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Nanostructured lipid carriers as nitroxide depot system measured by electron paramagnetic resonance spectroscopy

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ABSTRACT

Various nanometer scaled transport systems are used in pharmaceutics and cosmetics to increase penetration or storage of actives. Nanostructured lipid carriers (NLCs) are efficient drug delivery systems for dermatological applications. Electron paramagnetic resonance (EPR) spectroscopy was used for the determination of TEMPO (2,2,6,6-tetramethyl-1-piperidinyloxy) distribution within the carrier and to investigate the dynamics of skin penetration. Results of ex vivo penetration of porcine skin and in vivo data – forearm of human volunteers – are compared and discussed to previously obtained results with invasomes under comparable conditions. W-band measurements show 35% of TEMPO associated with the lipid compartments of the NLC. Application of TEMPO loaded NLC to skin ex vivo increases the observation time by 12 min showing a stabilisation of the nitroxide radical. Moreover, stabilisation is also seen with data generated in vivo. Thus, same as invasomes NLCs are a suitable slow release depot system.

1. Introduction

Various nanometer scaled carrier systems, e.g. invasomes and nanostructured lipid carriers (NLCs) are efficient drug delivery and storage systems. Invasomes, highly fluidic liposomes, promote the penetration of drugs with unfavourable log *P* value (octanol-water partition coefficient) and molecular weight, e.g. the lipophilic drug temoporfin (Chen et al., 2011; Dragicevic-Curic et al., 2008, 2009) and the hydrophilic model agent carboxyfluorescein (Chen et al., 2011). NLCs consist of a solid lipid matrix with a high content of liquid lipid. The solid lipid matrix can be also advantageous for the topical route of administration, e.g. the controlled release from these nanocarriers becomes feasible as it was shown for the highly lipophilic drug clotrimazole (Souto et al., 2004) and skin penetration of the lipophilic dye Nile red can be enhanced too (Jores et al., 2005; Lombardi Borgia et al., 2005).

Previously, it was shown by means of EPR spectroscopy that the nitroxide free radical (aminoxyl radical) TEMPO (2,2,6,6-tetramethyl-1-piperidinyloxy; log *P* = 2.3, MW = 156 g/mol) is stabilised when applied by an invasome dispersion at the surface of porcine ear skin and the skin of human volunteers (Haag et al., 2011). TEMPO as an amphiphilic nitroxide and as one of the few nitroxides which can be applied to the skin of human volunteers (Fuchs et al., 1998) penetrates the skin well without the need of a special transport system. Yet, as soon as TEMPO begins to interact with the skin, it is reduced to the corresponding EPR-silent hydroxylamine. This process occurs at the surface, as well as inside the skin due to enzymatic and non-enzymatic reductions by physiologically reducing agents, in particular the thioredoxin/thioredoxin reductase system, glutathione, ascorbic acid and vitamin E (Herrling et al., 2003; Schallreuter and Wood, 1986, 2001; Thiele, 2001; Thiele et al., 1998). Hydroxylamines as well as secondary amines have been detected as nitroxide metabolites in human keratinocytes (Kroll et al., 1999) indicating that various reductants are involved

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in the reduction of nitroxides. Thus, TEMPO is a good candidate to investigate the stabilising effect of nanosystems because of efficient penetration and its fast degradation by the skin antioxidant system. TEMPO loaded to carrier systems, e.g. NLCs or invasomes is protected, resulting in a delayed interaction and degradation by the skin antioxidant system. This is a good prerequisite to distinguish penetrated from carrier-stabilised and, therefore, protected TEMPO.

TEMPO loaded NLCs were compared to TEMPO solution for their ability to store the nitroxide TEMPO in the stratum corneum or viable epidermis. The relevant parameter was prolonged EPR measurement time. First, the NLCs were measured with W-band EPR spectroscopy which determines the polarity of the nitroxide microenvironment and gives information about its distribution within the NLC dispersion. Then the NLC dispersion and TEMPO solution were applied on porcine skin ex vivo and in vivo on the skin of human volunteers and the decay of TEMPO was measured. For monitoring of the stabilising effect the ^{14}N hyperfine coupling constant (a_{iso}) of the spin probe as a measure for the polarity of the immediate surrounding was determined, too, results are presented and discussed in comparison to the investigations of invasomes under comparable conditions (Haag et al., 2011).

2. Materials and methods

Three different TEMPO concentrations were used in this study. First, for the determination of TEMPO distribution within the nanocarrier dispersions at 94 GHz W-band frequency a low concentration (2 mmol l^{-1}) was used to avoid broadening of the spectrum due to spin exchange which occurs at higher concentrations and makes simulation of EPR spectra difficult. Due to the lowered sensitivity with decreasing frequency the TEMPO concentration had to be raised to 10 mmol l^{-1} for the ex vivo measurements at low frequency L-band (1.3 GHz). The setup for in vivo measurements additionally reduces sensitivity due to the high water content. To get detectable signals from a human arm mounted underneath the surface coil type resonator of the low frequency L-band spectrometer a higher TEMPO concentration (30 mmol l^{-1}) had to be used.

2.1. Preparation of nanocarriers

Invasomes were prepared by mechanical dispersion as described previously (Haag et al., 2011) with a mean size of 86 nm and a polydispersity index (PDI) of 0.13 measured by photon correlation spectroscopy (PCS; Zetasizer Nano series, Malvern Instruments, UK) and a zeta potential of −23 mV by laser Doppler electrophoresis using the same instrument.

NLCs were prepared by hot high pressure homogenisation (HPH) (Müller et al., 2000). The lipid matrix of the NLCs was composed of medium chain triglycerides (Miglyol® 812, Caelo, Hilden, Germany) as liquid lipid and of Dynasan® 118 (Cognis, Monheim, Germany) as solid lipid. The ratio of liquid lipid to solid lipid was 2:8 and the total amount of lipid phase in the dispersion was 10% (w/w). The dispersion was stabilised by 2% (w/w) Plantacare® 818 (C8–16 fatty alcohol glycoside, Cognis). TEMPO was loaded onto the NLCs with 2, 10 or 30 mmol l^{-1}. Milli-Q water (Millipore, Billerica, MA, USA) was used as dispersant. HPH was carried out using a LAB 40 (APV Deutschland, Unna, Germany) by applying 3 homogenisation cycles at 500 bar and 80 °C. NLCs had a mean particle size of 170 nm with a PDI of 0.16 measured by PCS and a zeta potential of −43 mV measured by laser Doppler electrophoresis.

2.2. EPR spectroscopy

Continuous wave EPR spectra of the test preparations (NLCs and invasomes) were recorded at an ambient temperature (22 °C) on the W-band EPR spectrometer Elexsys680 with Teraflex EN600-1021H probehead (Bruker Biospin, Karlsruhe, Germany). Filled into quartz sample tubes (0.9 mm o.d., 0.3 mm i.d.; Vitrocom, Mountain Lakes, New Jersey, USA) the samples were measured using parameter settings as summarised in Table 1. For measurements at W-band frequency the magnetic field was calibrated against a LiLiF g standard (Stesmans and van Gorp, 1989) and spectra were normalized to 94.0 GHz. Ex vivo and in vivo skin measurements were performed on a L-band spectrometer (LBM MT 03, Magnettech, Berlin, Germany) with a surface coil type resonator. Parameter settings are also listed in Table 1. Different sample holders were used to guarantee a reproducible skin to surface coil distance. For in vivo measurements, the arm of the volunteers was pressed against a spacer in order to lock the arm in position.

Table 1
Parameter settings for W- and L-band EPR spectroscopy.

	W-band	L-band
Frequency (GHz)	94	1.3
Central magnetic field B_0 (mT)	3348	46
Magnetic field sweep width (mT)	10	8
Modulation amplitude (mT)	0.05	0.15
Lock-in time constant (ms)	41	10
Microwave power (mW)	0.05	10

The evaluation of magnetic parameters for the estimation of TEMPO distribution within the nanocarrier dispersions was done using the EasySpin toolbox (Stoll and Schweiger, 2006), a Matlab package for EPR spectra simulation. The fraction of TEMPO in the two phases was achieved by calculating the sum of lipid and aqueous phase spectra intensity and weighing them to achieve the best fit.

2.3. Ex vivo study

Porcine ear skin was used for ex vivo EPR investigations because it is a suitable model for human skin (Haag et al., 2010; Meyer et al., 1978). The ears were delivered from a nearby butcher on the day of slaughter with approval by the Veterinary Office Berlin-Treptow. Ears were washed with cold water, dried using paper towels and hairs were completely removed by clipping, taking care to avoid skin injury. Punch biopsies of full thickness skin (approximately 1.5 mm) were taken in the center of the ear and fixed onto microscopic glass slides using cyanoacrylate glue (Uhu, Bühl, Germany). TEMPO (10 mmol l^{-1}) loaded NLCs and the reference formulation with TEMPO dissolved in water (10 µl cm^{-2}) were spread evenly on the skin surface and the tissues were immediately subjected to L-band EPR measurements which were repeated up to 30 min. Both formulations were tested on three porcine ears with two samples each. For all experiments the signal intensity was calculated by a double integration of the spectra. As a measure of polarity of the TEMPO environment, hyperfine coupling constants (a_{iso}) were derived by the distance between high field and low field line divided by two (Analysis 3.3 software, Magnettech, Berlin, Germany).

2.4. In vivo study

The penetration of 30 mmol l^{-1} TEMPO loaded NLCs, invasomes and TEMPO in water/ethanol (1:1, v:v) was studied in duplicate using the left and right forearm of seven healthy Caucasian volunteers without dermatological diseases, aged 20–50 years. All volunteers have given their written informed consent. The study protocol had been approved by the ethics committee of the Charité – Universitätsmedizin Berlin in accordance with the Declaration of Helsinki as revised in 1983. Previous studies have shown that the spin probe TEMPO does not cause any irritating effects in human skin at the used concentration and application time (Fuchs

et al., 1998). The procedure was conducted as described previously (Haag et al., 2011) allowing a comparison of the results. Briefly, 50 μl of TEMPO solution was applied on a filter paper disc and covered with a Chamber (Epitest Oy, Tuusula, Finland), in order to avoid ethanol evaporation. The nanocarrier dispersions (50 μl) were applied directly to the skin within a marked area. After an application time of 10 min, surplus material was removed from the skin with a paper towel and EPR measurements were started. In order to improve signal to noise ratio, four scans per minute were recorded over a period of 20 min and the mean spectrum of 8 scans covering 2 min of measurement time was used. Measurement was stopped after 20 min because the arm of the volunteers was pressed against a spacer and therefore a longer measurement time would have been unreasonable for the volunteers. The relative amount of TEMPO in the skin was derived from the height of the central line of the spectra.

2.5. Statistical analysis

Arithmetical mean values and standard errors of mean (SEM) of the data are reported. For explorative data analysis, PASW for Windows (SPSS, Chicago, IL, USA) was used. The generalized estimating equations (GEE) function was used to test for significant differences between the mean values obtained; $p \leq 0.05$ was regarded to indicate a difference.

3. Results

Data obtained from invasome measurements were previously published in Haag et al. (2011). Nevertheless, to allow comparison of the two nanocarriers the results of both nanocarriers are presented in this paper.

3.1. Distribution of TEMPO within the nanocarriers

Fig. 1 shows the spectra obtained from W-band measurements of NLCs and invasomes. The lines from each of the two phases are clearly resolved for both nanocarriers. Subsequent simulation revealed 35 ± 5% TEMPO within the lipid compartments of the NLC and 56 ± 3% associated with the invasome membrane, respectively. Since the amount of lipid used is 10% for both nanocarriers, the local concentration of TEMPO amounts to 7.0 mmol l^{-1} in the NLC lipids and 11.3 mmol l^{-1} in the invasome membrane. Also the spectrum belonging to the hydrophilic NLC environment is shifted up-field

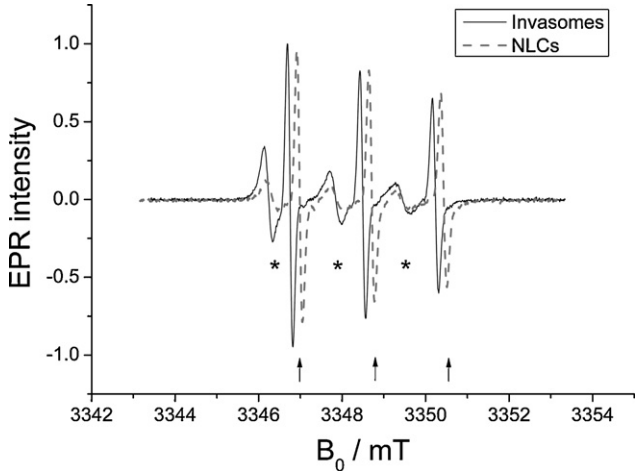

Fig. 1. High frequency W-band EPR spectra of TEMPO within the invasome and NLC dispersions. The arrows indicate TEMPO signals from hydrophilic environments and the asterisks from lipophilic environments.

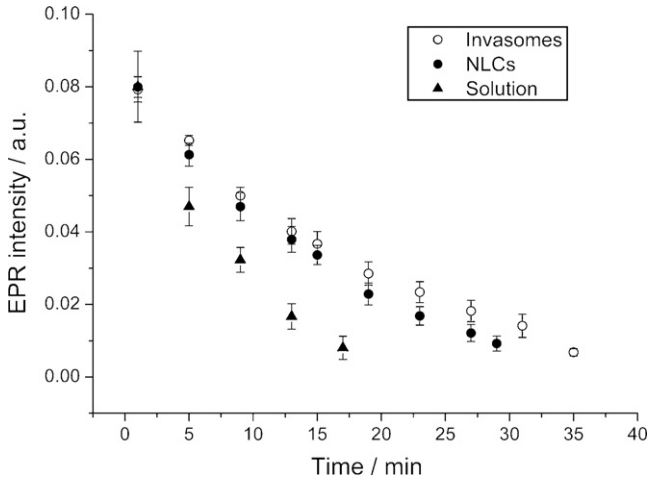

Fig. 2. EPR intensity decline following the application of TEMPO invasomes, NLCs and solution onto porcine skin ex vivo (mean ± SEM, $n = 6$).

and the spectrum attributed to the lipophilic part differs in nitrogen hyperfine coupling when compared to the invasome spectrum. Field position of the spectral lines gives information about the polarity of the TEMPO environment.

3.2. Stabilisation of TEMPO ex vivo

In Fig. 2 the EPR intensity decline of TEMPO measured at L-band is shown for solution and NLCs as well as for invasomes as investigated previously (Haag et al., 2011). EPR intensity rapidly decreases when applied in solution and is below the detection limit after 17 min. Invasomes and NLCs delayed TEMPO interaction with the skin resulting in prolonged signal detection by 18 min and 12 min, respectively, when compared to solution. Thus, invasomes increase the measurement time ex vivo twofold and NLCs 1.7-fold. Statistical analysis revealed significance between solution and nanocarriers, also NLCs and invasomes differ significantly ($p \leq 0.05$).

The change of a_{iso} over time is presented in Fig. 3. High values of a_{iso} represent a hydrophilic and low values indicate a lipophilic microenvironment. Whereas invasomes exhibit a linear decline of a_{iso} over time, NLCs show a slower decline until approximately 15 min followed by an enhancement.

Since the intensity of the L-band signal following the application of nanocarriers and solution does not decline linearly with time,

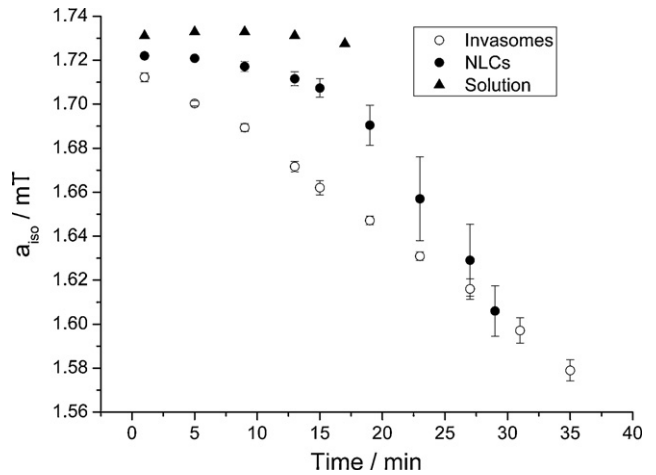

Fig. 3. Change of a_{iso}, as a measure of the polarity of the immediate surrounding of TEMPO, of the NLC and invasome dispersions as well as of solution during penetration into porcine skin ex vivo (mean ± SEM, $n = 6$).

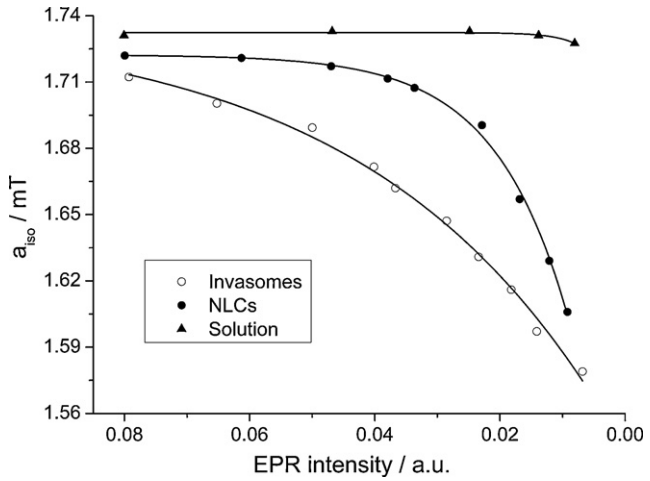

Fig. 4. Plot of EPR intensity versus a_{iso} of the nanocarriers and solution during penetration into porcine ear skin ex vivo.

intensity versus a_{iso} was plotted (Fig. 4). Both nanocarriers exhibit a strong correlation of intensity change with hyperfine coupling constant decrease in contrast to solution which remains almost constant.

3.3. Stabilisation of TEMPO in vivo

For comparison with previously published invasome data (Haag et al., 2011) in vivo investigations were performed after a 10 min TEMPO exposure and subsequent removal of surplus material from the skin. This had to be done due to technical reasons and therefore, the procedure differs from in vitro set-ups. Fixation of the arm causes a surface curvature of the skin and the nanocarriers cannot be distributed homogeneously thus leading to an accumulation of nanocarrier dispersion and TEMPO solution at the silicone barrier. Fig. 5 shows the intensity decline after application of TEMPO solution and TEMPO within the NLC and invasome dispersion. After application of TEMPO solution the intensity decline is most pronounced and differs significantly ($p \leq 0.05$) from invasomes and NLCs, whereas no significance between invasomes and NLCs could be obtained. The results obtained in human volunteers are in agreement with those obtained ex vivo regarding the prolonged intensity decline.

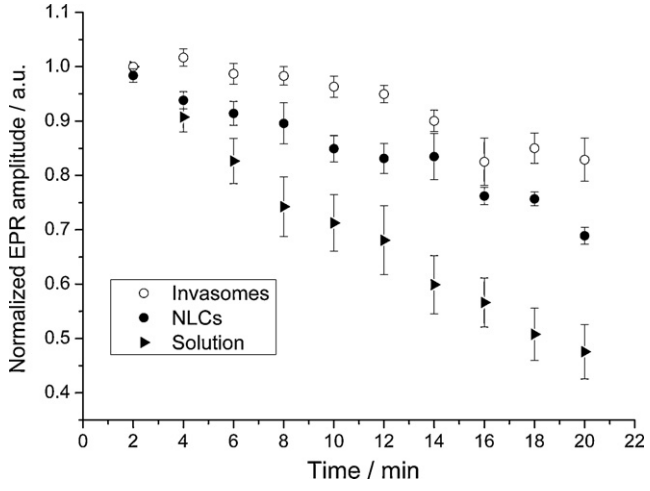

Fig. 5. TEMPO intensity (mean ± SEM) decline following the exposure of the 3 formulations for 10 min to the skin of 7 healthy volunteers.

4. Discussion

For the application of EPR methods the use of spin probes or spin-labelled molecules/drugs is necessary. The use of different spectrometers operating at different microwave frequencies allows comprehensive studies of pharmaceutical formulations. At high frequency W-band (94 GHz) the localisation of a spin probe or a spin-labelled drug within a carrier matrix becomes feasible allowing the observation of drug-carrier interactions. Especially interesting for the investigation of topical dermatics is low frequency L-band (1.3 GHz) EPR spectroscopy, as measurements of formulations applied to the skin ex vivo and in vivo are feasible, allowing investigations of drug–target and carrier–target interactions.

4.1. TEMPO distribution within the carriers

As previously described for invasomes (Haag et al., 2011), 2 mmol l^{-1} TEMPO within the NLCs lead to sharp spectral lines at W-band frequency (94 GHz) without noticable spin-exchange broadening of the lipophilic signals. The field position of the signal lines reflects the polarity of the microenvironment of TEMPO, e.g. a shift down-field is correlated with a rather lipophilic and a shift up-field with a more hydrophilic environment. Fig. 1 shows for both nanocarriers clearly resolved signals from both compartments. The shift of the NLC hydrophilic lines up-field can be explained by the composition of the aqueous phase, which consists of water for the NLCs and phosphate buffer including 10% ethanol for the invasomes. The latter is less polar and therefore the invasome lines are shifted down-field and the NLC lines up-field. The different hyperfine coupling of the lipophilic lines of the carriers indicates a slightly lower polarity in the lipid compartments of the NLCs. Although both carriers were prepared with 10% lipid there is a significant difference regarding the distribution of the spin probe. NLCs exhibited a smaller fraction of TEMPO associated with the lipid matrix than invasomes with the lipid membrane. This could be due to TEMPO being located in the surfactant shell of the NLCs as it was shown for solid lipid nanoparticles (SLN) by Braem et al. (2007) who determined two distinct sub compartments of the spin probe 4,5,5,-trimethyl-1-yloxy-3-imidazoline-2-spiro-3′-(5′*-cholestane) on the surface of the SLN but rather no incorporation in the solid lipid matrix. Furthermore, when loading the spin probe to NLCs they could demonstrate that it is located in the liquid lipid compartments as well as in the surfactant shell. The same distribution profile was found for the spin probe tempol benzoate after loading to NLCs (Jores et al., 2003). Results from TEMPO loaded SLN demonstrated that the spin probe is located in the surfactant shell but not in the solid lipid matrix (Küchler et al., 2010). For the spin probe 4-hydroxy-TEMPO no incorporation could be observed, it was located in the dispersant. Only spin probes with a C_{13} fatty acid residue could be incorporated into the lipid core of SLN (Ahlin et al., 2000, 2003). Since incorporation of TEMPO in the solid lipid can be rather excluded the lipophilic signals are assumed to originate from TEMPO in the liquid lipid compartments of the NLC matrix. The same holds true when loading Nile red (Jores et al., 2005; Lombardi Borgia et al., 2005).

Regarding the development of lipid based nanocarriers for a specific purpose the distribution of the drug within the carrier dispersion is important e.g. for sustained release the localisation of the drug in the lipid core and for burst release in the surfactant shell is favourable. The determination of drug distribution becomes feasible by either labelling the drug with a spin probe or using a spin probe with similar physicochemical properties. Subsequent high frequency W-band EPR measurements can distinguish between populations differing in polarity and/or mobility, thus allowing localisation of the drug within the carrier. This is a promising tool

for optimising the loading process of drugs to nanocarriers in order to achieve the desired distribution profile.

At L-band frequency (1.3 GHz) the signals from phases differing in polarity are not clearly separated, an overlap occurs resulting in a three line spectrum. Yet the different hyperfine coupling values as a measure for polarity cause a broadened low- and high-field line and therefore, allow investigations of changes in polarity after application of TEMPO containing nanocarriers to the skin.

4.2. Stabilisation and monitoring of stabilisation

The decrease in EPR spectra intensity (Fig. 2) of TEMPO and the change in a_{iso} (Fig. 3) give information about the interaction of the nanocarriers with the skin. TEMPO penetrating the skin reacts with reducing agents in the stratum corneum, which turn the spin probe EPR silent. The slower reduction rate of TEMPO containing nanocarriers compared to solution indicates a stabilisation of TEMPO in the lipid compartments of the carriers and, subsequently, a delayed reduction of lipid associated spin probe. Significant difference between the two applied carrier systems regarding signal intensity decline is due to longer signal detection after invasome application, yet, calculating statistics for both nanocarriers up to 27 min NLCs and invasomes do not differ significantly. For both systems a change of a_{iso} towards a more lipophilic microenvironment of TEMPO could be observed over time. The differences in a_{iso} decline can be explained by the different distribution profiles of TEMPO within the nanocarrier dispersions. The results from W-band measurements (Fig. 1) show that NLCs have more TEMPO associated with hydrophilic environments than invasomes. This becomes obvious when plotting intensity versus a_{iso} as shown in Fig. 4. After application of the nanocarriers and upon interaction of unprotected TEMPO with the skin, it is reduced quickly to the corresponding EPR-silent hydroxylamine which results in a rapid change of signal intensity and first a slow decline in a_{iso}. With decreasing amounts of TEMPO in the hydrophilic compartments a_{iso} is strongly influenced by lipid protected TEMPO. Then reaction of TEMPO from both phases leads to a rather linear decrease of a_{iso} with intensity. Fig. 4 clearly indicates that the decline of a_{iso} with intensity depends on TEMPO distribution within the carrier which is especially in the beginning of penetration steeper for invasomes than for NLCs. This can be explained by more hydrophilic associated TEMPO of the latter.

In contrast to ex vivo investigations, measurements of intensity and a_{iso} during the first 10 min after TEMPO application to the human forearm was not possible. Fixation of the arm for EPR recordings causes a surface curvature of the skin and the nanocarriers cannot be distributed homogeneously on the skin surface thus leading to an accumulation of nanocarrier dispersion and TEMPO solution at the silicone barrier. To avoid this cause of error, the nanocarriers were applied for 10 min and EPR measurements were performed after removal of surplus material. Therefore, the in vivo experiments only give new insights into the behaviour of spin probe and lipid compartments after TEMPO penetration into the skin, whereas ex vivo investigations can also monitor the events directly after application. Furthermore, for the in vivo experiments, the use of a higher TEMPO concentration was necessary due to a significant sensitivity loss. Placing a human arm in the magnetic field to the surface coil resonator causes non-resonant, dielectric loss of microwave power due to the high water content and subsequently to a reduced EPR signal intensity. For a stable TEMPO solution, a mix of water and ethanol (1:1, v:v) had to be used as solvent. Although the study protocol and the concentration of TEMPO and ethanol were different in the ex vivo and in vivo experiments and between the carrier dispersions, the results can be compared and regarding TEMPO stabilisation in vivo and in vitro investigations are in agreement.

Ex vivo a more pronounced increase in measurement time could be achieved with invasomes, whereas TEMPO stabilisation by NLCs and invasomes in vivo does not differ significantly. Yet, considering shelf life, production costs, and large scale production, NLCs are the superior nanocarriers for TEMPO stabilisation. Production can be from lab scale to large scale with 2000 kg/h output when produced continuously (Müller et al., 2002). Shelf life can exceed one year at room temperature (Souto et al., 2006). In contrast, invasomes have a rather limited shelf life of approximately 4 weeks long when kept at 8 °C. Furthermore, invasomes are sensitive to microbiological contaminations. On the other hand, invasomes can serve as penetration enhancer for hydrophilic and highly lipophilic agents (Chen et al., 2011) and function as sustained release depot systems as it was shown for the drug pergolide (Honeywell-Nguyen and Bouwstra, 2003) for similar elastic vesicles.

5. Conclusion

The distribution of the spin probe TEMPO within the nanocarrier dispersions depends on the physical and chemical properties of the lipid matrix. The results obtained ex vivo on porcine skin and on human volunteers regarding a reduced intensity decline are in agreement for both nanocarriers compared to the solution.

The use of different microwave frequencies allows comprehensive investigations from the distribution of the spin probe within the carrier to the dynamics of penetration.

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