

Polyoxyethylene/polyoxypropylen dimethyl ether (EPDME) improves the structure of intercellular lipids in SDS-induced dry skin

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Synopsis

The dimethyl ether of an amphiphilic random ethylene oxide/propylene oxide copolymer (EPDME) is useful for the preparation of finely dispersed micro-emulsions. We examined whether EPDME is effective for skin moisturization by means of electron paramagnetic resonance (EPR) studies of *ex vivo* specimens of stratum corneum (SC) obtained by successive stripping. The values of the order parameter S obtained by EPR measurement indicated that EPDME treatment improved sodium dodecyl sulfate (SDS)-induced disruption of SC lipid structures. This effect appeared to be related to improved hydration of the epidermis, not occlusion by EPDME, since there was no significant change in transepidermal water loss (TEWL).

INTRODUCTION

Development of novel functional molecules for improved cosmetic or pharmaceutical formulations that have a physiological effect on skin is an important task for cosmetic scientists. We have developed a random copolymer, polyoxyethylene/polyoxypropylen dimethyl ether (EPDME) as a functional amphiphilic polymer suitable for the preparation of finely dispersed micro-emulsions. EPDME was found to be very effective for skin moisturization to protect dry skin or to improve poor skin conditions (1). When EPDME is combined with glycerin in a formulation, EPDME increases the amount of glycerin held in the SC (1). There are many empirical data indicating that EPDME is effective in cosmetics for people with sensitive skin. The objective of this study is to elucidate the mechanism of EPDME's effect on the skin condition, by means of an electron paramagnetic resonance (EPR) analysis of lipid structure in successively stripped SC layers, along with measurements of SC hydration and of transepidermal water loss (TEWL) from the skin, with or without SDS treatment.

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MATERIALS AND METHODS

MATERIALS

The EPDME used in this study was synthesized as previously reported (1). Figure 1 shows the chemical formula of EPDME. Liposomes (1 mg/ml), prepared from lecithin, were a gift from Professor Kumazawa of Ibaraki University.

METHODS

Experimental dry skin and preparation of stripped SC samples. The Shiseido Research Center Internal Review Board approved all protocols used in this study. The ethical principles for non-clinical biomedical research involving human subjects, as stated in the Declaration of Helsinki Principles, were adhered to in every respect. The mid-volar forearms of ten volunteers (male, average age 38) were treated with 150 μ l of 1% SDS (Wako Pure Chemical Industries, Osaka, Japan) and covered with adhesive tape for one hour. The application site was washed with distilled water, and then 10% EPDME aqueous solution or water was applied in appropriate amounts. Application of 10% EPDME aqueous solution was carried out before the subjects' going to bed and on rising the following morning. On the following day, the sample-application area was washed with soap, and the physiological parameters of the skin were evaluated 20 min later at 22°C and 45% RH. The SC sheets for *ex vivo* measurement were prepared as reported (2,3). A surface biopsy was conducted with a quartz glass plate (8 mm \times 70 mm; Matsunami, Tokyo, Japan) on which a single drop of cyanoacrylate resin had been spread uniformly. Four SC specimens were successively removed in this way from the mid-volar arm treated with SDS and EPDME, SDS and distilled water, or without treatment (control).

TEWL was measured using a Vapometer SWL3N (Delfin, Finland) before and after stripping the SC. The water content of the SC was measured with a Corneometer CM825 (C+K Electric, Koln, Germany) and a Skicon 200 (ISBS Co, Hamamatsu, Japan) before and after stripping the SC. These instruments utilize measurements of capacitance and conductance, respectively, to determine water content.

Preparation of SC sheets for ex vivo measurements. A single-chain aliphatic spin probe, 5-doxylstearic acid (5-DSA) was purchased from Aldrich-Sigma Chemical Co. Inc., and was used as received. A 5-DSA ethanol solution (26 mM) was prepared, and diluted 1000-fold with distilled water. Stripped SC samples were incubated with 100 μ l of the 5-DSA ethanol-aqueous solution (26 μ M) for 1 hour at 37°C. Excess spin probe was washed off with distilled water, and the SC samples were mounted in the EPR cavity (3).

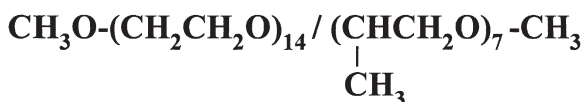


Figure 1. Chemical formula of polyoxyethylene/polyoxypropylene dimethyl ether (EPDME). EPDME is a random copolymer of ethylene oxide and propylene oxide, and the numbers 14 and 7 represent the average molecular ratio of the two monomers.

Preparation of EPDME-loaded liposomes. Liposomes (0.5 mg/0.5 ml) were incubated with the 5-DSA ethanol-aqueous solution (26 μM final concentration) in a microtube for 1 hour at 37°C; then the EPDME solution was added (1% final concentration) and the mixture was incubated for ten minutes at 37°C. SDS aqueous solution was added (0.02% final concentration) and incubation was continued for 30 minutes at 37°C. Excess spin probe was washed off with distilled water by centrifugation, and dispersions were transferred to 50 μl capillaries, which were used as sample cells for the EPR measurement.

EPR measurements and spectral analysis. A JEOL JES-RE1X X-band (9 GHz) EPR spectrometer was used to measure all samples. Before measurements of the SC from volunteers, *ex vivo* specimens were treated with distilled water for five minutes to have sufficient hydration (3,4). Typical spectrometer settings were as follows: microwave power, 10 mW; time constant, one second; sweep time, eight minutes; modulation, 0.2 mT; sweep width, 15 mT. All measurements were performed at ambient temperature. The obtained EPR spectra were analyzed by conventional calculation of the order parameter.

The order parameter S provides a measure of the flexibility of the spin label in the membrane. The flexibility reflects motility and orientation, and $S = 1$ for a highly ordered system and $S = 0$ for completely isotropic motion (5). The conventional S value is determined from the hyperfine couplings of the EPR signals according to the following relations (6):

$$S = \frac{A_{\parallel} - A_{\perp}}{A_{zz} - \frac{1}{2}(A_{XX} + A_{YY})} \cdot \frac{a}{a'}, \quad a' = \frac{A_{\parallel} + 2A_{\perp}}{3}, \quad a = \frac{A_{XX} + A_{YY} + A_{ZZ}}{3}.$$

In calculation from experimental spectra, the principal components of $(A_{XX} + A_{YY} + A_{ZZ}) = (0.66, 0.55, 3.45)$ mT were used (7).

Multiple comparison assays were carried out using Fisher's protected least significant difference (Fisher's PLSD) in the case of the order parameter S of SC from volunteers, and Dunnett's test in the case of the order parameter S of liposomes. Multiple linear regression analysis was carried out using JMP Ver.6 (SAS Institute Inc.).

RESULTS

Figure 2 shows EPR spectra of SC from the first stripping from one volunteer. Water treatment resulted in an order parameter (S) value of 0.57, while SDS treatment decreased it to 0.53 and EPDME treatment restored it to 0.56. Figure 3 shows the average values of the order parameter of ten subjects. The parameter was decreased significantly upon SDS treatment, but was significantly restored when EPDME was applied.

Figure 4 shows the changes in the mean values of the order parameter of SC obtained in successive strippings (i.e., increasing depth). In all four strippings, SDS treatment significantly decreased the order parameter S , showing that the structural order of the SC lipids was disrupted, while EPDME significantly, although not completely, restored the order parameter.

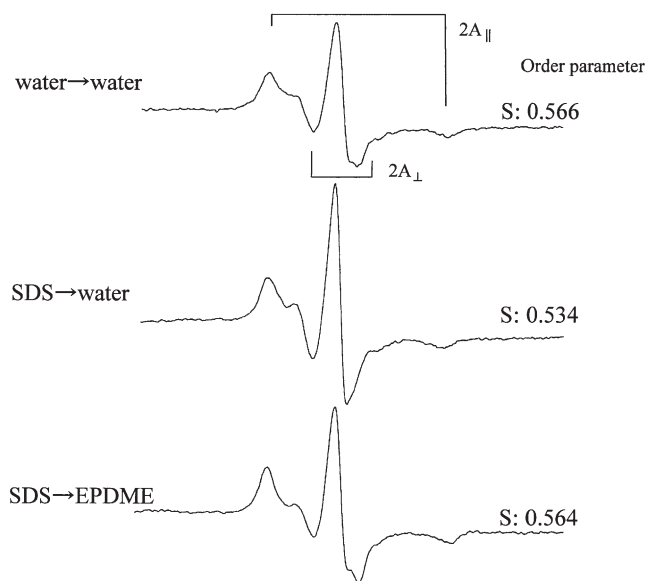


Figure 2. EPR spectrum of 5-DSA incorporated into SC lipids from the first stripping of a volunteer's mid-volar forearm.

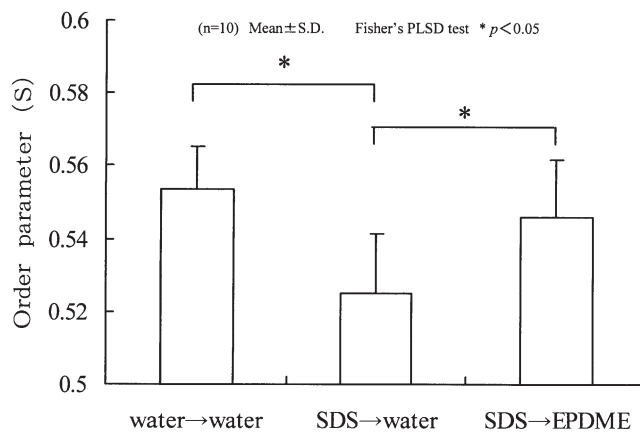


Figure 3. Values of the order parameter S obtained from the EPR spectrum of SC removed by single strippings with cyanoacrylate from the forearms of ten volunteers.

Table I shows the average values of the water content, TEWL, and the order parameter S , for each stripping. The water content in the SC, measured with a Corneometer, showed a tendency to decrease upon SDS treatment but to increase following EPDME post-treatment. The water content measured with the Skicon showed similar results. TEWL, which is considered to be a parameter of the barrier function of the skin, increased after SDS treatment, indicating impaired barrier function, while it tended to be reduced with EPDME post-treatment.

Stepwise regression analysis by null hypothesis was conducted to find influential predictor variable(s) to the order parameter S as an outcome variable. Within the three predictor variables analyzed, i.e., water content by Skicon or Corneometer and

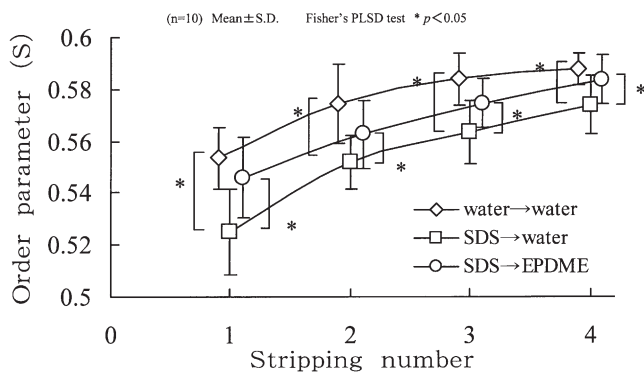


Figure 4. Values of the order parameter S obtained from the EPR spectrum of SC removed in successive strippings from the forearms of ten volunteers.

barrier disruption by TEWL, only water content by Corneometer showed a level of significance of 0.099.

Figure 5 shows typical spectra of liposomes treated with SDS, and Figure 6 summarizes the results. Although the order parameter S shows only a slight change, and SDS treatment significantly increased the fluidity of the membranes, this increase was suppressed by treatment of the liposomes with EPDME beforehand.

DISCUSSION

Skin cleansers are widely used to keep skin clean and sanitized, but excessive cleansing can remove natural moisturizers and SC lipids. It is also well established that surfactants, such as SDS, cause skin irritation (8–11). Imokawa *et al.* (8) reported a significant decrease in SC hydration in skin treated with 5% SDS, though there was no inflammation. The amount of released SC lipid constituents, such as cholesterol, cholesterol ester, and fatty acids, increased time-dependently, leading to disruption of lamellar lipid structure. Ohmori *et al.* (1) applied 5% SDS solution to the mid-volar forearm for ten minutes and observed a reduction of SC hydration and barrier function. We used 1% SDS solution in order to investigate changes in lipid structure in the SC without causing significant barrier disruption, as determined by means of TEWL and SC hydration measurements (Table I).

Electron microscopy is a powerful tool to investigate structural changes in SC lipid layers directly, e.g., in skin obtained by biopsy (12), and the stripping technique has been successfully applied to electron microscopic evaluations (13). EPR measurement using *ex vivo* SC obtained by surface biopsy with cyanoacrylate is also suitable to examine depth-dependent changes in the ordering profile of SC lipid layers (2). An advantage of EPR is that the operation is very simple and easy in comparison to electron microscopy.

Following the application of EPR to measure the membrane fluidity of model compounds (14), its use has recently been extended to the structural analysis of human SC (15–17). The EPR spectrum of hydrophobic spin probes incorporated into a lipid membrane reflects the properties of the membrane, and it is sensitive to the rotational mobility of the spin probes, the polarity of the environment, and the orientation of the probe molecules. The order parameter (S) is determined from the hyperfine couplings of the EPR signals

Table I
Physiological Parameters of SC Obtained in Successive Strippings (mean \pm S.D)

| Stripping number | Skicon 200 (μ s) | | | | Corneometer (a.u.) | | | | TEWL (g/m^2h) | | | | Order parameter S | | |
|------------------|--------------------------------|------------------------------|------------------------------|--------------------------------|------------------------------|------------------------------|--------------------------------|------------------------------|--------------------------------|------------------------------|--------------------------------|------------------------------|--------------------------------|------------------------------|------------------------------|
| | Water \downarrow water | SDS \downarrow water | SDS \downarrow EPDME | Water \downarrow water | SDS \downarrow water | SDS \downarrow EPDME | Water \downarrow water | SDS \downarrow EPDME | Water \downarrow water | SDS \downarrow water | Water \downarrow water | SDS \downarrow water | Water \downarrow water | SDS \downarrow water | SDS \downarrow EPDME |
| 1 | 78* \pm 13 | 39 \pm 7 | 74 [†] \pm 17 | 39.3 \pm 2.5 | 33.7 \pm 1.4 | 42.0 \pm 2.4 | 7.0 \pm 0.6 | 8.0 \pm 0.9 | 8.0 \pm 0.9 | 7.0 \pm 0.6 | 0.553* \pm 0.004 | 0.525 \pm 0.005 | 0.546* \pm 0.005 | | |
| 2 | 103 \pm 18 | 58 \pm 12 | 104 \pm 26 | 41.3 \pm 2.2 | 35.4 \pm 1.6 | 45.2 \pm 2.9 | 8.5 \pm 0.9 | 9.7 \pm 1.2 | 9.7 \pm 1.2 | 8.0 \pm 1.0 | 0.574* \pm 0.005 | 0.552 \pm 0.003 | 0.563* \pm 0.004 | | |
| 3 | 94 \pm 14 | 108 \pm 30 | 146 \pm 30 | 40.0 \pm 2.7 | 37.9 \pm 2.4 | 45.6 \pm 3.0 | 10.9 \pm 1.1 | 12.8 \pm 1.3 | 10.6 \pm 1.3 | 10.6 \pm 1.5 | 0.584* \pm 0.003 | 0.564 \pm 0.004 | 0.575* \pm 0.003 | | |
| 4 | 174 \pm 50 | 174 \pm 57 | 274 \pm 113 | 44.1 \pm 2.8 | 39.3 \pm 2.9 | 52.2 \pm 3.6 | 14.4 \pm 2.3 | 19.4 \pm 3.0 | 17.8 \pm 5.1 | 17.8 \pm 5.1 | 0.588* \pm 0.002 | 0.574 \pm 0.004 | 0.584* \pm 0.003 | | |

* $p < 0.05$, [†] $p < 0.1$.

SDS \rightarrow water vs water \rightarrow water or SDS \rightarrow EPDME.

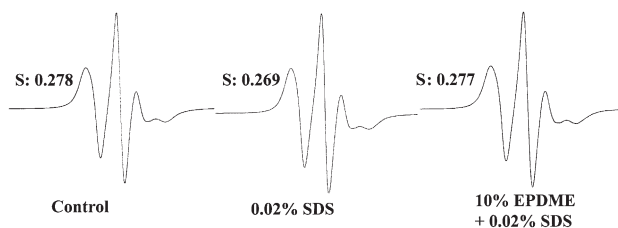


Figure 5. EPR spectra of 5-DSA-labeled liposomes.

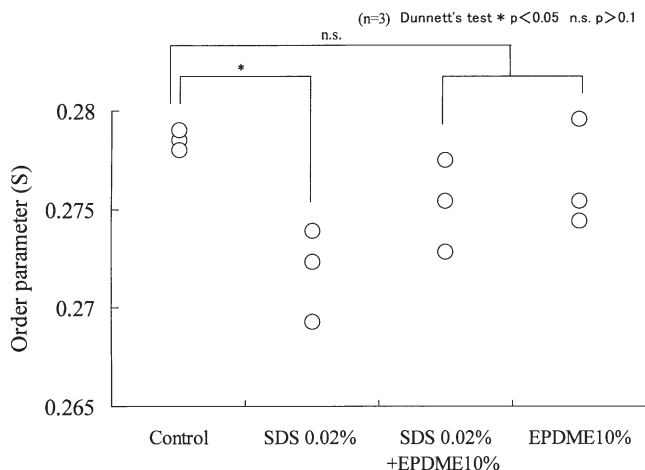


Figure 6. Values of the order parameter S obtained from the EPR spectra of 5-DSA-labeled liposomes.

(6). Precise analysis of experimental spectra by means of slow-tumbling simulation has also been developed (5,18,19), and we have employed this approach to investigate quantitatively the ordering of SC lipid structure as a function of depth (2). Multilamellar lipids of the first stripped (outermost) layers show relatively low values of the order parameter (S_0), while the middle to lower SC layers show higher values of the order parameter (S_0), reflecting the presence of well-ordered intercellular lipid structures. The order parameter values obtained for normal untreated skin by slow-tumbling simulation (S_0) showed more significant differences between each SC layer compared with those indicated by the conventional order parameter (S) calculated geometrically from hyperfine couplings. In the present study, we used SDS-treated SC, in which marked structural change was expected. In this case, the conventional method for calculating S is adequate to evaluate the structural changes, and therefore we evaluated the order parameter using the conventional method.

For the skin treated with water, the value of the order parameter S increased gradually from 0.553 for the first stripping to 0.588 for the third stripping, but there was no further change at the fourth stripping, as shown in Table I and Figure 4. These values are consistent with our previous results (2). For the skin treated with SDS and EPDME post-treatment, the profiles of the order parameter with increasing depth of SC were similar, but there were significant differences between “water vs SDS” and “SDS vs EPDME post-treatment”. The effects of SDS treatment correspond well to those reported by Mizushima *et al.* (15), confirming that conventional order parameter calculation is sufficient for further analysis (2). The decrease in the S value by SDS (Table I; Figure 4) indicates disruption of

the SC lipid lamellar structure, especially at the surface SC layers (first strip). EPDME post-treatment clearly restored these structures, although not completely (Figure 4).

TEWL also changed with depth in the SC, as shown in Table I, in agreement with our previous data (2). The values were from 7 to 8 ($\text{g}/\text{m}^2\text{h}$) for the top layer and 14 to 19 for the fourth stripping. All the TEWL values are well within the normal range for skin with intact barrier function, and there were no significant differences among treatments. This discrepancy between changes in TEWL, as a measure of barrier function, and the order parameter S indicates that the lamellar lipid structures are not the single decisive determinant of skin barrier function, as we have previously suggested (2).

Stepwise regression analysis was applied to investigate factors influencing the changes in the S value between treatments. As a result, the extent of hydration measured with the Corneometer was confirmed as a predictor variable at the 10% level of significance. On the other hand, TEWL and the hydration value measured with the Skicon were not predictively valuable. The Skicon, based on the conductance principle, measures very superficial hydration (to 20 microns of depth), corresponding to the SC hydration, while the Corneometer, based on the capacitance principle, measures hydration at deeper levels, including the epidermal region (20). It is noteworthy that surface treatment with EPDME, a large-molecular polymer that is expected to remain at the surface of the SC, alters epidermal hydration and improves the lipid-layer structure in the outer SC.

Figure 7 shows the relationship between the water content of the SC measured by Corneometer and order parameter (S). The treatment with SDS leads to the loss of water in the SC and the loss of the ordering in the SC lipid structure, both in all four strippings. The EPDME post-treatment for SDS-treated skin led the restoration of water content even higher than the control (water), and the ordering of SC lipid structures was also repaired. Hence EPDME, which remains on the surface of skin as a large-molecular-weight polymer, assumes to stimulate epidermal moisturization to enhance the structuring of SC lipid disrupted by SDS treatment.

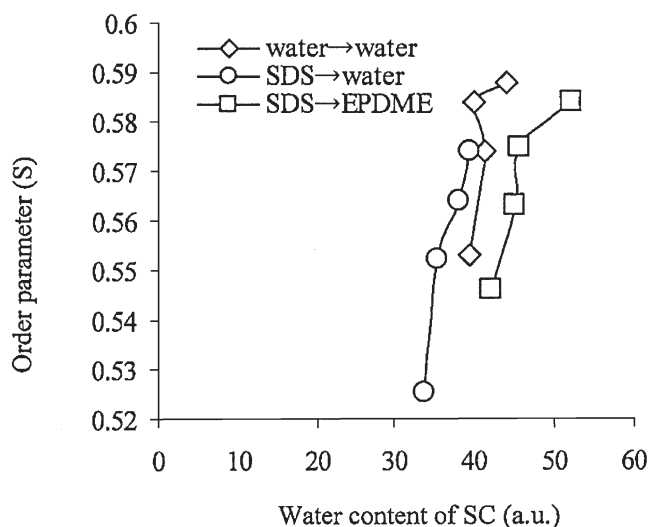


Figure 7. Relationship between the water content of SC measured by Corneometer and order parameter (S).

To evaluate further the effect of EPDME on lipid membranes, we conducted experiments using liposomes. There are many reports of EPR studies on the fluidity of liposome membranes (14,21–24), and the spectra that we obtained with 5-DSA were consistent with these reports (Figure 5). It was confirmed that, although the lipid membrane structure was disrupted by SDS (the fluidity is increased, as indicated by a decrease in S of *ca.* 0.007), pre-treatment with EPDME suppressed this effect (the reduction in S was only 0.003), as shown in Figure 6.

Mizushima *et al.* (16) examined the moisture retention capacity of SC under various conditions and suggested that water in untreated SC hydrates the lipid layers and maintains the S value even under very dry external conditions. On the other hand, the amount of water in SC treated with SDS varied, depending on environmental humidity, with corresponding changes in the S value (16). It can be speculated that SDS disrupts lamellar structures by solubilizing the lipid components, releasing water of hydration as free water. Thus, the improvement in membrane structural order by EPDME in SDS-treated SC and liposomes in this study may be attributable to the ability of EPDME to provide bound water at the lipid layers.

CONCLUSIONS

EPR spectroscopy was used to examine changes in the order parameter of lipid structure in SC successively stripped from SDS-treated dry skin. Application of EPDME partially reversed the disruption of lipid structure due to SDS treatment. Similar improvement was found in SDS-treated liposomes. The low concentration of SDS used did not result in any decrease in TEWL, indicating that EPDME may preserve the ordered lamellar structure of lipids by increasing the level of hydration.

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