Nitroxide spin probes in dermatopharmacology – method development using electron paramagnetic resonance spectroscopy

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1. Gutachter: PD Dr. Martina Meinke
2. Gutachter: Professor Dr. Monika Schäfer-Korting

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Selbstständigkeitserklärung

Hiermit erkläre ich, Stefan Haag, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Verwendung anderer als der angegebenen Hilfsmittel angefertigt habe.

Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Berlin,

Stefan Haag

Abbreviations

a_{iso}	^{14}N hyperfine coupling constant
AO	Antioxidant
AOP	Antioxidant potential
AU	Arbitrary units
B_0	Magnetic field strength
CMS	Core-multishell
EPR	Electron paramagnetic resonance
g	Landé g-factor
HPH	High pressure homogenisation
IR	Infrared
log*P*	Octanol-water partition coefficient
LSM	Laser scan microscopy
mPEG	monomethyl poly(ethylene glycol)
MW	Molecular weight
NLCs	Nanostructured lipid carriers
NMR	Nuclear magnetic resonance
PBS	Phosphate buffered saline
PCA	3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxy
PCS	Photon correlation spectroscopy
PDI	Polydispersity index
PDT	Photodynamic therapy
RPF	Radical protection factor
SD	Standard deviation
SEM	Standard error of mean
TEMPO	2,2,6,6-tetramethyl-1-piperidinyloxy
UV	Ultra violet
τ_c	Rotational correlation time
ω_{HE}	Heisenberg spin exchange

Publications

1) [*]Haag SF, Bechtel A, Darvin ME, Klein F, Groth N, Schäfer-Korting M, Bittl R, Lademann J, Sterry W, Meinke MC: Comparative study of carotenoids, catalase and radical formation in human and animal skin. *Skin Pharmacol Physiol* **2010**;23:306-312.

2) [*]Darvin ME, Haag SF, Lademann J, Zastrow L, Sterry W, Meinke MC: Formation of free radicals in human skin during irradiation with infrared light. *J Invest Dermatol* **2010**;130:629-631.

3) [*]Haag SF, Fleige E, Chen M, Fahr A, Teutloff C, Bittl R, Lademann J, Schäfer-Korting M, Haag R, Meinke MC: Skin penetration enhancement of core-multishell nanotransporters and invasomes measured by electron paramagnetic resonance spectroscopy. *Int J Pharm* **2011**;416:223-228.

4) [*]Haag SF, Taskoparan B, Darvin ME, Groth N, Lademann J, Sterry W, Meinke MC: Determination of the antioxidative capacity of the skin in vivo using resonance raman and electron paramagnetic resonance spectroscopy. *Exp Dermatol* **2011**;20:483-487.

5) [*]Haag SF, Taskoparan B, Bittl R, Teutloff C, Wenzel R, Fahr A, Chen M, Lademann J, Schäfer-Korting M, Meinke MC: Stabilization of reactive nitroxides using invasomes to allow prolonged electron paramagnetic resonance measurements. *Skin Pharmacol Physiol* **2011**;24:312-321.

6) *Haag SF, Chen M, Peters D, Keck CM, Taskoparan B, Fahr A, Teutloff C, Bittl R, Lademann J, Schäfer-Korting M, Meinke MC: Nanostructured lipid carriers as nitroxide depot system measured by electron paramagnetic resonance spectroscopy. *Int J Pharm* **2011**;421:364-369.

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8) Darvin ME, Haag SF, Meinke MC, Sterry W, Lademann J: Determination of the influence of IR radiation on the antioxidative network of the human skin. *J Biophotonics* **2011**;4:21-29.

9) Darvin ME, Haag S, Meinke M, Zastrow L, Sterry W, Lademann J: Radical production by infrared A irradiation in human tissue. *Skin Pharmacol Physiol* **2010**;23:40-46.

10) Popov AP, Haag S, Meinke M, Lademann J, Priezzhev AV, Myllyla R: Effect of size of TiO(2) nanoparticles applied onto glass slide and porcine skin on generation of free radicals under ultraviolet irradiation. *J Biomed Opt* **2009**;14:021011.

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Oral presentations

Haag SF, Teutloff C, Bittl R, Lademann J, Meinke MC.
Applications of electron paramagnetic resonance (EPR) spectroscopy in dermatology and pharmacology.
Seminar an der **Freien Universität Berlin**, Fachbereich Physik, 18.10.2011, Berlin

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Application of electron spin resonance spectroscopy to investigate interactions between free radicals and antioxidants in human skin.
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Haag SF, Fahr A, Bittl R, Haag R, Lademann J, Meinke M.
Einsatz der ESR-Spektroskopie in der Dermatologie – Untersuchungen an Haut und topisch applizierten Nanotransportsystemen.
Symposium der **Gesellschaft für Dermopharmazie**, 20.11.2009, Berlin

Haag SF, Lademann J, Meinke M.
Determination of antioxidants and free radicals in the skin using Electron Paramagnetic Resonance (EPR)-spectroscopy.
Forschungsseminar der **Klinik für Dermatologie, Venerologie und Allergologie, Charité – Universitätsmedizin Berlin**, 04.05.2009, Berlin

Poster presentations

Meinke MC, Haag SF, Chen M, Fahr A, Bittl R, Teutloff C, Fleige E, Haag R,
Peters D, Keck C, Lademann J.
Evaluation of stability and penetration effects of nano transporters in the skin using Electron Paramagnetic Resonance spectroscopy
International Conference of the **Schwerpunktprogramm 1313** on Biological Responses to Nanoscale Particles, 11.09 – 15.09.2011, Essen

Haag SF, Lademann J, Meinke MC.
Electron Paramagnetic Resonance measurements of the skin to evaluate stability and penetration effects of nanocarriers.
25. **Treffpunkt Medizintechnik**, 26.05.2011, Berlin

Haag SF, Chen M, Fahr A, Bittl R, Teutloff C, Fleige E, Haag R, Peters D, Keck C, Lademann J, Schäfer-Korting M, Meinke MC.
Electron Paramagnetic Resonance measurements of the skin to evaluate stability and penetration effects of nanocarriers.
15. Jahrestagung der **Gesellschaft für Dermopharmazie**, 04.04. – 06.04.2011, Vaals, Netherlands

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Electron Paramagnetic Resonance study of nitroxide loaded invasomes – penetration and drug delivery ex vivo and in vivo.
XXXVIII. Jahrestagung der **Arbeitsgemeinschaft Dermatologische Forschung e.V.** 17.02. – 19.02.2011, Tübingen

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1 Introduction

The thesis focuses on the use of stable spin probes in dermatology and pharmacology. The detection of free radicals and the antioxidative capacity (AOC) are important diagnostic methods in dermatology. Up to now, non-invasive methods have been rare. Therefore, in this thesis methods for the detection of free radicals and the determination of the antioxidative capacity of the skin are developed using electron paramagnetic resonance (EPR) spectroscopy. Depending on the desired application, different spin probes must be used. Moreover, these spin probes also have to penetrate the skin. For penetration enhancement of hydrophilic spin probes, nanocarriers may be an option. Furthermore, lipid based nanocarriers may allow the stabilization of reactive spin probes.

This chapter outlines the fundamentals of EPR spectroscopy, spin probes, skin structure and nanocarriers for topical application. Furthermore, purpose, aim and challenge form a part of this chapter.

1.1 Electron paramagnetic resonance spectroscopy

Electron paramagnetic resonance (EPR) or electron spin resonance spectroscopy is a powerful tool for the detection of free radicals and other paramagnetic molecules (molecules with one or more free, unpaired electrons in the outer orbital shell). The concept of EPR spectroscopy is similar to nuclear magnetic resonance (NMR) spectroscopy. Both are based on the interaction of electromagnetic radiation with magnetic moments. In EPR spectroscopy the magnetic moment originates from free unpaired electrons of a molecule, whereas in NMR spectroscopy, the magnetic moment arises from the proton spin of hydrogen nuclei.

Therefore, EPR spectroscopy is concerned with resonant absorption of microwave energy by an unpaired free electron within an applied magnetic field. The energy of a free electron in a magnetic field splits into two distinct energy levels, which is termed the Zeeman Effect (figure 1). If the energy of the microwave radiation corresponds to the energy difference of the two distinct energy levels of an electron in a magnetic field, resonance conditions are fulfilled and spin conversion occurs. This results in a change of microwave energy, which is detected in EPR spectroscopy.

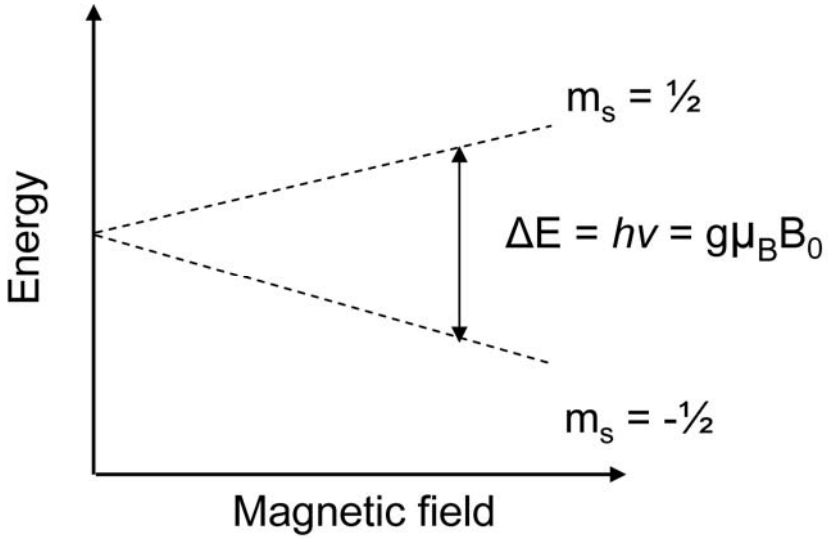

Figure 1 Zeeman Effect. The magnetic field splits the energy of a free electron into two distinct energy levels. Resonance conditions are fulfilled, if the microwave energy fits the energy difference between the two energy levels.

The fundamental equation of EPR spectroscopy is given in formula 1:

$$h\nu = g\mu_B B_0 \quad (1)$$

with h as the Planck constant, ν the microwave frequency, g the Landé g-factor, μ_B the Bohr magneton and B_0 the magnetic field strength.

EPR spectrometers are classified by the microwave frequency which ranges from 0.3 GHz (Alecci et al., 1994) to 360 GHz (Fuchs et al., 2002). L-band EPR spectrometers operate at a frequency of 1.3 GHz, X-band at 9.4 GHz, Q-band at 34 GHz and W-band at 94 GHz, the frequency determines the measuring sensitivity of an EPR spectrometer. Sensitivity is directly related to the square of operating frequency (Fuchs et al., 2001). Moreover, the dependency of microwave frequency with microwave penetration into biological tissue is of importance. At L-band frequency, microwave penetration amounts to 20 mm and is reduced exponentially at X-band frequency to below 1 mm (Fuchs et al., 2001). This can be explained by the high water content of biological samples, which causes high non-resonant, dielectric absorption of microwaves. Non-resonant absorption is a function of frequency and is enhanced with increasing frequency.

Besides detection and quantification of nitroxides or other paramagnetic molecules, EPR spectroscopy can provide information on the physicochemical properties of the molecule as well as information about its immediate surroundings, i.e., polarity and

viscosity. This information can be derived from the line shape of the EPR spectrum. EPR spectra of nitroxides in solution comprise of three resonant lines that are due to the magnetic moment of the neighbouring nitrogen nucleus. Interactions of free, unpaired electrons with the magnetic moment of the nitrogen nucleus are described as ^{14}N hyperfine coupling, which splits the EPR spectrum into three lines. The ^{14}N hyperfine coupling constant (a_{iso}) is strongly influenced by hydrogen bonds between solvent molecules and the oxygen of the nitroxide and therefore, gives information about the polarity of the immediate surrounding of the paramagnetic molecule. It was found that a_{iso} increases proportionally with the concentration of hydrogen donor groups of the solvent (Gagua et al., 1978).

1.2 Stable nitroxide spin probes

Nitroxides are versatile spin probes for various applications in biology, medicine, and pharmaceutics. Nitroxides are paramagnetic species, possessing a free, unpaired electron in the outer shell of the molecule and are therefore detectable by EPR-spectroscopy. Whilst all have the free electron at the nitrogen in common, the chemical structures and thus physicochemical properties can differ significantly. The most commonly applied nitroxides are six ring systems (piperidine) and five ring systems (pyrrolidine). The stability of the free radical arises from the delocalized electron from the NO bond and steric hindrance by the four methyl groups.

In this thesis, the two spin probes 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxy (PCA; log*P* = -1.7; MW = 186 g/mol) and 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO; log*P* = 2.3; MW = 156 g/mol) were used (figure 2). The stability of the nitroxide in biological tissue is mainly determined by its ring structure; PCA with its five ring system is significantly more stable than TEMPO with its six ring system (Fuchs et al., 1993). Therefore, TEMPO is used for the determination of the antioxidant capacity and PCA for the detection of oxidative stress (free radical production). TEMPO reacts well with the skin antioxidant system and is rapidly reduced to the EPR-silent hydroxylamine. This reduction is correlated with the antioxidant capacity (Fuchs et al., 1997). PCA hardly reacts with skin antioxidants, but well with induced short-lived free radicals, which recombine with PCA turning the spin probe EPR-silent. Therefore, PCA reduction is correlated with free radical production (Herrling et al., 2003).

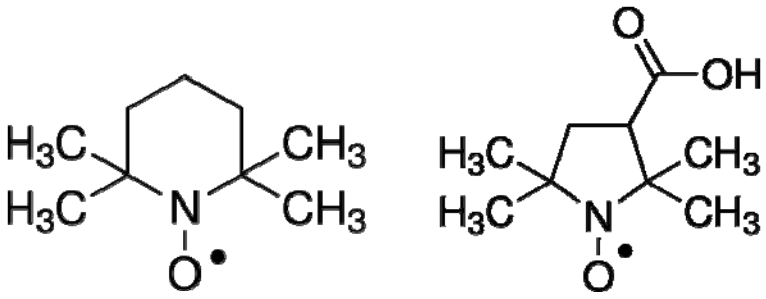

Figure 2 Chemical structure of the spin probes TEMPO (left) and PCA (right).

1.3 The skin

The skin, as the outermost organ of the human body, weighs approximately 10% of the total body mass and represents the barrier of the human body to the environment. Its primary function is the prevention of extensive water loss, protection against microorganisms e.g., bacteria and viruses and other noxious agents. The skin consists of three layers: epidermis, dermis and subcutis. The epidermis comprises of the stratum corneum and the viable epidermis. The stratum corneum, 10-20 µm thick, is the (non-viable) upper layer of the epidermis, built up by 15-20 layers of corneocytes (horny cells) which are flat, non-nucleated cells with a diameter of approximately 40 µm and a thickness of 0.5 µm (Marks and Barton, 1983). The corneocytes are surrounded by intercellular lipids which form the only continuous matrix of the stratum corneum (Nemanic and Elias, 1980). A relatively simple concept of the stratum corneum structure is a brick wall with the bricks representing the corneocytes and the mortar the intercellular lipids (Elias, 1983). Furthermore, this concept divides the stratum corneum into protein- and lipid-rich domains. The former is correlated with the corneocytes and the latter with the intercellular lipids. Corneocytes are mainly composed of a core of bundled keratin surrounded by a cell envelope and stabilized by cross-linked proteins and covalently bound lipids. The protein composition is insoluble in most solvents. Furthermore, corneocytes are cross-linked by corneodesmosomes, which contribute significantly to the tight bond of corneocytes within the stratum corneum. The intercellular lipids consist mainly of ceramides, free fatty acids, cholesterol, and triacylglycerol (Yardley, 1987). Therefore, the position of the skin barrier is in the stratum corneum

The viable epidermis is located exactly underneath the stratum corneum. It is composed of 10-20 layers of keratinocytes and forms the stratum corneum by keratinocyte differentiation, degradation of phospholipids and ceramide formation.

Furthermore, the epidermis contains melanocytes responsible for skin pigmentation, Langerhans cells as antigen presenting immune cells, and Merkel cells responsible for sensory perception.

Underlying the epidermis, the dermis provides a matrix for nerve and vascular networks that consists of collagen, elastin, fibrillin, laminin, fibronectin and vitronectin. Besides fibroblasts, which synthesize the extracellular matrix, endothelial cells and mast cells are the cellular residents of the dermis. Due to its vast vascular system, the dermis is responsible for heat exchange, repair, thermal regulation and skin nutrition. The latter is especially important since the epidermis does not have an independent vascular system; nutrients are transported via diffusion from the dermis to the epidermis. The extracellular matrix, in particular elastin, provides skin elasticity and cross-linking of collagen provides protection from tensile strength.

The subcutis, located underneath the dermis, is composed of a network of adipocytes. In addition to the importance for energy storage, the subcutis functions as an insulation layer and provides protection from injury.

1.3.1 Antioxidant network of the skin

The skin, as the barrier of the human body to the environment, is permanently exposed to external stress such as sun irradiation, especially to light in the UV, visible and infrared (Zastrow et al., 2009) spectral range. Furthermore, the skin is frequently exposed to a prooxidative environment, including air pollutants, i.e., ozone, chemical oxidants, other noxious agents (Burke and Wei, 2009) and microorganisms. As well as external processes, internal processes, such as metabolic activity (Iannone et al., 1993) and inflammation (Maeda and Akaike, 1998) can give rise to the production of free radicals and other reactive species. Free radicals are involved in the destruction of viruses and bacteria (Akaike, 2001; Victor et al., 2004), as well as in signalling processes inside cells and between cellular systems (Droge, 2002; Jackson, 1999). Extensive and permanent free radical production can cause oxidative cell damage, leading to accelerated skin ageing and cancer (Dhalla et al., 2000; Hagen et al., 1994; Halliwell, 2006; Sander et al., 2004).

Therefore, the skin consists of a comprehensive, synergistically acting antioxidant network, including endogenous antioxidants, as well as antioxidants taken up by nutrition. The antioxidant network can be divided into enzymatic and non-enzymatic antioxidants. Vitamin E, an important antioxidant of the latter group, is the most

important lipophilic antioxidant (Traber and Sies, 1996). It provides protection against lipid peroxidation and is also alleged to stabilize intercellular lipids of the stratum corneum (Thiele et al., 2001). The distribution of vitamin E differs significantly within the skin; a twofold higher concentration was found in the epidermis than in the dermis (Shindo et al., 1994). Furthermore, a vitamin E gradient was found in the stratum corneum of human skin, with lower amounts in the outer layers and higher amounts in the layers close to the viable epidermis, the ratio between outer and inner concentration is approximately 1:47 at the cheek and 1:2 at the inner upper arm (Thiele et al., 1999). This can be explained by vitamin E depletion due to sun exposure, in particular UV-irradiation. The intensity of UV-irradiation is particularly high on the skin surface and decreases significantly in the lower stratum corneum layers (Thiele et al., 1998). Furthermore, oxygen partial pressure decreases from the outer to the inner stratum corneum layers due to the high diffusion resistance (Hatcher and Plachy, 1993). As a result, there is more oxygen available in the outer stratum corneum layers for the production of UV-induced reactive oxygen species. Ubiquinol 10, a further lipophilic antioxidant, exhibits a 9-fold higher epidermal concentration compared to the dermis (Shindo et al., 1994).

Vitamin C is the most important hydrophilic antioxidant species. Its distribution within the skin differs even more than the distribution of vitamin E; the epidermis exhibits a 6-fold higher vitamin C concentration than the dermis (Shindo et al., 1994). The vitamin C concentration of the stratum corneum close to the viable epidermis exceeds the concentration at skin surface 10-fold (Thiele et al., 2001). Uric acid also exhibits a 6-fold higher concentration in the epidermis than in the dermis (Shindo et al., 1994), whereas, its distribution within the stratum corneum is more homogeneous (Thiele et al., 2001). The important endogenous antioxidant glutathione is 5 to 6-fold more concentrated in the epidermis than in the dermis (Shindo et al., 1994).

Antioxidant enzymes are part of the endogenous antioxidant network and neutralize reactive species specifically. Superoxide dismutase catalyzes the dismutation of superoxide to oxygen and hydrogen peroxide and is twofold more active in the epidermis than in the dermis (Shindo et al., 1994). Catalase catalyzes the reaction of hydrogen peroxide to water and oxygen and its activity is 8-fold higher in epidermal tissue than in the dermis (Shindo et al., 1994). The distribution within the stratum corneum of both superoxide dismutase and catalase exhibits a gradient across the first 10 layers, once more with lower activity towards the outer layers, whereas the

activity towards the viable epidermis remains constant. Furthermore, catalase activity is seasonal dependent; its activity is significantly lower in the summer than in the winter. Catalase activity is close to the detection limit on sun-exposed sites during the summer, whereas on sun protected sites it can be well quantified. The recovery after UVA irradiation takes 4 weeks. In contrast, superoxide dismutase activity is independent of seasonal influences and also independent of whether the examined site was protected from or exposed to the sun (Hellemans et al., 2003). The antioxidant enzyme glutathione peroxidase is only slightly (1.7-fold) more active in the epidermis (Shindo et al., 1994). Substrates are, e.g., hydrogen peroxide, organic peroxides and lipid peroxides. Glutathione reductase catalyzes the reaction of oxidized glutathione to the biological active form, containing a sulfhydryl group. It is 3-fold more active in the epidermis (Shindo et al., 1994).

Summarizing the distribution profiles of antioxidants, the concentrations and in the case of enzymes the activities are significantly higher in the epidermis than in the dermis. Furthermore, the distribution profile within the SC exhibits a gradient with lower concentration and activity towards the skin surface and higher ones to the viable epidermis. This fact remains decisive, in particular for vitamin C and vitamin E, as well as for the enzymes catalase and superoxide dismutase. A further explanation is the protective function of the viable epidermis and the stratum corneum, which need to counteract the destructive actions of reactive oxygen species, as well as being a target for antioxidant depletion due to solar irradiation.

1.3.2 Skin penetration and skin types

Due to the strong barrier of the stratum corneum, the human body is protected from extensive water loss but also against invading compounds from the outside. Yet, for several skin diseases a local therapy is desirable, in order to avoid side effects resulting from systemic drug exposure. In general, there are two pathways for skin penetration of substances: through the stratum corneum (in particular the intercellular passage) and the underlying epidermis and dermis, as well as along the skin appendages. Skin penetration is best for substances with a log*P* between 1 and 3 and a molecular weight < 500 g/mol (Korting and Schäfer-Korting, 2010; Potts and Guy, 1992), favouring the intercellular passage.

In this thesis, only the skin of animals was used, after being slaughtered for meat production. Besides human skin, porcine ear and bovine udder skin were used.

Porcine skin is widely used as a substitute for human skin since it closely resembles human skin (Simon and Maibach, 2000). Bovine udder skin exhibits a weaker barrier strength than human and porcine skin (Schäfer-Korting et al., 2006). This might be due to morphological reasons as bovine udder skin (same as furry animal skin) exhibits many times higher amounts of hair follicles than human and porcine skin. Furthermore, the amount of triglycerides, free fatty acids and ceramides was low in bovine udder, yet high in porcine and particularly high in human skin (Netzlaff et al., 2006). The biochemical composition of the skin might also influence the barrier strength, e.g., ceramides were found to be decreased in humans suffering from atopic dermatitis (Yamamoto et al., 1991).

1.4 Nanocarriers

Besides penetration enhancers like ethanol and dimethylsulfoxide, various nano- and micrometer scaled carrier systems are used in pharmaceutics and cosmetics for enhanced skin penetration. Liposomes were the first nano-sized drug carriers for the topical route of administration with triamcinolone, as the first drug encapsulated and applied to the skin of rabbits (Mezei and Gulasekharam, 1980). Furthermore, microemulsions and nanoemulsions are used as drug carriers as well as lipid based nanoparticles. Polymer-based macromolecules, e.g., dendrimers appear to be promising nanocarriers.

1.4.1 Invasomes

Invasomes are highly fluidic, ultra-deformable, elastic liposomal vesicles which consist of phosphatidylcholine, ethanol and terpenes (Dragicevic-Curic et al., 2008). Due to their fluidic membrane, liquid-state vesicles exhibit superior drug penetration enhancement compared to conventional, rigid gel-state liposomes (El Maghraby et al., 2001). Figure 3 shows cryo-electron microscopy images of conventional liposomes (b) and invasomes (f). Liposomes are of spherical shape due to their rigid gel-state membrane, whereas invasomes are deformed due to their highly fluidic membrane.

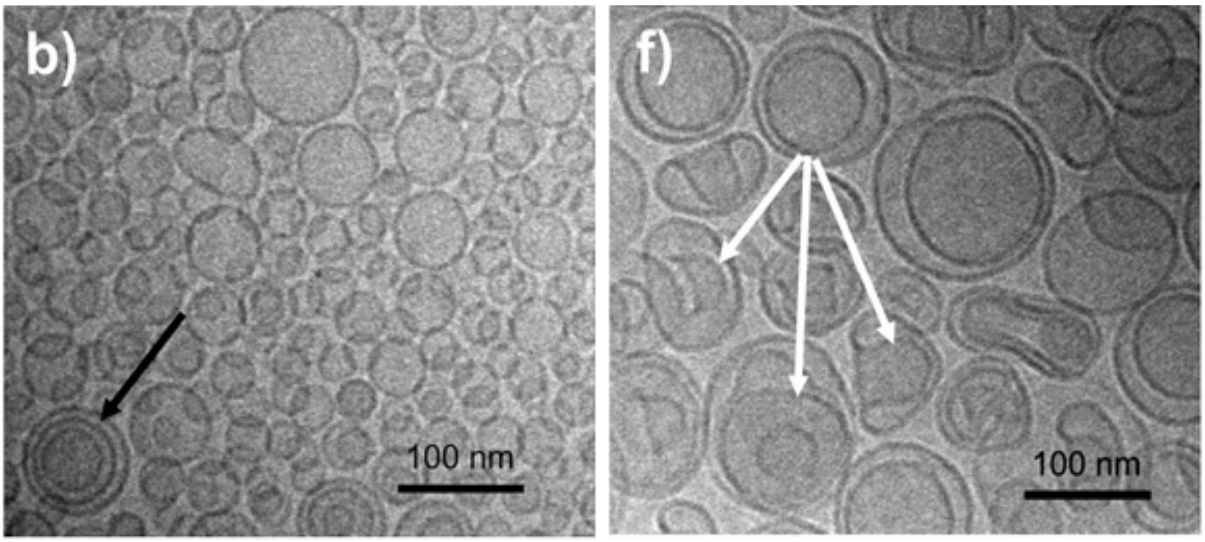

Figure 3 Cryo-electron microscopy images of temoporfin-loaded liposomes with 3.3% ethanol (b) and temoporfin-loaded invasomes with 1% terpenes (f). The black arrow represents oligolamellar vesicles, whereas the white arrows represent deformed vesicles (Dragicevic-Curic et al., 2008)

Furthermore, it was shown that the combination of ethanol and terpenes can enhance skin penetration of drugs, e.g., the highly lipophilic drug midazolam (Ota et al., 2003) as well as the water-soluble diclofenac sodium (Obata et al., 1991; Obata et al., 1990). Therefore, invasomes promote the penetration of drugs with unfavourable log*P* value and molecular weight, e.g., the lipophilic drug temoporfin (MW = 681 g/mol, log*P* = 9.3), (Chen et al., 2011; Dragicevic-Curic et al., 2008, 2009) and the hydrophilic model agent carboxyfluorescein (MW = 378 g/mol, log*P* = -1.5), (Chen et al., 2011). There are two mechanisms described in the literature, which explain how the interaction of elastic vesicles with the skin appears to enhance penetration. The first mechanism proposes that vesicles or vesicle constituents disorganize and disrupt inter-cellular lipids, affecting the ultra structure of the stratum corneum by the creation or modification of pathways for possible drug penetration, thus leading to increased skin permeability. However, treatment with rigid vesicles did not affect stratum corneum ultra structure or permeability (van den Bergh et al., 1999). The second mechanism postulates that ultra-flexible vesicles can penetrate the skin unfragmented on areas with low penetration resistance, e.g., between neighbouring corneocyte clusters with no lateral overlap of the corneocytes (Cevc et al., 2002; Schätzlein and Cevc, 1998). Moreover, it was found that ultra-flexible vesicles penetrate intact into deeper stratum corneum layers, however only small amounts were found in the deepest stratum corneum layers. These findings could not

be observed after the application of rigid gel-state vesicles (Honeywell-Nguyen et al., 2002).

Visualization of invasomes revealed that they appear unilamellar and bilamellar, which is independent of the loaded agent (Chen et al., 2011).

1.4.2 Core multishell nanotransporters

Core multishell (CMS) nanotransporters are chemical chameleons, which can encapsulate both hydrophilic and lipophilic guest molecules. Additionally, they can be dissolved in a variety of solvents, ranging form water to toluene. The unimolecular architecture of CMS nanotransporters contains a hydrophilic core, followed by a hydrophobic middle layer, and a hydrophilic outer shell (figure 4). This architecture mimics the structure of liposomes, which are lipid vesicles that comprise of an aqueous inner phase, enclosed by a phospholipid membrane and surrounded by an exterior aqueous phase. The liposome architecture was found to have a penetration enhancing effect. It was suggested that liposomes adhere to the skin surface and destabilize, fuse or mix the lipid matrix, resulting in a loosening of the lipid structure, which lowers the barrier strength of the skin and enhances skin penetration of loaded drugs or agents (Elsayed et al., 2007; Kirjavainen et al., 1996). Due to their liposome-like architecture, CMS nanotransporters might exhibit a similar penetration efficiency and additionally, as polymer-based nanotransporters, an improved stability.

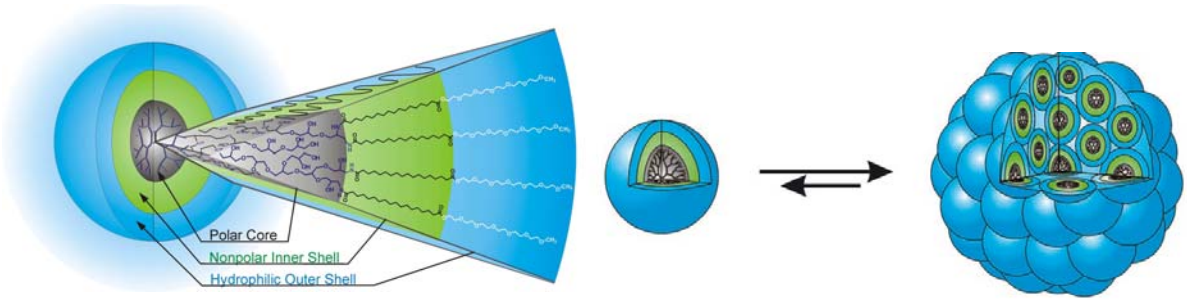

Figure 4 Schematic illustration of CMS nanotransporters – unimer and aggregates (Radowski et al., 2007).

The straightforward three-step synthesis uses cheap, commercially available building blocks. The core of the CMS nanotransporters consists of hyperbranched poly(ethylene imine) or polyglycerol. The hydrophobic inner shell consists of linear, amphiphilic building blocks formed by alkyl diacides (C_6, C_{12}, or C_{18}) which are covalently bond to the hyperbranched core and to a terminal hydrophilic shell,

consisting of monomethyl poly(ethylene glycol) (mPEG) with up to 14 glycol units on average (Haag, 2004; Radowski et al., 2007).

The terminal mPEG shell provides a good solubility of the particle in water as well as in organic solvents and a high degree of biocompatibility. Furthermore, as well as the core, it serves as a matrix for encapsulation of hydrophilic agents. The nonpolar inner shell allows incorporation of hydrophobic guest molecules (Radowski et al., 2007).

CMS nanotransporters comprise of small unimers, 5 nm in size, but if the critical aggregation concentration of 0.1 g/l is reached, larger aggregates are formed with a diameter of approximately 30-50 nm. ß-carotene loaded CMS nanotransporters form aggregates up to 144 nm in size (Radowski et al., 2007).

CMS nanotransporters were shown to enhance skin penetration of the lipophilic dye Nile red (Küchler et al., 2009b) and the hydrophilic dye rhodamine B (Küchler et al., 2009a) and thus appear to be of outstanding versatility. Furthermore, CMS nanotransporters were used for tumour targeting (Quadir et al., 2008), cellular copper uptake by yeast cells (Treiber et al., 2009) or as stabilizers for catalytic platinum nanoparticles (Keilitz et al., 2010).

1.4.3 Nanostructured lipid carriers

Nanostructured lipid carriers (NLCs) consist of a solid lipid matrix formed by, e.g., Dynasan 118 and are loaded with liquid lipids of, e.g., medium chain triglycerides, such as Miglyol 812. NLCs are dispersed in water and stabilized by an emulsifying agent. However, the structure of NLCs is described differently in the literature. One theory describes NLCs as spherical nanoparticles with droplets of liquid lipid within the solid lipid matrix (Müller et al., 2002), whereas another group describes them as lipid platelets with oil spots located on the surface of the solid lipid matrix (Jores et al., 2004). Preparation of NLCs is performed by high-pressure homogenisation (HPH), applying two to three cycles at 500 bar. HPH can be performed at high temperatures, e.g., 80°C but also cold HPH is known (Müller et al., 2000). HPH at room temperature is particularly interesting for the loading of temperature sensitive agents to NLCs. The disadvantage of cold HPH is the increase in particle size. Mean particle size exhibits a wide range from 50 to 1000 nm. The lipid content of a formulation/nano-dispersion can be from 5 to 40% (Schäfer-Korting et al., 2007). NLCs were used for the controlled release of the highly lipophilic drug clotrimazole (Souto et al., 2004). Moreover, topical application of NLCs results in the formation of

an occlusive film which improves skin hydration (Müller et al., 2002). Nevertheless, NLCs failed to enhance the uptake of Nile red (Lombardi Borgia et al., 2005) and cyproterone acetate (Stecova et al., 2007) when compared to the uptake from solid lipid nanoparticles.

1.5 Purpose, aim and challenge

Stable nitroxide free radicals are versatile spin probes for research in dermatology and pharmacology, and are detectable by electron paramagnetic resonance (EPR) spectroscopy. In dermatology, investigations in the redox state, including the antioxidative and oxidative status are possible using spin probes. This is important, because oxidative stress like free radical production is associated with skin inflammation and can lead to skin cancer and accelerated skin ageing. Additionally, the antioxidative status reflects the skin protection system against external and internal oxidative stress, e.g., due to solar irradiation, noxious agents, smoking or alcohol consumption. The evaluation of both positive effects, such as a healthy, balanced diet as well as negative effects like oxidative stress can be studied. Yet, up to now non-invasive in vivo measurements are rare. For example, the antioxidative enzymes catalase and superoxide dismutase can be quantified by a chromogenic assay using tape strips taken from the stratum corneum (Hellemans et al., 2003), and carotenoids can be determined non-invasively by Raman spectroscopy (Darvin et al., 2004). A versatile method capable of detecting a variety of antioxidant species of the skin simultaneously is desirable.

In pharmacology, nitroxides can be used as model agents, or drugs can be nitroxide labelled. After incorporation into carrier systems, nitroxide spin probes give information about the polarity of their immediate surrounding, e.g., incorporation within a lipid based carrier system can reveal the nitroxide distribution profile between lipid and aqueous domains. This is a promising tool for optimizing the loading process of drugs to carrier systems. Furthermore, skin penetration efficiency of carrier systems can be evaluated after nitroxide incorporation and skin application.

The development of EPR based ex vivo and in vivo techniques is quite challenging. Several restrictions occur: i) the application of EPR detectable nitroxides is necessary, ii) these nitroxides must penetrate the human skin, and iii) depending on the desired application they have to remain stable in the skin over a certain time period. Therefore, before approaching in vivo measurements, in vitro and ex vivo

investigations were performed initially in this thesis. Figure 5 shows schematically the structure of the thesis.

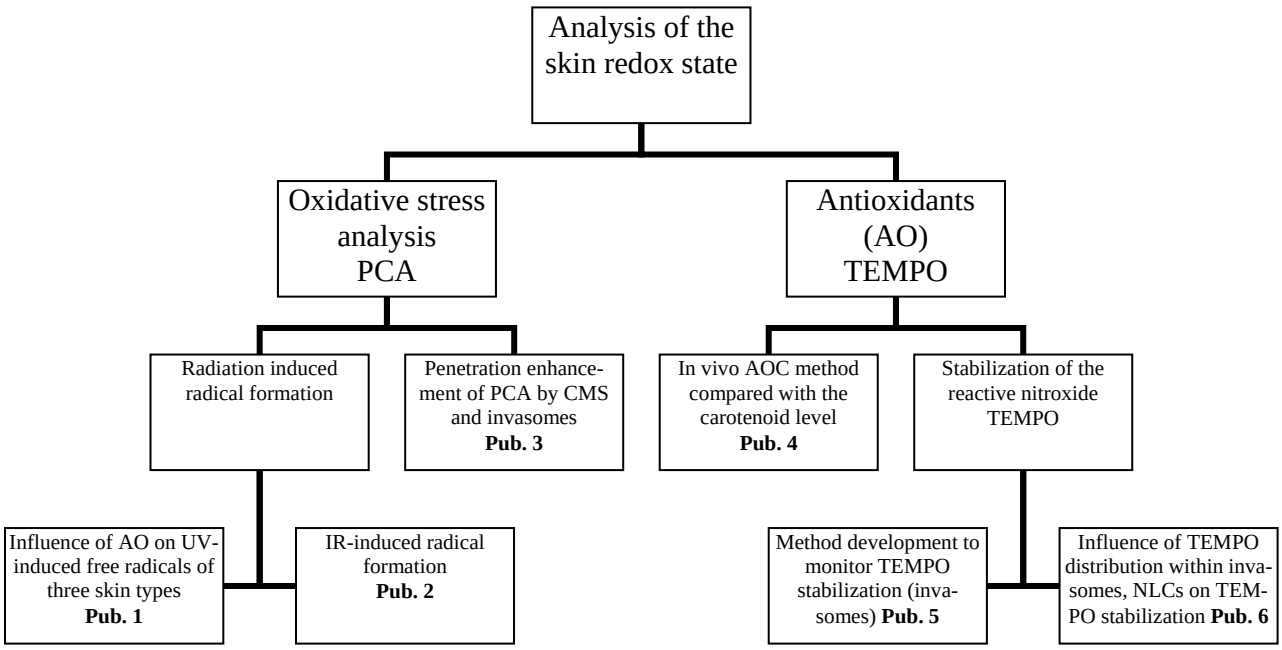

Figure 5 Schematic illustration: Spin probes to investigate the redox properties of the skin as well as their penetration enhancement and stabilization (AOC, antioxidative capacity).

The spin probe PCA was found to be particularly suitable for the detection of short-lived free radicals (Herrling et al., 2003). Therefore, the suitability of human, porcine and bovine udder skin for the detection of UV-induced free radicals was investigated. Due to the limited availability of human skin it was studied, whether it is necessary to use human skin for free radical detection, or whether animal skin is adequate. Furthermore, the activity of the antioxidant enzyme catalase and the carotenoid content of human and animal skin were investigated with regard to their influence on the formation of UV-induced free radicals (publication 1).

Recently, it was found that the production of free radicals in the skin is not only induced in the UV- but also in the visible- and IR-spectral range (Zastrow et al., 2009). Furthermore, investigations regarding the influence of IR-light on the carotenoid substances ß-carotene and lycopene resulted in carotenoid degradation in human skin (Darvin et al., 2007). Therefore, using the method for radical detection developed in publication 1, radical formation in the IR-spectral range was investigated afterwards (publication 2).

Due to poor skin penetration of the hydrophilic nitroxide PCA, CMS nanotransporters and invasomes were used to achieve penetration enhancement. It was investigated whether the distribution of PCA within the nanocarriers or the size of the nanocarriers themselves had an impact on penetration efficiency and penetration profile of the stratum corneum. Moreover, it was evaluated whether EPR spectroscopy is a promising tool for the determination of penetration enhancement by means of nanocarriers (publication 3).

The spin probe TEMPO was found to readily react with reducing agents in the skin (Fuchs et al., 1997) and, therefore was used for the development of an EPR-based method for the detection of the antioxidative capacity of the skin of human volunteers. Additionally, it was investigated if the reduction of TEMPO correlates with the carotenoid content of the skin (publication 4).

As mentioned previously, the nitroxide TEMPO was found suitable for the detection of the antioxidative capacity since its reaction with reducing agents is considerably fast. In order to study free radical production during inflammation, it is necessary to deliver the spin probe protected to the site of inflammation. During inflammation, mast cells and leukocytes exhibit an increased uptake of oxygen and, therefore, an increased release of free radicals, which is termed the “respiratory burst” (Hussain et al., 2003). Therefore, aiming for longer measurement times and protected delivery, the stabilization of TEMPO by nanocarriers was investigated. Initially, invasomes were used, because it is known that the lipid membrane of elastic vesicles can penetrate the skin either fragmented or unfragmented (Cevc et al., 2002; Honeywell-Nguyen et al., 2002). TEMPO was used since its log*P* value indicates accumulation in the lipid membrane of the invasomes. The distribution of TEMPO within an invasome suspension was determined by high frequency W-band measurements, followed by simulations of W-band spectra and determination of magnetic parameters. These magnetic parameters were applied to L-band spectra and a method was developed to follow the distribution of TEMPO between the lipid and aqueous phases during skin penetration. The stabilizing effect was investigated ex vivo on porcine skin and in vivo on the forearm of human volunteers. Furthermore, the results were compared to the hyperfine coupling constant, which is a measure for the polarity of the TEMPO microenvironment (publication 5).

In publication 6, the results of invasomes were compared with those of nitroxide loaded NLCs. It was investigated whether the distribution of TEMPO within the nanocarriers had an impact on the measurement times and on the stabilizing effect observed.

2 Results

In this chapter the results and applied methods are presented as publications in peer reviewed journals.

2.1 Evaluation of human and animal skin for free radical detection

Haag SF, Bechtel A, Darvin ME, Klein F, Groth N, Schäfer-Korting M, Bittl R, Lademann J, Sterry W, Meinke MC, 2010. Comparative study of carotenoids, catalase and radical formation in human and animal skin. Skin Pharmacol. Physiol. 23, 306-312.

Available online: http://dx.doi.org/10.1159/000313539

Abstract:

Animal skin is widely used in dermatological free radical research. Porcine ear skin is a well-studied substitute for human skin. The use of bovine udder skin is rare but its high carotenoid content makes it particularly appropriate for studying the redox state of the skin. Yet, information on the suitability of animal skin for the study of external hazard effects on the redox state of human skin has been lacking. In this study, we investigated the activity of the antioxidant enzyme catalase and the carotenoid content defining the redox status as well as UV-induced radical formation of human, porcine ear and bovine udder skin ex vivo. In human skin only low levels of radical formation were detected following UV irradiation, whereas bovine skin contains the highest amount of carotenoids but the lowest amount of catalase. Porcine ear skin does not exhibit a carotenoid signal but its catalase activity is close to human skin. Therefore, radical formation can neither be correlated to the amount of catalase nor to the amount of carotenoids in the skin. All skin types can be used for electron paramagnetic resonance-based detection of radicals, but porcine skin was found to be the most suitable type.

2.2 Infrared light induced free radicals

Darvin ME, Haag SF, Lademann J, Zastrow L, Sterry W, Meinke MC, 2009. Formation of free radicals in human skin during irradiation with infrared light. J. Invest. Dermatol. 130, 629-631.

Available online: http://dx.doi.org/10.1038/jid.2009.283

No abstract available

2.3 Skin penetration enhancement of a hydrophilic nitroxide

Haag SF, Fleige E, Chen M, Fahr A, Teutloff C, Bittl R, Lademann J, Schäfer-Korting M, Meinke MC, 2011. Skin penetration enhancement of core-multishell nanotransporters and invasomes measured by electron paramagnetic resonance spectroscopy. Int. J. Pharm. 416, 223-228.

Available online: http://dx.doi.org/10.1016/j.ijpharm.2011.06.044

Abstract:

In order to cross the skin barrier several techniques and carrier systems were developed to increase skin penetration of topical dermatics and to reduce systemic adverse effects by avoiding systemic application. Ultra-flexible vesicles, e.g. invasomes and core-multishell (CMS) nanotransporters are efficient drug delivery systems for dermatological applications. Electron paramagnetic resonance (EPR) spectroscopic techniques were used for the determination of localization and distribution of the spin label 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxy (PCA; logP=-1.7) within the carrier systems and the ability of the carriers to promote penetration of PCA into the skin. The results show an exclusive localization of PCA in the hydrophilic compartments of the invasome dispersion and the CMS nanotransporter solution. PCA penetration was enhanced 2.5 fold for CMS and 1.9 fold for invasomes compared to PCA solution. Investigation of penetration depth by step-wise removal of the stratum corneum by tape stripping revealed deepest PCA penetration for invasomes. UV-irradiation of PCA-exposed skin samples revealed that the spin label is still reactive. In conclusion novel polymer-based CMS nanotransporters and invasomes can favor the penetration of PCA or hydrophilic drugs. This offers possibilities for e.g. improved photodynamic therapy.

2.4 Determination of the skin antioxidative capacity in vivo

Haag SF, Taskoparan B, Darvin ME, Klein F, Groth N, Lademann J, Sterry W, Meinke MC, 2011. Determination of the antioxidative capacity of the skin in vivo using resonance Raman and electron paramagnetic resonance spectroscopy. Exp. Dermatol. 20, 483-487.

Available online: http://dx.doi.org/10.1111/j.1600-0625.2010.01246.x

Abstract:

BACKGROUND: Non-invasive measurements are of major interest for investigating the effects of stress, nutrition, diseases or pharmaceuticals on the antioxidative capacity of the human skin. However, only a few non-invasive methods are available.

MATERIAL AND METHODS: The resonance Raman spectroscopy is well established to monitor carotenoids in the skin, but correlations with other antioxidants have not yet been described. Electron paramagnetic resonance spectroscopy used for measurements of free radicals has already been used elsewhere to investigate the reduction of applied long-living nitroxide radicals, caused by skin antioxidants and UV irradiation, but only a single or up to four volunteers were included in these studies. Therefore, in this study, the two methods were applied in parallel on 17 volunteers, and the rate constant of the nitroxide decrease was correlated with the cutaneous carotenoid concentration.

RESULTS AND DISCUSSION: A correlation with R = 0.65 was found, supporting the thesis that different antioxidants protect each other and build an antioxidative network in the skin. The results also give first indications that the carotenoids serve as marker substances for the antioxidative capacity, if the nutrition is well balanced.

2.5 Stabilization of TEMPO using invasomes

Haag SF, Taskoparan B, Bittl R, Teutloff C, Wenzel R, Fahr A, Chen M, Lademann J, Schäfer-Korting M, Meinke MC, 2011. Stabilization of reactive nitroxides using invasomes to allow prolonged electron paramagnetic resonance measurements. Skin Pharmacol. Physiol. 24, 312-321.

Available online: http://dx.doi.org/10.1159/000330235

Abstract:

The detection of the antioxidative capacity of the skin is of great practical relevance since free radicals are involved in many skin damaging processes, including aging and inflammation. The nitroxide TEMPO (2,2,6,6-tetramethyl-1-piperidinyloxyl) in combination with electron paramagnetic resonance spectroscopy was found suitable for measuring the antioxidative capacity since its reaction with reducing agents is considerably fast. Yet, in order to achieve longer measurement times, e.g. in inflammatory skin diseases, the stabilizing effect of an invasome (ultraflexible vesicle/liposome) suspension with TEMPO was investigated ex vivo on porcine skin and in vivo on human skin. Invasomes increased the measurement time ex vivo 2-fold and the reduction was significantly slowed down in vivo, which is due to membrane-associated and therefore protected TEMPO. Furthermore, TEMPO accumulation in the membrane phase as well as the decreasing polarity of the ultimate surroundings of TEMPO during skin penetration explains the stabilizing effect. Thus, an invasome suspension with TEMPO exhibits stabilizing effects ex vivo and in vivo.

2.6 Stabilization of TEMPO using nanostructured lipid carriers

Haag SF, Chen M, Peters D, Keck CM, Taskoparan B, Fahr A, Teutloff C, Bittl R, Lademann J, Schäfer-Korting M, Meinke MC, 2011. Nanostructured lipid carriers as nitroxide depot system measured by Electron Paramagnetic Resonance spectroscopy. Int. J. Pharm. 421, 364-369.

Available online: http://dx.doi.org/10.1016/j.ijpharm.2011.10.009

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 12min 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.

3 Final discussion

The availability of human skin in sufficient size and quality is rare and is mainly obtained from patients undergoing abdominal skin reduction surgery. Therefore, in publication 1, it was investigated whether porcine ear and bovine udder skin can be used for oxidative stress measurements instead of human skin. Porcine ear skin was used since it is a highly suitable substitute for human skin in dermatopharmacology (Jacobi et al., 2007; Jacobi et al., 2005). Bovine udder skin is also an interesting candidate for oxidative stress measurements, as its ß-carotene content is particularly high (Vahlquist et al., 1987). On account of ethical reasons, only the skin of animals was used which had been slaughtered for meat production. In this study, the carotenoid content, the catalase activity of the stratum corneum and the radical formation after UV-irradiation of the three skin types were investigated. It was found that neither carotenoids nor catalase influenced radical formation. These results suggest that other antioxidant substances or enzymes contribute considerably to the antioxidant defence system. In the case of human skin, the antioxidant composition of the skin is known (Shindo et al., 1994) but data for the animal skin has only been published sporadically. UV-induced radical formation was lowest in human skin, which can be explained by the human diet. It is suspected that humans have a healthier, more varied diet than animals whose diet is optimized for maximum meat production.

These investigations have shown, once more, that PCA is suitable to detect free radicals but is not influenced by the antioxidant system of the skin, which results in PCA signal detection for a sufficient time period.

Nevertheless, all investigated skin types can be used for oxidative stress measurements. Regarding availability and handling, porcine ear skin is the most suitable skin type. The developed method has been used for the detection of free radicals after UV-irradiation of titanium dioxide applied on glass slides as well as on porcine skin (Popov et al., 2009).

Besides radical formation in the UV-spectral range, Zastrow et al. (2009) have shown free radical generation also in the visible and infrared spectral ranges. Therefore, in publication 2, IR-induced free radicals were detected using the same procedure as described in publication 1. Although the energy of the photon is not sufficient to break covalent bonds, radical formation could be observed. One explanation may perhaps

be that enzymatic processes are involved which transfer energy from IR light, heat shock-induced formation of reactive oxygen species was reported as having been promoted via enzymes and mitochondria (Shin et al., 2008).

In order to cross the stratum corneum several techniques and carrier systems were developed. Among these, mechanical barrier disruption can be performed, e.g., by microneedle application (Bal et al., 2010) or tape stripping. Tape stripping had been used in the former studies, in order to ease PCA penetration. Without barrier disruption, penetration of sufficient PCA amounts could not be achieved, thus, no significant PCA degradation due to UV-induced free radicals could be obtained. Skin penetration of most substances depends on their physicochemical properties. Substances penetrate best with a log*P* of 1-3 and a MW < 500 g/mol (Korting and Schäfer-Korting, 2010). PCA penetration is low due to its unfavourable log*P* value of -1.7. Therefore, in publication 3, PCA penetration enhancement was studied using nanocarriers. PCA was incorporated in CMS nanotransporters as well as in an invasome dispersion. Invasomes and CMS nanotransporters were chosen, because they were shown to enhance skin penetration of hydrophilic agents (Chen et al., 2011; Küchler et al., 2009a). First, the distribution of PCA within the nanocarriers was investigated and due to its hydrophilicity PCA was found exclusively in the hydrophilic compartments of the nanocarriers. Neither partitioning into the membrane of invasomes, nor into the hydrophobic regions of the CMS polymers could be observed. Application onto porcine skin revealed the best penetration for CMS nanotransporters, resulting in a 2.5-fold higher penetration compared to PCA application in solvent. PCA application within an invasome dispersion enhanced penetration by 1.9-fold compared to PCA in solution. The results are well in accordance with penetration enhancement of rhodamine B by CMS nanotransporters (Küchler et al., 2009a) and with the drug temoporfin by invasomes (Dragicevic-Curic et al., 2008). Additionally, PCA degradation due to UV-induced free radicals was significant, if applied within the nanocarriers. This is important because the removal of the stratum corneum in part results in a loss of skin protection. Especially the outer layers of the stratum corneum protect the skin from UV-irradiation and UV-induced damage. This allows more realistic investigations on the impact of radiation and other noxious agents. The most efficient penetration of PCA after application within CMS nanotransporters can be explained by the size of CMS nanotransporters of only 5 nm. Due to their small size, CMS nanotransporters may penetrate the less densely

packed superficial parts of the stratum corneum which also explains the strong intensity decline after stepwise removal of the stratum corneum, already after the first strip taken. On the other hand, invasomes deliver PCA in particular to deeper layers of the stratum corneum, which can be explained by their penetration mechanism. The membrane or other membrane constituents of invasomes and other highly fluidic liposomes can disintegrate the stratum corneum lipids, forming channel-like pathways through which molecules penetrate (van den Bergh et al., 1999). EPR imaging was not used for the determination of PCA distribution within the stratum corneum, because its resolution is too low.

The determination of penetration efficiency by EPR spectroscopy is a fast alternative to fluorescence dye techniques. Furthermore, high frequency EPR allows the determination of spin probe distribution within the carrier and at low frequencies ex vivo, and in vivo skin measurements become feasible.

Besides oxidative stress and free radical production, the oxidative status of the skin is also determined by the antioxidative capacity. Up to the present time, only a few methods are available for the non-invasive determination of single compounds, e.g., carotenoids (Darvin et al., 2004) and the enzymes catalase and superoxide dismutase (Hellemans et al., 2003). Therefore, in publication 4, an in vivo EPR-method was developed, capable of detecting a variety of antioxidants simultaneously. Up to now, in vivo applications using EPR spectroscopy have been e.g., the non-invasive determination of oxygen concentrations in tissue and the assessment of radiation exposure (Swartz et al., 2004). The spin probe TEMPO was used since it can be applied in vivo (Fuchs et al., 1998) and reacts considerably fast with the skin antioxidant system (Fuchs et al., 1997). In this study, the TEMPO decay was measured on the forearm of 17 volunteers and the rate constant was determined using a first order exponential function. Moreover, Raman spectroscopy was applied for the determination of the carotenoids ß-carotene and lycopene. A correlation between TEMPO decay by means of antioxidants and carotenoids could be shown for the first time. The Pearson coefficient is not high but the tendency is remarkable. This is a first indication that carotenoids can serve as a marker for the whole antioxidant system, if the nutrition is well balanced regarding fruit and vegetables and the life style of the volunteer is stable.

In the case of carotenoids, the bioavailability of oral supplements in the skin can be determined using Raman spectroscopy, as shown recently (Meinke et al., 2010). Oral supplements, especially natural extracts comprise of a variety of antioxidants, e.g., vitamins C and E as the most important hydrophilic and lipophilic antioxidant, respectively, and polyphenols. Yet, non-invasive methods for the detection of these compounds in the skin are not available. Therefore, the testing of skin bioavailability must be extended from carotenoids to more antioxidant compounds (e.g., vitamins C and E) either by single compound detection or the detection of different antioxidants simultaneously. Since TEMPO reacts with a variety of antioxidant substances, the developed method could be the first step to investigate the effects of antioxidant supplements, with regard to their appearance in the skin and their radical scavenging capacity before performing further studies regarding their clinical effects, e.g., tumour prevention or inflammation. Recently, it was shown that the uptake of a natural chokeberry extract and vitamin C to the skin could be determined by means of TEMPO application followed by EPR measurements (Meinke et al., 2011).

For the determination of the antioxidative capacity, the rather fast reaction of TEMPO with the skin antioxidant system is advantageous. For the measurement of radical formation during inflammatory skin diseases longer measurement times and protected TEMPO delivery to the site of inflammation would be favourable. Since the membrane of elastic vesicles can penetrate the skin and, due to its log*P* value, TEMPO was assumed to accumulate in the membrane, invasomes containing TEMPO were used to achieve longer measurement times and protected delivery, and to study the stabilizing effect of elastic vesicles (publication 5). First, invasomes were measured with high frequency W-band in order to determine the magnetic parameters. Also, the signal lines of TEMPO from the aqueous phase are clearly separated from those attributed to the lipid phase. This allows the determination of TEMPO distribution within the invasome suspension. As expected, TEMPO accumulated in the lipid membrane of invasomes. After application to porcine skin, the measurement time could be increased two-fold and a method was developed to study stabilization. Spectral simulation of the relative amount of TEMPO in the membrane phase during penetration could be performed using the EasySpin toolbox (Stoll and Schweiger, 2006). This was made possible by applying magnetic parameters derived from W-band measurements to simulations performed with L-band spectra recorded during invasome penetration. The advantage of the simulation

approach is the determination of the exact amount of TEMPO in each phase at any time during penetration. The results of the simulation approach correlate well with the data generated by the determination of the hyperfine coupling constants, which is a measure of the polarity of the TEMPO microenvironment. Importantly, for the first time, in vivo measurements on the forearms of human volunteers were performed. The results regarding stabilization are in accordance with the results obtained from ex vivo measurements.

Finally, the influence of TEMPO distribution within the nanocarriers on the stabilizing effect was studied in publication 6. TEMPO was loaded to NLCs and the same investigations were performed as previously with invasomes. NLCs had less TEMPO associated with the lipid compartments of the carrier, which could be derived from W-band measurements. This is due to the fact that TEMPO cannot be incorporated in the solid lipid matrix as it was shown for solid lipid nanoparticles with TEMPO being located in the surfactant shell (Küchler et al., 2010). The lipophilic signals of TEMPO originate from the liquid lipid droplets. It could be shown that the different distribution profiles had an impact on the stabilizing effect ex vivo. Measurement time was increased 1.7-fold compared to two-fold with invasomes. The same as with invasomes, NLCs also show TEMPO stabilization if applied in vivo.

In publications 4 – 6 the spin probe TEMPO was applied to the skin of volunteers. This could be performed, because it was found that TEMPO had no irritating effect on human skin at the concentration (30 mmol/l) used in this thesis (Fuchs et al., 1998). The authors reported that TEMPO caused moderate skin irritation after a single application of 100 mmol/l TEMPO on human skin and slight skin irritation after repetitive applications of 10 mmol/l for 21 days. Besides TEMPO, it was found that oxazolidine nitroxides caused slight irritations after a single application (100 mmol/l) but no irritation after repetitive applications. Pyrrolidine and imidazoline type nitroxides did not cause any irritations at the used concentration or application type (single and repetitive), (Fuchs et al., 1998). Furthermore, it was found that TEMPO did not exhibit any mutagenic activity after incubation with different strains of *S. typhimurium*, which also holds true for different aromatic nitroxides (Damiani et al., 2000). The same was found for the spin probe PCA and other nitroxides (Sosnovsky, 1992). Cytotoxicity tests using Chinese hamster ovary cells revealed that the induced level of toxicity of TEMPO and three aromatic nitroxides was lower than the toxicity of

butylated hydroxytoluene, a synthetic antioxidant widely used in the food industry (Damiani et al., 2000).

The TEMPO derivative 4-hydroxy-TEMPO was even suggested as an antiproliferative agent for the treatment of malignant gliomas. Tumours from mice treated with 4-hydroxy-TEMPO showed a decrease in growth, with an increase in the number of apoptotic cells and a decrease in neo-vascularisation, compared to tumours from control mice. Moreover, treated mice did not show any signs of general or organ toxicity (Gariboldi et al., 2003).

Summarizing the results of the thesis, it could be shown that EPR spectroscopy with the spin probe PCA is suitable for the detection of free radicals in the UV- and IR-spectral ranges. The results obtained are in agreement with previously published data using the EPR spin trapping technique (Zastrow et al., 2009). Spin traps are EPR-silent molecules, which turn paramagnetic upon reaction with short-lived free radicals. Both EPR-based methods are capable of measuring free radicals in tissue samples, whereas the frequently applied dichlorofluorescein assay (Wölfle et al., 2011) cannot be used with tissue samples; it is restricted to in-vitro measurements of e.g., intracellular radical formation in cultured mammalian cells. EPR-based detection of radical formation using PCA offers the possibility for human in vivo measurements. Penetration enhancement of the spin probe PCA could be achieved using CMS nanotransporters and invasomes. EPR spectroscopy is a fast alternative to fluorescence dye techniques for the quantification of penetration enhancement. Fluorescence techniques are somewhat time consuming as the skin has to be cryosectioned, followed by laser scan microscopy (LSM) and picture analysis (Küchler et al., 2009a). In contrast to EPR spectroscopy, LSM does not provide information about the localization of the dye within the nanocarrier or nanoparticle. Nevertheless, it has to be kept in mind that LSM allows the detection of the dye in the viable epidermis and dermis. EPR based investigations can serve as an additional method to obtain information about the localization of the spin probe and its microenvironment, e.g., polarity and mobility as well as the quantification of penetration enhancement.

Stabilization of the reactive nitroxide TEMPO was achieved using invasomes and NLCs. EPR spectroscopy at high frequency W-band (94 GHz) allows the localisation of a spin probe or a spin-labelled drug within a carrier matrix, allowing the

observation of drug-carrier interactions. This is a promising tool for optimising the loading process of drugs to nanocarriers, in order to achieve a certain distribution, e.g., in the surfactant shell for burst release or in the lipid phase for sustained release. At low frequency L-band ex vivo measurements during penetration become feasible, allowing the observation of drug-target and drug-carrier interactions. EPR spectroscopy is particularly interesting to gain mechanistic insights into drug-target and drug-carrier interactions, e.g., cleavage of a covalently bond spin probe can be observed by the determination of spin probe mobility, polarity of the microenvironment of a spin probe can reveal the localization within a carrier or a formulation and give information regarding the affinity of a spin probe to a carrier.

4 Summary

The thesis focuses on the use of spin probes for EPR-based method development in dermatology and pharmacology. Spin probes can be used for the detection of free radicals and the antioxidative capacity, which are important diagnostic methods in dermatology. Primarily, it was evaluated whether it is necessary to use human skin or if animal skin is suitable for UV-induced free radical detection using the spin probe PCA. It was found that the antioxidant network of the skin does not influence free radical detection and that all skin types can be used, whereas porcine skin was found to be the most suitable skin type. Using porcine skin and the developed method for free radical detection, radical formation could also be measured in the infrared spectral range.

In order to improve the poor skin penetration of the spin probe PCA, it was incorporated into nanocarriers. W-band measurements revealed an exclusive PCA localization in the hydrophilic compartments of the carriers. CMS nanotransporters enhanced PCA penetration most excellently followed by invasomes. Moreover, it was found that CMS nanotransporters mainly penetrate the superficial, less dense layers of the stratum corneum, whereas invasomes deliver PCA in particular to deeper stratum corneum layers.

In addition to the ability of free radical detection, spin probes can be used for the determination of the antioxidative capacity of the skin. An in vivo method was developed by using the spin probe TEMPO, which is reduced by the skin antioxidant system. Quantification of the carotenoid antioxidant substances ß-carotene and lycopene unravelled a correlation between carotenoid content and TEMPO reduction. This is a first indication that carotenoids can serve as a marker for the entire antioxidant system.

The fast reduction of TEMPO is advantageous for the determination of the antioxidative capacity. For the detection of free radicals produced by inflammatory skin diseases, longer measurement times and protected spin probe delivery to the site of inflammation are necessary. Therefore, TEMPO was applied within an invasome suspension. High frequency W-band measurements demonstrated that TEMPO is located in both the lipophilic and hydrophilic compartments. Studies on

porcine skin ex vivo revealed a two-fold increased measurement time compared to TEMPO solution and the reduction was significantly slowed down in vivo on human volunteers. The stabilizing effect is due to membrane located and therefore protected TEMPO. Additionally, during penetration, the amount of TEMPO associated with the membrane phase increased and the immediate polarity of TEMPO decreased.

TEMPO was also loaded to NLCs and the same experiments were performed as with invasomes. It was found that the distribution of TEMPO within the nanocarriers had an impact on prolonged measurement times. Compared to invasomes, NLCs had less TEMPO associated with the lipid compartments, which lead to a reduced measurement time. However, compared to solution, NLCs also increased the measurement time, indicating stabilization and slow release of TEMPO.

This thesis has shown that EPR spectroscopy using nitroxide spin probes is able to monitor (a) the antioxidative capacity in vivo, (b) oxidative stress induced by irradiation or other stressors, and can investigate (c) the penetration enhancement and stabilization of spin probes by nanotransporters from in vitro to in vivo.

For (a) and (b) an L-band EPR spectrometer is sufficient, for (c) multi-frequency EPR is necessary.

4.1 Zusammenfassung

Die vorliegende Arbeit beschäftigt sich mit dem Einsatz von Spinsonden für die EPR-basierte Methodenentwicklung in der Dermatologie und Pharmakologie. Spinsonden können für den Nachweis von freien Radikalen und zur Bestimmung der antioxidativen Kapazität verwendet werden, welche beide wichtige diagnostische Verfahren in der Dermatologie sind. Zunächst wurde untersucht, ob es notwendig ist, Humanhaut für den Nachweis UV-induzierter freier Radikale zu verwenden, oder ob Tierhäute eine Alternative bieten. Zum Nachweis der Radikalbildung wurde die Spinsonde PCA verwendet. Es zeigte sich, dass die Antioxidantien der Haut keinen Einfluss auf den Nachweis freier Radikale haben und dass alle untersuchten Hauttypen geeignet sind, wobei sich Schweinehaut als besonders geeignet herausstellte. Mit Hilfe der entwickelten Methode zum Nachweis freier Radikale, konnte die Radikalbildung in Schweinehaut nach IR-Bestrahlung gemessen werden.

Um die Penetration der Spinsonde PCA in die Haut zu verbessern, wurde diese in Nanocarrier eingearbeitet. Messungen im W-Band ergaben eine ausschließliche Lokalisation von PCA in den hydrophilen Kompartimenten der Nanocarrier. CMS Nanotransporter verbesserten die PCA Penetration am stärksten, gefolgt von Invasomen. Darüber hinaus zeigte sich, dass CMS Nanotransporter hauptsächlich in die oberflächlichen, weniger dicht gepackten Schichten des Stratum corneums penetrieren, wohingegen Invasomen die PCA Penetration in tiefere Schichten des Stratum corneums begünstigen.

Neben der Messung von freien Radikalen, können Spinsonden auch für die Bestimmung der antioxidativen Kapazität der Haut verwendet werden. Mit Hilfe der Spinsonde TEMPO, die schnell durch die Antioxidantien der Haut neutralisiert wird, wurde eine in vivo Methode zur Bestimmung der antioxidativen Kapazität der Haut etabliert. Durch zusätzliche Bestimmung der Carotinoide ß-Carotin und Lycopin zeigte sich, dass der Gehalt an Carotinoiden mit der Reduktionsrate von TEMPO korreliert. Dies ist ein erster Hinweis darauf, dass die Carotinoide als Marker für das gesamte antioxidative System dienen können.

Für die Bestimmung der antioxidativen Kapazität ist die schnelle Reaktion von TEMPO mit den Antioxidantien der Haut von Vorteil. Für die Bestimmung von freien Radikalen, die bei entzündlichen Hauterkrankungen produziert werden, sind jedoch längere Messzeiten sowie ein geschützter Transport der Sonde zum

Entzündungsherd wichtig. Aus diesem Grund wurde TEMPO in einer Invasomen-suspension eingesetzt. Messungen im W-Band zeigten, dass sich TEMPO sowohl in der lipophilen als auch in der hydrophilen Phase der Invasomen befindet. Ex vivo Untersuchungen an Schweinehaut zeigten eine zweifach erhöhte Messzeit im Vergleich zur TEMPO-Lösung. Auch in vivo Messungen an Probanden zeigten, dass TEMPO nach Applikation in Invasomen langsamer reduziert wird als nach Applikation in Lösung. Die stabilisierende Wirkung beruht darauf, dass sich TEMPO teilweise in der Membran der Invasomen befindet und dadurch geschützt ist. Dies zeigte sich auch während der Penetration in Schweinehaut. Hierbei stieg während der Penetration der TEMPO Anteil in der Membran an, während gleichzeitig die Polarität der Mikroumgebung von TEMPO deutlich abnahm.

Des Weiteren wurden NLCs mit TEMPO beladen und die gleichen Untersuchungen wie mit den Invasomen durchgeführt. Es zeigte sich, dass die Verteilung von TEMPO in den Nanocarriern die Messzeit beeinflusst. Im Vergleich zu den Invasomen befand sich bei den NLCs weniger TEMPO in der lipidischen Phase der Nanocarrier. Dies führte im Vergleich zu den Invasomen zu einer verminderten Messzeit. Im Vergleich zur TEMPO-Lösung führten aber auch die NLCs zu einer verlängerten TEMPO-Detektion, was auf Stabilisierung und langsame Freisetzung von TEMPO hinweist.

Diese Arbeit hat deutlich gezeigt, dass die EPR Spektroskopie mit der Verwendung von Spinsonden die Messung (a) der antioxidativen Kapazität in vivo und (b) von oxidativem Stress (induziert durch Strahlung oder andere Stressfaktoren) ermöglicht sowie die Untersuchung der (c) Penetrationsverstärkung und Stabilisierung von Spinsonden durch Nanotransporter erlaubt.

Für (a) und (b) sind Messungen an einem L-Band EPR Spektrometer ausreichend, für (c) sind Messungen bei unterschiedlichen Frequenzen notwendig.

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