Clinics in Dermatology (2008) **26**, 326–333

ELSEVIER

Clinics in Dermatology

The multifunctional role of ectoine as a natural cell protectant

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Abstract The protective properties of ectoine, formerly described for only extremophilic microorganisms, can be transferred to human skin. Our present data show that the compatible solute ectoine protects the cellular membrane from damage caused by surfactants. Transepidermal water loss measurements in vivo suggest that the barrier function of the skin is strengthened after the topical application of an oil in water emulsion containing ectoine. Ectoine functions as a superior moisturizer with long-term efficacy. These findings indicating that ectoine is a strong water structure-forming solute are explained in silico by means of molecular dynamic simulations. Spherical clusters containing (1) water, (2) water with ectoine, and (3) water with glycerol are created as model systems. The stronger the water-binding activity of the solute, the greater the quantity of water molecules remaining in the cluster at high temperatures. Water clusters around ectoine molecules remain stable for a long period of time, whereas mixtures of water and glycerol break down and water molecules diffuse out of the spheres. On the basis of these findings, we suggest that the hydrogen bond properties of solutes are not solely responsible for maintaining the water structure form. Moreover, the particular electrostatic potential of ectoine as an amphoteric molecule with zwitterionic character is the major cause for its strong affinity to water. Because of its outstanding water-binding activity, ectoine might be especially useful in preventing water loss in dry atopic skin and in recovering skin viability and preventing skin aging.
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Introduction

Ectoines, as small organic molecules, occur widely in aerobic, chemoheterotrophic, and halophilic organisms that enable them to survive under extreme conditions. These organisms protect their biopolymers (biomembranes, proteins, enzymes, and nucleic acids) against dehydration caused by high temperature, salt concentration, and low water activity by substantial ectoine synthesis and enrichment within the cell.

The organic osmolyte ectoine (Fig. 1) and hydroxyectoine are amphoteric, water-binding, organic molecules. They are generally compatible with the cellular metabolism without adversely affecting the biopolymers or physiologic processes and are so-called compatible solutes.[1]

The protective function of the compatible solutes in a low-water environment may be explained by the "preferential exclusion model": The solutes are excluded from the immediate hydration shell of, for example, a protein because of an unfavorable interaction with the protein surface. The

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doi:10.1016/j.clindermatol.2008.01.002

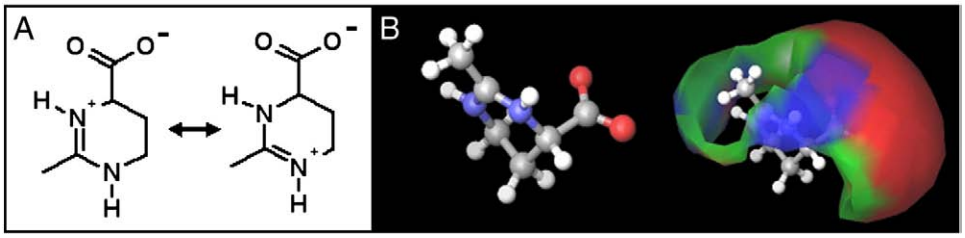

Fig. 1 Molecular structure of ectoine with the two tautomeric forms (A) and its hydrophilic surface colored according to the corresponding atomic partial charges (B).

consequence is preferential hydration of the protein, thus promoting its native conformation. Because compatible solutes do not interact directly with the protein surface, the catalytic activity remains unaffected.[2,3]

Yu and Nagaoka[5] reported interesting results on molecular dynamic simulations performed for water-ectoine mixture models around chymotrypsin inhibitor 2. According to their statement, ectoine maintains water at the surface by slowing down the water diffusion around a protein, where it is most needed, whereas it does not directly interact with macromolecules themselves. Thus, ectoine plays an indirect role in the alteration of the solvent properties and the modification of the stability of proteins.[4]

Ectoine minimizes the denaturation that occurs on the removal of water molecules by making the unfolding less favorable.[6] In addition, hydroxyectoine, with its OH group, can at least partly replace those water molecules lost from the hydrate shell (replacement hypothesis); in this way, the native structure of the biopolymers can be further stabilized. Compatible solutes are amphiphilic in nature and capable of "wetting" hydrophobic proteins, thus improving their hydration capability.[7] The structure-forming and breaking properties of compatible solutes indirectly influence the hydration shells and thus the activities of the proteins involved.[8]

In this way, halophilic organisms and other bacteria use ectoine to protect their cytoplasmic biomolecules against heat, freezing, dryness, and osmotic stress.[9] Ectoine and hydroxyectoine can be isolated from halophilic bacteria on a large scale and thus are available as active ingredients for skin care.[10]

The protective properties of ectoine, formerly described only for microorganisms, could be transferred to human skin. Human skin is situated at the interface of the organism and its environment and therefore is exposed to a variety of environmental assaults. The stratum corneum in particular provides a barrier to the evaporation of water from the viable epidermis. Many factors work to compromise this barrier and increase the rate of water loss from the skin. Exposure to extreme environmental conditions, including cold, dry winter weather, frequent washing with soap and hot water, or the exposure to surfactants, may cause skin dryness. In addition to dryness, the cumulative effect of external factors, such as radiation, wind, and temperature extremes, leads to accelerated skin aging.[11,12]

Various investigations underline the outstanding anti-aging properties of ectoine. Epidermal dendritic Langerhans cells are the single most important antigen-presenting cell population in the skin. The number of Langerhans cells decreases significantly in aged skin, whereas the decrease in skin exposed to the sun is greater than that in skin protected from the sun.[13-15] Topically applied ectoine shows an immunoprotective potential on the sun-exposed skin of healthy subjects. The ultraviolet-induced reduction of Langerhans cells has been prevented by pretreatment with ectoine before sun exposure.[16]

The exposure of primary human keratinocytes to ultraviolet A provokes the formation of ceramide by a singlet oxygen-mediated mechanism. As a consequence of the increased ceramide level, an intracellular signaling cascade is activated, leading to expression of the proinflammatory intercellular adhesion molecule-1. These negative effects are effectively prevented by ectoine as a result of its singlet oxygen-quenching properties.[17,18] Because the activity of antioxidant enzymes and the levels of nonenzymatic antioxidants decrease with age,[19,20] ectoine could prevent such oxidative damage in skin.

Skin in particular, which is susceptible to water loss because of the absence of an optimal skin barrier (eg, the skin of the elderly, atopic skin, or after surfactant treatment), shows increased transepidermal water loss (TEWL) and diminished moisturization.[21]

The goal of the present study was to investigate the effect of ectoine on the moisturization status and barrier function of the skin after topical application in vivo. Furthermore, different molecular dynamic simulation systems were created in silico to compare models of water, water-ectoine, and water-glycerol. The outstanding activity of ectoine as a strong water structure former was evaluated against glycerol as a commonly used humectant in cosmetics.

Materials and methods

Membrane assay

The membrane assay is based on the photometric quantification of free hemoglobin released from erythrocytes with a partially damaged membrane provoked by surfactants. For the different experiments, the erythrocytes are treated as

follows: (1) Human erythrocytes (2×10^8 cells/mL) are treated for 1 hour with 0%, 0.1%, 0.5%, 1%, and 5% ectoine to determine the effect of ectoine concentration; and (2) 2×10^8 erythrocytes/mL are treated for 0 (control), 6, 18, and 24 hours with 1% (w/v) ectoine to determine the effect of the incubation time. Both sets of cells are stressed for 10 minutes with 0% to 0.04% sodium dodecyl sulfate (SDS) solution, and the number of cells in lysis is determined spectroscopically via the content of free hemoglobin. With two absorption peaks at 540 and 575 nm, hemoglobin can be quantified by the determination of absorption at 575 nm, with the molar absorbance coefficient of 0.125 mmol/L oxyhemoglobin at A_{575nm}= 2.0.[22] The results are shown as the difference (%) of cells in lysis as a function of the concentration of ectoine against an untreated control. The experiment is repeated five times.

Determination of the transepidermal water loss in vivo

The volar forearm of five volunteers is treated twice daily for 1 week with an oil in water emulsion (2 mg/cm^2) containing 0% (placebo), 2%, and 5% ectoine. To achieve a synthetic increase in TEWL by damaging the skin barrier, the skin is occlusively treated with 80 µL SDS (2% in water) in an aluminium chamber for 24 hours. The TEWL is determined in an acclimatized room at 22°C with an air humidity of 60% using a TEWAmeter TM210 (Courage + Khazaka, Koeln, Germany). The TEWL values are visualized before and after treatment with ectoine-containing emulsion and after damaging the skin barrier with SDS.

Determination of skin moisture by corneometry

Ectoine treatment and subsequent dehydration with silica

The skin of the volar forearm of five volunteers is treated twice daily for 1 week with a cosmetic formulation (2 mg/cm^2) containing 0% (placebo), 2%, and 5% ectoine. The moisture content of the skin is determined with a Corneometer before application and, after 1 week, 4 hours after the final application. Silica gel 60 (0.2 g/cm^2) is applied under occlusion for 2 hours (dehydration step). On removal of the silica gel, the skin moisture is determined after 10 minutes, 2 hours, 4 hours, and 24 hours.

Ectoine treatment for long-term hydration

The skin of the volar forearm of five volunteers is treated twice daily for 12 days with a cosmetic formulation (2 mg/cm^2) containing 0% (control), 0.5%, and 1% ectoine. The skin hydration is determined by corneometry starting at day 8 until day 12. On day 12, the application is stopped for 7 days, finalizing this experiment on day 19 with a last measurement of hydration. The measurements are carried out in an acclimatized room at 22°C with an air humidity of 60%.

Molecular dynamic simulations

The Schrödinger package Impact (Integrated Modelling Program using Applied Chemical Theory[23]) is used for molecular dynamic simulations (with OPLS-2005 force field parameters and partial charges). The OPLS-2005 force field uses experimental data from the liquid state and quantum mechanical calculations. It is calculated from the sum of the intramolecular bond, angle, and torsion motions to set the constituent parameters and the nonbonded interaction as a van der Waals term together with an electrostatic term.

Three spheres have been created for: (1) water only; (2) an ectoine-water mixture; and (3) a glycerol-water mixture. The creations of spheres are as follows: For each ingredient (ectoine and glycerol), a $3 \times 3 \times 3$ matrix is created. For this purpose, 27 molecules of each ectoine or glycerol are clustered per sphere. A minimization is performed using the surface generalized born method with 500 steps of steepest descent, followed by 500 steps of conjugated gradient.

Ectoine and glycerol are placed in a rectangular box, and soaking of simple point charge water with a dimension of $70 \times 70 \times 70$ Å is performed.

The spheres are cut out with a radius of 30 Å away from the centroid atoms. The size of spheres of 30 Å in radius is sufficient to cover more than one solvation shell for solutes calculated in spheres. The reason for having so many water molecules is to ensure that there are at least two shells of water molecules around the solutes. In addition, we can examine and compare the indirect effect of solutes on water molecules on such a large scale.

The shake algorithm is used to constrain the X-H bond, which allows time steps of 2 fs. Elaborate equilibration runs of 50 ps at 298.15 K are performed to allow for a careful accommodation of water structure around the solutes (ectoine and glycerol). Water oxygen atoms are fixed beyond 25 Å from the defined centroid atoms in each created sphere in the equilibration. For the dynamic simulations, these constraints are removed.

The dynamic simulations are performed for water and water-glycerol for 200 ps and 500 ps at the temperature of 370 K with a temperature relaxation constant value of 0.01 ps. For the water-ectoine mixture, the simulation is performed for 1 ns to demonstrate the effect of ectoine with regard to water cluster formation in a long time frame. The trajectories are recorded every 50 time steps.

Results and discussion

Barrier-improving effects

The membrane of the skin cell can become damaged, for example, by exposure to surfactants present in washing and skin-cleansing solutions. Thus, the use of active cleansing

surfactants also leads to removal of fat from the skin, increased TEWL, and dry skin.

For the evaluation of the membrane-protecting properties of ectoine, the red blood cell (RBC) test was applied. This assay is a biologic in vitro test for the rapid estimation of membrane and protein-denaturing properties of surfactants. The standard protocol uses erythrocytes, non-nucleated blood cells containing hemoglobin. Because hemoglobin is incapable of crossing the RBC membrane, it is not detectable outside erythrocytes as long as the RBC membrane is intact. The assay is based on the photometric quantification of the hemoglobin released as a consequence of RBC plasma membrane damage after exposure to surfactants, thus providing a measure of surfactant aggressiveness.

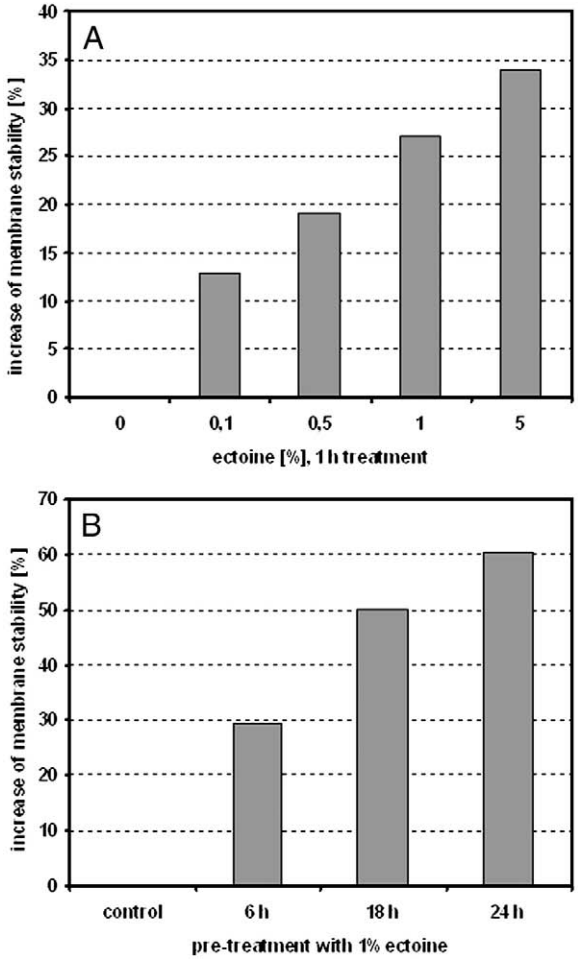

Fig. 2 Evaluation of the membrane-stabilizing effect of ectoine in surfactant-stressed cells. Human erythrocytes (2×10^8 cells/mL) are treated (A) for 1 hour with 0%, 0.1%, 0.5%, 1%, and 5% ectoine and (B) for 0 (control), 6, 18, and 24 hours with 1% ectoine. Both sets of cells are stressed for 10 minutes with 0% to 0.04% SDS solution, and the number of cells in lysis is determined spectroscopically via the content of free hemoglobin. The diagrams illustrate the difference (%) of cells in lysis as a function of the concentration of pretreated ectoine against an untreated control. The experiment is repeated five times.

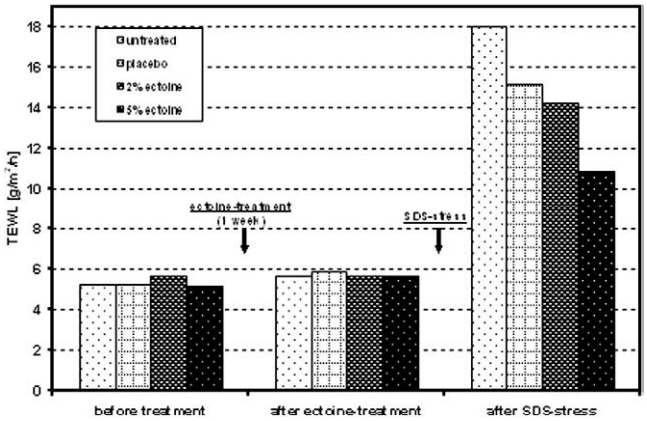

Fig. 3 In vivo determination of TEWL after damage of the skin barrier by SDS. The forearm skin of the volunteers (n = 5) is treated twice daily for 1 week with an oil in water emulsion (2 mg/cm^2) containing 0% (placebo), 2%, and 5% ectoine. To achieve a synthetic increase in TEWL by damaging the skin barrier, the skin is subsequently treated with 2% SDS in water for 24 hours and the TEWL is determined. The diagram shows the TEWL before and after treatment with emulsion containing ectoine and after damage of the skin barrier with SDS.

The stabilization effect on cell membranes pretreated with ectoine was evaluated. The erythrocytes were incubated for 10 minutes with SDS. SDS destabilizes the membranes of untreated cells in such a way that lysis occurs in part and cell components (eg, hemoglobin) are released. The hemoglobin released serves as an indicator for the spectrophotometric determination of the degree of cell membrane damage provoked by SDS. Detecting the released hemoglobin enabled the number of destroyed erythrocytes to be determined in our experiments. A modified version of the RBC test was used to determine the membrane stabilization achieved by a test substance versus surfactant lysis. This assay includes the RBC preincubation with a stabilizer before the addition of surfactant as the lytic agent.

Fig. 2 shows that ectoine protects the cells from damage caused by SDS treatment. The erythrocytes pretreated with ectoine are shown to be more resistant to membrane damage by SDS than those of untreated cells. No stabilizing effect was observed in cells without ectoine, in which maximum erythrocyte damage occurred (0% increase of membrane stability). The higher the ectoine concentration, the greater the protective effect against membrane damage (Fig. 2A).

Furthermore, the influence of prolonging the incubation time was investigated. The membrane stability increased to 30% after 6 hours of pretreatment and to approximately 60% after 24 hours. Thus, the longer the cells are pretreated with ectoine, the greater the protective effect against membrane damage by the surfactant SDS (Fig. 2B). The degree of cell protection that has been linked with the degree of membrane stabilization depends directly on the ectoine concentration and the duration of ectoine pretreatment.

Ectoine thus protects the skin barrier against the damaging effect (water loss) of SDS.

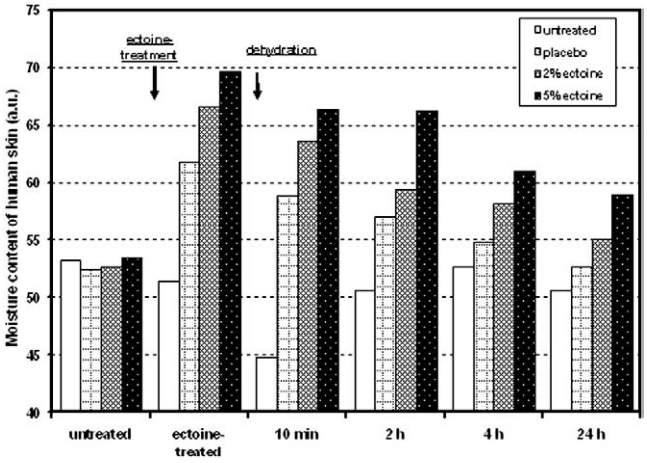

Fig. 4 In vivo determination of skin moisture after treatment with ectoine and subsequent dehydration with silica gel. The forearm skin of the volunteers (n = 5) is treated twice daily for 1 week with an oil in water emulsion (2 mg/cm^2) containing 0% (placebo), 2%, and 5% ectoine. The moisture content of the skin is determined before application and, after 1 week, 4 hours after the final application. Silica gel 60 (0.2 g/cm^2) is applied under occlusion for 2 hours (dehydration). On removal of the silica gel, the skin moisture is determined after 10 minutes, 2 hours, 4 hours, and 24 hours.

These data confirm our previous studies of further cosmetically relevant surfactants in which ectoine showed a stronger protective effect compared with the well-known membrane stabilizer phosphatidylcholine.[24]

These in vitro findings should also be approved in vivo. Surfactants have also been used to cause dry skin.[25] For this reason, after SDS treatment of the skin, the TEWL is determined as a read-out parameter for the integrity of the skin barrier. The barrier disruption can be expressed as a change in TEWL, and the influence of ectoine can be measured. The study is performed on the lower forearm of healthy volunteers.

The application of a cosmetic emulsion containing different amounts of ectoine leads to a remarkable reduction of TEWL to 40% (Fig. 3). Fig. 3 shows that skin pretreated with ectoine becomes less susceptible to damage by the surfactant SDS. The ectoine emulsion thus protects the skin against surfactant damage and the consequent loss of water.

Protection against dehydration

One of the major goals of cosmetics is still the protection of the skin against stress factors that lead to dehydration. Dry air, particularly during periods of freezing or hot weather and air conditioning, tends to dry out the skin considerably.

To demonstrate the protective effect of ectoine on skin moisture, two cosmetic formulations with and without ectoine were topically applied to the lower forearm of volunteers twice daily for 1 week. The moisture content of the skin was determined by corneometry, and the results are shown in Fig. 4.

The diagram illustrates that ectoine in a cosmetic oil in water emulsion protects the skin against dehydration. In addition to this protection, ectoine also produces a higher moisture content than the basic (placebo) formulation that already contains 3% glycerol. The results also show that ectoine, even after 24 hours, maintains a considerably greater degree of skin moisture than untreated or placebo-treated skin. Ectoine even protects skin against rapid dehydration after direct application of hygroscopic silica gel. Skin moisture can be maintained for a longer period of time by topically applying ectoine.

Low humidity has been shown to stimulate epidermal DNA synthesis and amplify the hyperproliferative response to barrier disruption.[26] Stratum corneum morphology is also

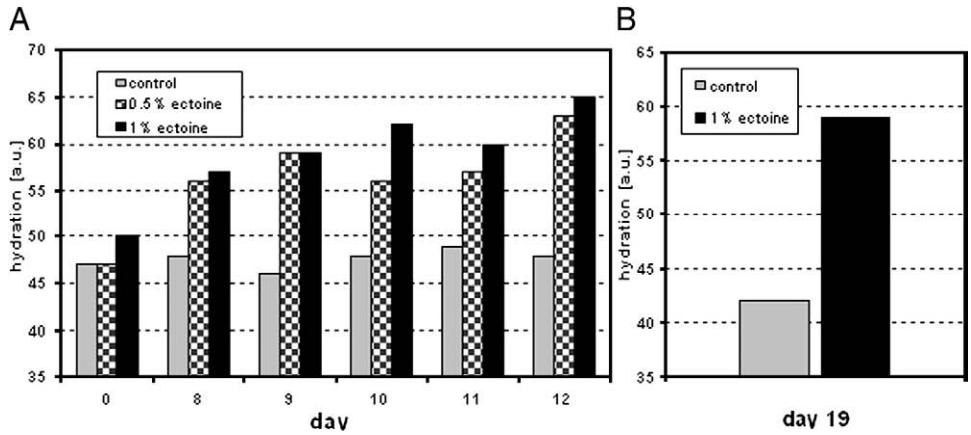

Fig. 5 Long-term moisturizing effect with ectoine. The skin of the volar forearm of five volunteers is treated twice daily for 12 days with a cosmetic formulation (2 mg/cm^2) containing 0% (control), 0.5%, and 1% ectoine. The skin hydration is determined by corneometry starting at day 8 until day 12 (A). On this day the application is stopped for 7 days, finalizing this experiment on day 19 with a last measurement of hydration (B).

Table 1

t (ps)	Water	Water-glycerol	Water-ectoine
0	3618	3429	3139
200	3026	2339	3138
500	NC	1288	3112
1000	NC	NC	3103

The number of water molecules retained in spherical water clusters during the dynamic simulation time. The simulation is carried out at 370 K, and the water molecules are counted after 0, 200, 500, and 1000 ps. *NC,* Not calculated.

influenced by a dry environment, and abnormal desquamation is observed under low humidity.[27,28] With respect to our findings in the "silica-dried skin model," formulations containing ectoine have a prophylactic effect against such adverse processes in dry skin.

Moisture boost with long-term effect

In a further series of experiments, ectoine was evaluated according to its long-term effect on skin moisture. The test was carried out on the volar forearm of volunteers. Twice-daily applications of 0.5% and 1% ectoine were applied for 12 days. The skin hydration was measured with a Corneometer starting at day 8 until day 12. On day 12, the application of ectoine was stopped for 7 days, detecting the skin hydration finally at day 19. The results of this placebo-controlled study underline the outstanding activity of ectoine: After 8 days of application, the hydration increased markedly up to 200% compared with the placebo-treated skin and remained constant until the end of the testing period (Fig. 5A). Although the topical application was stopped on day 12, the actual hydration status was preserved for approximately 7 days, underlining a significant long-term moisturizing effect of ectoine (Fig. 5B).

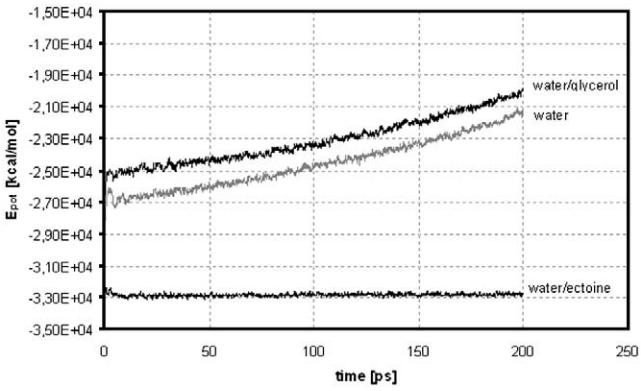

Fig. 6 Evaluation of the E_{pot}-value of different water clusters. During the dynamic simulation at 370 K, water molecules diffuse out of the spheres and the total amount of water molecules decreases. To explain this phenomenon, the total potential energy has been calculated and plotted as the E_{pot}-value. In this experimental setup, the E_{pot}-value can be adopted as the stored energy or the energy of position of each system.

Ectoine retains the power of water

The protein-stabilizing effects of ectoine can be explained by the preferential exclusion model as a consequence of entropically favored surface minimization. The ability of ectoine as a strong water structure-forming solute is further processed in comparison with glycerol as a commonly used humectant in cosmetics.[11]

After the dynamic simulation time of 200 ps, as well as 1000 ps, the number of water molecules in the water-ectoine complex remained unexpectedly constant. In contrast, the performance of the water-glycerol complex: an extreme corrosion was observed. The total number of water molecules decreased significantly after 200 ps of dynamic simulation, and only 2339 water molecules remained in the sphere (Table 1).

To explain this phenomena, the total potential energy (E_{pot}) was calculated for the spheres containing water, water-

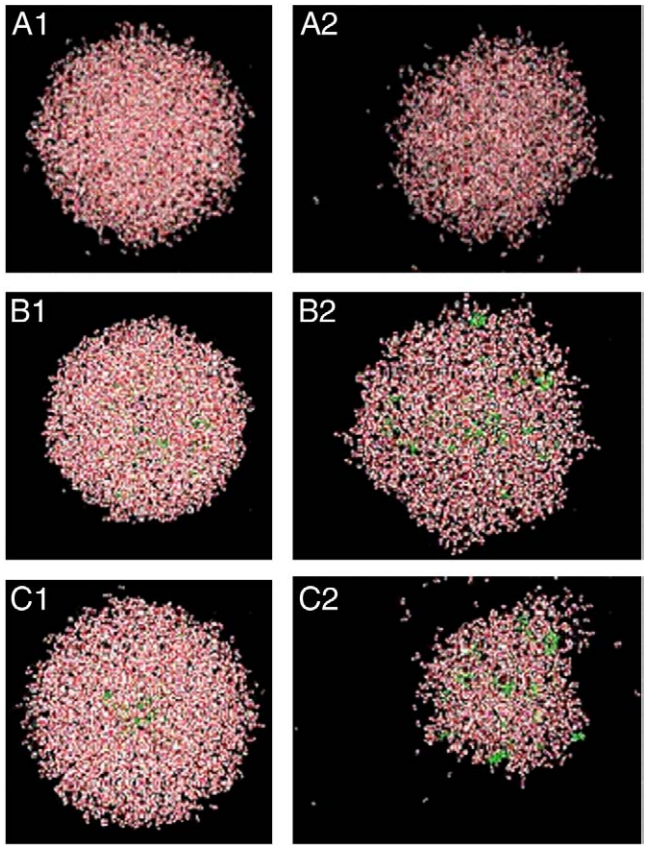

Fig. 7 Molecular dynamic simulation of different models containing (A) water, (B) water and ectoine, and (C) water and glycerol. The pictures are taken at the beginning of the simulation (t = 0, A1, B1, C1) and after 200 ps (A2), 1000 ps (B2), and 500 ps (C2) at a constant temperature of 370 K. Water clusters around ectoine molecules remain stable for a long period of time, whereas the cluster of water and glycerol breaks down and water molecules diffuse out of the spheres. The pictures represent the number of water molecules counted during the dynamic simulation as shown in Table 1. The solutes are green.

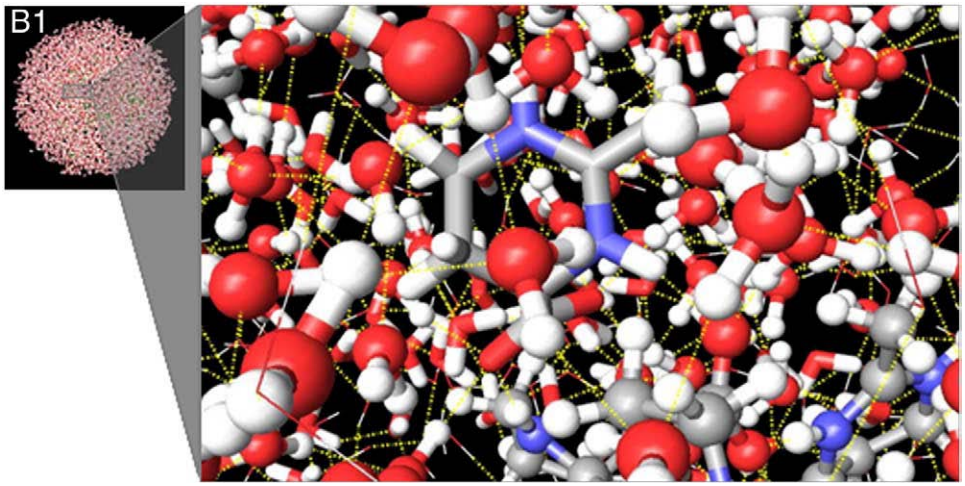

Fig. 8 Stick representation of atoms in the ectoine-water sphere. The grey area of the ectoine-water cluster is presented at a higher resolution to illustrate the molecular composition of the cluster. The small picture corresponds with Fig. 7, B1.

glycerol, and water-ectoine. In this experimental setup, the E_{pot}-value can be adopted as the stored energy or the energy of position in such a system.

With regard to water and the water-glycerol complexes, the E_{pot}-values decreased dramatically during the simulation time, whereas the E_{pot}-value of the water-ectoine sphere remained constant even throughout a longer simulation time (Fig. 6). The E_{pot}-value of the water-ectoine sphere remained constant at the level indicated in the diagram (data not shown). It is remarkable that the E_{pot}-value of regular water molecules per se was greater than that of the water-ectoine mixture, indicating the strong organizing and complexing properties of ectoine.

The dynamic simulation and animations, and the statistical analysis, demonstrated that the water diffusion out of the spheres was limited and decreased enormously by adding ectoine molecules to the sphere (Fig. 7A and B; see also the stick presentation of water and ectoine atoms in Fig. 8). Even a 5-fold longer simulation time showed a stable water structure form attributable to ectoine properties, which is superior compared with water itself and outstanding compared with a water-glycerol complex (Fig. 7).

We propose that the hydrogen bond properties of solutes are not solely responsible for maintaining the water structure form. Moreover, the particular electrostatic potential of a compatible solute, such as ectoine, as an amphoteric molecule with zwitterionic character is the major reason for its affinity to water.

Conclusions

Our recent studies demonstrate the outstanding role of the compatible osmolyte ectoine in preventing water loss caused by surfactant-induced barrier damage. Ectoine functions as a more potent moisturizer than glycerol and features long-term moisturizing efficacy. These in vivo findings were explained in silico by means of molecular dynamic simulations. Water clusters around ectoine molecules remain stable for a long period of time, whereas mixtures of water and glycerol are disintegrated by the diffusion of water molecules out of the spheres. Because of its strong water-binding activity, ectoine may be especially useful in the prevention of dehydration in dry atopic skin and the recovery of skin viability and prevention of skin aging.

Acknowledgments

We thank Dr Jianxin Duan, of Schrödinger GmbH, Mannheim, Germany, for the fruitful discussions and technical support for the dynamic simulations.

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