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Assessment of fluidity of different invasomes by electron spin resonance and differential scanning calorimetry

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ABSTRACT

The aim of this study was to investigate the influence of membrane-softening components (terpenes/terpene mixtures, ethanol) on fluidity of phospholipid membranes in invasomes, which contain besides phosphatidylcholine and water, also ethanol and terpenes. Also mTHPC was incorporated into invasomes in order to study its molecular interaction with phospholipids in vesicular membranes. Fluidity of bilayers was investigated by electron spin resonance (ESR) using spin labels 5- and 16-doxyl stearic acid and by differential scanning calorimetry (DSC). Addition of 1% of a single terpene/terpene mixture led to significant fluidity increase around the C16 atom of phospholipid acyl chains comprising the vesicles. However, it was not possible to differentiate between the influences of single terpenes or terpene mixtures. Incorporation of mTHPC into the bilayer of vesicles decreased fluidity near the C16 atom of acyl chains, indicating its localization in the inner hydrophobic zone of bilayers. These results are in agreement with DSC measurements, which showed that terpenes increased fluidity of bilayers, while mTHPC decreased fluidity. Thus, invasomes represent vesicles with very high membrane fluidity. However, no direct correlation between fluidity of invasomes and their penetration enhancing ability was found, indicating that besides fluidity also other phenomena might be responsible for improved skin delivery of mTHPC.

1. Introduction

The main barrier that limits the skin penetration of drugs, and therefore the access of the drug to its therapeutic target, is the most apical layer of the skin, the stratum corneum (SC). The SC is composed of keratin-filled corneocytes, which are embedded in a lipid-enriched intercellular matrix. The permeability barrier is located within lipid bilayers in the intercellular spaces of the SC (Elias and Friend, 1975; Landmann, 1986). However, some of the SC properties can be influenced (Wang et al., 1998; Cevc et al., 2003; Lopez et al., 2003; Cancel et al., 2004). One possibility is the use of vesicular systems, i.e. liposomes, which are being intensively studied as drug carrier systems for dermal and transdermal administration of drugs (Schreier and Bouwstra, 1994; Touitou et al., 2000; Cevc et al., 2002; Song and Kim, 2006).

In earlier studies (Dragicevic-Curic et al., 2008a,b, 2009a,b,c) different vesicular systems were investigated in order to find an efficient delivery system for the most potent second-generation photosensitizer temoporfin (mTHPC) (Hopper, 2000; Biel, 2002). mTHPC was reported to be effective in the treatment of primary non-melanomatous skin tumors of the head and neck after its intravenous administration (Kübler et al., 1999). Unfortunately, mTHPC (Fig. 1) is a highly hydrophobic drug with a large molecular weight (680 Da), and it is practically insoluble in all aqueous media. Thus, its percutaneous penetration is very low and the selection of a suitable vehicle, which could affect drug release and percutaneous penetration, is very important.

It has been reported in the literature (van Kuijk-Meuwissen et al., 1998; El Maghraby et al., 1999, 2001) that liquid-state vesicles are superior to rigid gel-state vesicles in terms of enhanced drug penetration. Furthermore, elastic vesicles have been shown to be superior to conventional gel-state and even liquid-state vesicles in terms of interactions with human skin (van den Bergh et al., 1999) and enhanced drug penetration (El Maghraby et al., 1999). Therefore, a series of liquid-state vesicles with elastic membranes were developed (Cevc et al., 1998; van den Bergh et al.,

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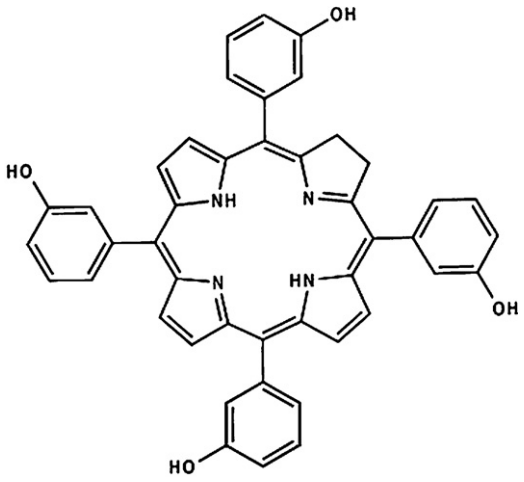

Fig. 1. Chemical structure of mTHPC.

1999; Touitou et al., 2000). The membranes of elastic vesicles are usually composed of phospholipids (i.e. phosphatidylcholine (PC)) and membrane-softening components. In the case of the so-called Transferosomes®, these membrane-softening components are sodium cholate, polysorbate 80 or polysorbate 20 (Cevc et al., 2002; Cevc and Blume, 2003, 2004), whereas high amounts of ethanol are used for ethosomes (Touitou et al., 2000) and small amounts of ethanol and terpenes for invasomes (Verma et al., 2004; Dragicevic-Curic et al., 2008a,b, 2009a). Another type of elastic vesicles is composed of the bilayer-forming surfactant L-595 (sucrose laurate ester) and the micelle-forming surfactant PEG-8-L (octaoxyethylene laurate ester) (van den Bergh et al., 1999). According to Cevc et al. (1998), such vesicles are able to squeeze themselves through the intercellular regions of the SC due to their highly elastic membranes and due to the transepidermal water-gradient. Thus, compared to more rigid liquid-state vesicles, elastic vesicles might further increase the drug transport across the skin.

In order to find the most efficient delivery system for mTHPC, different vesicles with elastic membranes were investigated. Among them ethanol-containing liposomes were able to deliver high amounts of mTHPC into the SC and into deeper skin layers, thus being superior to conventional liposomes (Dragicevic-Curic et al., 2009b). Compared to conventional liposomes also invasomes (Dragicevic-Curic et al., 2008a, 2009a) and neutral, cationic and anionic flexible vesicles containing polysorbate 20 (Dragicevic-Curic et al., 2009c) led to a significantly enhanced skin deposition of mTHPC. However, among these different elastic vesicles, invasomes (e.g. containing 1% of a single terpene or of a terpene mixture) delivered the highest amount of mTHPC into the SC and into deeper skin layers.

Since the penetration enhancing ability of vesicles depends on the fluidity and elasticity of their bilayers, the aim of this study was to investigate the interaction of membrane-softening components (terpenes, mixtures of terpenes and ethanol) with phospholipid membranes in invasomes. The influence of the membrane-softening components on the membrane fluidity should be evaluated and compared for different invasomes in order to explain their different penetration enhancing ability, which was previously studied (Dragicevic-Curic et al., 2008a, 2009a). Another goal was to study the molecular interaction between mTHPC and membrane phospholipids. Therefore, the fluidity of the bilayers of vesicles as well as the molecular interactions were investigated by electron spin resonance (ESR) using the spin labels 5- and 16-doxyl stearic acid (5- and 16-DSA) and by differential scanning calorimetry (DSC). ESR has been widely used to investigate lipid mobility in biological membranes and vesicles (Korstanje et al., 1989, 1990). It can also provide decisive information on the localization of the drug molecules in the liposomal membranes and reflect the changes in the membrane mobility (Budai et al., 2003, 2004). In bilayers, the spin labels 5- and 16-DSA are oriented with their longest axis parallel to the acyl chains, yielding information on the environment of the C5 and C16 carbons and on the fluidity across the bilayer, respectively. Furthermore, for the DSC measurements, dimyristoylphosphatidylcholine (DMPC) was used to prepare the formulations which were investigated. Besides dipalmitoylphosphatidylcholine (DPPC) also DMPC is used as a model phospholipid, which is widely used to study the thermotropic phase behaviour of liposomes and biological membranes using DSC, since its transition temperature can be measured easily. Furthermore, information about molecular interactions of a drug and the membrane phospholipids can be derived.

2. Materials and methods

2.1. Materials

Unsaturated soybean phosphatidylcholine (PC) dissolved in ethanol (NAT 8539 = PC:ethanol = 75:25, w/w) was a gift from Phospholipid GmbH (Germany). Dimyristoylphosphatidylcholine (DMPC) was a gift from Lipoid GmbH (Germany). 5- and 16-doxyl stearic acid (5-DSA and 16-DSA), cineole, citral and D-limonene were obtained from Sigma Chemicals (USA). Temoporfin, i.e. 7,8-dihydro-5,10,15,20-tetrakis-(3-hydroxyphenyl) porphyrin (mTHPC) was a gift from Biolitec (Germany). Methanol (HPLC grade) was purchased from Carl Roth (Germany). The phosphate buffered saline (PBS) pH 7.4 was prepared according to the European Pharmacopoeia. All other chemicals were of analytical grade.

2.2. Liposome preparation

The composition of the different formulations prepared for the ESR measurements is given in Table 1. Liposomes without mTHPC, but with the stearic acid spin labels (5- or 16-DSA) were prepared by the conventional film method. The molar ratio of the spin labels 5-DSA/16-DSA to lipid was 1:100. PC in ethanol (NAT 8539) and one of the spin labels (5- or 16-DSA) were dissolved in methanol:chloroform (2:1, v/v). This mixture was dried to a thin film by slowly reducing the pressure from 500 to 1 mbar at 50 °C using the rotary evaporator (BÜCHI Vacubox B-177, BÜCHI, Switzerland). The film was kept under vacuum (1 mbar) for 2 h at room temperature and subsequently flushed with nitrogen. Then, the film was hydrated for 30 min at 50 °C using PBS (pH 7.4). After cooling to room temperature, ethanol and a single terpene or a terpene mixture were added in order to obtain the liposome and invasome formulations given in Table 1. The obtained vesicles were vortexed, ultrasonicated and subsequently sized by 21 times extrusion through polycarbonate membranes of different pore sizes (400 nm, 200 nm, 100 nm and 50 nm) using the LiposoFast® mini-extruder (Avestin, Canada).

Liposomes containing mTHPC and spin labels (Table 1) were prepared in the same manner. The mTHPC was added to the lipid dissolved in methanol:chloroform prior the evaporation step. Liposomes containing mTHPC and ethanol were prepared by ethanol addition after the hydration step and after cooling to room temperature. Regarding invasomes containing mTHPC, ethanol and terpenes/terpene mixtures were also added, as described above, after the hydration step and cooling to room temperature. Among different terpene mixtures, only two terpene mixtures were chosen for the preparation of mTHPC-containing invasomes (Table 1).

Table 1
Composition of the different liposomes and invasomes containing the spin labels for ESR measurements. The molar ratio of the spin labels 5-DSA/16-DSA to lipid was 1:100.

Formulation	mTHPC %, w/v	PC (NAT8539) %, w/v	Ethanol %, w/v	Terpenes %, w/v	PBS %, w/v
5-DSA/16-DSA liposomes without mTHPC					
Liposomes 0% ethanol	–	10	–	–	ad 100
Liposomes 3.3% ethanol	–	10	3.3	–	ad 100
Invasomes 3.3% ethanol 0.5% terpene mix[a]	–	10	3.3	0.5	ad 100
Invasomes 3.3% ethanol 1% terpene mix[a]	–	10	3.3	1.0	ad 100
Invasomes 3.3% ethanol 1% terpene mix 1[b]	–	10	3.3	1.0	ad 100
Invasomes 3.3% ethanol 1% terpene mix 2[c]	–	10	3.3	1.0	ad 100
Invasomes 3.3% ethanol 1% terpene mix 3[d]	–	10	3.3	1.0	ad 100
Invasomes 3.3% ethanol 1% terpene mix 4[e]	–	10	3.3	1.0	ad 100
Invasomes 3.3% ethanol 1% citral	–	10	3.3	1.0	ad 100
Invasomes 3.3% ethanol 1% cineole	–	10	3.3	1.0	ad 100
Invasomes 3.3% ethanol 1% D-limonene	–	10	3.3	1.0	ad 100
5-DSA/16-DSA liposomes with mTHPC					
Liposomes 0% ethanol	0.15	10	–	–	ad 100
Liposomes 3.3% ethanol	0.15	10	3.3	–	ad 100
Invasomes 3.3% ethanol 1% terpene mix[a]	0.15	10	3.3	1.0	ad 100
Invasomes 3.3% ethanol 1% terpene mix 3[d]	0.15	10	3.3	1.0	ad 100
Invasomes 3.3% ethanol 1% citral	0.15	10	3.3	1.0	ad 100
Invasomes 3.3% ethanol 1% cineole	0.15	10	3.3	1.0	ad 100
Invasomes 3.3% ethanol 1% D-limonene	0.15	10	3.3	1.0	ad 100

[a] Terpene mix = cineole:citral:D-limonene = 0.45:0.45:0.10, v/v = standard.
[b] Terpene mix 1 = cineole:citral:D-limonene = 0.66:0.17:0.17, v/v.
[c] Terpene mix 2 = cineole:citral:D-limonene = 0.17:0.66:0.17, v/v.
[d] Terpene mix 3 = cineole:citral:D-limonene = 0.17:0.17:0.66, v/v.
[e] Terpene mix 4 = cineole:citral:D-limonene = 0.33:0.33:0.33, v/v.

In contrast to the formulations used for the ESR studies, formulations being prepared for DSC measurements were composed of the saturated phospholipid dimyristoyl-phosphatidylcholine (DMPC) instead of the unsaturated PC. DMPC-containing liposomes (for composition see Table 2) were prepared in the same manner as described above, but without adding the spin labels.

2.3. Photon correlation spectroscopy (PCS)

Particle size and polydispersity index (PDI) were determined by PCS using the Zetasizer Nano ZS (Malvern Instruments, UK). The formulations were diluted in filtered PBS (pH 7.4) at 25 °C and measured at an angle of 173°. The particle size given as the *z*-average diameter was calculated from the autocorrelation function of the intensity of light scattered from the particles, assuming a spherical particle shape, a medium viscosity of 0.89 mPa s and a refractive index of 1.33. The results are given as the mean value of three consecutive measurements.

2.4. Electron spin resonance (ESR) measurements

The spin labels 5- and 16-DSA were used for each vesicle dispersion (Table 1). Electron spin resonance spectra were taken with a Bruker ESP 300 E spectrometer at about 10 GHz (X-Band) at room temperature (24 °C). Modulation frequency, modulation amplitude and microwave power were 100 kHz, 0.5 G and 15 mW, respec-

Table 2
Composition of the different liposomes and invasomes for DSC measurements.

Formulation	mTHPC %, w/v	DMPC %, w/v	Ethanol %, w/v	Terpenes %, w/v	PBS %, w/v
DMPC-liposomes without mTHPC					
DMPC-liposomes 0% ethanol (1)	–	10	–	–	ad 100
DMPC-liposomes 3.3% ethanol (2)	–	10	3.3	–	ad 100
DMPC-liposomes 10% ethanol (3)	–	10	10.0	–	ad 100
DMPC-invasomes 3.3% ethanol 0.5% terpene mix[a] (4)	–	10	3.3	0.5	ad 100
DMPC-invasomes 3.3% ethanol 1% terpene mix[a] (5)	–	10	3.3	1.0	ad 100
DMPC-invasomes 3.3% ethanol 1% terpene mix 1[b] (6)	–	10	3.3	1.0	ad 100
DMPC-invasomes 3.3% ethanol 1% terpene mix 2[c] (7)	–	10	3.3	1.0	ad 100
DMPC-invasomes 3.3% ethanol 1% terpene mix 3[d] (8)	–	10	3.3	1.0	ad 100
DMPC-invasomes 3.3% ethanol 1% terpene mix 4[e] (9)	–	10	3.3	1.0	ad 100
DMPC-invasomes 3.3% ethanol 1% citral (10)	–	10	3.3	1.0	ad 100
DMPC-invasomes 3.3% ethanol 1% cineole (11)	–	10	3.3	1.0	ad 100
DMPC-invasomes 3.3% ethanol 1% D-limonene (12)	–	10	3.3	1.0	ad 100
DMPC-liposomes with mTHPC					
mTHPC-invasomes 3.3% ethanol 1% terpene mix[a] (13)	0.15	10	3.3	1.0	ad 100
mTHPC-invasomes 3.3% ethanol 1% terpene mix 3[d] (14)	0.15	10	3.3	1.0	ad 100
mTHPC-invasomes 3.3% ethanol 1% citral (15)	0.15	10	3.3	1.0	ad 100
mTHPC-invasomes 3.3% ethanol 1% cineole (16)	0.15	10	3.3	1.0	ad 100
mTHPC-invasomes 3.3% ethanol 1% D-limonene (17)	0.15	10	3.3	1.0	ad 100

[a] Terpene mix = cineole:citral:D-limonene = 0.45:0.45:0.10, v/v = standard.
[b] Terpene mix 1 = cineole:citral:D-limonene = 0.66:0.17:0.17, v/v.
[c] Terpene mix 2 = cineole:citral:D-limonene = 0.17:0.66:0.17, v/v.
[d] Terpene mix 3 = cineole:citral:D-limonene = 0.17:0.17:0.66, v/v.
[e] Terpene mix 4 = cineole:citral:D-limonene = 0.33:0.33:0.33, v/v.

Table 3
Particle size, PDI, and calculated order parameter S (experimental uncertainty ± 5%) and rotational correlation time τ_c (experimental uncertainty ± 5%) from the ESR spectra of different liposomes without mTHPC. The molar ratio of the spin labels 5-DSA/16-DSA to lipid was 1:100.

Formulation	z-average (nm)	PDI	S	τ_c (ns)
Liposomes with 5-DSA				
Liposomes 0% ethanol	132.3 ± 0.8	0.112 ± 0.004	0.43	–
Liposomes 3.3% ethanol	110.5 ± 1.2	0.109 ± 0.004	0.41	–
Invasomes 3.3% ethanol 0.5% terpene mix[a]	111.3 ± 0.6	0.108 ± 0.003	0.40	–
Invasomes 3.3% ethanol 1% terpene mix[a]	123.8 ± 0.5	0.098 ± 0.005	0.40	–
Invasomes 3.3% ethanol 1% terpene mix 1[b]	120.8 ± 0.9	0.109 ± 0.003	0.40	–
Invasomes 3.3% ethanol 1% terpene mix 2[c]	130.1 ± 1.2	0.104 ± 0.007	0.39	–
Invasomes 3.3% ethanol 1% terpene mix 3[d]	155.9 ± 0.1	0.118 ± 0.006	0.40	–
Invasomes 3.3% ethanol 1% terpene mix 4[e]	153.2 ± 1.1	0.119 ± 0.008	0.39	–
Invasomes 3.3% ethanol 1% citral	132.7 ± 0.9	0.157 ± 0.004	0.38	–
Invasomes 3.3% ethanol 1% cineole	109.3 ± 0.4	0.096 ± 0.003	0.39	–
Invasomes 3.3% ethanol 1% D-limonene	135.5 ± 0.8	0.090 ± 0.010	0.40	–
Liposomes with 16-DSA				
Liposomes 0% ethanol	126.2 ± 1.1	0.111 ± 0.002	0.31	1.01
Liposomes 3.3% ethanol	108.4 ± 1.2	0.113 ± 0.006	0.29	0.94
Invasomes 3.3% ethanol 0.5% terpene mix[a]	109.0 ± 1.2	0.099 ± 0.011	0.28	1.02
Invasomes 3.3% ethanol 1% terpene mix[a]	118.2 ± 0.6	0.072 ± 0.009	0.27	0.52
Invasomes 3.3% ethanol 1% terpene mix 1[b]	123.1 ± 0.4	0.089 ± 0.003	0.27	0.56
Invasomes 3.3% ethanol 1% terpene mix 2[c]	124.1 ± 0.2	0.114 ± 0.007	0.27	0.45
Invasomes 3.3% ethanol 1% terpene mix 3[d]	151.9 ± 0.1	0.113 ± 0.007	0.27	0.47
Invasomes 3.3% ethanol 1% terpene mix 4[e]	158.2 ± 2.8	0.209 ± 0.008	0.27	0.47
Invasomes 3.3% ethanol 1% citral	129.7 ± 0.9	0.081 ± 0.008	0.27	0.46
Invasomes 3.3% ethanol 1% cineole	105.8 ± 0.8	0.084 ± 0.005	0.27	0.58
Invasomes 3.3% ethanol 1% D-limonene	130.8 ± 0.4	0.096 ± 0.010	0.27	0.51

[a] Terpene mix = cineole:citral:D-limonene = 0.45:0.45:0.10, v/v = standard.
[b] Terpene mix 1 = cineole:citral:D-limonene = 0.66:0.17:0.17, v/v.
[c] Terpene mix 2 = cineole:citral:D-limonene = 0.17:0.66:0.17, v/v.
[d] Terpene mix 3 = cineole:citral:D-limonene = 0.17:0.17:0.66, v/v.
[e] Terpene mix 4 = cineole:citral:D-limonene = 0.33:0.33:0.33, v/v.

tively. The scan time was 167 s or 335 s with a resolution of 1024 points on a field interval of 100 G. All parameters were estimated with the Bruker WIN-EPR program. The vesicles bilayer dynamics and ordering are characterized by the order parameter S and the rotational correlation time τ_c. Both parameters were used in order to characterize the fluidity of the lipid membrane in 16-DSA-containing vesicles, whereas only the order parameter S was used for the characterization of 5-DSA-containing vesicles.

2.5. Differential scanning calorimetry (DSC) measurements

The phase behaviour of the formulations (for composition see Table 2) was investigated in a Micro DSC III (Setaram, France). Approximately 300 mg of the samples were measured at a scan rate of 0.5 °C/min. A crucible filled with undecane was used as reference. The crucibles were introduced into the device at ambient temperature and subsequently cooled to −5 or −10 °C, respectively. A measurement consisted of two heating runs and two cooling runs with alternating heating (−5/−10 °C to 55 °C) and cooling steps (55 °C to −5/−10 °C). Between heating and cooling an isothermal phase of 5 min was entered.

3. Results and discussion

3.1. Characterization of different vesicles (particle size and PDI)

Tables 3–5 summarize the results obtained for particle size and PDI of the vesicles used for ESR and DSC measurements. The results

Table 4
Particle size, PDI, and calculated order parameter S (experimental uncertainty ± 5%) and rotational correlation time τ_c (experimental uncertainty ± 5%) from the ESR spectra of different mTHPC-containing liposomes. The molar ratio of the spin labels 5-DSA/16-DSA to lipid was 1:100.

Formulation	z-average (nm)	PDI	S	τ_c (ns)
mTHPC-liposomes with 5-DSA				
mTHPC-liposomes 0% ethanol	133.6 ± 0.9	0.108 ± 0.014	0.43	–
mTHPC-liposomes 3.3% ethanol	95.6 ± 0.6	0.172 ± 0.004	0.42	–
mTHPC-invasomes 3.3% ethanol 1% terpene mix[a]	120.1 ± 2.9	0.078 ± 0.004	0.42	–
mTHPC-invasomes 3.3% ethanol 1% terpene mix 3[b]	165.3 ± 0.8	0.108 ± 0.003	0.42	–
mTHPC-invasomes 3.3% ethanol 1% citral	151.2 ± 1.1	0.101 ± 0.007	0.41	–
mTHPC-invasomes 3.3% ethanol 1% cineole	109.1 ± 0.9	0.070 ± 0.003	0.40	–
mTHPC-invasomes 3.3% ethanol 1% D-limonene	149.2 ± 0.6	0.085 ± 0.002	0.43	–
mTHPC-liposomes with 16-DSA				
mTHPC-liposomes 0% ethanol	127.7 ± 1.1	0.125 ± 0.003	0.32	1.19
mTHPC-liposomes 3.3% ethanol	99.3 ± 1.2	0.136 ± 0.006	0.30	1.17
mTHPC-invasomes 3.3% ethanol 1% terpene mix[a]	121.1 ± 1.7	0.086 ± 0.005	0.28	0.70
mTHPC-invasomes 3.3% ethanol 1% terpene mix 3[b]	160.0 ± 0.9	0.101 ± 0.004	0.28	0.67
mTHPC-invasomes 3.3% ethanol 1% citral	146.1 ± 0.8	0.085 ± 0.003	0.28	0.62
mTHPC-invasomes 3.3% ethanol 1% cineole	117.6 ± 0.6	0.086 ± 0.007	0.28	0.65
mTHPC-invasomes 3.3% ethanol 1% D-limonene	152.6 ± 0.4	0.077 ± 0.003	0.28	0.91

[a] Terpene mix = cineole:citral:D-limonene = 0.45:0.45:0.10, v/v = standard.
[b] Terpene mix 3 = cineole:citral:D-limonene = 0.17:0.17:0.66, v/v.

Table 5
Particle size, PDI, and transition temperatures of different liposomes without and with mTHPC.

Formulation	*z*-average (nm)	PDI	Peak temperatures (°C)
DMPC-liposomes without mTHPC			
DMPC-liposomes 0% ethanol (1)	152.9 ± 0.5	0.122 ± 0.004	14.2 24.3
DMPC-liposomes 3.3% ethanol (2)	136.1 ± 0.6	0.124 ± 0.029	9.9 23.2
DMPC-liposomes 10% ethanol (3)	108.0 ± 0.9	0.099 ± 0.008	21.9
DMPC-invasomes 3.3% ethanol 0.5% terpene mix[a] (4)	143.7 ± 0.5	0.166 ± 0.009	17.1
DMPC-invasomes 3.3% ethanol 1% terpene mix[a] (5)	145.4 ± 0.1	0.125 ± 0.002	3.8 8.9 13.8
DMPC-invasomes 3.3% ethanol 1% terpene mix 1[b] (6)	148.5 ± 3.2	0.178 ± 0.003	8.9 13.1
DMPC-invasomes 3.3% ethanol 1% terpene mix 2[c] (7)	144.6 ± 4.0	0.129 ± 0.004	5.4 14.7
DMPC-invasomes 3.3% ethanol 1% terpene mix 3[d] (8)	155.8 ± 2.6	0.126 ± 0.008	0.0 14.0
DMPC-invasomes 3.3% ethanol 1% terpene mix 4[e] (9)	159.0 ± 5.1	0.160 ± 0.005	−1.0 10.4 14.4
DMPC-invasomes 3.3% ethanol 1% citral (10)	152.1 ± 4.7	0.113 ± 0.005	9.3 12.9 16.7
DMPC-invasomes 3.3% ethanol 1% cineole (11)	131.9 ± 3.7	0.168 ± 0.007	12.8
DMPC-invasomes 3.3% ethanol 1% D-limonene (12)	156.2 ± 3.4	0.125 ± 0.004	0.8 13.8
DMPC-liposomes with mTHPC			
mTHPC-invasomes 3.3% ethanol 1% terpene mix[a] (13)	110.1 ± 1.8	0.103 ± 0.005	14.6 20.7
mTHPC-invasomes 3.3% ethanol 1% terpene mix 3[d] (14)	123.8 ± 2.8	0.104 ± 0.006	22.2
mTHPC-invasomes 3.3% ethanol 1% citral (15)	127.9 ± 3.1	0.144 ± 0.011	9.3 14.2 23.4 26.1
mTHPC-invasomes 3.3% ethanol 1% cineole (16)	120.1 ± 1.5	0.135 ± 0.009	16.3 22.9
mTHPC-invasomes 3.3% ethanol 1% D-limonene (17)	126.5 ± 2.5	0.108 ± 0.006	22.3

[a] Terpene mix = cineole:citral:D-limonene = 0.45:0.45:0.10, v/v = standard.
[b] Terpene mix 1 = cineole:citral:D-limonene = 0.66:0.17:0.17, v/v.
[c] Terpene mix 2 = cineole:citral:D-limonene = 0.17:0.66:0.17, v/v.
[d] Terpene mix 3 = cineole:citral:D-limonene = 0.17:0.17:0.66, v/v.
[e] Terpene mix 4 = cineole:citral:D-limonene = 0.33:0.33:0.33, v/v.

revealed that all vesicles were of sufficiently small particle size and satisfactory PDI (i.e. homogeneity).

3.2. ESR measurements

3.2.1. ESR measurements of different unloaded liposomes

In the present study, 5-doxyl stearic acid (5-DSA) and 16-doxyl stearic acid (16-DSA) were used as spin labels. These spin labels are oriented like the lipids in the bilayer, and can determine local motional profiles in the two main regions of the lipid bilayer. Thus, the radical in the position 5 of the acyl chain (5-DSA) can determine motional profiles near the polar head group, while the radical in the position 16 of the acyl chain (16-DSA) determines motional profiles at the end of the hydrophobic chain. The typical ESR spectra obtained for liposomes with 5-DSA and 16-DSA, which were investigated in this study, are represented in Fig. 2a and b.

ESR spectra of 5-DSA incorporated into the lipid membranes of vesicles show an anisotropic motion. The fluidity of the vesicle membranes can be estimated from the order parameter *S* (Fig. 2a). The order parameter *S* is a measure of the average angular deviation of the fatty acid' acyl chain of the spin label at the nitroxide group from the average orientation of the fatty acids in the membrane (Seelig, 1970; Hubbel and McConnel, 1971). It reflects in the case of vesicles with 5-DSA the rotational freedom of PC close to the polar head groups in the bilayer and it decreases with the increase in membrane fluidity. An *S* value of 1.0 is characteristic for rigid lipid membranes, while a reduction in this value indicates an increase in the fluidity of the membranes. The order parameter *S* is calculated from the ESR-spectra (Gaffney, 1975):

$$S = 1.723 \left[\frac{T'_{II} - T'_{\perp} - C}{T'_{II} + 2T'_{\perp} + 2C} \right] \quad (1)$$

where the correction factor is $C = 1.4\,G - 0.053\,(T'_{II} - T'_{\perp})$, $2T'_{II}$ is the outer-peak separation and $2T'_{\perp}$ is the separation between inner hyperfine lines (Fig. 2a).

Regarding 5-DSA liposomes without mTHPC, a low *S* value (in the range 0.38–0.43) was observed for all samples (Table 3), sug-

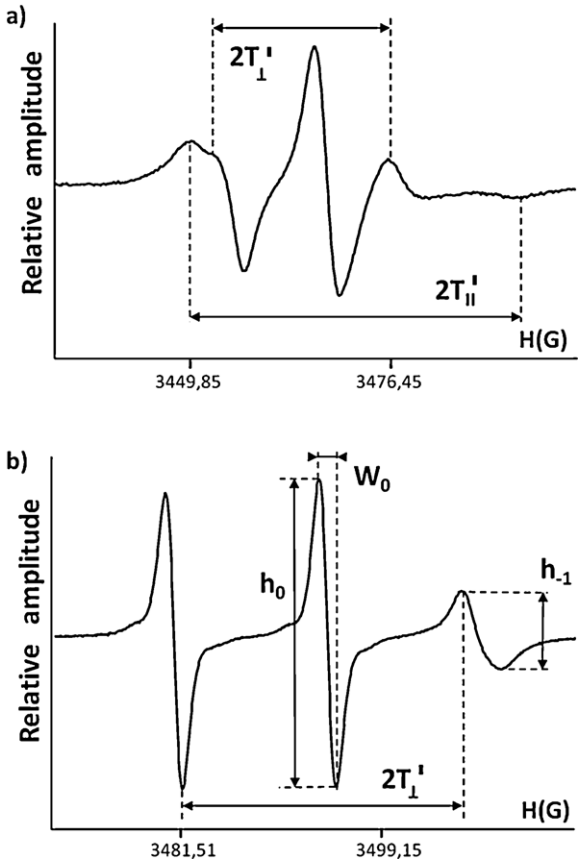

Fig. 2. Representative ESR-spectra. (a) ESR-spectrum of the 5-DSA spin label incorporated into different vesicles; (b) ESR-spectrum of the 16-DSA spin label incorporated into different vesicles.

gesting a high bilayer fluidity in the region of the phospholipid acyl chains close to the polar head groups of the phospholipids. The highest S value was detected for the liposomes without ethanol (conventional liposomes). However, this value of 0.43 is still small, indicating fluid phospholipid membranes. This small value was expected since unsaturated PC with a low transition temperature was used for the preparation of the liposomes. Addition of 3.3% ethanol further decreased the S value (i.e. increased the bilayer fluidity). Another decrease of the S value was achieved by the addition of 1% terpenes/terpene mixtures. The lowest value for S was obtained for invasomes with 1% citral. However, due to the experimental uncertainty (±5%), the differences in values of the order parameter S of conventional liposomes, liposomes with 3.3% ethanol and different invasomes containing 1% terpene/terpene mixture were not considered significant. An exception was the order parameter S of invasomes with 1% citral, delivering the highest overall mTHPC-amount to the skin (Dragicevic-Curic et al., 2009a), which was significantly smaller than the order parameter S of conventional liposomes. Thus, the addition of terpenes to liposomes, with the exception of citral, did not increase significantly the fluidity of acyl chains near the polar head groups of phospholipids in the vesicle bilayers (compared to the fluidity of conventional liposomes).

The ESR spectra of 16-DSA incorporated into the phospholipid bilayers of liposomes reflect an isotropic motion of the phospholipid acyl chains (Fig. 2b). Since the outer features of T'_{II} of the ESR spectra of 16-DSA samples were not resolved, and therefore the deduction of the order parameter S by Eq. (1) is less reliable, the approach of Bales et al. (1977) was used, and an estimation of S was obtained by fixing $T'_{II} - 2T'_{\perp}$ and C. Following Gaffney (1975), $T'_{II} - 2T'_{\perp} = 44.5$ G and $C = 0.8$ G was set. Thus, Eq. (2) was obtained (Bales et al., 1977):

$$S = 1.723 \left[\frac{43.7\,\text{G} - 3T'_{\perp}}{46.1\,\text{G}} \right] \quad (2)$$

Regarding liposomes without mTHPC containing the 16-DSA spin label, very low S values were obtained for all samples, suggesting an extremely high fluidity of the phospholipid acyl chains near their hydrophobic end (Table 3). The values were in the range 0.27–0.31. As in the case of samples with the 5-DSA spin label, also in samples with the 16-DSA spin label the highest S value among investigated vesicles was obtained for liposomes without ethanol. However, also this value was generally very low. Again, addition of 3.3% ethanol led to a decrease of the S value. A further decrease was observed after the addition of 0.5% of the standard terpene mixture. The addition of 1% of a single terpene or a terpene mixture decreased further the S value, implying that the fluidity near to the C16 atom of the phospholipid acyl chains of the invasome bilayer was remarkably higher compared to conventional liposomes. Taking the level of experimental uncertainty (±5%) of the determination of the order parameter S into account, it was concluded, that the fluidity of the phospholipid acyl chains near their hydrophobic end increased, but not significantly, by the addition of 3.3% ethanol and 0.5% of the standard terpene mixture, while it increased significantly by the addition of 1% terpenes/terpene mixtures. However, since the values of the order parameter S of different invasomes with 1% terpenes/terpene mixtures did not differ among themselves, it was not possible to observe a difference in the effect of different terpenes/terpene mixtures on the fluidity of the liposomal bilayers.

More detailed information should be obtained from the rotational correlation time τ_c. Since in the rapid motion regime, in the case of 16-DSA samples, the calculation of S is less reliable, and molecular freedom of motion is related quantitatively to the rotational correlation time τ_c of the nitroxide spin-labeled molecule, also this parameter was calculated and used to measure the motion of the phospholipid acyl chains near their hydrophobic end. The τ_c was calculated by the following equation of Keith et al. (1970):

$$\tau_c = (6.5 \times 10^{-10}) W_0 \left[\left(\frac{h_0}{h_{-1}} \right)^{0.5} - 1 \right] \quad (3)$$

where W_0 is the width of the midfield line of the spectrum in Gauss (G), h_0 the height of the midfield line of the spectrum and h_{-1} being the height of the highfield line (Fig. 2b). A lower value of τ_c reflects an increased rotational motion of the label.

A low value of τ_c was observed for all samples (in the range 0.45–1.02), suggesting an increased molecular dynamics of the phospholipid acyl chains near to their hydrophobic end (Table 3). The highest value for τ_c was detected for liposomes without ethanol, which was in accordance with the S value obtained for the same formulation. Invasomes containing 1% of a single terpene/terpene mixture possessed significantly lower τ_c values than conventional liposomes, liposomes with 3.3% ethanol and invasomes with 0.5% standard terpene mix. This indicated that the addition of 1% of a terpene/terpene mixture to liposomes led to a significant increase in the fluidity of the phospholipid acyl chains near to their C16 atom. The lowest value for τ_c was obtained for invasomes with 1% terpene mix 2, which contains the highest amount of citral. The next higher value for τ_c was determined for the invasomes containing 1% citral. Regarding the highest value of τ_c, it was obtained for invasomes with 1% cineole. Thus, among invasomes, invasomes with 1% terpene mix 2 revealed the highest fluidity, while invasomes with 1% cineole revealed the lowest fluidity near to the hydrophobic end of phospholipid acyl chains of the vesicle bilayers. However, since the τ_c values for all invasomes

were very low and did not differ remarkably between themselves, it could be concluded that all invasomes showed a high fluidity near to the hydrophobic end of the phospholipid acyl chains of the vesicle bilayer, being similar for all invasomes. Thus, it is not possible to differentiate between the influences of each terpene/terpene mixture on the fluidity of invasomes. A correlation of the fluidity of the different invasomes to their penetration enhancing ability is thus also not possible (Dragicevic-Curic et al., 2008a, 2009a). In addition, ESR showed that all terpenes/terpene mixtures increased the fluidity of vesicles bilayers, in contrast to the penetration studies (Dragicevic-Curic et al., 2009a), which showed that not all terpenes/terpene mixtures increased the penetration enhancing ability of invasomes compared to conventional liposomes and liposomes containing 3.3% ethanol. This also disables a correlation between the invasome bilayer fluidity and the mTHPC-amount delivered by invasomes.

Considering the results obtained for 5- and 16-DSA samples, it can be concluded that compared to conventional liposomes, the addition of 1% terpenes/terpene mixtures did not increase significantly the fluidity of the bilayers near the polar head groups of the phospholipids, while it increased significantly the fluidity of the vesicle bilayers at the hydrophobic ends of the phospholipid acyl chains (i.e. near the C16 atom of the acyl chains) in invasome bilayers. Further, there was no direct correlation between the bilayer fluidity (at the hydrophobic ends of the phospholipid' acyl chains as this region was perturbed) of different invasomes and their penetration enhancing ability (Dragicevic-Curic et al., 2009a). However, conventional liposomes possessed the lowest fluidity in both regions of the liposomal bilayer, and showed, next to invasomes containing the terpene mix 3, the smallest penetration enhancing ability among all tested vesicles (Dragicevic-Curic et al., 2009a). Invasomes with 1% citral, which showed the highest penetration enhancing ability (Dragicevic-Curic et al., 2009a), revealed after invasomes with 1% terpene mix 2 also the highest bilayer fluidity (near to the C16 atom of phospholipid acyl chains), which would explain the efficacy of these invasomes in improving the penetration of mTHPC. In contrast, invasomes with 1% cineole possessed among invasomes the smallest bilayer fluidity (near to the C16 atom of phospholipid acyl chains), revealed however after invasomes with 1% citral the highest penetration enhancing ability (Dragicevic-Curic et al., 2009a). Thus, this discrepancy confirms, that it might be not possible to correlate directly between invasome fluidity and their ability to improve the skin penetration of mTHPC.

3.2.2. ESR measurements of different mTHPC-loaded liposomes

In an attempt to obtain more accurate information on the connection between the fluidity of invasomes and their penetration enhancing ability regarding mTHPC, as well as on the influence of mTHPC on the fluidity of the vesicles (i.e. on the localization of mTHPC in the vesicle bilayer), ESR measurements were also performed with mTHPC-containing invasomes (Table 4).

For samples containing 5-DSA, the incorporation of mTHPC into the liposomal bilayers increased the S value (Tables 3 and 4) compared to the formulations without mTHPC (except for the conventional liposomes). However, the determined increase of the S value due to mTHPC incorporation was very small and within the limits of the experimental precision (±5%). Thus, the incorporation of mTHPC did not induce significant perturbations in the region of acyl chains close to the polar head groups of the phospholipids in the bilayers of the vesicles. Among mTHPC-loaded vesicles, the highest S values were determined for the conventional mTHPC-containing liposomes and the invasomes containing 1% D-limonene. The lowest values were obtained for invasomes containing 1% cineole followed by those containing 1% citral. However, as already noticed for vesicle dispersions without mTHPC, the differences among the formulations containing mTHPC, regarding S values, (i.e. vesicle fluidity near to the C5 atom of acyl chains of phospholipids), were not significant and thus no direct correlation of the fluidity of this bilayer region to the penetration enhancing ability of the vesicles could be found (Dragicevic-Curic et al., 2009a).

For samples containing 16-DSA (Table 4), the incorporation of mTHPC induced a minor increase of the S values, which was within the limits of the experimental precision (±5%). Only the calculation and comparison of the τ_c values (Tables 3 and 4), which are more reliable than the S values in the case of samples containing the 16-DSA spin label, revealed that the incorporation of mTHPC led to a remarkable decrease (considering the experimental uncertainty ± 5%) of the fluidity of all investigated liposomes in the region near the hydrophobic end of the phospholipid acyl chains.

Therefore, the influence of the incorporation of mTHPC into the vesicles on their fluidity was more pronounced in the 16-DSA labeled samples than in the 5-DSA labeled samples, i.e. mTHPC causes more disturbances at the hydrophobic ends of the acyl chains of the phospholipids in liposomes than in the region near the polar head groups of the phospholipids. This observation suggests that the binding site of mTHPC is in the inner hydrophobic cooperative zone of the bilayer, i.e. in the region near to the C16 atom of the acyl chains. Such a location of mTHPC could be anticipated from the structure of the drug (Fig. 1).

Further, among 16-DSA labeled vesicles containing mTHPC, the highest τ_c value and therefore the least fluidity was found for the conventional liposomes, followed by liposomes with 3.3% ethanol. The τ_c values of these two formulations were significantly higher compared to τ_c values of all invasomes. This indicated that mTHPC-loaded invasomes were of significantly higher fluidity in the region near to the C16 atom of the phospholipid acyl chains of the vesicle bilayers compared to the aforementioned vesicles. The τ_c value for mTHPC-loaded invasomes containing 1% D-limonene was significantly higher than the τ_c values obtained for other mTHPC-loaded invasomes, which did not differ significantly between themselves. The lowest τ_c values and thus the highest fluidity was determined for the mTHPC-loaded invasomes containing 1% citral, followed by invasomes with 1% cineole, invasomes with 1% terpene mix 3 and invasomes with 1% standard terpene mix. However, due to the experimental uncertainty of ±5% the difference in the τ_c value between the aforementioned four formulations was not considered significant.

Those mTHPC-containing formulations with a lower fluidity (Table 4) in the hydrophobic bilayer region (i.e. conventional liposomes, liposomes with 3.3% ethanol and invasomes with 1% D-limonene) also showed a very low penetration enhancing ability (Dragicevic-Curic et al., 2009a). On the other hand, mTHPC-containing invasomes with a higher fluidity in the hydrophobic bilayer region (e.g. invasomes with 1% citral, invasomes with 1% cineole and invasomes with 1% standard terpene mix) showed a good penetration enhancing ability and delivered higher amounts of mTHPC to the skin (Dragicevic-Curic et al., 2009a). Due to their low penetration enhancing ability, mTHPC-loaded invasomes with 1% terpene mix 3 (Dragicevic-Curic et al., 2009a) were expected to yield a τ_c value in the range of e.g. the mTHPC-containing conventional liposomes. However, according to the actually calculated τ_c value for this formulation, which was significantly lower than the τ_c value of conventional liposomes, a good skin penetration should be expected. At this stage, we are not able to explain this discrepancy on the basis of these experiments, but further studies will be done in order to clarify this finding.

As observed for the mTHPC-free 16-DSA labeled formulations, the different effects of the terpenes on the membrane fluidity could not be lined out clearly, and thus a direct correlation between the fluidity of invasomes and their penetration enhancing ability (Dragicevic-Curic et al., 2009a) could not be found.

Although the incorporation of mTHPC led to a decrease of the fluidity, the overall trend that the addition of terpenes increases the fluidity compared to conventional liposomes was observable as well. Thus, invasomes with and without mTHPC represent vesicles with a very high fluidity, which is desirable, since they are aimed to be used for improving the skin penetration of drugs. However, since no direct correlation between the fluidity of invasomes and their penetration enhancing ability (Dragicevic-Curic et al., 2009a) was found, it was proposed that besides the vesicle fluidity, also other phenomena might participate in the mechanism being responsible for the penetration enhancement by invasomes. One possibility could be the penetration enhancing effect of the invasome constituents (ethanol, terpenes and phospholipids) by direct action on the skin structure, which should be further investigated. Hofland et al. (1994) proposed that two mechanisms play an important role in the vesicle-skin interactions, being responsible for an enhanced drug penetration induced by nonionic vesicles. These mechanisms are the penetration enhancing effect of the surfactant molecules from the vesicles and the effect of the vesicular structures (acting as carriers).

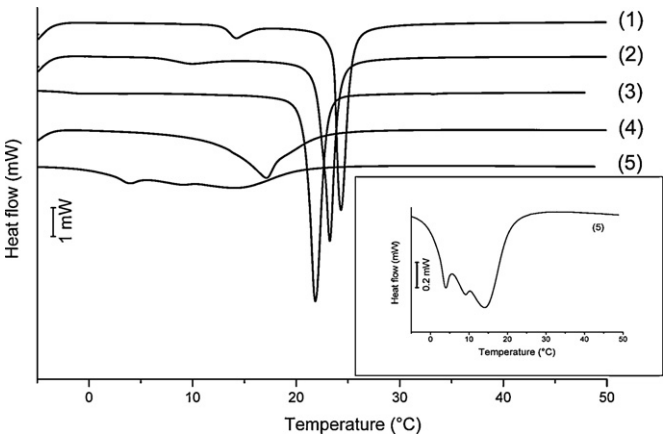

Fig. 3. Effects of ethanol and the standard terpene mixture on the phase behaviour of DMPC liposomes.

3.3. DSC measurements

The low transition temperature of the phospholipid used for the preparation of the ESR samples would not be detectable during DSC measurements according to the described setup. Thus, DMPC was used as a model phospholipid with a transition temperature of 23 °C. Upon heating of a sample of conventional DMPC liposomes (labeled as "DMPC-liposomes 0% ethanol") two thermal transitions were observable (Fig. 3 and Table 5). At about 14 °C the transition from the highly ordered gel state into the rippled gel state was observable in the form of a small broad pre-transition peak, whereas at about 24 °C the transition from the rippled gel state into the liquid crystalline state was observable as a sharp peak. With the transition into rippled gel state or liquid crystalline state, the fluidity of the lipids increases, and the lower the transition temperature is, the higher is the fluidity of the membrane at e.g. ambient conditions (New, 1990).The addition of ethanol (Fig. 3) led to a decrease of the transition temperatures and the peak of the pre-transition was broadened (addition of 3.3% ethanol) or had disappeared completely (addition of 10% ethanol). Beni et al. (2006) proposed that a shift of the pre-transition temperature indicates molecular interaction between the added drug (Imatinib) and the polar head groups of the phospholipids, which results in an increased mobility of that region. Therefore, the decreased pre-transition temperature of the DMPC vesicle dispersion upon addition of ethanol may point to molecular interactions between ethanol and the polar head groups of DMPC, resulting in an increased mobility in the head group region of the phospholipid bilayer. The decreased main transition temperature indicated an increased molecular motion of the acyl chains and thus looser and more flexible bilayers. Also the addition of ter-

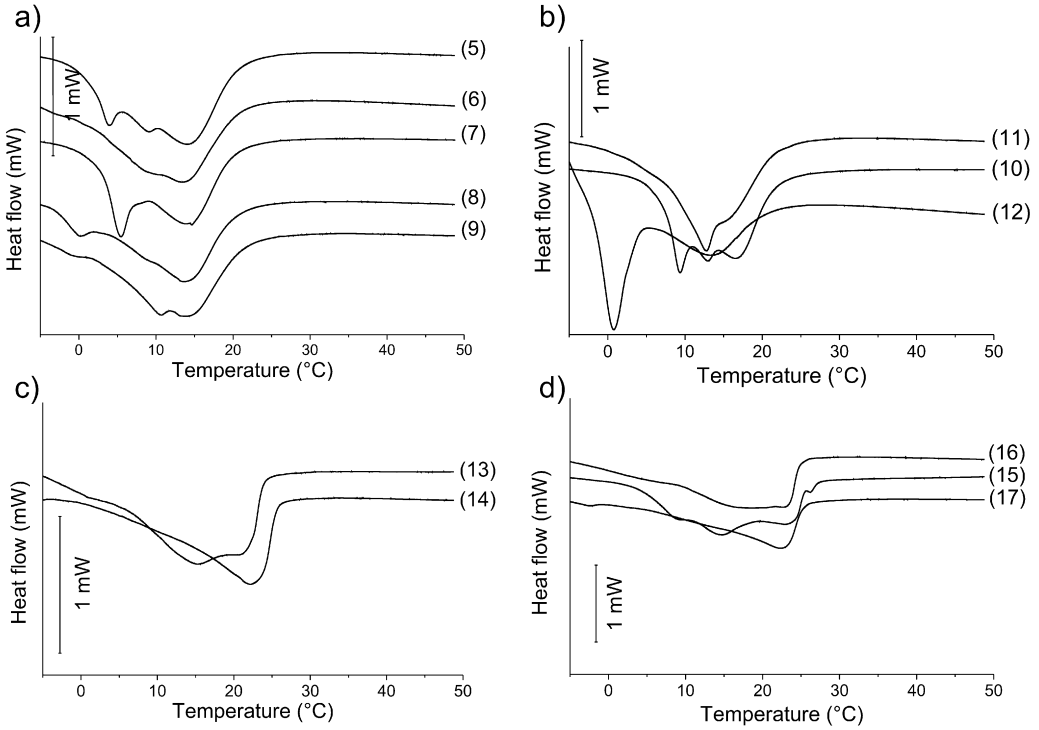

Fig. 4. Phase behaviour of different DMPC-invasomes. (a) invasomes without mTHPC containing different terpene mixtures; (b) invasomes without mTHPC containing different single terpenes; (c) mTHPC-loaded invasomes containing different terpene mixtures; (d) mTHPC-loaded invasomes containing different single terpenes. The labels of the samples are listed in Table 5.

penes, i.e. 0.5% of the standard terpene mixture, to liposomes with 3.3% ethanol eliminated the pre-transition peak (Fig. 3). Furthermore the main transition peak was broadened significantly and shifted to lower temperatures. Thus, the terpene mixture exerted a stronger effect on the bilayer structure than ethanol alone, even when used at higher concentrations. An increase of the amount of the standard terpene mixture to 1% exerted even stronger perturbational effects on the liposomal bilayer (Fig. 3). The peak shape was altered (see also Fig. 4a) compared to the lower terpene concentration and instead of showing one single minimum the peak showed three minima at about 4, 9 and 14 °C. According to El Maghraby et al. (2004), who investigated the interaction of surfactants and different skin penetration enhancers (including d-limonene) with DPPC liposomes, the occurrence of multiple peaks may suggest the formation of new species. However, in another study (Koutsopoulos et al., 2006) it was proposed that such a phenomenon could be a result of a hindrance of the hydration of the head groups of lipids in liposomes that are held assembled by strong intermolecular hydrogen bonds. Such multiple peaks were also seen when ions perturbed only the outer monolayer with little or no effect on the non-accessible inner lipid monolayer or bilayers of liposomes (Biltonen and Lichtenberg, 1993). Further, it was reported that broadening of the peaks as well as their splitting into two or more peaks is indicative of the formation of clusters, e.g. clusters of different lipid classes when using mixtures of different lipids and water, or clusters containing unaffected lipid and other containing lipid-metal ion complex in the case when metal ions are added to lipid-water mixtures, respectively (Chapman et al., 1974). Thus, the multiple peaks observed in this study could be indicative of the formation of clusters with phospholipids and clusters with the terpenes. However, these are only assumptions and the origin of the peaks observed in this study should be investigated in future studies.

A transformation of the peak shape and thus an alteration of the transition behaviour of the phospholipid bilayers with new minima or peak shoulders arising were also observable for all the other terpene mixtures under investigation (Fig. 4a), as well as after addition of single terpenes (Fig. 4b). Since the transition temperatures were shifted to lower temperature ranges a higher fluidity of the invasome phospholipid bilayers compared to conventional liposomes and liposomes with 3.3% ethanol is assumed. Unfortunately, due to the formation of multiple peak minima and the large diversity of the resulting peaks and transition temperatures no clear conclusion regarding the effect of the terpenes can be drawn. Further investigations are necessary regarding the origin of the multiple peaks. Thus, on the basis of the obtained results only the high fluidizing effect of single terpenes and terpene mixtures on the phospholipids bilayers of vesicles can be claimed.

However, what becomes clear is the influence of mTHPC on the transition behaviour of DMPC liposomes (Fig. 4c and d and Table 5). Addition of mTHPC shifted the transition peak to higher temperatures due to the incorporation of the drug into the bilayer. The thermograms indicate that mTHPC significantly influences the molecular motions of the phospholipid acyl chains, which results in a tighter and less flexible bilayer compared to mTHPC-free invasomes. Thus, the DSC results are in good agreement with those of ESR. mTHPC-containing formulations for the DSC measurements were chosen according to their penetration enhancing ability. Invasomes showing a high penetration enhancing ability (invasomes with 1% citral, invasomes with 1% cineole, invasomes with 1% standard terpene mix) and a low enhancing effect (invasomes with 1% D-limonene, invasomes with 1% terpene mix 3) were selected and investigated (Dragicevic-Curic et al., 2008a,b, 2009a). The goal was to evaluate their fluidity in order to establish whether there is a correlation between the fluidity of mTHPC-loaded invasomes and their ability to improve the skin delivery of mTHPC. This correlation, if confirmed, could possibly explain the different penetration enhancing ability of invasomes. The mTHPC-invasomes with 1% citral, mTHPC-invasomes with 1% cineole and mTHPC-invasomes with 1% standard terpene mix, which delivered the highest amount of mTHPC into the skin among different invasomes (Dragicevic-Curic et al., 2009a), showed broad multiple transition peaks, especially the mTHPC-invasomes with 1% citral. The main transition peaks of the mTHPC-invasomes with 1% D-limonene and mTHPC-invasomes with 1% terpene mix3, which provided the least mTHPC-amount in the skin, were also broad and in the same temperature range as the aforementioned peaks. Hence, all mTHPC invasomes were of high and similar membrane fluidity and it was not possible to differentiate between the fluidity of different invasomes, neither to put the invasomes fluidity into correlation with their penetration enhancing ability. Hence, some other phenomena in addition to vesicle fluidity may be responsible for the high penetration enhancing ability of invasomes. This has to be evaluated in further studies.

4. Conclusion

ESR studies revealed that the addition of 1% terpenes or terpene mixtures had an influence on the fluidity of the phospholipid bilayers of liposomes. The terpenes induced strong perturbations in the region of the C16 carbon atom of the phospholipid acyl chains in the vesicle bilayers, resulting in a very high fluidity of these vesicles compared to vesicles without terpenes. However, it was not possible to differentiate between the influences of single terpenes or terpene mixtures on the fluidity of vesicle bilayers, and thereby no direct correlation could be found between fluidity and penetration enhancing ability of individual invasome formulations, but there was found a direct correlation in the case of vesicles without terpenes. The incorporation of mTHPC into the vesicle bilayers decreased the fluidity in the region of the C16 carbon atom of the phospholipid acyl chains in the vesicle bilayers, indicating that mTHPC is localized in the inner hydrophobic zone of the bilayer. Also, in the case of mTHPC-loaded invasomes it was in most cases not possible to differentiate between the influences of certain terpenes or terpene mixtures on the bilayer fluidity. However, ESR confirmed that the addition of terpenes transforms conventional vesicles (being also fluid, but significantly less than invasomes) to vesicles, i.e. invasomes, of very high fluidity, which is desirable since invasomes are aimed to be used for improving the skin penetration of drugs. DSC measurements also showed the overall trend, that terpenes are able to increase the bilayer fluidity, since they decreased the transition temperatures and led to transformations of peak shapes. The DSC studies confirmed the results of the ESR measurements. Incorporation of mTHPC into the vesicles increased the transition temperature of the phospholipid bilayers, i.e. decreased the fluidity of the acyl chains. This again is an indication for the localization of mTHPC in the inner hydrophobic region of the bilayer. Both ESR and DSC measurements could not point out a direct correlation between individual invasome fluidity and their penetration enhancing ability. Thus, beside the invasome fluidity also other properties or effects of invasomes might be involved in the penetration enhancing ability and should be further investigated.

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