

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.

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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 methyl dibromo 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 Tóutou (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 $\pm$ 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

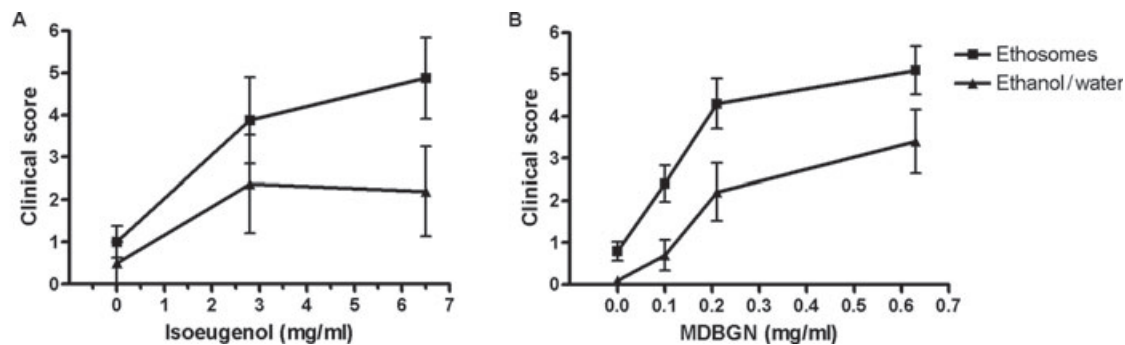


Fig. 1. Patch test results for methyldibromo glutaronitrile (MDBGN) ($n = 19$) and isoeugenol ($n = 8$) encapsulated in ethosomes (100 mg/ml) as compared with the same concentrations of allergen in ethanol/water. Significant increases in patch test reactions are seen for both allergens encapsulated in 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.

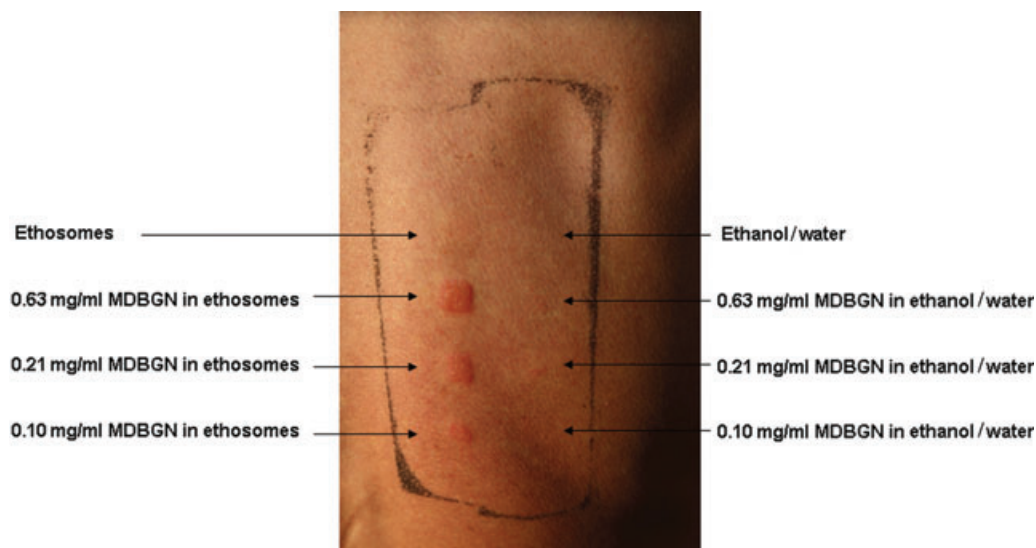


Fig. 2. Result of a serial dilution patch test in a sensitized volunteer with methyldibromo glutaronitrile (MDBGN), using IQ-chambers and 15 μ l of test substance formulated in ethosomes and ethanol/water.

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 ± 13 and 463 ± 13 nm, and the PI ranged from 0.06 ± 0.04 to 0.22 ± 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

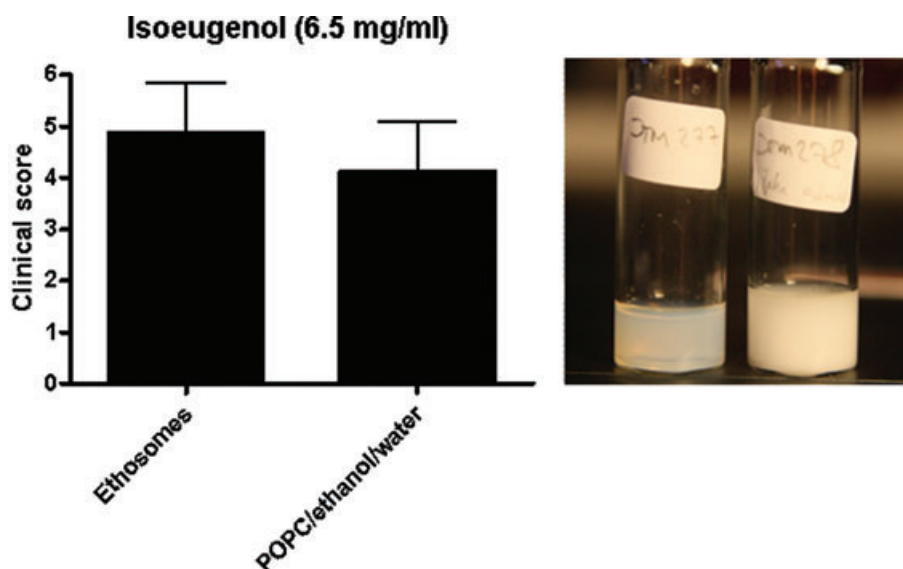


Fig. 3. Patch test results with 6.5 mg/ml isoeugenol formulated in ethosomes (400 nm) and in 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC)/ethanol/water ($n = 8$). No significant difference was observed (Wilcoxon signed rank test). Results are expressed as means $\pm$ standard error of the 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[®] (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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