Interaction of drug molecules with carrier systems as studied by parelectric spectroscopy and electron spin resonance

C. Braem\textsuperscript{a} T. Blaschke\textsuperscript{b} G. Panek-Minkin\textsuperscript{b} W. Herrmann\textsuperscript{a} P. Schlupp\textsuperscript{a,c} T. Paepenmüller\textsuperscript{d} C. Müller-Goyman\textsuperscript{d} W. Mehnert\textsuperscript{a} R. Bittl\textsuperscript{b} M. Schäfer-Korting\textsuperscript{a} K.D. Kramer\textsuperscript{b,\ast},

\textsuperscript{a}Freie Universität Berlin, Fachbereich Pharmazie
\textsuperscript{b}Freie Universität Berlin, Fachbereich Physik
\textsuperscript{c}Charité Berlin, Universitätsklinikum Benjamin Franklin
\textsuperscript{d}Technische Universität Braunschweig, Fachbereich Pharmazie

Abstract

According to recent investigations of nanoparticular carrier systems the mode of drug-particle interaction appears to influence drug penetration into the skin. For a more detailed insight into the molecular structure of drug loaded particles the two independent analytical methods, namely the parelectric spectroscopy (PS) and the electron spin resonance (ESR) have been applied to 4,5,5,-trimethyl-1-yloxy-3-imidazoline-2-spiro-3\textsuperscript{-} (5\textsuperscript{*}-cholestane) as a model drug. Spectra have been analyzed in dependence on the concentration of the spin label. Changes in the concentration-dependent dipole mobility and dipole density given by PS and the concentration-dependent rotational correlation time (ESR) which are a measure of the vicinity of carrier and / or the surfactant and guest molecule were studied with cholestane-labelled solid lipid nanoparticles (SLN), nanoparticular lipid carriers (NLC) and nanoemulsions (NE). The spin probes were attached to the SLN surface which consists of two distinct subcompartments: the rim and the flat surface of the disk-like shapes. The shape could be observed by freeze- fraction electron microscopy. Spin probes, however, were incorporated into the carrier matrix in the cases of NLC and NE. Results of PS are verified by ESR which
allows a more detailed insight. Taking the results together a detailed new model of 'drug'-particle interaction could be established.

Key words: Solid lipid nanoparticles, nanostructured lipids, nanoemulsions, parelectric spectroscopy, electron spin resonance

1. Introduction

Although most interesting due to a reduction of systemic drug load and thus potential side effects, the topical treatment of skin disease is often excluded by poor skin penetration of the drug [1]. To improve the uptake, nanoparticulate carrier systems enhancing the absorption are of great interest. Particular carrier systems applicable to the skin are liposomes [2; 3], solid lipid nanoparticles (SLN), nanostructured lipid carriers (NLC), nanoemulsions (NE) and microemulsions (ME) [4] as well as (biodegradable) polymers and dendrimers [5]. Above that, nanoparticulate carriers such as liposomes and lipid nanoparticles may have the potential to induce drug targeting and increase the benefit risk ratio of topical therapy of skin diseases [4; 6]. Recently a strong dependency of the penetration depths of topically applied nanoparticulate systems was observed when loaded with glucocorticoids [7; 8], antiandrogens [9] or a model dye [10] and the location of these agents with respect to the carrier matrix [8]. The penetration efficiency of loaded drugs has been quantified by High Performance Liquid Chromatography (HPLC) and by using dyes able to display fluorescence human skin [11]. Parelectric spectroscopy (PS) allowed to distinguish between the cases of incorporation of the agents into the particle matrix and an attachment to their surface, independent of labeling the drug or replacing it by dye molecules. This physical method, has been described before [8] in all its experimental details. However, it is unable to distinguish between various possible sites of the guest molecule in the case of incorporation into the lipid matrix surrounded by the surfactant shell necessary to avoid particle aggregation. A first step in the direction of a better understanding was the inspection of the above listed systems with fluorescence spectroscopy. Studying nile red loaded NLC, fluorescence light emission indicated that platelets composed of solid lipids were embedded into fluid lipids and tensids [12] as well as an enrichment of the dye in the fluid lipid compartment [10]. For such carrier systems as SLN, NLC and NE loaded with spin-labels it is known from the literature [13; 14] that electron spin resonance (ESR) allows to study the individual surrounding of the rotational part of the spin-labeled molecule in terms of its rotational correlation time $\tau_R$. Besides that, ESR has been used to study the distribution of spin-labeled (Tempol$^{\text{R}}$) analogues of fatty acids in SLN [15; 16]. Incorporation of the spin label into the lipid matrix increased with the lipophilicity of the fatty acid.

Topically applied glucocorticoids still are of high importance for dermatitis treatment. By loading glucocorticoids into nanoparticles [7; 8] skin uptake and distribution within the skin can be influenced. For a detailed insight into glucocorticoid and lipid matrix interaction, we decided to apply ESR using the commercially available spin label 4,5,5,- trimethyl-1-yloxy-3-imidazoline-2-spiro-3'-(5'*-cholestane) (Cholestane$^{\text{R}}$) as a model drug, which is close in its structure to steroidal drugs. To the best of our knowledge, the results of the PS and the ESR are compared and used together to unravel the structure of the drug carrier system for the first time. Since the results asked for a detailed knowledge of the geometric structure of the systems, freeze-fraction TEM analysis was performed in addition. Integrating the results, we were able to establish a
model able to explain the existence of at least two clearly distinct sub-compartments of the drug-carrier system with SLN as a host.

2. Materials and Methods

2.1. Materials

Compritol® 888 ATO (glycerol behenate) was a gift from Gattefosse (Weil a. Rh., Germany), Miglyol® 812 (medium chain triglycerides) was obtained from Caelo (Hilden, Germany) and the emulsifier Lutrol® F 68 (Poloxamer® 188) was supplied by BASF (Ludwigshafen, Germany). The spin label 4,5,5-trimethyl-1-yloxy-3- imidazoline-2-spiro-3’-(5’*-cholestane) (Fig. 1) is purchased from Magnettech (Berlin, Germany) and ascorbic acid was obtained from Sigma-Aldrich (München, Germany). Preparation of lipid dispersions: Solid lipid nanoparticles (SLN), nanostructured lipid carriers (NLC) and nanoemulsions (NE) were prepared as described previously [10]. Briefly, the total lipid concentration in the dispersions was 10% (w/w), the NE formulations were made up from Miglyol 812 and SLN from Compritol. In the case of NLC, Compritol was replaced by increasing amounts of Miglyol 812 (5%, 10%, 15%, 20% and 25% referring to the total lipid phase). In general, it is recommended to destroy any crystal center of the bulk material by a sufficiently long heating period above the melting point in order to avoid the lipid memory effect and to enable a new crystallization. An amount of 1 mmol or 0.1% up to 0.3% of the spin label was dissolved in the hot lipid phase and given to an aqueous solution of 2.5% Lutrol F 68 at the same temperature. The dispersion was formed using a rotor-stator mixer at 8000 rpm for 30 s. This premix was passed through a high pressure homogenizer (Emulsiflex® C5 Avestin, Mannheim, Germany) at 95°C. The hot dispersions were filled in silanized glass vials, cooled down to room temperature and stored at 8°C.

Fig. 1. Structure of Cholestane (4,5,5-,trimethyl-1-yloxy-3- imidazoline-2-spiro-3’-(5’*-cholestane))
Table 1

<table>
<thead>
<tr>
<th>Formulation</th>
<th>LD 50 / nm</th>
<th>LD 95 / nm</th>
<th>PCS / nm</th>
<th>PI / nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLN</td>
<td>116 ± 1</td>
<td>331 ± 6</td>
<td>151 ± 2</td>
<td>0.223 ± 0.026</td>
</tr>
<tr>
<td>NLC</td>
<td>128 ± 1</td>
<td>328 ± 2</td>
<td>192 ± 2</td>
<td>0.136 ± 0.018</td>
</tr>
<tr>
<td>NE</td>
<td>325 ± 1</td>
<td>544 ± 11</td>
<td>377 ± 7</td>
<td>0.260 ± 0.040</td>
</tr>
</tbody>
</table>

Particle size of the tested systems determined by Laser Diffraction (LD) and Photon Correlation Spectroscopy (PCS) together with the Polydispersity Index (PI).

2.3. Transmission electron microscopy

Both physical methods, PS and ESR, have to be accompanied by further information about the carrier system in order to develop models for the correct interpretation of the spectra obtained. Such information concerning the size and shape of the particles was obtained by transmission electron microscopy (TEM). The samples were quick-frozen at the boiling nitrogen temperature between two gold supports. Subsequently the samples were fractured in the vacuum chamber of a Balzers BAF 400 (Balzers, Wiesbaden, Germany) freeze-fracture device at 173 K and 5 x 10^-6 Bar. The fractured surface was shaded with a 2 nm platinum-carbon layer at an angle of 45°. For mechanical stabilization an additional layer of pure carbon had to be applied to the surface at an angle of 90°. After cleaning with sulphuric acid and water, the replica was transferred to a copper grid and viewed using a LEO EM 922 device (Leo, Oberkochen, Germany) at 200 kV.

2.4. Parelectric spectroscopy

Under the influence of an external electric radiofrequency field of frequency f, the permanent electric dipole moments of the drug carrier system give a macroscopic polarization answer, the amplitude of which is frequency-dependent. A frequency analyzer (Type ZVR, Rohde & Schwarz, München, Germany) feeds an electromagnetic wave into an open-ended coaxial probe in contact with the sample. In the frequency region 0.1 – 100 MHz the reflected wave is analyzed as to their amplitude and phase, thus yielding the dispersion $\varepsilon'(f)$ and the absorption $\varepsilon''(f)$ of the complex permittivity of the sample. The fundamentals of this approach, experimental set-up and evaluation algorithms have been described in detail [8]. Both curves allow to extract the two parameters of interest, the dipole mobility $f_0(c)$ and the dipole density $\Delta\varepsilon(c)$, dependent on the drug or spin label concentration c. As long as the loaded agent is incorporated into the carrier matrix, solely the increasing mass of the dipole-carrying system influences the quantities $f_0(c)$ and $\Delta\varepsilon(c)$. If the agents are attached to the carrier surface, the above given functions drastically change. The theory of rate processes [18] can be the basis of a model to explain this behavior as depicted in Fig. 2. The frequency region 0.1 - 100 MHz was found best for all preparations under test. Nevertheless, an extension up to 4 GHz was used in order to answer the question whether
Fig. 2. Distinction between the cases of incorporation and attachment: For agents of mass $m_A$ incorporated with a percentage $c$ into the carrier of mass $m_C$, we find the relation $f_0 \sim 1/(m_C + 100c m_A)$ resulting in a slightly falling curve. The density $\Delta \varepsilon(c)$ depends in addition on the agent dipole moment. In the case of attachment, only for the agent concentrations $c = 0$ and $c = c_{\text{max}}$ neighbouring particles have to overcome their maximum jump energies $\Delta E_0$ when the drug-carrier system moves under the electric field influence. In between these limiting cases, the neighbouring particles find places on the surface besides the agent sites and have to overcome energies $\Delta E < \Delta E_0$ resulting in higher mobilities $f_0$.

performed at room temperature. To test the polarity of the environment additional measurements using low temperature high field (94 GHz) ESR have been carried out. The resulting hyperfine tensors showed virtually identical splittings, indicating a hydrophobic environment, for all samples. In order to compare the ESR spectra of the Cholestane (CS) spin label in the different formulations SLN, NLC and NE, spin label rotational mobility is basis to develop a model describing the striking differences in the results of the formulations under test. It is well known that in sufficiently viscous systems the restricted rotational tumbling of the spin label results in incompletely averaged $g$- and $A$-tensors governing the ESR spectra. There are two effects which can serve to extract the rotational correlation time $\tau_R$, the quantity expressing the mobility of the agent part that can undergo hindered rotation. The sample spectra submitted to a complete line-shape analysis with Freed’s interpretation \[19\] allowed to extract two distinct values of the quantity $\tau_R$ in the SLN formulations. This had to be explained by a model yielding both the difference in size of the two values and the relative weights of the number of spin labels involved. An aqueous solution of ascorbic acid was used to measure the rate of reduction of the unpaired spins. This rate is closely related to the degree of access of the ascorbic acid and thus is well suited to draw conclusions of the position of the spin label depending on the type of formulation.
particular micelles. This is in agreement with the CMC as given in the literature [20]. To underline the TEM results we also carried out additional PS measurements. Knowing the PS response of water molecules with their molar mass of 18 at 20GHz, we should have found an answer from micelles with their molar masses at least two orders higher in the frequency span below 4GHz. In accordance to TEM no micelles could be observed in this frequency range. Thus, the combination of PS, ESR, and TEM have been shown to be a powerful tool to decide which models (see Fig. 7) can be applied to describe our systems and their behaviour.

Figure 4 shows the dipole density $\Delta \varepsilon(c)$ and the dipole mobility $f_0(c)$ derived by PS measurement for the CS concentrations $c = 0, 0.025, 0.05$ and $0.1\%$ for all systems (SLN, NLC and NE). The curves $f_0(c)$ and $\Delta \varepsilon(c)$ for NE obey the classical Einstein-Debye behavior: This means that the spin label is almost completely incorporated in the lipid matrix. In the frame of the PS measurement for the CS concentrations $c = 0, 0.025, 0.05$ and $0.1\%$ for all systems (SLN, NLC and NE). The curves $f_0(c)$ and $\Delta \varepsilon(c)$ for NE obey the classical Einstein-Debye behavior: This means that the spin label is almost completely incorporated in the lipid matrix. In the frame of the PS

![Fig. 3. TEM pictures and corresponding ESR spectra of the Cholestane spin probe (0.025%) of nanoparticular systems. A: SLN (Compritol 10%) B: NLC (Compritol (8%, Miglyol 812 2%), the outer surface of the spherical carrier structures begins to be mantled by Miglyol 812 shells C: NE (10% Miglyol 812)](image-url)
In dependence on the Cholestane concentration dipole density $\Delta \varepsilon$ and dipole mobility $f_0$ are presented. In the case of NE there is a pure incorporation, whereas for NLC and even more pronounced for SLN we have to interpret the results as an attachment of the agents to the carrier surface.

The correlation time $\tau_R$ to be extracted from the individual three-line spectra. For a first inspection, the different line shapes for SLN, NLC and NE as carrier matrices are depicted in Fig. 3. The smallest line-widths are found for NE yielding a value $\tau_R = 3.12\,\text{ns}$ which is very close to the value $\tau_R = 3.07\,\text{ns}$ obtained with pure Miglyol 812. According to this value we have to assume a pure incorporation of the agent in the liquid lipid phase. Thus, the results obtained with PS and ESR are well in accordance. The change in line widths and relative heights with decreasing Miglyol 812 content show a decreasing mobility of the spin labels in the NLC. This indicates a gradual change in the location of the agent within the system: Part of the label is located in the liquid lipid (Miglyol 812) phase whereas another part appears to interact with the surface of the solid lipid matrix resulting in a higher immobilization in the latter sites. The SLN spectra display, again in good accordance with the PS results, the highest degree of immobilization in the matrix surface. Interestingly an additional distinction between two sub-compartments can be made: A smaller contribution of 23.7% (referred to as 'site 1' in Fig. 5) is characterized by a value of $\tau_R = 4.85\,\text{ns}$ and a larger contribution of 76.3% ('site 2') with a value of $\tau_R = 5.8\,\text{ns}$. The underlying geometric model as extracted from the corresponding TEM pictures allows to attribute the smaller contribution to spin label sites on the rim of the disc-like SLN shapes, whereas the larger contribution with the longer rotational correlation time appear to be the agents fixed on the lower and upper flat surface. Using the average dimensions, $r \approx 10\,\text{nm}$ and $R \approx 100\,\text{nm}$, a good...
approximation of the surface ratio 'site 2' / 'site 1' yields a value of 3.5, which is in good accordance with the ratio 76.3%/23.7%. For a further affirmation of the models describing the site of the spin labels relative to the carriers in the SLN, NLC and NE preparations, the ESR signals were quenched by reduction of the CS with an aqueous solution of ascorbic acid. The signal decreased very fast in the SLN formulations, suggesting the spin labels being in close contact with the aqueous phase (Fig. 6). This once more excludes a possible incorporation within the particle matrix. In contrast, there was an extremely slow reduction of the CS loaded to NE preparations. Thus the agent should be incorporated into the Miglyol 812 droplets, protected from the action of the aqueous solution of ascorbic acid by the additional surfactant shell. In between these two extreme situations, the NLC formulations with varying Miglyol 812 content as an independent parameter showed a clear decrease in accessibility for the ascorbic acid with increasing Miglyol 812 content. These findings, along with the TEM results, are the basis for models to describe the different preparations.

4. Models

The different models for the carrier systems NE, NLC and SLN including possible other structures in minor amounts [1; 22] are shown in Figure 7. NE consists of a liquid lipid distributed in
Fig. 6. Reduction velocity of the labeled Cholestane by an aqueous solution of ascorbic acid demonstrates the accessibility of the label and thus confirms the position of these molecules in or on the carrier matrix. Incorporation in the case of NE and an attachment in the case of SLN is on the hand. Mixing the lipid content from 0.5% Miglyol and 9.5% Compritol continuously to 2.5% Miglyol 812 and 7.5% Compritol allows interpolating between the cases of attachment and incorporation.

The water phase with the boundary layer stabilized by a surfactant. The shape of the lipid drops is generally assumed to be spherical because spheres have the largest volume / surface ratio. In addition to the existence of the lipid drops the formation of micelles has to be regarded as possible. However, micellar structures as additional carriers for the model drugs can be excluded, as their contribution would add to the ESR signal with far smaller values of the correlation time \(\tau_E\), and micelles could neither be found by TEM (Fig. 3) nor by PS in the 4 GHz range. The slow quenching effect of the ascorbic acid assay (Fig. 6) shows that the agent is located in the liquid lipid droplets of NE formulations with only small quantities reaching the surface. The NE organization retards the reduction because the droplets are surrounded by a surfactant shell as mentioned above. SLN are made up from solid lipid particles suspended in the water phase. The dispersion, too, is stabilized by a surfactant shell. Once more, a micelle formation should be possible but such sub-structures could not be found in the TEM pictures. In the process of producing the SLN formulations the originally solid lipid is molten, thus giving a system similar to the NE structure. When annealing the formulation the lipid crystallizes to yield disc-like structures as shown by the TEM inspection (Fig. 3). From the PS results (Fig. 4) and the ascorbic acid reduction procedure in the ESR experiments (Fig. 6) we have to assume the CS to be rather attached to the carrier surface instead of being incorporated. We have to conclude that there is an only weak
Cholestane, in contrast, has its nitroxide group attached to a five-ring-system in an imidazoline structure. In this highly lipophilic plane arrangement the neighbouring methyl groups form a larger hindrance for the aqueous ascorbic acid solution. This explains, at least qualitatively, the faster reduction dynamics found by Jores et al.

5. Discussion

Progress in the understanding of signaling pathways and of the pathophysiology of diseases at the molecular level allowed to identify highly active agents. Yet not infrequently these are highly toxic, too. Drug carrier systems are developed to introduce these agents into therapy while improving tolerability. For a better understanding of the potential of drug carrier systems the interactions of drug and particle have to be characterized which includes the effects of particle surface modifications, the interactions of particles and proteins as well as the influence on drug distribution in vitro and in vivo. Loading to nanoparticulate systems can also influence skin penetration and the benefit to risk ratio of topical dermatics. To further explain the close relation between the skin penetration behavior of drug-carrier systems and the site of the agents incorporated into the carrier matrix or attached to its surface, the methods of PS, ESR, and TEM have been combined to describe the behavior...
are: In the SLN formulations the agent molecules have to be assumed to be attached to the carrier matrix surface, whereas in the NE preparations the spin labels are fixed inside the Miglyol 812 droplet system and sheltered from the aqueous surrounding by the surfactant shells. In the case of NE as carriers with variable Miglyol 812 content, a continuous switching from the NE-like to the SLN-like behavior with the Miglyol 812 content as an independent parameter could be observed. Spin labeled fatty acids were accessible to ascorbic acid reduction by 20–60% and were reported to be incorporated into the lipid core of SLN by 46–10% with increasing lipophilicity. The hydrophilic Tempol label itself was not incorporated and freely accessible to ascorbic acid [31]. To understand these different results, the TEM pictures could serve as a basis for models to explain the differences in shape from spherical structures of the NE carrier matrices to the disc-like shape of the SLN carriers. Moreover, the existence of two sub-compartments as postulated from the ESR spectra for SLN carriers could be verified from the geometrical parameters of the discs, thus explaining the ratio of the two contributions with different rotational correlation times. The differences in particle form and drug-particle interaction, namely incorporation into the lipid matrix (NE, NLC) or association with the particle surface (SLN) may influence the interaction of drug and skin, too, and thus explain differences in the efficiency of drug delivery into the skin.

6. Conclusion

The combination of TEM, PS, and ESR allows the description of the interaction of a model drug with lipid nanoparticulate systems which can be understood by assuming one type of interaction in the case of NE, an intermediate behavior in the case of NLC, and more than one type of interaction in a formally single-type compartment in the case of SLN. Future experiments have to focus on the relevance for skin penetration efficiency.

7. Acknowledgement

Christian Braem was scholarship holder of the NaFÖG founded by the city of Berlin. Moreover, financial support of the German Research Foundation (FG 463) is gratefully acknowledged.
Abbreviations

A tensor describing the spatial dependence of the electron hyperfine interaction (ESR)
c concentration
CMC critical micelle concentration
DSC differential scanning calorimetry
ESR electron spin resonance
$\varepsilon'(f)$ frequency dependent paraelectric dispersion
$\varepsilon''(f)$ frequency dependent paraelectric absorption
$\Delta\varepsilon(c)$ concentration dependent dipole density
$f_0(c)$ concentration dependent dipole mobility
f continuously varied radiofrequency (PS)
g tensor describing the spatial dependence of the electron spin-orbit coupling (ESR)
HPLC high performance liquid chromatography
LS laser scattering
NE nanoemulsions
NLC nanostructured lipid carriers
PCS photon correlation spectroscopy
PS paraelectric spectroscopy
r $1/2$ smallest diameter of the SLN discs
R $1/2$ largest diameter of the SLN discs
SLN solid lipid nanoparticles
$\tau_R$ rotational correlation time (ESR)
TEM transmission electron microscopy
X-band microwave frequency band (6.6 - 13.2) GHz
References


[31] H. Fesq, J. Lehmann, A. Kontny, I. Erdmann, K. Theiling, M. Rother, J. Ring, G. Cevc,