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Influence of nanosized delivery systems with benzyl nicotinate and penetration enhancers on skin oxygenation

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ABSTRACT

Many novel nanosized delivery systems have been designed for topical application of drugs since they can overcome the skin barrier and improve drug bioavailability. The increased absorption is often a consequence of a reversibly disrupted barrier function of the skin by the vehicle itself or by specific ingredients that act as penetration enhancers. This paper reports the effects of two nanosized systems (microemulsion and liposomes), in the presence and absence of penetration enhancers (PE), on the topical delivery of a lipophilic drug *in vivo* and compares that to classical hydrogel formulation. A vasodilator benzyl nicotinate (BN), which increases the blood flow of the skin, was incorporated into the formulations, and skin oxygenation was followed by electron paramagnetic resonance oximetry. It was found that microemulsions and liposomes (with or without PE) accelerate the rate of BN action when compared to hydrogel. However, incorporation of PE in microemulsion also improves the effectiveness of BN action. To understand why PE enhances the action of BN, its effect on the structure of the stratum corneum was investigated *in vitro*. The increased fluidity of the stratum corneum lipids provides an explanation for the greater penetration of BN into the skin when the drug and PE are together incorporated into the appropriate formulation.

1. Introduction

Skin is the largest organ easily accessible for local and systemic drug administration. Topical application of drugs has many advantages compared to oral delivery or injection, such as high patient compliance, no first-pass metabolism of drugs, and sustained and controlled delivery over long time periods. For successful dermal or transdermal delivery it is necessary to reversibly overcome the skin barrier. However, skin is an excellent barrier that is naturally adapted to prevent transport of molecules into and out of the body (Mukhtar, 1992). Thus, even after decades of searching for appropriate ways to deliver drugs through skin, very few transdermal products are available on the market (Prausnitz et al., 2004).

Different strategies have been proposed to overcome the skin barrier, from more complex enhancement methods such as iontophoresis, electroporation, ultrasound, patches with microneedles and laser treatment, to classical approaches with optimization of the dermal formulation (Barry, 2001; Fang et al., 2004; Prausnitz et al., 2004; Trommer and Neubert, 2006). Nanosized delivery systems, such as liposomes, nanoparticles and microemulsions, contribute to better skin delivery of drugs (Schubert and Muller-Goymann, 2004; Kogan and Garti, 2006; Liu et al., 2006; Elsayed et al., 2007). These colloidal systems have attracted attention as potential delivery systems for targeting the drug, controlling its release, and increasing its availability. With the appropriate composition of nanosized carriers, enhanced permeation of drugs to deeper layers of skin or the systemic circulation has been achieved relative to classical dermal formulations (Lasic, 1993; Sentjurc et al., 1999; Schubert and Muller-Goymann, 2004; Kogan and Garti, 2006). Some of the intrinsic ingredients in these systems, such as fatty acids, phospholipids and surfactants, enhance penetration through the skin, increasing absorption of the drug (Williams and Barry, 2004). Many new penetration enhancers (PE) have been developed to improve percutaneous absorption of drugs. Certain combinations of enhancers (so called synergistic combinations) have been found to be highly successful in delivering drugs, but cause mild irritation of the skin, which is a frequent problem with many of the older enhancers (Karande et al., 2004; Prausnitz et al., 2004; Williams and Barry, 2004; El Maghraby et al., 2005).

In order to identify improved carriers for dermal or transdermal drug delivery, we have investigated the effect of incorporating chemical penetration enhancers into nanosized delivery systems

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(microemulsion and liposomes) on drug absorption into the skin of mice and compared it to classical hydrogel formulation. Previous studies have shown that liposomes containing hydrogenated soy lecithin with 30 mol% cholesterol are appropriate system for drug delivery into the deeper layers of the skin (Vrhovnik et al., 1998; Sentjurc et al., 1999; Kristl et al., 2003). We have used this specific liposome carrier and also a water-in-oil microemulsion to study the effect of added penetration enhancers. A particular mixture of sorbitan laurate (SL) and *N*-lauroyl sarcosine (NLS) has been shown to be an effective synergistic combination of penetration enhancers (Karande et al., 2004). Both the penetration enhancers are already used as cosmetic ingredients in different products on the market (Wenninger et al., 2001). This combination was added to the liposomal formulation and to a microemulsion and the efficacy of the two systems in promoting drug penetration, together with the stability of the systems was studied.

The vasodilator benzyl nicotinate (BN) has been selected as model drug, on the basis of its lipophilicity that makes it good candidate for particulate encapsulation, skin penetration and detection. BN crosses the skin and, by enzymatic hydrolysis, releases nicotinic acid. This increases cutaneous blood flow locally and, as a consequence, skin oxygenation (Wilkin et al., 1985). Electron paramagnetic resonance (EPR) oximetry, previously applied for a similar purpose (Kristl et al., 2003), was used to evaluate skin penetration of BN and to measure the response of the skin to the action of topically applied BN incorporated into different colloidal formulations in combination with penetration enhancers.

To obtain an insight into the mechanism of PE action, its effect on stratum corneum (SC) lipid structure was also investigated by electron paramagnetic resonance (EPR) spectroscopy, using a spin labelled fatty acid as a probe (Marsh, 1981; Anjos et al., 2007).

2. Materials and methods

2.1. Materials

Benzyl nicotinate, sorbitan laurate, and *N*-lauroyl sarcosine were obtained from Fluka, Buchs, Switzerland, PEG-8 caprylic/capric glyceride (Labrasol®) and polyglyceryl-6-dioleate (Plurol oleique®) from Gattefosse, Saint-Priest Cedex, France, caprylic/capric triglyceride (Mygliol 812®) from Hüls, Witten, Germany, colloidal silica (Aerosil 200®) from Degussa AG, Düsseldorf, Germany, hydrogenated soy lecithin (Emulmetik® 320) from Lucas Meyer, Hamburg, Germany, cholesterol from Merck, Darmstadt, Germany, and hydroxyethylcellulose (Natrosol®) 250, from Aqualon, Wilmington, USA.

2.2. Preparation of microemulsions

BN was incorporated into the oil phase of microemulsions at a concentration of 2.5% (w/w). It was dissolved in a mixture of PEG-8 caprylic/capric glyceride surfactant (47.5%), polyglyceryl-6-dioleate (11.9%) cosurfactant, and caprylic/capric triglyceride (22.3%) oil. Finally, water was added to 15.8%. A water-in-oil type of microemulsion was spontaneously formed after gentle mixing on a magnetic stirrer for 2 min at room temperature. Microemulsions with 1.25%, 4%, and 6% BN were also prepared by the same procedure. Amount of oil was appropriately corrected on the account of added BN. The quantiative composition of microemulsion was selected on the basis of the pseudoternary phase diagram constructed previously with the same composition (Gasperlin and Spiclin, 2001) where microemulsion regions were identified as transparent, low viscous and isotropic mixtures. This microemulsion with lipophilic as well as hydrophilic active substances has been previously extensively characterized by others in our laboratory (Spiclin et al., 2001).

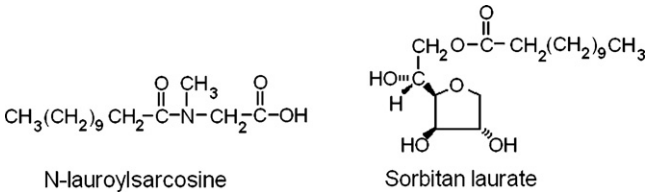

Fig. 1. Chemical structure of penetration enhancers used in this work.

A combination of two penetration enhancers, sorbitan laurate (SL) and *N*-lauroyl sarcosine (NLS) (Fig. 1) in weight fraction 3:2 was used. They were dissolved in oil, added to surfactant and cosurfactant and further treated as above. 1% of PE was incorporated in microemulsion and the amount of oil phase decreased for appropriate mass.

The microemulsions were insufficiently viscous and could be therefore quickly removed from the skin. Spiclin et al. (2003) showed that colloidal silica was the best choice as thickening agent for this type of microemulsion. Colloidal silica was therefore added to microemulsion, at a final concentration of 4.0% to increase the viscosity. After mixing on magnetic stirrer the colloidal silica was equally distributed in microemulsion and no aggregation of particles was seen. The obtained microemulsion remained transparent with increased viscosity.

2.3. Preparation of liposomes

Multilamellar liposomes were prepared by the thin film method. Hydrogenated soy lecithin, cholesterol and BN were dissolved in chloroform:methanol (2:1, v/v), and dried in a rotary evaporator (4.74×10^4 Pa, 45 °C) in a round-bottom flask. The remaining solvent was removed by vacuum pump at room temperature (10–15 min at 100 Pa). The dry film was hydrated with distilled water above the phase transition temperature of pure phospholipids (above 80 °C). The flask was shaken until the film was completely removed from the walls. The liposome dispersion was stabilized by stirring for 2 h on a magnetic stirrer (300 turns/min) at room temperature. To obtain liposomes of smaller size and homogeneity, the dispersion was homogenized at 10,000 rpm with a rotor-stator homogenizer (LabTek, Omni International, Gainesville, USA) for 5 min. A 1-ml sample of liposome dispersion contained 48 mg of lipids (33.6 mg of lecithin and 14.4 mg cholesterol) and 37.5 mg BN.

For easier topical application of liposomes, they were mixed into the hydrogel. Quantitative compositions of hydrogels, composed of hydroxyethylcellulose, water and liposome dispersion, with or without PE, are presented in Table 1. For hydrogel with penetration enhancers, SL and NLS were first dissolved in ethanol and the solution mixed with hydrogel. Liposome dispersion containing BN was then added and mixed to yield a homogeneous mixture. The final concentration of BN was 2.5%.

Table 1
Composition of hydrogel with encapsulated BN in liposomes with (HGPE) or without penetration enhancers (HG)

Component	HG (%)	HGPE (%)
Hydroxyethylcellulose	2	2
Distilled water	31	28
Liposome dispersion	67	67
Ethanol	–	2
N-lauroyl sarcosine	–	0.6
Sorbitan laurate	–	0.4

2.4. Hydrogel with BN

Hydrogel with BN was used as classical dermal formulation for comparison of BN action incorporated to liposomes or microemulsions. Two grams of hydroxyethylcellulose were dispersed in 2/3 of the total mass (98 g) of distilled water with continuous stirring to give a homogeneous mixture. BN was mixed with the hydroxyethylcellulose dispersion and the remaining water to give homogeneous dispersed BN in hydrogel.

2.5. Characterization of liposomes

The size, polydispersity index (PI) and zeta potential of liposomes were determined by photon correlation spectroscopy (PCS, Zetasizer 3000, Malvern, UK, using software for Malveren Instruments Dispersion Technology and Light Scattering systems, Version 1.32) (Kristl et al., 2003).

The entrapment efficiency of BN in the liposomes was evaluated by ultracentrifugation separation of liposomes from solution and HPLC chromatographic analysis of supernatant. After 3-h centrifugation on Centrikon T 2070 centrifugation machine (Kontron Instruments, Zurich, Switzerland) at 270 000 × *g* and room temperature liposomes formed the sediment, which was clearly separated from supernatant. Supernatant was applied to chromatograph with the UV–vis detector and LiChrospher-60 Si chromatographic colon (both Knauer, Germany) with methanol as mobile phase. The detection was made at the wavelength 254 nm (Pyka and Sliwiok, 2004).

The internal volume of liposomes, V_i, (the volume of aqueous space inside the liposomes expressed as a percentage of the total volume of liposome dispersion) was determined by EPR spectroscopy. Liposomes were prepared as described above except that the lipid film was hydrated with a 10 mM aqueous solution of the spin probe *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)-*N*,*N*-dimethyl-*N*-hydroxyethylammonium iodide (ASL). The liposomes described in this section were not applied to the mouse skin. ASL is readily soluble in water and has positive charge, which prevents it from crossing the intact liposome membrane once incorporated inside the liposome. For determination of V_i the dispersion was mixed with a 0.1 M aqueous solution of sodium ascorbate (NaASC) in a ratio 1:1 (v/v) to reduce the spin probe to the EPR invisible hydroxylamine derivative. NaASC is a charged molecule that does not easily penetrate intact liposomes and therefore only reduces ASL that is outside the liposome bilayer (Sentjurc et al., 1999). EPR spectral intensity was measured before, (I_0), and immediately after addition of NaASC, (I_{NaASC}), and V_i calculated from the equation:

$$V_i = \left(\frac{I_{NaASC}}{I_0}\right) \times 100 \quad (1)$$

Signal intensity was determined by double integration of the EPR spectra measured on a Bruker X-band EPR spectrometer ELEXSYS E500 at room temperature. Spectrometer settings were: modulation frequency, 100 kHz; magnetic field, 0.332 T; incident microwave power, 10 mW; modulation amplitude, 0.05 mT.

For stability measurements, liposomes including spin probe were prepared as described for the determination of V_i. Uncaptured spin probe was removed by overnight dialysis at room temperature. The liposome dispersion was mixed with NaASC as described above and the EPR spectral intensity was measured immediately and over a 7-day period. Liposome instability allows release of the spin probe that is then reduced by NaASC, resulting in lowered EPR intensity.

2.6. In vivo EPR measurements of the skin oxygenation

For the measurements of oxygen concentration in tissues (usually given as partial pressure of oxygen pO_2), an oxygen-sensitive paramagnetic probe has to be implanted into the tissue. After initial insertion of the paramagnetic probe into the tissue, measurements are completely non-invasive and can be performed from the same site for longer period of time (Dunn and Swartz, 2003).

EPR oximetry is based on the fact that molecular oxygen is paramagnetic and interacts with the inserted paramagnetic probe by Heisenberg spin exchange interaction (Sentjurc et al., 2004). As a consequence, the spectral line-width of the paramagnetic probe is broadened, to an extent that depends on the oxygen concentration. Changes in pO_2 were determined by measuring the peak-to-peak line-widths of the EPR spectra of the paramagnetic probe (ΔB in Fig. 2) and the relation between pO_2 and line-width can be calculated from a calibration curve. A paramagnetic probe used in this experiment was lithium phthalocyanine (LiPc), and its calibration curve is presented in Fig. 2. At zero oxygenation the initial line-width of the EPR spectra of LiPc is around 0.005 mT. LiPc is good probe for *in vivo* measurements of oxygenation by EPR due to its high sensitivity to oxygen. Although its sensitivity can decrease in certain tissues with time after implantation, we have shown that it is stabile more that 2 weeks in mouse skin (Sentjurc et al., 2004). Probe can be shattered during the insertion into the tissue, however this does not change its sensitivity to oxygen (Norby et al., 1998; Ilangovan et al., 2001).

Female mice (Balb C, 20–25 g) of the ages between 3 and 6 months were used. Guidelines and legislative regulations on the use of animals for scientific purposes were followed. The protocol was approved by the Veterinary Administration of the Republic of Slovenia (323-02-154/2005/5). Mice were anaesthetized by isoflurane delivered through a nose cone at 1.5% in pure oxygen at a flow rate of 1 l/min. The hair on the thigh was cut off, and the remainder removed with a depilatory cream (Vitaskin®, Krka, Slovenia). Several crystals (approximately 40 μg) of LiPc were inserted into the skin using 25 G needle. The position of the paramagnetic probe in the skin was confirmed by histological examination (hematoxylin and eosin staining) at the end of the experiment. Microscope pictures showed that the LiPc was appropriately located in the skin hypodermis (Abramovic et al., 2007).

Measurements were started 3 days after depilation and LiPc implantation, to allow the stratum corneum to recover (Honzak et al., 2000). There was not significant difference in time course of BN action if measurement were performed on the same animal 3 days or more after depilation, additionally proving that stratum corneum was completely recovered 3 days after depilation. The mice were anaesthetized and placed between the poles of the magnet. The loop of the resonator was placed gently over the skin where the LiPc was implanted (Fig. 2). Baseline of partial pressure of oxygen (pO_2) in skin was measured for at least 30 min prior to the application of vasodilator formulation. When a stable baseline was achieved, 0.25 g of formulation was applied to the skin and EPR spectra were recorded continuously for 90 min. In control measurements, formulations without BN were applied. Each treatment was performed on 4–5 animals. In the *in vivo* experiments microemulsions and liposomes were prepared at least 1 day before the application on mouse skin, however they were always less than a week old. Liposomes were mixed into hydrogel immediately before the *in vivo* experiments. The temperature of animals was measured rectally and was maintained at 37.0 ± 0.5 °C during measurements by a thermostatically controlled heated pad and a flow of warm air.

In vivo EPR measurements were performed on a Varian E-9 EPR spectrometer (Varian, Palo Alto, CA) with a home-built low-frequency microwave bridge operating at 1.2 GHz with a surface loop resonator (11-mm). Optimal spectrometer settings for

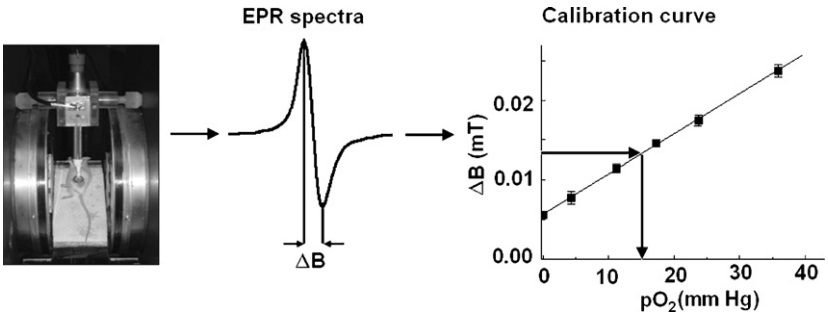

Fig. 2. *In vivo* measurement of the skin pO_2 in mouse placed between the poles of the magnet. From the line width (ΔB) of the EPR signal and calibration curve of LiPc tissue pO_2 is measured.

LiPc were used: modulation frequency, 27 kHz; central magnetic field, 43 mT; scan range, 0.1–0.4 mT; incident microwave power, 4 mW; modulation amplitude not exceeding one third of the peak-to-peak line-width (typical 0.005–0.015 mT), and a scan time of 30 s (usually 2–3 scans were averaged to increase signal to noise ratio).

2.7. *In vitro EPR investigation of lipid structure in stratum corneum*

Stratum corneum (SC) was prepared from skin, obtained from Balb/C mice. Animals were sacrificed; the skin of the back area was excised and fat removed by scalpel. The skin was immersed in a 60 °C water bath for a minute and the epidermis then separated from the dermis mechanically. The epidermis was placed on filter paper (SC side up) immersed into 0.2% trypsin (Sigma–Aldrich, Steinheim, Germany) for 48 h at 37 °C. The SC was washed with distilled water and dried in air.

A piece of SC (0.5 cm^2) was incubated in 2 ml of 5-doxyl stearic acid spin probe (Sigma–Aldrich) water solution (26 μM) for 1 h at 37 °C. For elucidation of SC with penetration enhancers, NLS and SL in final concentrations of 0.6% and 0.4% (w/w) respectively were added to the water solution of spin probe. SC was treated with PE simultaneously with the labelling. The SC was then well washed in water to remove unattached spin probe and placed onto the flat surface of a Teflon tissue cell. EPR measurements were carried out on a Bruker X-band EPR spectrometer ELEXSYS E500 at room temperature with modulation frequency, 100 kHz; magnetic field, 0.332 T; incident microwave power, 16 mW; modulation amplitude, 0.2 mT.

2.8. *Statistical evaluation*

Comparisons were performed by standard Two-Sample Student's *t*-test. Significance was tested at the 0.05 level of probability.

3. Results and discussion

3.1. *Characterization of nanosized systems with BN*

3.1.1. *Microemulsions*

Microemulsion of the chosen composition could stably incorporate BN at all concentrations used (1.25–6%) and 1.0% PE and maintained microemulsion characteristics. Transparent water/oil systems were formed. Systems were checked visually for any signs of physical instability during the investigated period of 3 weeks. They showed no signs of phase separation or precipitation of components and remained stable over observed period of time at room temperature.

3.1.2. *Liposomes*

BN is a lipophilic substance (partition coefficient (octanol/water)=254) that is expected to intercalate within the hydrophobic region of a lipid bilayer (Guy et al., 1986). We have additionally proven this by HPLC analysis of supernatant, which was separated from the liposome sediment in the process of ultracentrifugation. Less that 6% of added BN into the liposome dispersion was in water phase and more that 94% were detected in liposome sediment. This is clear evidence that majority of BN is entrapped in liposomes (most likely in lipid bilayer).

Inhomogeneous suspension of multilamellar vesicles was obtained with average diameter of liposomes with BN 430 ± 80 nm, while empty liposomes were larger (850 ± 300 nm; mean ± S.D.). In spite of homogenization procedure PCS measurements still showed quite broad distribution of vesicles size (polydispersity index was above 0.6 for both liposome suspensions). The zeta potential, which is a measure of the surface charge on the membrane, was negative in liposomes without (−51 ± 4 mV) and with BN (−45 ± 1 mV). The negative values are probably consequence of negatively charged phosphatidic acid which is present in hydrogenated soy lecithin used (Sentjurc et al., 1999). Similar values of zeta potential for soy lecithin vesicles were reported also by other authors (Foco et al., 2005; Manconi et al., 2007).

BN incorporation also influenced the internal volume of the liposomes. V_i is smaller in liposomes with BN (35 ± 4%) than without BN (48 ± 1%) what is consistent with the size measurements. In liposomes with BN, the EPR signal intensity of ASL decreased with time after the addition of NaASC, which was not the case in liposomes without BN (data not shown). Although both ASL and NaASC are charged molecules and therefore should not penetrate the liposome membrane, nitroxide reduction is clear evidence that the membrane of liposomes containing BN is leaking. This was not surprising as the incorporation of hydrophobic molecules into the liposome membrane is known to perturb the packing characteristics of phospholipids, produce a looser structure and destabilize liposomes (Lasic, 1993; El Maghraby et al., 2005).

3.2. *Stability of liposomes in hydrogel*

Usually, liposomes are applied to the skin in hydrogel in order to increase the viscosity of the formulation and in this way help to minimise loss of material from the skin surface. Hydrogels are more suitable for liposome incorporation than conventional creams because emulsifiers in creams destroy the vesicles (Handjani-Vila et al., 1993). However, the type and concentration of the polymer in hydrogel can negatively affect the liposome stability (Gabrijelcic and Sentjurc, 1995). To evaluate the effect of hydrogel and incorporation of enhancers into hydrogel on liposome stability the relative EPR spectral intensity in presence of reducing agent sodium ascor-

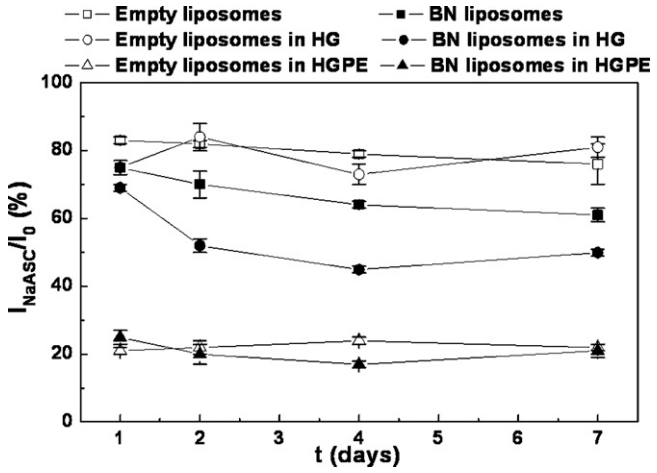

Fig. 3. Changes in relative EPR spectral intensity (I_{NaASC}/I_0) of liposome formulations with time after preparation (mean of 3 measurements ± S.D.). I_0 is the intensity measured immediately after dialysis before adding NaASC and I_{NaASC} is intensity measured after addition of NaASC.

bate (I_{NaASC}/I_0) was measured as a function of time after preparation of liposome formulation (Fig. 3).

In control experiments hydrogel with ASL (freely mixed into the hydrogel) was mixed with NaASC and no EPR signal was detected 2 min after the addition of reducing agent, proving that hydrogel itself without liposomes does not protect ASL from reduction. In Fig. 3, an initial decrease (17 ± 1%, mean ± S.D.) of EPR signal intensity in empty liposomes on day 1 was observed. As ASL has a positive charge and liposomes rather high negative electrostatic potential we suppose that some ASL molecules remained adsorbed on the outer surface of liposome membrane even after dialysis. These molecules are reduced to EPR silent hydroxylamine after addition of sodium ascorbate causing decrease in EPR signal intensitiy. In all other cases the initial decrease of EPR signal after addition of NaASC reflects not only the reduction of the adsorbed ASL molecules but also release of ASL from liposomes. In empty liposomes incorporated into hydrogel the decrease was slightly more pronounced comparing to empty liposomes (EPR signal diminished for 25 ± 2%), indicating that during mixing of liposomes with HG some ASL molecules are released. However, after that the EPR intensity remained stable for a week and we can conclude that hydrogel from hydroxyethylcellulose is a good system for liposome incorporation that does not significantly change the stability of empty liposomes.

In liposomes with BN, slightly more pronounced decreases of EPR spectra intensity were observed first day after preparation for the liposome dispersion (25 ± 2%) and for liposomes mixed in hydrogel (31 ± 1%). However with time after preparation EPR spectra intensity was further decreasing. One week after preparation already 39 ± 2 and 50 ± 1% of ASL was released from BN liposomes and BN liposomes in hydrogel. This could be explained by the higher permeability of the membrane of liposomes with BN to ASL and/or NaASC in comparison to liposomes without BN, reported in the preceding section.

The presence of PE in hydrogel affected the EPR intensity of empty liposomes and liposomes with BN much more than hydrogel without enhancers. A high percentage (79 ± 1% of empty liposomes and 75 ± 2% of BN liposomes) of liposomes disintegrated immediately after mixing of liposomes with HGPE (Fig. 3). After an initial decrease the EPR spectra remained stable for a week. The negative influence of PE on liposome stability is not surprising, since both NLS and SL are surfactants. They most probably penetrate from the hydrophilic surroundings of the hydrogel into the lipid domains of liposome membrane (at least with their lipophilic tail) and disrupt the membrane structure. Increased ASL and/or NaASC flux through such disrupted membranes would explain the faster reduction of spin probe after mixing liposomes into HGPE (El Maghraby et al., 2005). Similar is the effect of PE on liposomes in the absence of the hydrogel (results not shown).

3.3. Influence of colloidal carriers and penetration enhancers on the effect of BN in skin

As a control, formulations without BN were applied onto the skin of animals and no significant increase in skin pO_2 was observed (Fig. 4A). In the case of empty hydrogel, the oxygenation of skin even decreased (for about 5 mm Hg) which could be explained with cooling effect of hydrogel and transitional decrease of skin temperature (in average for 2 °C within first 8 min after application).

The effect of BN was evaluated by four pharmacokinetic parameters determined from the individual ΔpO_2 *vs. t* curves: the lag-time, t_{lag}, the time from application of the formulation to the first increase of pO_2, t_{max}, the time for achieving maximal increase in pO_2, AUC, the area under the response–time curve, and ΔpO_{2max}, the relative maximal increase in pO_2 after application of BN. The baseline of pO_2 varied from animal to animal, from 19 to 65 mmHg, depending on the physiological state of the mouse, the vessels' dilatory ability, and the different locations of LiPc in the skin. The difference, ΔpO_2, between the pO_2 baseline and the measured pO_2 after application, was therefore derived for each mouse experiment.

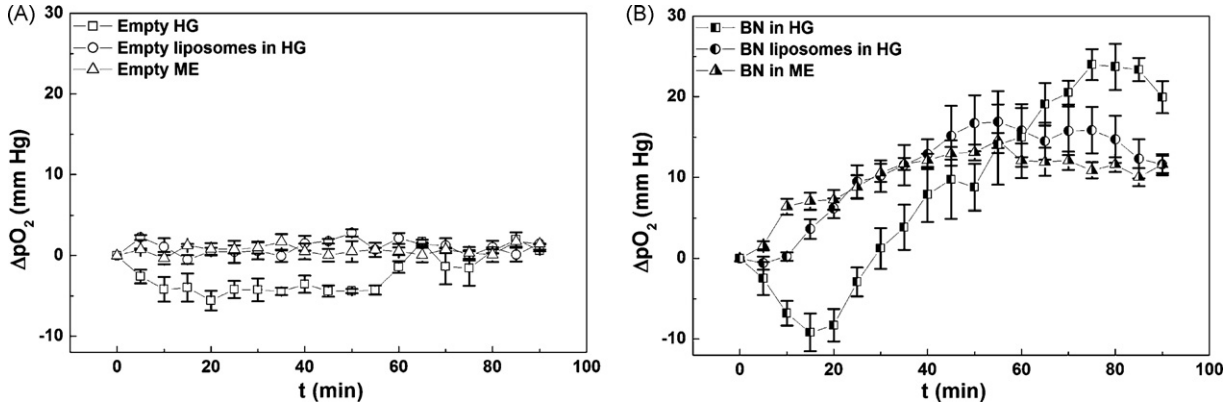

Fig. 4. The time course of relative increase in oxygen level (ΔpO_2) comparing to baseline values in mouse skin after application of BN or control (empt) carriers: (A) empty hydrogel (HG), liposomes that were mixed with hydrogel and microemulsions (ME); (B) BN hydrogel, BN liposomes that were mixed with hydrogel and BN microemulsions. Average of at least 5 measurements on 4–5 animals ± S.E. is presented.

Table 2
Influence of different formulations on the oxygenation of mouse skin

	HG	Liposomes in HG	ME	Liposomes in HGPE	MEPE
ΔpO_{2max} (mm Hg)	$23 \pm 1^{3,5}$	19 ± 3^{5}	$15.4 \pm 0.4^{1,5}$	20 ± 2^{5}	$27 \pm 2^{1,2,3,4}$
t_{lag} (min)	$26 \pm 1^{2,3,4,5}$	$14 \pm 1^{1,3,5}$	$10 \pm 1^{1,2,4}$	$15.2 \pm 0.2^{1,3,5}$	$8 \pm 1^{1,2,4}$
t_{max} (min)	$80 \pm 3^{2,3}$	$53 \pm 4^{1,5}$	$57 \pm 1^{1,5}$	63 ± 7	$79 \pm 4^{2,3}$
$AUC_{0\text{-}90}$ (mm Hg × min)	727 ± 140^{5}	956 ± 151^{5}	859 ± 58^{5}	868 ± 115^{5}	$1346 \pm 73^{1,2,3,4}$

BN was applied topically, free in hydrogel (HG), incorporated into liposomes that were mixed either in hydrogel (HG) or hydrogel with PE (HGPE) and microemulsion with (MEPE) and without (ME) penetration enhancers.
AUC is the area under the curve ($=1/2\sum(t_{i+1} - t_i)(pO_{2i} + pO_{2i+1})$, where t_i is the time of measurement and pO_{2i} is the corresponding partial pressure for $i = 0$–90).
Each value represents the mean ± S.E.M. of measurements on at least five measurements on 4–5 different animals.
Statistically different from [1]HG, [2]Liposomes in HG, [3]ME, [4]Liposomes in HGPE and [5]MEPE group respectively.

Concentration dependence of BN action in hydrogel previously showed a dose dependant increase in oxygen level in skin up to 2.5% BN and saturation at 6% BN (Krzic et al., 2001). Similar results were obtained also in our study with microemulsions were 4% and 6% BN did not resulted in higher pO_2 increase compared to 2.5% BN. Therefore in our further experiments 2.5% BN was used in all formulation tested.

Skin oxygenation changed significantly compared to baseline values following BN application in all the formulations tested (Table 2, Figs. 4 and 5). Nanosized delivery systems significantly affect the lag time and the time when maximal pO_2 is achieved. Time when pO_2 started to increase was the shortest for microemulsions followed by liposomes and hydrogel (differences among all three formulations were significant). For BN incorporated in liposomes and microemulsions the skin oxygenation reached maximal value significantly sooner as compared to the BN free in hydrophilic gel, while no difference was observed between liposomes and microemulsion. The skin oxygenation rose very slowly after application of BN in HG above the baseline (after 30 min) and maximal pO_2 was achieved only 80 min after application (Fig. 4). Slow increase of pO_2 could be partially explained by the observed cooling effect of HG, which caused pO_2 decrease (Fig. 4A). However, in the presence of BN, pO_2 decrease in first 20 min after application was the same as in empty HG, indicating that BN vasodilatory action started only 20 min after application (Fig. 4B). In microemulsion and liposomes BN action started after 10 and 14 min, respectively, what shows that both colloidal carriers accelerate penetration of BN into the skin compared to hydrogel. When comparing the maximal increase of pO_2 in the skin after BN application it was only significantly higher when BN was incorporated in hydrogel compared to microemulsions, while the overall effectiveness of BN (expressed as AUC) did not significantly differ among microemulsion, liposomes or hydrogel (Table 2).

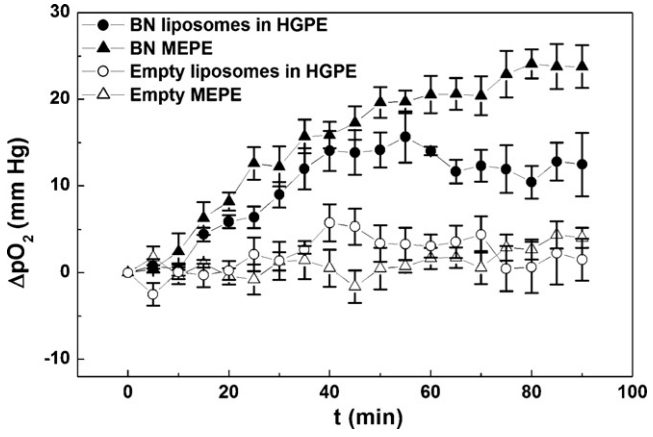

Fig. 5. The time course of relative increase in oxygen level (ΔpO_2) comparing to baseline values in mouse skin after application of BN or control empty carriers: empty and BN liposomes that were mixed with hydrogel with penetration enhancers (HGPE); empty and BN microemulsions with penetration enhancers (MEPE). Average of at least 5 measurements on 4–5 animals ± S.E. is presented.

The results indicate that colloidal carriers of tested composition do not significantly increase the effectiveness of BN action but only accelerate its penetration. Colloidal carriers enable penetration of the encapsulated BN closer to the vessels in the dermis where the main site of drug action is. The diffusion path of BN is therefore shortened and the effect appears sooner. This correlates with the results of other authors who also show that colloidal carriers enable faster penetration of the encapsulated drug deeper into the skin (Honzak et al., 2000; Alvarez-Román et al., 2004; Dubey et al., 2007; Teichmann et al., 2007). It is supposed that by incorporation into the colloidal carrier better contact with the skin is achieved, which could be the reason why the effect appears sooner. Both liposomes and microemulsions have better solubilization capacity for lipophylic drugs as aqueous solutions, which leads to high concentration gradients towards the skin and could be also the reason for faster drug diffusion (Kreilgaard et al., 2001).

The PE at a final concentration of 1% significantly improved the effectiveness of BN action when dissolved in microemulsion, but not in liposomes (Fig. 5). Negative result obtained with liposomes could be explained in part by the low solubility of PE in the liposome-hydrogel formulation. Even though ethanol was added to the hydrogel in order to increase the solubility of PE, the actual solubility and diffusion of enhancers within the hydrogel is still uncertain. It is interesting to note that the parameter presented in Table 2 for liposome samples with and without PE are in the range of experimental error although after addition of PE ca. 80% of liposomes disintegrate. We can explain this by the fact that lipophilic ingredients from disintegrated liposomes formed a lipid film on the surface of the skin. BN, as highly hydrophobic molecule remained incorporated in this phospholipid layer of disintegrated liposomes, which provided better contact of BN with skin as empty HG, and enable faster penetration of BN through the stratum corneum (Sentjurc et al., 1999). It was also proven by some authors that even when drug is outside the liposomes it penetrates better and deeper (to stratum corneum and possibly deeper) into the skin as drug in solution (Verma et al., 2003).

On the other hand, in microemulsion with PE the maximal increase in oxygen level and area under the curve (AUC) calculated in the time range from 0 to 90 min were significantly larger as in microemulsions without PE and other preparations tested (Table 2). After application of BN in microemulsion with PE skin oxygenation reached a maximal value 79 min and stayed at this higher level for at least 100 min post-application (data not shown), while after application of microemulsion without PE skin oxygenation was decreasing after 57 min, and maximal pO_2 was significantly lower. Comparing to hydrogel maximal skin pO_2 was achieved at similar time however with HG it was decreasing after that. Besides, with microemulsion oxygenation rose above the baseline after only

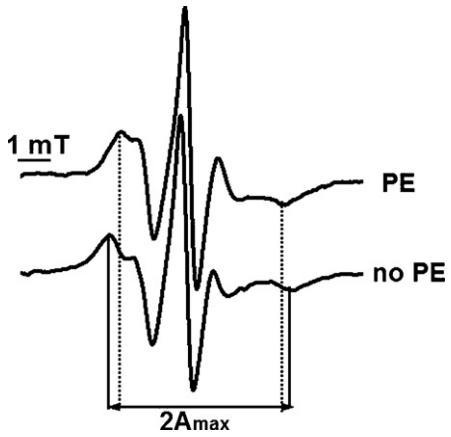

Fig. 6. EPR spectrum of 5-doxyl stearic acid in stratum corneum of mouse skin before and after treatment with penetration enhancers (SL and NLS) in final concentrations of 1%. The outer maximal hyperfine splitting $2A_{max}$ is indicated for both spectra.

8 min. This is much faster compared to hydrogel as well as to both liposome formulations (with or without PE).

Unlike the hydrogel where PE were poorly soluble, the oil phase in microemulsion is a good solvent for PE. Since in the water-in-oil microemulsion oil is also a continuous phase, PE can easily diffuse through the formulation, reach the stratum corneum and enable better penetration of BN into the skin.

However, alongside the improved delivery of BN in MEPE we also observed some irritation of mouse skin. This negative side effect was not seen in response to liposomes-hydrogel, with or without PE, or in microemulsion without PE, and can therefore be attributed to the application of PE onto the skin, in combination with high concentrations of other surfactant molecules in ME. This drawback will have to be addressed before the system proposed here can be applied in practice.

3.4. Effect of penetration enhancers on the structure of stratum corneum lipids

The enhanced action of BN observed in microemulsions with PE might be explained by a change in the structure of skin, which could contribute to more efficient delivery and action of the lipophilic drug within the skin. This possibility was explored by *in vitro* experiments on isolated mice stratum corneum using EPR spectroscopy with a fatty acid spin probe, 5-doxyl stearic acid, 5-DSA, which is located into the lipid part of stratum corneum. The EPR spectra are sensitive to the mobility and orientation of the probe, as well as to the polarity of the environment which surrounds the spin probe molecules (Marsh, 1981; Sentjurc et al., 2002).

The EPR spectra of 5-DSA incorporated in SC, both native and treated with penetration enhancers, gave values of the outer maximal hyperfine splitting, $2A_{max}$, of 5.5 and 4.8 mT respectively (Fig. 6). The significant decrease of $2A_{max}$ in response to penetration enhancers shows that the lipids in stratum corneum become more fluid, which is reflected in increased mobility of the spin probe and consequently changed shape of its EPR spectra. Both penetration enhancers used are amphiphilic (Fig. 1) and are probably penetrated with their alkyl chains in lipid layers of SC, causing disruption of lipid structures and increased mobility. The results are consistent with previous studies on the effects of anionic surfactants, ethanol and other penetration enhancers (Kawasaki et al., 1997; Hendrich et al., 2002; Anjos et al., 2007). The perturbation of the lipid structures of SC following treatment with penetration enhancers can be the reason for the positive effect of penetration enhancers on the action of BN incorporated in microemulsion, as was observed in the *in vivo* experiments.

4. Conclusion

In this work liposomes and microemulsions with a synergistic combination of penetration enhancers and a vasodilator drug benzyl nicotinate (BN) were prepared and the effectiveness of BN penetration into the skin was followed by EPR oximetry *in vivo*. The results show that tested liposomes and microemulsions accelerate the penetration of the vasodilator into the skin; however, they did not improve BN action compared to classical dermal formulation (hydrogel). On the other hand, addition of the penetration enhancers to microemulsion influences not only the time course but also improves the effectiveness of BN action. No such positive effect of PE was observed in liposome-hydrogel preparation. It can be concluded therefore that the application of these PEs in microemulsion is capable of significantly improving the delivery of a drug through the stratum corneum barrier, presumably by rearranging the lipid structures of SC. Additionally, the negative influence of PE on liposome stability should be considered at design of dermal formulations.

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