SUPPLEMENTARY MATERIALS AND METHODS

Human SC lipids extraction

For SC lipids extraction, the epidermal layer of human skin was isolated mechanically after 60°C water treatment and the SC was obtained after 12 h of trypsin treatment (at 37°C). Separated SC was washed and dried over P_2O_5. Skin from ≥10 donors was combined. Human SC lipids were obtained by extraction of the dried SC by a series of chloroform/methanol mixtures. The extracts were combined, dried, and purified by column chromatography (Nováčková *et al.*, 2021). Before use, the composition of the mixture was examined by HPTLC.

High-performance liquid chromatography (HPLC)

In permeability experiments, the concentration of model permeants in acceptor samples was assayed by HPLC using Shimadzu Prominence instrument (Shimadzu, Kyoto, Japan). Indomethacin concentration was determined using a LiChroCART 250-4 column (LiChrospher 100 RP-18, 5 μm particle size, Merck) according to a validated method (Školová *et al.*, 2017) using 90:60:5 acetonitrile/water/acetic acid mobile phase at 2 ml/min flow rate. The column was maintained at 40 °C. Indomethacin was detected at 260 nm and its retention time was 3.5 ± 0.1 min.

For FITC-inulin concentration detection a method using Supelco BIOshell A400 Protein C18 column (3 cm x 4.6 mm, 3.4 um particle size, Merck) was developed and validated. The column was maintained at 35 °C. 1:1 acetonitrile/water mobile phase pH 8 (adjusted using NaOH) at 0.4 ml/min flow rate was used, FITC-inulin was detected at 520 nm with 493 nm excitation, at 1.4 ± 0.2 min.

Mica-deposited lipid layers

For AFM measurements the lipid monolayers were compressed to 30 mN/m surface pressure, and after 15 min Langmuir-Blodgett deposited onto freshly cleaved mica support (15 × 15 mm^2, SPI Supplies, West Chester, PA USA) by uplifting mica from the subphase through the lipid monolayer at a speed of 1 mm/min and constant surface pressure. In experiments focusing on the effect of mean molecular area the monolayers were compressed to different surface pressures corresponding to the required area per lipid. Mica substrates are molecularly flat, which was verified in our experimental setting. The samples were left to dry for 20 min, further dried under a stream of nitrogen and stored in dark at laboratory temperature under argon atmosphere before measurement.

Infrared spectroscopy of propylene glycol vs. water treated human SC lipids

0.9 mg of extracted purified human SC lipids were sprayed onto glass coverslip using Linomat V (Camag Muttenz, Switzerland), annealed in a sealed container at 70°C at 100% RH for 20 min, and allowed to cool down slowly to 32°C for 4 h. 100 µl of either water or 60% propylene glycol was applied on the lipid film to cover it entirely for the next 15 h at 32°C. Following this treatment the lipids were placed onto a single-reflection MIRacle ATR ZnSe crystal preheated to 20°C in an abundance of either water or 60% propylene glycol and infrared spectra were collected while gradually increasing the temperature as described earlier.

SUPPLEMENTARY FIGURE LEGENDS

Figure S1. Effect of propylene glycol and hydration on human SC lipids thermal phase behavior. (**a-b**) Relative OR phase content (intensity ratio of ~730 cm^{-1} and ~720 cm^{-1} bands) (**a**) and lipid chain conformation (methylene symmetric stretching vibration, $\nu_{sym(CH2)}$; lower values indicate more ordered all-*trans* chains) (**b**) around the OR-HEX transition, approximately indicated by the gray rectangle, in SC lipids that were treated by and measured in abundance of either water (black circles) or propylene glycol (blue circles). Mean ± SD, n=3.

Figure S2. Lipid chain conformation in isolated human SC lipids as a function of temperature and hydration. (**a-b**) Evolution of the lipid chain conformation indicated as methylene symmetric stretching vibration ($\nu_{sym(CH2)}$; lower values indicate more ordered all-*trans* chains) in SC lipids that were fully hydrated and allowed to dehydrate freely at 10-15% relative humidity (blue line) and in dry SC lipids (black line) at 32°C (**a**) and 37°C (**b**). Mean ± SD, n≥3.

Figure S3. Monolayers of isolated SC lipids show transition behavior concerning stability and elasticity. (**a**) Area per lipid and surface pressure at collapse of a monolayer of SC lipids at the air-buffer interface at 23-46°C; mean±SD, n=3-6, * $P < 0.05$. The vertical lines delineate the approximate extent of the OR-HEX lipid transition. (**b**) Compressibility moduli as a function of area per lipid at 23-46°C. Representative curves, n=3-6. (**c**) Maximum compressibility moduli of SC lipids monolayers at 23-46°C; mean±SD, n=3-6, * $P < 0.05$. The blue area shows the relative content of the OR phase at these temperatures and the vertical lines delineate the approximate extent of the OR-HEX lipid transition.

Figure S4. Effect of mean molecular area on human SC lipid assembly. Representative atomic force micrographs of lipid samples prepared at 27 $Å^2$/23°C (**a**) and 22 $Å^2$/32°C (**b**) (n=3, each scanned at least 5×), blue lines indicate analyzed height profiles. (**c-d**) height profiles of the respective lipid samples prepared at 23 (**c**) or 32°C (**d**) (representative of at least 10 measurements).

SUPPLEMENTARY REFERENCES

Nováčková A, Sagrafena I, Pullmannová P, Paraskevopoulos G, Dwivedi A, Mazumder A, *et al.* (2021) Acidic pH is required for the multilamellar assembly of skin barrier lipids in vitro. *Journal of Investigative Dermatology* 141:1915-1921. e4.

Školová B, Kováčik A, Tesař O, Opálka L, Vávrová K (2017) Phytosphingosine, sphingosine and dihydrosphingosine ceramides in model skin lipid membranes: permeability and biophysics. *Biochimica et Biophysica Acta (BBA)-Biomembranes* 1859:824-834.