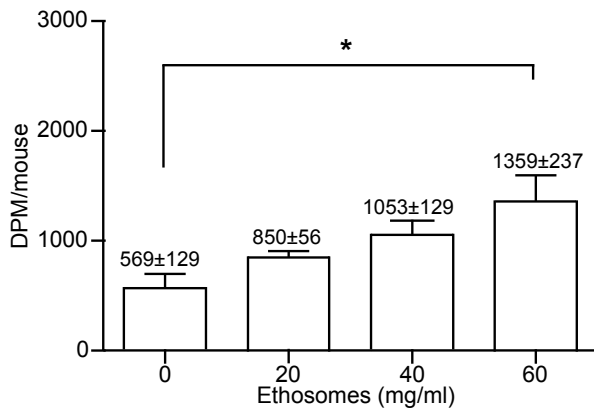


Fig. 3. Encapsulated isoeugenol in ethosomes reaches a significant sensitizing potency by increasing the amount of phosphatidylcholine (POPC) to 60 mg/ml ethosomes compared with the control vehicle (0 mg/ml POPC) in the local lymph node assay (LLNA). * $p < 0.05$ ($n = 5$ in each group) (Mean \pm standard 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 EC₃ 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 EC₃ values reported from other LLNA experiments with isoeugenol using ethanol/water as vehicle. In accordance with this,

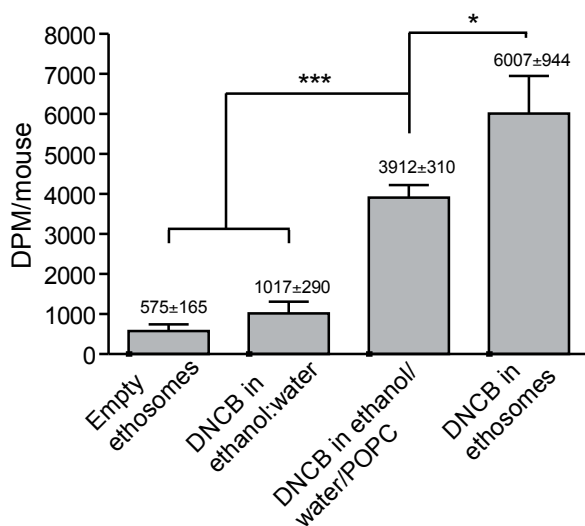


Fig. 4. Encapsulated dinitrochlorobenzene (DNCB) (0.04% w/v) in ethosomes increases the sensitizing capacity significantly (* $p < 0.05$, *** $p < 0.001$) compared with DNCB in ethanol/water/phosphatidylcholine (POPC) solution. ($n = 4-6$ in each group) (Mean \pm standard error of the mean).

isoeugenol did not sensitize in the solution, only in the ethosome formulation. DNCB is a more potent allergen, which permitted a concentration above its EC₃ 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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