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Changes of the Properties in the Upper Layers of Human Skin on Treatment with Models of Different Pharmaceutical Formulations—An Ex vivo ESR Imaging Study

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The noninvasive method of spectral-spatial electron spin resonance imaging (ESRI) was used to obtain a polarity map of human skin. The spin probes TEMPO, TEMPOL, and CAT-1, which are considered to act as drug representatives, were applied as reporter molecules. The polarity in skin layers was described by means of the changes of the hyperfine splitting constant A_{iso}, which itself is a reflection of interactions at a molecular level, and the effect of polarity on the spatial distribution of spin probes in skin samples was studied. Analyses of ESR tomograms of two-phase systems finalized in a simplified description for the empiric interpretation of values of the isotropic hyperfine coupling constants A_{iso} of spin probes in different layers of human skin. The simplified statement provides values for the probability of interactions of water molecules with the NO group of spin probes. This allows conclusions concerning the state of hydration of the spin probes in different layers of the skin and introduces the spatial polarity function as additional and valuable information for existing skin models.

Introduction

Early investigations involving electron spin resonance imaging (ESRI) dealt with problems of the application of spin traps, their distribution, and reduction.[1–3] Kroll et al.[4] have used ESRI to determine pH changes in skin layers after the application of drugs. As the successful determination of the microacidity is based on the determination of the values of the hyperfine splitting constant A_{iso}, we were encouraged to think about the possibilities to monitor the polarity[1] in different depths of skin using polarity sensitive nitroxides and the method of ESRI.

In continuation of the examinations performed to date of polarities by means of spin probes in homogeneous solutions and homogeneously distributed particles,[5–7] respectively, one of the main objectives of this paper is the usage of ESRI as a nondestructive spectroscopic method for establishing a polarity map of the upper skin layers and based on this, the spatial distribution of model drugs in the skin.

As a result of the peculiarities of spectral-spatial ESRI, weighted averages of the isotropic hyperfine coupling constants in specific layers (slices) are obtained. After applying ESRI successfully to describe the changes of A_{iso} in two-phase systems, particularly at the phase boundary[8] and in heterogeneous systems,[9] it should be the next step to examine biological tissues, such as human skin, to get hyperfine coupling profiles, including the possibility to calculate probabilities for the interactions of water with the applied spin probe molecules. That means the presented paper shall describe the distribution of model drugs, including their interactions, spatially resolved in living human skin. Representatives of such model drug spin probes with different lipophilic/hydrophilic properties have been used. The advantage of this approach will be the possibility of its direct investigation by means of ESR spectroscopy and tomography because of their high sensitivity and selectivity.

By means of this, new and unique results can be obtained by evaluating the hyperfine splitting constant A_{iso} ($^{14}N/^{15}N$) and the signal intensities (which corresponds to the concentration) of TEMPO, TEMPOL, and CAT-1 as a function of the distance from the skin surface.

Altogether, it describes the behavior of the spin probes in the skin, including its dependence on the respective pharmaceutical formulation and, moreover, the effect of the formulation on the upper skin layers.

For that it was checked first, if ESR tomography is able to differentiate between stratum corneum, lower layers of the epidermis, and dermis. This was done by evaluating the changes

[1] *Solvent polarity should be understood as the action of all possible, specific and nonspecific, intermolecular forces between solvent and solute molecules, including Coulomb interactions between ions, directional interactions between dipoles, and inductive, dispersion, hydrogen-bonding, and charge transfer forces, as well as solvophobic interactions. In the context of the described systems polarity is primarily meant to be water-induced mainly due to hydrogen-bonding.*

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of A_{iso} on progression to deeper skin layers. Furthermore the following questions should be answered: 1) will the epicutaneous application of pharmaceutical formulations change the polarity of the skin layers, 2) is there an influence of the pretreatment of the skin with N-ethylmaleinimide (NEM), which had been used for inhibition of spin probe reduction, and 3) what is the influence of the polarity of the different skin layers on the spatial distribution of spin probes?

Results and discussion

Evaluation of the spin probe's surrounding (micropolarity) and establishment of a polarity map of the upper skin layers

Figure 1 displays the formal structures of the spin probes used, as they are expected in nonpolar (Figure 1 a) and polar hydrogen bond forming media (Figure 1 b). The polar surrounding results in states of the samples, which are characterized by macroscopic parameters, such as ε_r (relative dielectric constant, see Figure 1 c) and other specific parameters.[10] Figure 1 c shows the relative changes ΔA_{iso} of the isotropic hyperfine coupling constants as a function of the relative dielectric constant ε_r for selected solvents, which occur in the biological context whether directly as a metabolic product or as a constituent of pharmaceutical formulations, including a classification of polar and nonpolar regions. And it should be emphasized again, because of its quantity, water is the main contributor to the specific interactions. Hydrogen bonds (dashed line in Figure 1 b) and nondirectional electrostatic interactions (dotted line in Figure 1 b) with the solvent or matrix molecules, respectively, compete on stabilizing the polar structure. Compared to the nonpolar surrounding hydrogen bonds in particular increase the viscosity of the matrix as well as the spin density at the nitrogen atom of the spin probe, and a larger isotropic hyperfine coupling constant will be observed in the ESR experiment. As a result of the dominating single bonds in the spin probe molecules and related to this, the very small delocalization of the spin density, the substituent R does not influence the isotropic hyperfine coupling constant A_{iso} very much. However, the substituent does determine the solubility of the spin probe and therefore its localization in the presence of coexisting polar and nonpolar surroundings as for example, in biological matrices.

First the results shall be presented which have been obtained by using ESR tomography for the examination of skin samples after incubation with an aqueous solution of ^{15}N-TEMPO.

The spectral-spatial ESRI as applied herein supplies information about the localization (spatial information), the spectral properties (for example A_{iso}), and the intensity (concentration) of a paramagnetic sample (see Figure 2), that is, a 2D plot displays whether A_{iso} or the intensity as a function of the local position.

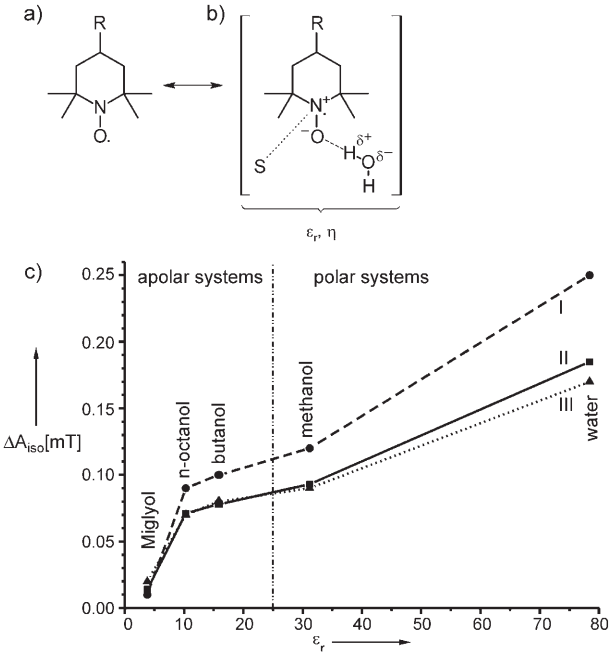

Figure 1. a) General and simplified structure of the used spin probes; R = H (TEMPO), R = OH (TEMPOL), R = $N^+(CH_3)_3$ (CAT-1). b) Polar structure induced by 1) the interaction of solvent molecules S with the NO group of the spin probes and 2) the interaction with water molecules forming hydrogen bonds. ε_r: relative dielectric constant, η: macroscopic viscosity. c) Changes of the hyperfine coupling constant A_{iso} (^{15}N) of ^{15}N-TEMPO (I), ^{14}N-TEMPO (II), and ^{14}N-TEMPOL (III) depending on the solvent expressed as $\Delta A_{iso} = A_{iso}$ (solvent) $- A_{iso}$ (toluene).

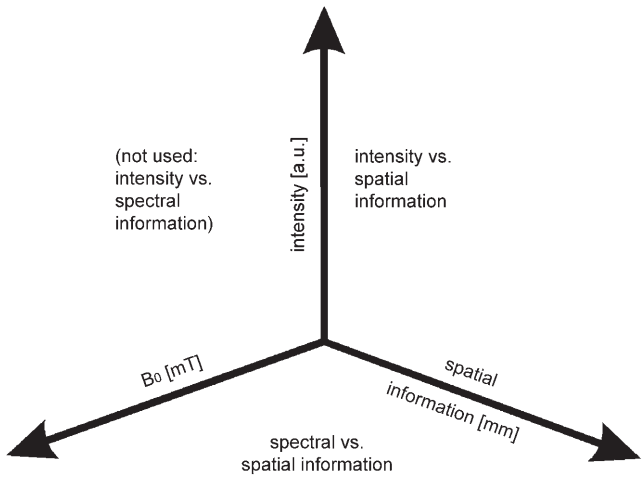

Figure 2. Principal sketch for deriving the desired information from a spectral–spatial ESR tomogram.

Figure 3 a shows the contour plot of an ESR tomogram indicating the change of the ^{15}N hyperfine coupling constant versus the sample dimension. The intensity distribution of the ESR signal versus the sample dimension is displayed in Figure 3 b. It is obvious that the spin probe TEMPO can be found with a higher amount (77 %) in deeper skin layers, as it is expected considering the lipophilic properties of the spin probe. A smaller amount (about 23 %) of TEMPO will be found in the region between the skin surface and up to a depth of about

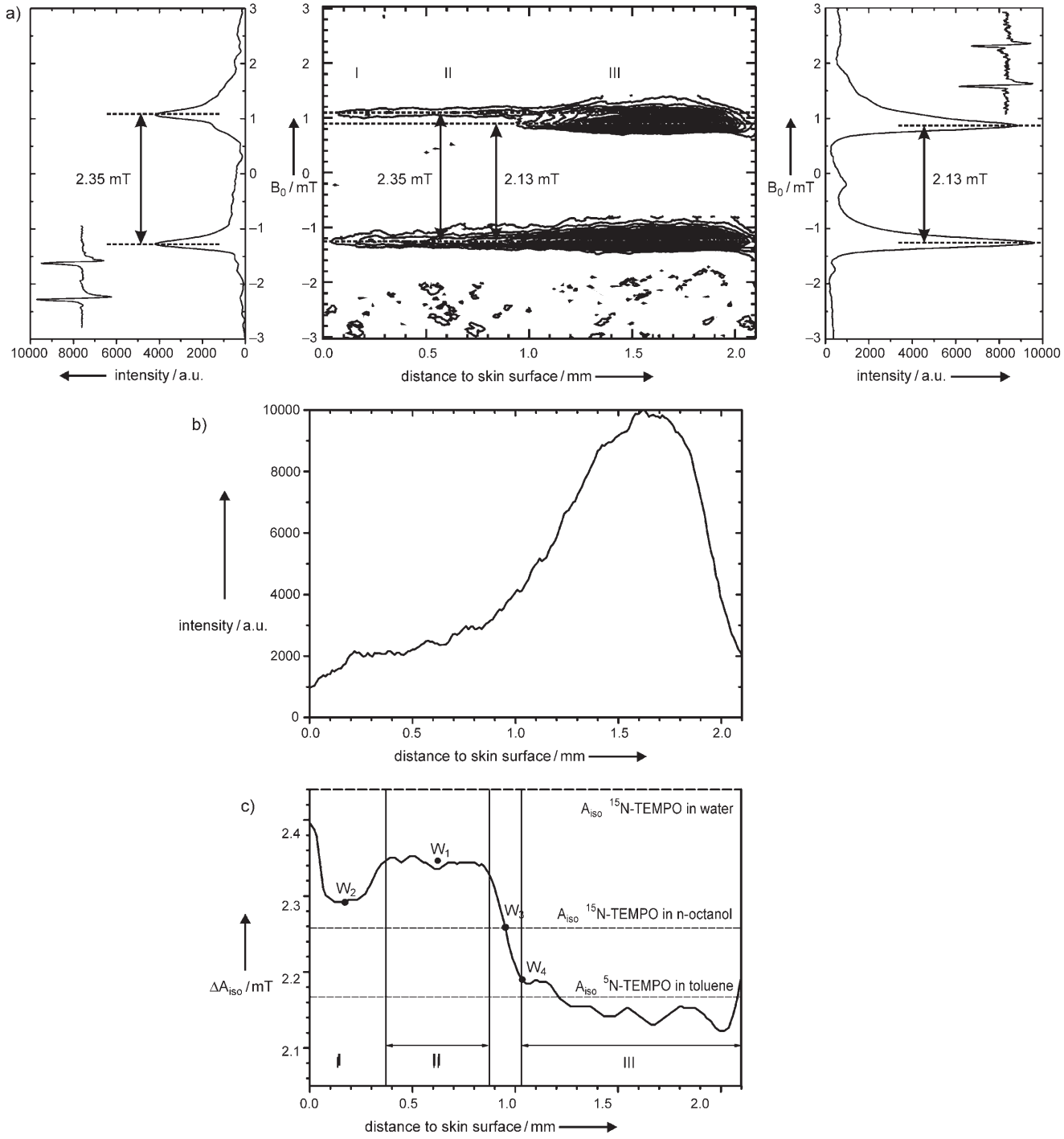

Figure 3. Penetration of the spin probe ^{15}N-TEMPO in human abdominal skin. a) Contour plot of the tomographic image, including an example of an integrated spectrum out of the region of largest (left) and smallest (right) polarity, respectively, b) distribution profile, after 30 min incubation of a 30 mmol aqueous solution, and c) the assignment of the hyperfine splitting constants to different skin layers [I: epidermis, II: upper dermis, III: lower dermis; probability W of the interaction of water molecules with the NO-group of the spin probe: $W_1 = 69.7\,\%$, $W_2 = 49\,\%$, $W_3 = 36\,\%$, and $W_4 = 8.5\,\%$, see Equation (2)].

0.6 mm. That spatial distribution, already observable in the contour plot, is also reflected in the changes of the hyperfine coupling constants A_{iso}. Figure 3c shows a (spatial) profile of the A_{iso} values versus the sample dimension.

The beginning of the curve (<0.1 mm) is an artefact caused by tiny remainders of the spin probe solution, which was used for incubation, in the papillary structure of the skin surface as can be seen in the intensity plot of Figure 3c.

The (dashed) horizontal lines represent the hyperfine coupling constants of TEMPO dissolved in water, n-octanol, and toluene. As can be seen in Figure 3c there is no region where the profile approaches the value of an aqueous solution,

whereas the smallest values in the A_{iso} profile are even below the value of toluene. Such a comparison supplies only a rough, preliminary picture concerning the characterization of the skin samples.

A semiquantitative interpretation of the obtained A_{iso} values can be done based on Figure 1b and the following considerations: the value of A_{iso} of the experiment depends mainly on the probability of interactions of the NO group of the spin probe with water molecules or other less polar constituents S of the system. If these constituents are represented by their mole fractions, that is, x (H_2O) and x_i (S_i), the experimental coupling constant can be expressed as follows:

$$A_{iso}\ (\text{exp.}) = x\,(H_2O)\ A_{iso}\ (H_2O)\ +\ \sum_i x_i(S_i)A_{iso}(S_i) \qquad (1)$$

Previous examinations have shown that the water content of the human skin plays a decisive part for transportation and distribution processes. Thus, the interactions of the NO group of the spin label whether with water and/or other constituents of the surrounding can be used as sensor for reflecting the effect of the pharmaceutical formulation. The second term in Equation (1) $\sum x_i(S_i)A_{iso}(S_i)$ stands for a manifold of different compounds, all of them having a small share in the overall interactions. Moreover, distribution processes in biological specimen are often modeled by means of water–octanol systems. Thus, a possibility to simplify Equation (1) is to reduce the right-hand term in Equation (1) to $x_2\ A_2$, or after replacing x_2 by $(1-x_1)$, to $(1-x_1)A_2$.

For the evaluation of the experimental hyperfine splitting values, obtained from the experiments with skin samples, Equation (1) will be rearranged

$$x_i = \frac{A_{iso}(\text{exp}) - A_{iso}(2)}{A_{iso}(H_2O) - A_{iso}(2)} \qquad (2)$$

with variable (2) representing the sum of interacting non-aqueous constituents.

Moreover, that approach is not in direct relation to the Nernst distribution law, which requires thermodynamic equilibrium, because the actual situation of the spin probe distribution after a fixed incubation period shall be described, which rarely will be in equilibrium under that condition.

To express the probability of interactions of water with the NO group of the spin probe we assume a direct proportionality to the values of the mole fraction.

The application of the simplified Equation (2) delivers the following picture as indicated in Figure 3c: The following relative probabilities for the NO group of the spin probes to interact with water result: $W_1 = 69.7\,\%$ for region II, $W_2 = 49\,\%$ for region I, $W_3 = 36\,\%$ for the intermediate region between II and III, and $W_4 = 8.5\,\%$ for region III. That means, the tomographic method does not only supply information about the localization of the spin probe in the skin, but also the respective micropolarity, and the degree of hydration of the spin probe in its respective spatial position.

Dependence of the hyperfine splitting constant on the local position

Further time-dependent effects, recorded by means of the changes in the isotropic hyperfine coupling constants A_{iso} (^{14}N), are displayed in Figure 4, which shows the changes of A_{iso} of

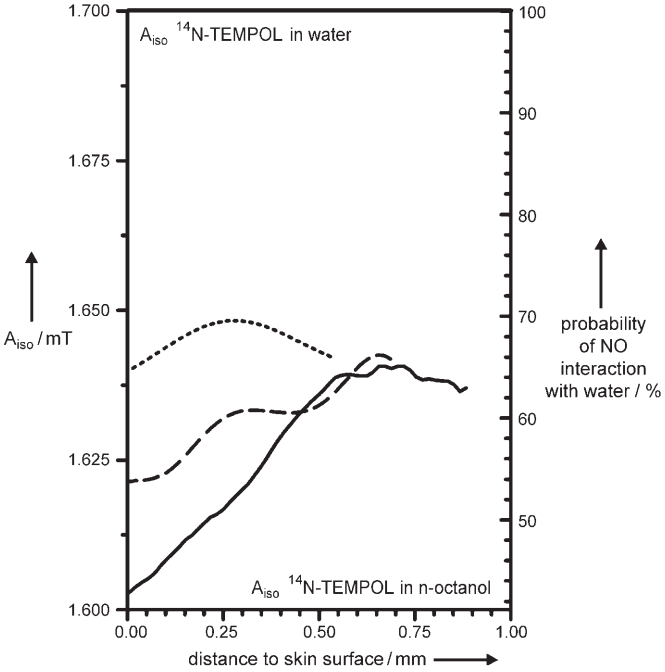

Figure 4. Changes of the hyperfine splitting constants A_{iso} in dependency on the penetration distance after 3.5 (solid line), 7 (dashed line), and 24 h (dotted line) application of a liposomal preparation containing the spin probe TEMPOL as a model drug on the surface of untreated human skin.

^{14}N-TEMPOL versus the sample dimension after epicutaneous application of a liposomal preparation on human skin. The tomograms have been recorded after 3.5, 7, and 24 h incubation time. It is obvious that the values of A_{iso} increase with increasing incubation time particularly near the skin surface as a consequence of the treatment with the liposomal formulation.

Using Equation (2) it can be stated, that the probability of the interaction of the NO group of the spin probe with water molecules will be about 43.5% near the skin surface after 3.5 h incubation time and increases continuously up to a maximum of 65.5% with increasing distance from the skin surface.

After an incubation period of 7 h the values will be 54.5% at the skin surface and increase to 66.5% with increasing distance from the surface. After 24 h incubation a probability of 65% can be determined at the skin surface and 70% at the maximum.

Generally it might be stated that the extension of the incubation period causes increasing probabilities of interactions of water molecules with the NO group of the spin probe. In other words, we observe an increasing degree of hydration in dependency on the time of incubation. Moreover, the spatial position of the maximum values for the interactions of water and the NO group of the spin probes shifts in the direction of the skin surface.

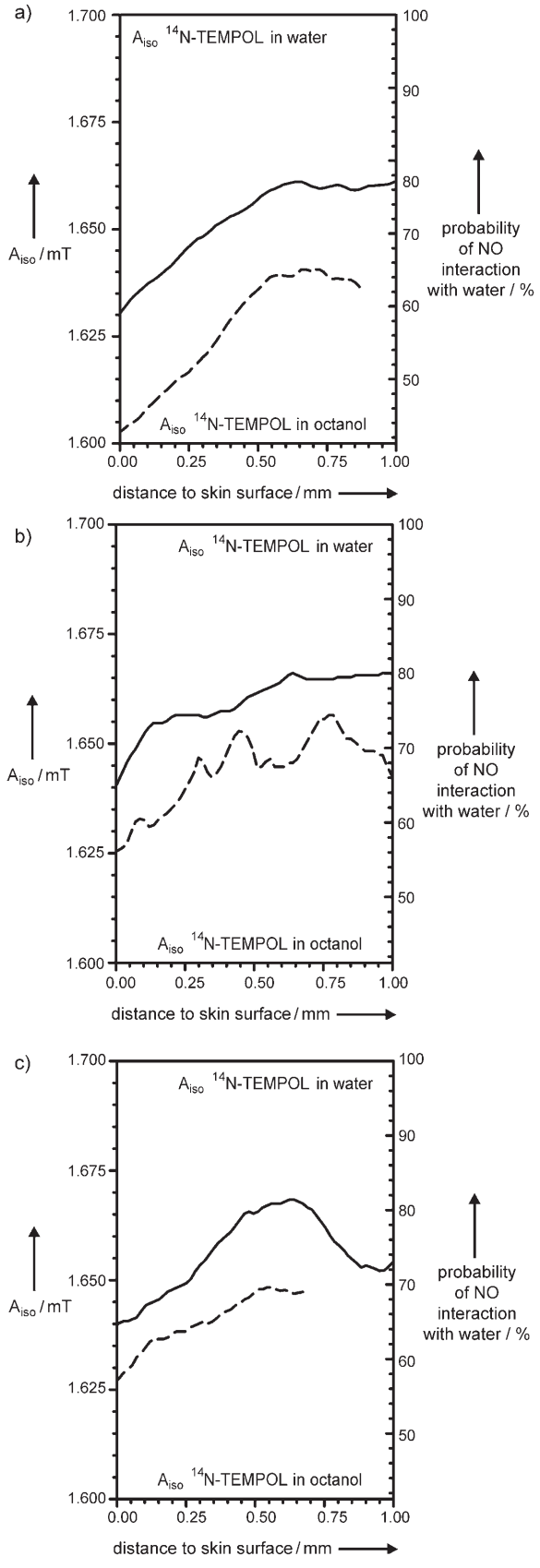

Figure 5. Changes of the hyperfine splitting constants A_{iso} in dependency on the penetration distance after 3.5 h application a) of a liposomal preparation on pretreated and untreated human skin, b) of an aqueous solution, and c) of a hydrophilic ointment containing the spin probe TEMPOL (solid lines: skin sample pretreated with NEM, dashed lines: no pretreatment of the skin sample with NEM).

Figure 5 a–c illustrates two effects: 1) the influence of the pretreatment of the skin with the inhibitor NEM and 2) the kind of spin probe application on the spatial distribution of the A_{iso} values of TEMPOL. At first the influence of the NEM pretreatment is remarkable. The degree of hydration increases considerably resulting in a higher polarity and thus in larger values of A_{iso}. A similar tendency was already observable in Figure 4 with increasing incubation time.

Generally the values approach a plateau level as in Figure 3 c (region II). There are similarities in the course of changes of the different preparations but differences in the extent of the hydration. For the examined formulations we observe the strongest degree of hydration for the application of an aqueous solution, followed by the hydrophilic ointment, whereas the influence of the liposomal preparation is weakest.

In general, the evaluation of A_{iso} as a function of the distance from the skin surface supplies a measure for the interaction of the model drug with water.

Dependence of intensity (concentration) on the distance from the skin surface

The acquired knowledge about the polarity profile, obtained from the incubation experiment described above, shall be used to examine the time-dependent phenomena of spin probe distribution after the application of a liposomal preparation on the skin surface. Figure 6 shows the normalized intensities of the spin probes CAT-1 and TEMPOL versus the sample dimension after different periods of epicutaneous application of a liposomal formulation. After 3.5 h incubation the main part of the charged spin probe CAT-1 (Figure 6 a) will be found in the region of highest polarity (Figure 3 c, region II). With increasing incubation time the maximum shifts somewhat towards the skin surface, most probably due to increasing degree of hydration in the upper skin layers. The highest probability to find the amphiphilic spin probe TEMPOL will be in a region of moderate polarity, that is, between region I and II. The further course is largely monotonic and somewhat similar to that of the spin probe CAT-1.

Based on the findings described above, it may be stated that the hydrophilic/lipophilic properties of the spin probes and the incubation time, including the time of pretreatment, determine the localization in the human skin.

Summary

The sensitivity and selectivity of ESRI allows the direct determination of the intensity, that is, the concentration, dependent on the local position by means of that spectroscopic technique.

Analyses of ESR tomograms of two-phase systems resulted in a simplified relation for the empiric interpretation of values of the isotropic hyperfine coupling constants A_{iso} of spin probes in different layers of human skin. It allows estimation of the probability of interactions of water molecules with the NO group of spin probes and to conclude the state of hydration of the spin probes, which are considered to act as drug represen-

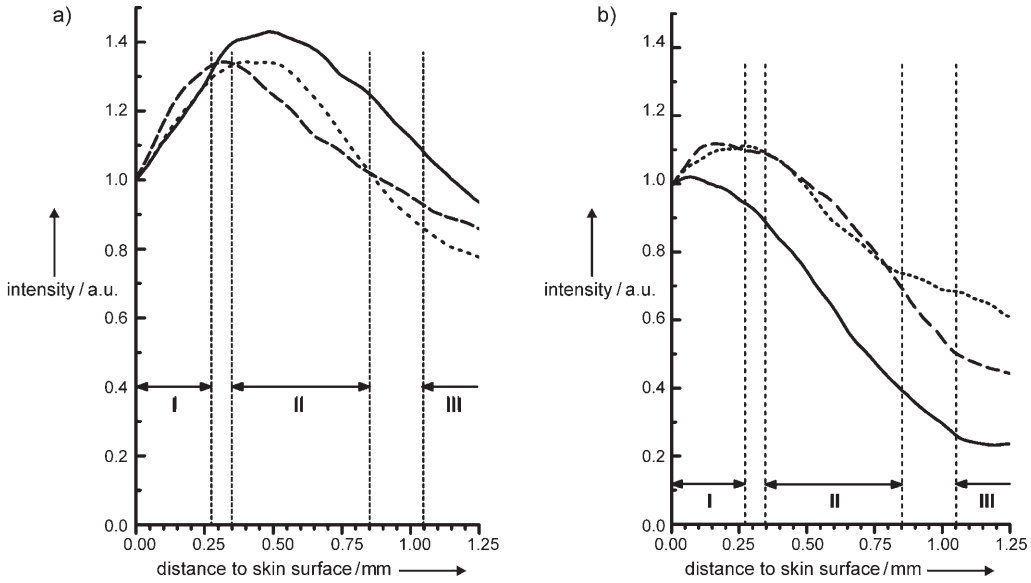

Figure 6. Normalized signal intensities of a) CAT-1 and b) TEMPOL in skin samples depending on the distance from the skin surface 3.5 (solid line), 7.0 (dashed line), and 24 h (dotted line) after epicutaneous application of the spin probes in P80H/CH liposomes (median, $n = 3$).

tatives, in different skin layers. It should be stated, that the tomographic method does not only supply statements about the localization of the spin probe in the skin and the corresponding micropolarity, but also about the degree of hydration of the spin probe in its respective spatial position, which again is a measure of the local degree of hydration of the respective tissue.

On using the spin probes TEMPO, TEMPOL, and CAT-1 a polarity map for a stationary state of the skin can be established, thus introducing the spatial polarity function as additional and valuable information for existing skin models. Moreover, time-dependent processes of spin probe distribution in pharmaceutical preparations as well as biological objects could be interpreted.

Spectral-spatial electron spin resonance imaging has been shown to be a suitable tool to monitor the polarity of skin layers and the underlying changes following the application of topical formulations.

Experimental Section

Materials

The nitroxide spin probes ^{15}N-2,2,6,6-tetramethylpiperidin-1-oxyl (^{15}N-TEMPO) and 2,2,6,6-tetramethyl-4-trimethylammoniumpiperidin-1-oxyl-iodide (CAT-1) were purchased from the Institute of Organic Chemistry, Russian Academy of Science, Novosibirsk, Russia. α,α'-diphenylpicrylhydrazyl (DPPH) was obtained from BDH Chemicals Ltd., Poole, UK. N-ethylmaleinimide (NEM) and 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPOL) were purchased from Sigma, St. Louis, USA. Modified Eagles Medium came from Sigma, Seelze, Germany, cholesterol (CH) and charcoal from Fluka, Buchs, Switzerland. The solvents methanol, n-octanol, and toluene were purchased from Merck, Darmstadt, Germany. The neutral oil Miglyol 812 was bought from Caelo, Hilden, Germany. Phospholipon 80 (P80) and Phospholipon 80 H (P80 H) were granted from Nattermann Phospholipid GmbH, Köln, Germany, and dipalmitoylphosphatidylcholine (DPPC), dioleylphosphatidylcholine (DOPC), dipalmitoylphosphatidylglycerol (PG) from Lipoid GmbH, Ludwigshafen, Germany. All other chemicals were of highest purity available.

Methods

Experimental settings for the ESRI and ESR studies. The ESR spectra were recorded using an X-band spectrometer ERS 220 (ZWG, Berlin-Adlershof, Germany) equipped with a home made imaging device. The following settings were used: 2 mW microwave power; 0.1 mT modulation amplitude; 8 mT scan width; 3.30 T/m maximum gradient; 10 s scan time per projection; 95 projections; 512 points per projection; image reconstruction: filtered backprojection giving an image matrix of 256×256 points. The temperature inside the resonator was set to 32 °C. The spatial distribution of the nitroxide spin probes was quantified using the central line of ^{14}N-spin probes and the high field line of ^{15}N-TEMPO, respectively. Conventional ESR spectra were recorded under similar conditions but without using a field gradient. The ^{14}N and ^{15}N hyperfine splittings were determined at low signal-to-noise ratios by measuring the distances between the hyperfine lines or by simulation of the ESR spectra.

Measurements of hyperfine splittings of nitroxides in solvents. The nitroxides ^{15}N-TEMPO and TEMPOL were dissolved in water, methanol, butanol, n-octanol, Miglyol, and toluene, respectively. The final concentrations were 1 mmol. Fifty microliters of the solutions were filled in disposable micropipettes, transferred to the spectrometer immediately and ESR measurements were carried out.

Liposome preparation. Liposomes were prepared by the film method which is described in reference [11]. The compositions used were: DOPC/CH/PG (6.5:3:0.5); DPPC/PG/CH (4:5:1), P80 and P80H/CH (6:4). The final lipid content was 50 mg mL^{-1}. The lipid films were hydrated above their phase transition temperatures with a 15 mmol solution of the spin probes TEMPOL and CAT-1 for two hours. The resulting liposomal dispersions were extruded through a polycarbonate filter using the LiposoFast membrane extruder (Avestin Europe GmbH, Mannheim, Germany).[12] The average diameters of the liposomes were determined by photon corre-

lation spectroscopy using a Malvern Zetasizer 4 (Malvern Instruments, Malvern, UK) and have been found to be between 158 and 188 nm.

Skin preparation. The human skin (healthy abdominal female skin; the authors confirm the existence of the signed consents of the respective patients) was obtained from cosmetic surgery. The fat tissue was carefully removed mechanically, and the skin was cleansed. Finally the skin samples were cut into pieces (diameter 1 cm) and kept on polycarbonate membranes in cell culture plates filled with Dulbeccos Modified Eagles Medium at 4 °C until treatment and measurement (four days maximum).

To prevent the reductive metabolism of nitroxide spin probes the inhibitor NEM[13] was used to pretreat one part of the skin samples. For that purpose the respective skin sample has been placed on a filter paper which was drenched with a 30 mmol solution of NEM in Dulbeccos Modified Eagles Medium for 5 h.

To study the distribution of model drugs in human skin 10 μL of the respective formulation were applied to the skin surface. Now, the skin samples were kept on the cell culture plates over Dulbeccos Modified Eagles Medium at 32 °C and 44 % rel. humidity for the desired incubation time. Before the measurements the residue solution was carefully removed from the skin surface and a sample ($d = 4.5$ mm) was punched out according to the method of Štolc et al.[14]

ESR spectra and hyperfine splittings of spin probes in skin layers. The skin samples were transferred to a modified tissue cell (minimal air volume, with a DPPH sample serving as intensity and spatial standard) and ESR tomograms recorded in a common H_{102}-cavity (for experimental arrangement see ref. [15]). The thickness of the skin samples was determined recording a second ESR tomogram and using the DPPH standard and a sample of micronized charcoal, placed on the skin surface, for marking the spatial depth. The applied method of spectral-spatial ESR tomography resolves an object in planar slices. The thickness of such a slice mainly depends on the technical parameters of the tomographic equipment. As it is well known, the extension of the different layers of the skin does not follow a strictly planar arrangement. Thus the uppermost layer of the skin, the stratum corneum, cannot be differentiated under the selected experimental conditions, but we can observe an averaged picture of the uppermost layers of the skin, which reflects the properties of the respective main constituents.

To study the effect of epicutaneously applied topical formulations on the polarity of the skin 10 μL of the liposomal formulations as well as a solution of the spin probe in phosphate buffer were used, containing the respective spin probe in a concentration of 15 mmol. According to the method described above, ESRI tomograms of skin samples were recorded after 3.5, 7.0, and 24 h. The effect of the skin layer polarity on the distribution of spin probes was investigated using liposomal preparations of the lipid mixture P80H/CH containing TEMPOL and CAT-1, respectively.

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Keywords: drug delivery · ESR imaging · skin layers · spatial polarity function

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