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Effect of surfactants on human stratum corneum: electron paramagnetic resonance study

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Abstract

Electron paramagnetic resonance (EPR) spectra of nitroxide spin probes are useful for studying biological membranes and chemical-membrane interactions. Recently, we established a stripping method to remove stratum corneum (SC) for this purpose. To assess this stripping method with EPR and correlate with standard methods, we quantified the irritant effects of three types of surfactants by measurements of visual score and transepidermal water loss (TEWL), SC hydration and chromametry and studied EPR spectra measurements of surfactant-treated cadaver SC (C-SC) and stripped off SC (S-SC) on patch tested sites. 5-Doxyl stearic acid was the spin label. The order parameter S obtained from the spectra of S-SC correlated with those of C-SC and TEWL values. The results suggest that this method is capable of evaluating the fluidity of SC and correlates with the above bioengineering parameters.

Keywords: Electron paramagnetic resonance spectroscopy; Human stratum corneum; 5-Doxyl stearic acid; Sodium lauryl sulfate; *N*-3-alkyl oxyhydroxypropyl-L-arginine

1. Introduction

Skin bioengineering techniques, such as transepidermal water loss (TEWL) and electrical capacitance, enable the assessment of the biophysical changes induced by chemicals causing skin irritation. Electron paramagnetic resonance (EPR) of nitroxide spin probes has also provided

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a powerful tool for studying biological membranes, and chemical-membrane interactions. Literature exists on the effects of detergents (Curtain and Gorden, 1984), percutaneous penetration enhancers (Quan and Maibach, 1994; Quan et al., 1995), anionic surfactants (Kawasaki et al., 1997) on stratum corneum (SC) from cadaver skin, penetration enhancers on dipalmitoyphosphatidylcholine liposomes (Gay et al., 1989) and on guinea pig epidermis (Kitagawa et al., 1998). Although SC from cadaver skin (C-SC) and guinea pig skin are models of human skin in vivo, it is desirable to study these effects on skin in vivo. Recently, we have established a method to measure the spectra from stripped SC (S-SC) with EPR (Mizushima et al., 1999). To evaluate the effectiveness of this method, we investigated the SC irritation potential of three types of surfactants, utilizing skin bioengineering techniques.

2. Materials and methods

5-Doxyl stearic acid (5-DSA) purchased from Sigma Chemical (St. Louis, MO, USA) was used as a spin-labeled reagent without further purification. Sodium lauryl sulfate (SLS; Sigma Chemical, USA; purity > 99%), stearyl trimethyl ammonium chloride (MSAC; Tokyo Chemical Industry, Tokyo, Japan; purity > 97%), and N-[3-alkyl (12.14) oxy-2-hydroxypropyl]-L-arginine hydrochloride (HEA; Ajinomoto, Kawasaki, Japan; Commercial Grade) were used as surfactants without further purification. These surfactants have anionic, cationic and amphoteric properties, respectively. The purity of all materials was as stated by the suppliers.

2.1. Preparation of C-SC

Human cadaver skin, excised from the abdomen within 24 h of death, dermatomed to a thickness of approximately 500 μm, was obtained from Northern California Transplant Bank (San Rafael, CA, USA). Epidermis was separated from dermis by immersing the skin in 60°C water bath, set for 2 min, followed by mechanical removal. Then the epidermis was placed SC side up on filter paper and floated on 0.5wt% trypsin (type II; Sigma) in a phosphate buffered saline (PBS; pH 7.4) for 2 h at 37°C. After incubation, any softened epidermis was removed by mild agitation of the SC sheet, which was then dried and stored at −20°C (Quan and Maibach, 1994).

2.2. Spin-labeling procedure and treatment with surfactants of C-SC

5-DSA was used as a stearic acid spin-labeling agent. A slice of the dried SC sheet (≈ 0.5 cm^2; 0.7×0.7 cm) was incubated in 1.0 mg/dl 5-DSA aqueous solution for 30 min at 37°C and then washed with deionized water. The labeling time for control EPR spectra is defined as 0 (zero) h just after incubation.

Treatment with surfactants was performed by immersing a piece of spin labeled C-SC in 1 wt% of surfactant aqueous solutions and incubating it at approximately 37°C for 1 h and 24 h. The labeling time for sample EPR spectra is defined as 0 (zero) h just after the incubation in the surfactant solutions.

2.3. EPR spectral measurements

Spectral measurements were performed with an ER/200D EPR spectrometer (Bruker, Billierica, MA, USA). X-band first derivative absorption spectra were obtained with a microwave power output of 25 mW; center field, 346 mT; time constant, 200 ms; sweep time, 50 s; modulation, 0.2 mT at a frequency of 100 kHz; and total sweep width, 12.6 mT. C-SC samples and S-SC with quartz cells, previously labeled with 5-DSA, were mounted on the flat surface of an EPR cell and located in the center of a TM cavity. Spectra were collected by a PC-486 using software written in PC/FORTH (version 3.2, Laboratory Microsystems, Marina del Rey, CA, USA). Each sample was scanned several times; EPR signals were averaged to give a single estimate for each sample. EPR measurements were performed at approximately 20°C in contact with atmospheric O_2. The line broadening due to the spin-spin interactions with O_2 will have a negligible effect on line width of 5% (Hyde and Subczynski, 1989).

Sharp triplet signals can be observed when the spin probe (doxyl group) moves freely. However, the EPR spectrum broadens, as shown in Fig. 1a, when the mobility of the spin probe is restricted by its interaction with other molecules.

Order parameters S were calculated according to standard methods (Hubbel and McConnell, 1971; Griffith and Jost, 1976; Marsh, 1981):

$$S = (A_{\|} - A_{\perp})/[Azz - 1/2\ (Axx + Ayy)](a'_o/a_o)$$

where $2A_{\|}$ is identified with the outer maximum hyperfine splitting, A_{max}, and $A_{\perp}$ is obtained from the inner minimum hyperfine splitting Amin (see Fig. 1a).

The symbol a'_o is the isotropic hyperfine splitting constant for nitroxide molecule in the crystal state.

$$a'_o = (Axx + Ayy + Azz)/3$$

The values used to describe the rapid anisotropic motion of membrane-incorporated probes of fatty acid type are:

$$(Axx,\ Ayy,\ Azz) = (0.61,\ 0.61,\ 3.24)\ \text{mT}$$

Similarly the isotropic hyperfine coupling constant for the spin label in the membrane a_o is given by:

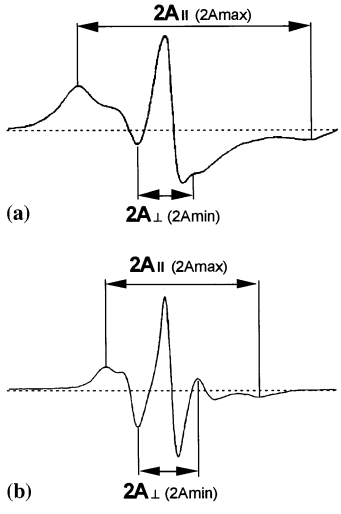

Fig. 1. (a) EPR spectrum of 5-DSA labeled C-SC. Order parameter S is calculated by the indicated splittings. (b) EPR spectrum of 5-DSA labeled C-SC treated with SLS for 24 h.

$$a_o = (A_{\|} + 2A_{\perp})/3$$

The a_o values are sensitive to the polarity of the environment of the spin labels since increases in a_o values reflect an increase in the polarity of the medium.

The order parameter S provides a measure of the flexibility of the spin labels in the membrane. It follows that $S = 1$ for highly oriented motion (rigid) and $S = 0$ for completely isotropic motion (liquid). Increases in order parameter S reflect decreases in flexibility, and decreases in order parameter S reflect increases in flexibility (Curtain and Gorden, 1984). Fig. 1b is a spectrum obtained from C-SC treated with SLS for 24 h in which $2A_{\|}$ narrowed and $2A_{\perp}$ broadened, and its order parameter S is smaller than that of Fig. 1a.

2.4. Subjects and test procedure

Five healthy male volunteers (aged 34–38, mean 35.2) provided informed consent. Institutional Research Board approval was from the University of California, San Francisco, Committee on Human Research. Two hundred microliters of aqueous solution of 1% of SLS, MSAC and HEA were applied to the mid-volar forearm using occlusive polypropylene chambers (1.8 cm diameter; Hilltop Laboratory, Cincinnati, OH, USA) for 24 h. Deionized water served as vehicle controls. Application sites for the different treatments were rotated to avoid an anatomical selection bias (Cua et al., 1990). Each site was examined by the same investigator.

2.5. Visual scoring

Visual scores were examined on day 1 and 2. Patches were removed after 24 h and the test sites were exposed to air for 30 min to allow deconvolution of excess water. Erythema was graded according to a five-point visual scoring system modified from the Frosch and Kligman method (Frosch and Kligman, 1979): 0, no erythema; 0.5, equivocal reaction; 1, slight redness, either spotty or diffuse; 2, moderate, uniform erythema; 3, intense erythema; 4, fiery redness with edema.

2.6. Instrumental measurements

The following non-invasive testing methods were used on days 0, 1 and 2. All measurements were conducted under standard ambient conditions (temperature 21 ± 2°C, relative humidity 50–60%). Volunteers rested at least 30 min before measurements. To minimize the effect of occlusion on the measurements on day 1, the following test reactions were measured 60 min after patch removal.

2.6.1. Transepidermal water loss

Transepidermal water loss (TEWL), as a marker of functional integrity of the SC water barrier, was measured with an evaporimeter (Tewameter; Courage and Khazaka, Cologne, Germany). This device works on the principle of vapor pressure gradient calculation described in detail by Nilsson (Nilsson, 1997).

2.6.2. Electrical capacitance

Electrical capacitance, as a parameter for SC hydration, was measured in triplicate with a Corneometer CM 820 PC (Courage and Khazaka, Cologne, Germany). The probe head (7×7 mm), consisting of a condenser, was applied to the skin surface at constant pressure (3.5 N). The measuring principle, based on the distinctly different dielectric constants of water (≈ 81) and most other materials (< 7), is described elsewhere (Courage, 1994).

2.6.3. Chromametry

Skin color was measured in triplicate with a Chromameter CR 300 (Minolta, Osaka, Japan), using the chromaticity coordinates of the CIE $L^*a^*b^*$ color space (CIELAB) defined by three vertical axes (Weatherall and Coombs, 1992). The parameter a^* was selected which represents the chromaticity between $-a^*$ (green) and $+a^*$ (red) as a sensitive measure to quantify erythema (Wilhelm and Maibach, 1989, 1995).

2.7. S-SC preparation from volunteers

After these non-invasive measurements were taken, samples of S-SC were removed on patch-tested sites by a single stripping with one drop of cyanoacrylate resin, onto quartz glass (5×15 mm; Nihon Denshi, Tokyo, Japan) in accordance with Imokawa's method (Imokawa et al., 1991).

2.8. Spin-labeling procedure of S-SC and EPR spectral measurement

S-SC (≈ 0.5–1.5 cm^2) attached to a quartz cell, was incubated with one to three drops of 1.0 mg/dl 5-DSA aqueous solution for 30 min at 37°C, then washed with deionized water. EPR measurements were similarly taken for C-SC.

2.9. Statistical analysis

Values of parameters obtained from testing different surfactants were analyzed for statistical significance using a two way analysis of variance (ANOVA, Fisher's exact test). The level of significance was taken as $P < 0.05$.

3. Results

3.1. EPR spectra analysis

3.1.1. Order parameter S from C-SC and S-SC treated with surfactants

Order parameters S from C-SC and S-SC treated with surfactants are summarized in Table 1. The order parameter S obtained from C-SC from the controls at 0, 1 and 24 h were 0.85, 0.83, 0.85, respectively. There was no significant difference among them. At 1 h, significant differences between the control, and SLS and MSAC, treated C-SC, respectively; and those between SLS and MSAC and HEA, respectively, were found. At 24 h, there were significant differences between the control and SLS, MSAC and HEA treated C-SC, respectively ($P < 0.01$); and those between SLS and MSAC and HEA, respectively, were found ($P < 0.01$). There were no significant differences between MSAC and HEA at 1 h, but there were at 24 h ($P < 0.01$) (Fig. 2).

As for the order parameter S obtained from S-SC, significant differences between control and SLS, MSAC and HEA treated sites were found

Table 1
The order parameter S obtained from C–SC and S–SC[a]

Treatment	C–SC						S–SC	
	Time (h)	AVE	SD	Time	AVE	SD	AVE	SD
SLS	1	0.51	±0.010	24	0.33	±0.013	0.72	±0.033
MSAC	1	0.68	±0.017	24	0.55	±0.011	0.77	±0.005
HEA	1	0.75	±0.018	24	0.69	±0.034	0.77	±0.010
Cont	1	0.83	±0.014	24	0.85	±0.020	0.82	±0.011
	0	0.85	±0.013					

[a] 0 h, measured just after labeling.

($P < 0.01$, $P < 0.01$, $P < 0.05$, respectively). There were significant differences between SLS and, MSAC and HEA treated S-SC ($P < 0.05$, $P < 0.01$), but there were no significant differences between MSAC and HEA (Fig. 3).

3.1.2. Correlation between order parameter S from S-SC and C-SC

The order parameter S from C-SC at 24 h was well correlated with those of S-SC and the functional relationship was estimated as 'C-SC = $-3.883 + 5.833$ S-SC' (Fig. 4).

3.2. Values from visual score and instrumental measurements

3.2.1. Visual score

SLS treated sites induced more intense erythema, and HEA showed the least erythema on day 1. No erythema was observed in the control (water) sites on day 1 and 2, and MSAC and HEA treated sites on day 2. The visual score of SLS treated sites on day 2 was the same as those of day 1. Statistical analysis was performed based on the data of day 1. The values from HEA and MSAC are significantly lower than SLS ($P < 0.01$), and HEA is lower than MSAC ($P < 0.05$) (Fig. 5).

3.2.2. Transepidermal water loss

SLS treated sites induced significantly higher increase than did MSAC and HEA on day 1 ($P < 0.01$). There were no significant differences between the control (water) and MSAC and HEA, respectively, nor between MSAC and HEA treated sites. On day 2, SLS induced significantly higher values than MSAC and HEA ($P < 0.01$); and SLS and MSAC were higher than the control ($P < 0.01$, $P < 0.05$, respectively); but there was no significant difference between the control and HEA, nor between MSAC and HEA (Fig. 6).

3.2.3. Capacitance

After 24 h application (day 1), SLS treated sites significantly increased SC water content over the control, but MSAC and HEA showed a decrease ($P < 0.01$). On day 2, the SLS values decreased

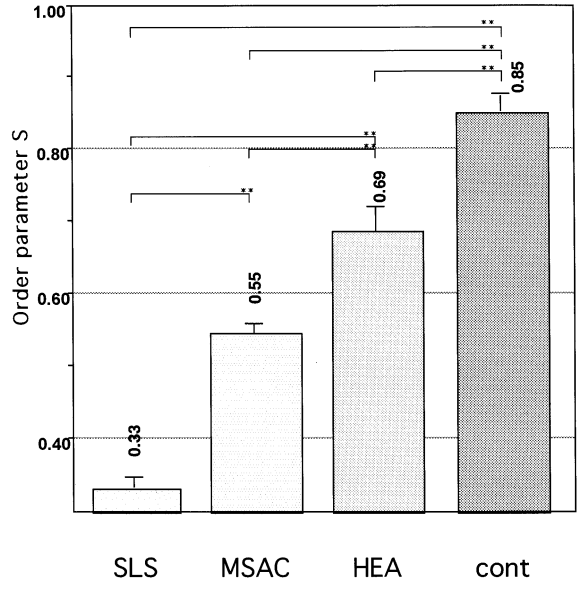

Fig. 2. Order parameter S obtained from C-SC ($n = 3$) treated with surfactants (SLS, MSAC and HEA) for 24 h and control; $^{**}P < 0.01$.

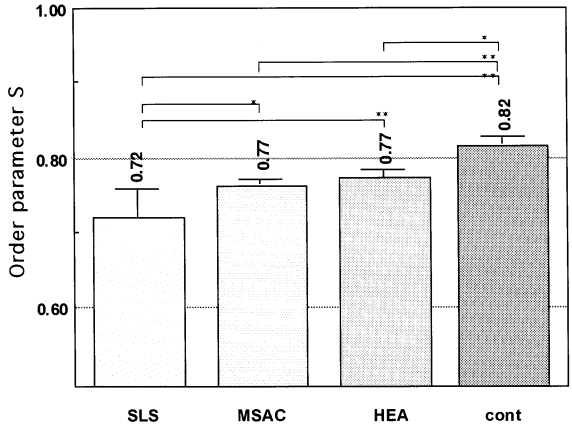

Fig. 3. Order parameter S obtained from S-SC ($n = 5$) treated with surfactants using occlusive patch testing for 24 h and control (water); $*P < 0.05$, $**P < 0.01$.

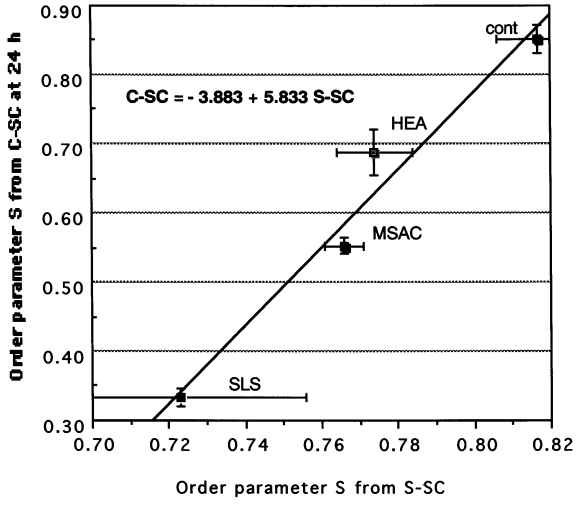

Fig. 4. Functional relationship between order parameter S from S-SC and those of C-SC.

and there was no significant difference between SLS and the control. The data of MSAC and HEA increased from day 1, but were still significantly less than SLS ($P < 0.01$). There were no significant differences between MSAC and HEA on day 1 and 2 (Fig. 7).

3.2.4. Colorimetry

SLS treated sites showed significantly higher a^* values than the control, MSAC and HEA on day 1 and 2 ($P < 0.01$). There were significant differences between the control, and MSAC and HEA ($P < 0.01$, $P < 0.05$) on day 1, but not on day 2. There was also significant difference between MSAC and HEA ($P < 0.05$) on day 1 (Fig. 8)

3.3. Correlation between order parameter S from S-SC and clinical readings

The correlation coefficient of the order parameter S from S-SC and visual score, TEWL, capacitance and colorimetry values on day 2 were 0.526, 0.708, 0.160 and 0.570, respectively. Fig. 9 shows

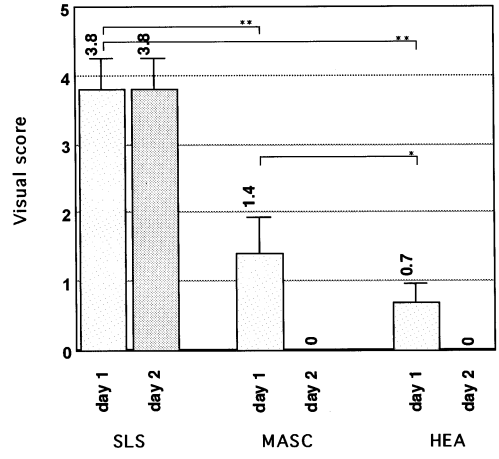

Fig. 5. Average visual scores of three test surfactants on day 1 and 2; $*P < 0.05$, $**P < 0.01$.

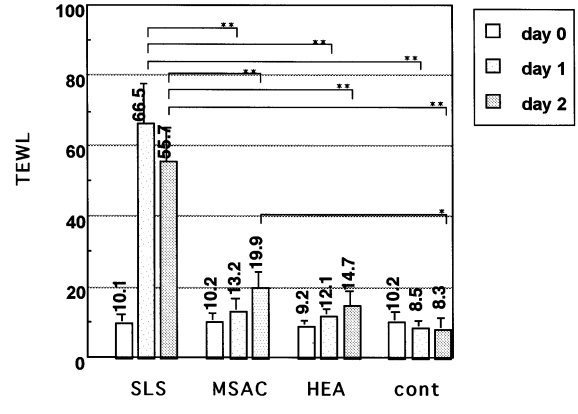

Fig. 6. Results of TEWL values (g/m^2/h), measured before (day 0) and day 1 and 2 ($n = 5$);$*P < 0.05$, $**P < 0.01$.

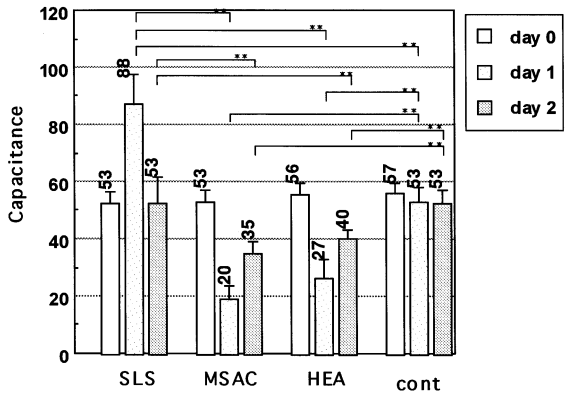

Fig. 7. Capacitance value (arbitrary capacitance units) measured before (day 0) and day 1 and 2 ($n = 5$); $^{**}P < 0.01$.

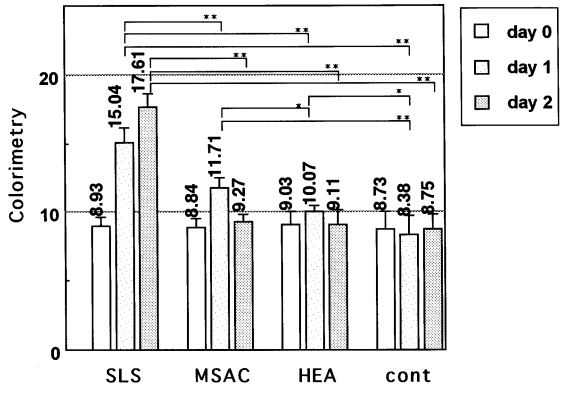

Fig. 8. Results of a^* color values (arbitrary a^* units) measured before (day 0) and day 1 and 2 ($n = 5$); $^{*}P < 0.05$, $^{**}P < 0.01$.

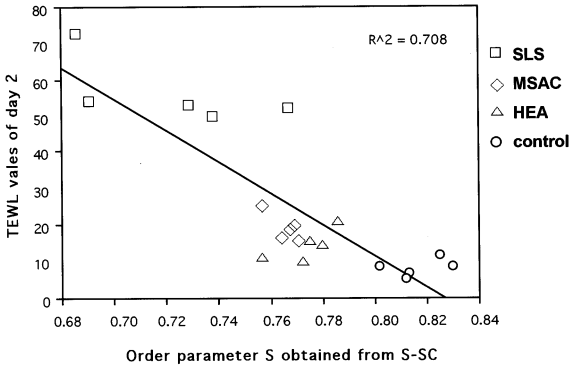

Fig. 9. Correlation between order parameters S obtained from S-SC and TEWL values of day 2.

the relation between the order parameter S and TEWL values on day 2, and Fig. 10. shows the relation between the order parameter S and capacitance values.

4. Discussion

4.1. Order parameter S from C-SC and S-SC

EPR spectra of nitroxide spin probes are useful tools for studying biological membranes and drug-membrane interaction. In 1990, Plachy et al. investigated S-SC obtained from normal human subjects by bonding the skin surface to the surface of a small quartz cell with cyanoacrylate, then labeled them with the spin probe perdeuterated di-tert-amyl nitroxide (Plachy et al., 1990). They concluded that this method provides a superior method to obtain S-SC, but there have been no further studies since this abstract. This is the first study to evaluate irritated S-SC skin utilizing EPR. Order parameter S, of the spin labels in 5-DSA, calculated from the observed spectra, is a measure of the distribution of orientations of the spin probe, and indicates the degree of order within the lipid bilayer. Recently, we examined EPR spectra utilizing normal S-SC with cyanoacrylate resin (Mizushima et al., 1999), containing at least five layers of SC (Imokawa et al., 1991). This sufficiently gains spectra, even though S-SC is thinner than C-SC and cyanoacrylate resin is attached to one side of the S-SC. Once the glue has solidified, no signals arise from the cured

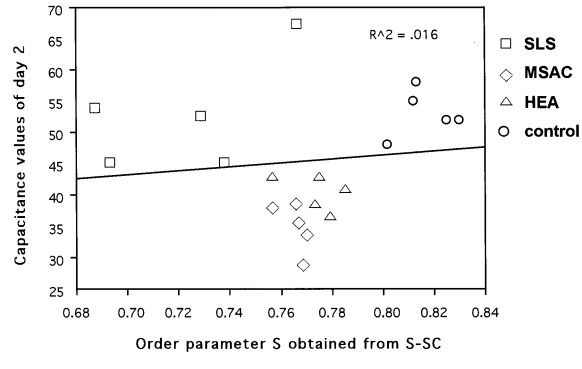

Fig. 10. Correlation between order parameter S obtained from S-SC and capacitance values of day 2.

resin or from the spin probe dissolved in the resin (Hou et al., 1991), but superfluous labeling may lead to false positives. There is another problem with 5-DSA aqueous solution. Water may influence fluidity of the lipid bilayer, especially in the region near the membrane–water interface (Alonso et al., 1995, 1996). To minimize the amount of labeling aqueous solution, we labeled S-SC with one to three drops of 5-DSA aqueous solution gaining sufficient spectra from S-SC.

We could detect decreases of order parameter S from S-SC and have shown that this new method effectively provides a quantitative measure of membrane properties for comparisons between different samples, even though order parameters S from S-SC are greater than those of C-SC. But there is a strong correlation between the order parameters S from C-SC and S-SC. The order parameter *S* from C-SC at 24 h was well correlated with those of S-SC and the functional relationship was estimated as 'C-SC = -3.883 $+5.833$ S-SC' (Fig. 4). The *P*-value for the null hypothesis that C-SC and S-SC is linearly related against an alternative hypothesis that they are not was 0.300, which reasonably supported the null hypothesis in spite of large variability appeared on the order parameters *S* obtained from S-SC treated with SLS. So this method may be useful to evaluate fluidity of the irritated in vivo skin. There are two explanations for this difference. It may depend on the ability of rapid recovery of in vivo skin. The epidermis can synthesize lipids immediately after water barrier disruption (Grubauer et al., 1989): this barrier is 80% repaired in 6–8 h after acetone treatment (Elias and Feingold, 1992). The epidermis responds with a lipid biosynthetic burst when disrupted by topical treatments with detergents (Menon et al., 1985). The other explanation is the difference in methodology used to induce irritated SC in C-SC versus in S-SC also contributes to the larger order parameter S in S-SC.

4.2. Correlation between order parameter S and skin bioengineering readings

We found a correlation between the order parameter *S* and TEWL consistent with in vitro data (Kawasaki et al., 1999). With in vitro data, the EPR spin-labeling method with human SC provided a robust technique to define the irritation potential of chemicals interacting with intercellular lipids. The order parameter *S* correlated to TEWL values. The order parameter *S* also correlated with cytotoxicity (NR 50) but hemoglobin denaturation did not correlate with order parameters. These results suggest that EPR measurement with 5-DSA relates to intercellular lipids, not proteins (Kawasaki et al., 1999). This relationship is consistent with the fact that water barrier function depends on lipid layers, and 5-DSA interposes itself into lipid bilayers.

Conversely, there was no relation between the order parameter S and capacitance which depends on natural moisturizing factors (NMF) in corneocytes. This discrepancy between the water holding capacity and lipid rigidity of SC may also be accounted for by a property of 5-DSA. In this study, we observed hyperhydration of SLS after 24 h application (day 1), which can be explained by the disruption of keratin protein exposing new water-bounding sites (Wilhelm et al., 1994). Inducing lower hydration may be due to decreasing NMF, such as amino acids, which play an important role in water retention in SC; treating with surfactants might affect keratin fiber which cooperate with NMF in holding bound water and does not correlate with the fluidity of the lipid bilayer. The visual score and colorimetry, showed similar correlation coefficients, which mainly reflect reactions of the skin including edema of the epidermis and upper dermis, perivascular infiltrates and vasodilation.

4.3. Irritation potential of anionic, cationic and amphoteric surfactant on human skin

In this study we used three types of surfactants: anionic (SLS), cationic (MSAC) and amphoteric (HEA). Tabohashi et al. reported that HEA, a new class of amphoteric surfactant, is less irritating than MSAC in a skin irritation assay (Tabohashi et al., 1998). In this human study, we found HEA has the lowest irritant potential in visual score, TEWL, capacitance, colorimetry, and intercellular lipid structure (order parameter *S*). Fur-

ther studies are needed to evaluate the different irritation potentials of these three types of surfactants.

5. Conclusion

The present work has demonstrated the perturbation induced by several surfactants on human skin in vivo and the order parameters obtained from S-SC correlated with those of C-SC and TEWL values. The results suggest that this stripping method is capable of evaluating the fluidity of lipid layers in the SC and the irritation potential of chemicals and may aid in investigating the effects of skin penetration enhancers and drug delivery systems.

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