Stimuli-Responsive Polyguanidino-Oxanorbornene Membrane Transporters as Multicomponent Sensors in Complex Matrices

Andreas Hennig, Gregory J. Gabriel, Gregory N. Tew,* and Stefan Matile*

Department of Organic Chemistry, University of Geneva, Geneva, Switzerland, and Polymer Science & Engineering Department, University of Massachusetts, 120 Governors Drive, Amherst, MA 01003, tew@mail.pse.umass.edu, stefan.matile@chiorg.unige.ch

Supporting Information

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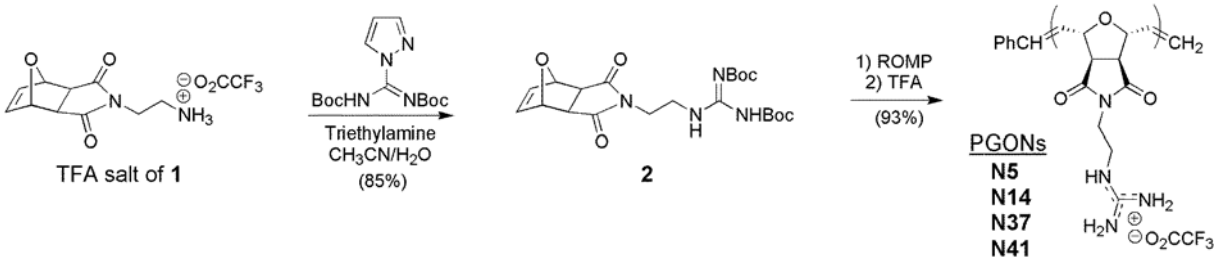
1. Materials. All salts and buffers were of the best grade available from Sigma or Fluka and used as received. 5(6)-carboxyfluorescein (CF) was from Fluka. 8-aminonaphthalene-1,3,6-trisulfonic acid, disodium salt (ANTS), *p*-xylene-bis-pyridinium bromide (DPX), and Cascade Blue hydrazide trisodium salt (CB hydrazide) were from Invitrogen, Eugene, Oregon, USA. Egg yolk phosphatidylcholine (EYPC), egg yolk phosphatidyl glycerol (EYPG), dipalmitoyl phosphatidylcholine (DPPC) and a Mini-Extruder used for vesicle preparation were from Avanti Polar Lipids. Fluorescence measurements were performed with a FluoroMax-2 spectrofluorometer (Jobin-Yvon Spex) equipped with a stirrer and a temperature controller (measurements at 25 °C unless otherwise noted). UV spectroscopy was carried out with a Jasco V-650 spectrophotometer.

2. Abbreviations. ADP: adenosine 5'-diphosphate; ANTS: 8-aminonaphthalene-1,3,6-trisulfonic acid, disodium salt; ATP: adenosine 5'-triphosphate; CB hydrazide: Cascade Blue hydrazide trisodium salt; CF: 5(6)-carboxyfluorescein; DMSO: dimethylsulfoxide; DAN: dialkoxynaphthalene; DPPC: dipalmitoyl phosphatidylcholine; DPX: *p*-xylene-bis-pyridinium bromide; EYPC: egg yolk phosphatidylcholine; EYPG: egg yolk phosphatidylglycerol, Hepes: *N*-(2-hydroxyethyl)piperazine-*N*'-(2-ethanesulfonic acid); LUVs: large unilamellar vesicles; PGON: polyguanidino-oxanorbornene; Tris: tris(hydroxymethyl)aminomethane.

3. Synthesis of Transporters



The synthesis of PGONs starts with the TFA salt of **1** from Scheme 1 in the text.[S1] To this salt (1.4 g, 4.34 mmol) *N*,*N*-Di-Boc-1*H*-pyrazole-1-carboxamidine (Aldrich, 4.0 g, 13.02 mmol) and triethylamine (Aldrich 4.1 g, 40 mmol) were added with 60 mL of solvent (90% CH_3CN, 10% H_2O). This solution was stirred at room temperature for 12 h. The solution was then diluted with ethyl acetate and washed twice each with H_2O and brine. The organic layer was evaporated and purified by silica column chromatography using a MeOH in CH_2Cl_2 gradient to afford the Boc-protected guanidinium oxanorbornene monomer, **2**.

This monomer was polymerized by ring-opening metathesis polymerization (ROMP) using a derivative of the 2nd generation Grubbs' catalyst, $[(H_2Imes)(3\text{-Br-py})_2\text{-}(Cl)_2Ru{=}CHPh]$.[S2] As a typical example, the polymerization of **N14** entailed adding to a test tube monomer (100 mg) plus catalyst (13.7 mg). The test tube was capped with a septum and purged with N_2 for 5 min, then 1 mL dry CH_2Cl_2 (Acros, packed under N_2 and molecular sieves) was injected. The N_2 line was removed and the clear, brown solution was stirred at 28 ºC for 30 min after which 0.4 mL ethyl vinyl ether was injected to endcap the polymer. After stirring for 15 min the solution was added dropwise to 300 mL of stirring pentane to precipitate the polymer. The pentane solution was stirred an additional 30 min and left standing undisturbed for an hour. The precipitate was then collected by a fine sinter funnel. The polymer was then redissolved in 1 mL of CH_2Cl_2 and reprecipitated and collected then dried by vacuum for 8 h.

The Boc-protected polymers were deprotected by stirring 100 mg in 8 mL of 1:1 TFA:CH_2Cl_2 for 2 h. The solution was dried to an oil by rotary evaporator set at 40 ºC and residual TFA was removed by sonicating the oil in more CH_2Cl_2 and evaporating the solvent again by rotary evaporator. The resulting solid was placed under vacuum for 2 h. Finally, the solid was fully dissolved in 4 mL H_2O and filtered through a PES syringe filter (Whatman, 25 mm diameter, 0.45 μm pore) and freeze-dried for 48 h to give an eggshell colored soft solid. Final deprotected polymers were protected from moisture and stored at -20 ºC.

Gel permeation chromatography (GPC) was performed on the Boc-protected polymers with a Polymer Lab LC1120 pump equipped with a Waters differential refractometer detector. The mobile phase was tetrahydrofuran with a flow rate of 1.0 mL/min. Separations were performed with 105, 104, and 103 Å Polymer Lab columns and molecular weights were calibrated versus narrow molecular weight polystyrene standards. The Boc-protected PGON is THF soluble and GPC (polystyrene-calibrated) was used to approximate molecular weight (M_n) and polydispersity index (PDI). ^{1}H-NMR and ^{13}C-NMR spectra were obtained on a Bruker DPX-300 NMR spectrometer. Spectra were calibrated to the DMSO-d_6 solvent signal.

DiBoc-protected monomer 2. ^{1}H-NMR (300 MHz, DMSO-d_6): δ 11.44 (1H, s), 8.35 (1H, t, J = 6.0 Hz), 6.56 (2H, s), 5.10 (2H, s), 3.52 (2H, m), 3.43 (2H, m), 2.85 (2H, s), 1.47 (9H, s), 1.36 (9H, s). ^{13}C-NMR (75 MHz, DMSO-d_6): δ = 176.3, 156.6, 153.0, 136.5, 83.3, 80.9, 47.6, 39.0, 38.4, 28.3, 28.1.

Boc-protected N14. ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 11.51 (1H, br), 8.47 (1H, br), 5.90 (trans) and 5.70 (cis) (2H total, br), 4.89 (cis) and 4.39 (trans) (2H total, br), 1.45 (9H, s), 1.38 (9H, br), cis:trans ratio = 47:53.

N14. ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 7.88 (1H, br), 7.36 (4H, br), 5.96 (1H, br), 5.74 (1H, br), 4.92 (1H, br), 4.43 (1H, br) 3.47 (4H, br).

4. Synthesis of Inactivators

Cascade Blue Hydrazide (CB Hydrazide).[S3] Concentrations of CB hydrazide stock solutions were determined by UV spectroscopy (ε_{377} = 22900 $M^{-1}cm^{-1}$, ε_{399} = 27500 $M^{-1}cm^{-1}$).[S4] The derivatizations were carried out in two variants. *Variant A:* Total reaction volume of derivatizations was 50 μl or 100 μl. A stock solution of CB hydrazide (50, 10, 2, 1, or 0.2 mM) in DMSO was diluted with DMSO to half of the desired total reaction volume. The other half was composed of either 10 or 2 mM of analyte (*i.e.* pyruvate, ketoglutarate, or oxaloacetate) in 0.4 M NaOAc/HOAc, pH 4.5. Subsequently, the mixtures were combined and incubated for 30 min at 50 °C. Aliquots from that solution were diluted with a 1:1 mixture of DMSO and 0.4 M NaOAc/HOAc (pH 4.5). These aliquots were subjected to fluorescence analysis as described below. *Variant B:* 50 μl CB hydrazide in DMSO (1 mM) was mixed with 10 μl analyte stock solution (pyruvate, ketoglutarate = 1 μM – 100 mM), and 40 μl 0.4 M NaOAc/HOAc (pH 4.5). The resulting series of reaction mixtures were incubated for 30 min at 50 °C, and 20 μl aliquots of the mixtures were subjected to fluorescence analysis as described above (see below). Consistent EC_{50}s for pyruvate with both variants exclude possible influences from incomplete derivatization. In the case of ketoglutarate, the EC_{50} with Variant A was significantly larger than with Variant B, which might limit practical applications.[S3]

Dialkoxynaphthalene Hydrazide (DAN Hydrazide).[S3] 580 μg (2 μmol) DAN hydrazide were suspended in 200 μl DMSO and mixed with 160 μl 0.4 M NaOAc/HOAc (pH 4.5) and 40 μl 100 mM pyruvate in H_2O. As a control, the pyruvate was substituted with 0.4 M NaOAc/HOAc (pH 4.5). The reaction mixtures were incubated for 2 h at 50 °C and subsequently centrifuged for 1 min at 2000 rpm. Aliquots from that solution were diluted with a 1:1 mixture of DMSO and 0.4 M NaOAc/HOAc (pH 4.5) and subjected to fluorescence analysis as described below.

5. Activity, Activators and Inactivators (EYPC-LUVs ⊃ CF)

Preparation of EYPC-LUVs ⊃ CF.[S3] A thin lipid film was prepared by evaporating a solution of 25 mg EYPC in 1 ml MeOH/$CHCl_3$ (1:1) on a rotary evaporator (40 °C) and then *in vacuo* overnight. After hydration (> 30 min) with 1.0 ml buffer (10 mM Tris, 10 mM NaCl, 50 mM CF, pH 7.5), the resulting suspension was subjected to >5 freeze-thaw cycles (liquid N_2, 40 °C water bath), and >15 times extruded through a polycarbonate membrane (pore size 100 nm). Extravesicular components were removed by size exclusion chromatography (Sephadex G-50, Sigma-Aldrich) with 10 mM Tris, 107 mM NaCl, pH 7.5. Final conditions: ~2.5 mM EYPC; inside: 10 mM Tris, 10 mM NaCl, 50 mM CF, pH 7.5; outside: 10 mM Tris, 107 mM NaCl, pH 7.5.

PGON Activity in EYPC-LUVs ⊃ CF.[S3,S5] 25 μl EYPC-LUVs ⊃ CF were added to 1975 μl gently stirred, thermostated buffer (10 mM Tris, 107 mM NaCl, pH 7.5) in a disposable plastic cuvette. The time-dependent change in fluorescence intensity I_t (λ_{exc} = 492 nm, λ_{em} = 517 nm) was monitored during the addition of PGON (20 μl stock solution in DMSO, 1 nM - 100 μM final concentration) at t = 0 min, and addition of 40 μl 1.2 % (aq.) triton X-100 at the end of every experiment. Time courses of I_t were normalized to fractional intensities I_f using equation S1.

$$I_f = (I_t - I_0) / (I_\infty - I_0) \qquad \text{(S1)},$$

where $I_0 = I_t$ before pore addition and $I_\infty = I_t$ after lysis. I_f at 200 s after the start of the experiment just before lysis was defined as transmembrane activity Y. For Hill analysis, Y was plotted against PGON concentration c_{PGON} and fitted to the Hill equation S2 to give effective concentration EC_{50} and the Hill coefficient n.

$$Y = Y_\infty + (Y_0 - Y_\infty) / \{1 + c_{PGON} / EC_{50})^n\} \qquad \text{(S2)},$$

where Y_0 is Y in absence of PGON, Y_∞ is Y with excess PGON.

PGON Inactivators.[S3,S5] 20 μl of inactivator stock solution (in H_2O or derivatization buffer, see above) and 25 μl EYPC-LUVs ⊃ CF were added to 1955 μl gently stirred,

thermostated buffer (10 mM Tris, 107 mM NaCl, pH 7.5) in a disposable plastic cuvette. When necessary, the pH was readjusted to 7.5 by addition of 1 M NaOH or 1 M HCl. Care was taken that volume changes were insignificant, i.e. below 1 %. The time-dependent change in fluorescence intensity I_t (λ_{exc} = 492 nm, λ_{em} = 517 nm) was monitored during the addition of 20 μl PGON stock solution (50 μM PGON14 or 10 μM PGON41 in DMSO, final concentrations 0.5 μM and 0.1 μM, respectively) at t = 0 min, and addition of 40 μl 1.2 % (aq.) triton X-100 at the end of every experiment. Fluorescence time courses were normalized and converted into transmembrane activity Y (equation S1) and further converted into fractional activity Y_f according to equation S3.

$$Y_f = (Y - Y_0) / (Y_\infty - Y_0) \qquad \text{(S3)},$$

where Y_0 is Y without inactivator, and Y_∞ is Y with excess inactivator. For Hill analysis, Y_f was plotted against inactivator concentration c_I and fitted to the Hill equation S4 to give the concentration at which inactivator was 50 % (IC_{50}) and the Hill coefficient n.

$$Y_f = Y_{\infty,f} + (Y_{0,f} - Y_{\infty,f}) / \{1 + c_I / IC_{50})^n\} \qquad \text{(S4)},$$

where $Y_{0,f}$ is Y_f without inactivator, and $Y_{\infty,f}$ is Y_f with excess inactivator.

PGON Activators.[S5] 20 μl of activator stock solution (in H_2O or DMSO depending on solubility) and 25 μl EYPC-LUVs ⊃ CF were added to 1955 μl gently stirred, thermostated buffer (10 mM Tris, 107 mM NaCl, pH 7.5) in a disposable plastic cuvette. The time-dependent change in fluorescence intensity I_t (λ_{exc} = 492 nm, λ_{em} = 517 nm) was monitored during the addition of 20 μl PGON stock solution (3 μM PGON14 or 30 μM PGON5 in DMSO, final concentrations 30 nM and 0.3 μM, respectively) at t = 0 min, and addition of 40 μl 1.2 % (aq.) triton X-100 at the end of every experiment. Fluorescence time courses were normalized and converted into fractional activity Y_f as described for inactivation. The obtained Hill plots were then analyzed in analogy to equation S4, in which the inactivator concentration was replaced by the activator concentration, and the IC_{50} by the EC_{50} (as in equation S2 but for varied activator concentration at constant transporter concentration).

Pyrene Excimers. Aqueous solutions of pyrenebutyrate were prepared by addition of 1 equivalent of NaOH to pyrenebutyric acid in water. Solutions of pyrenebutyrate (120 μM)

in buffer (2 ml, 10 mM $Na_mH_nPO_4$, pH 7.4) with **N14**, **N41** or polyarginine (0 - 50 μl of concentrated stock solutions) were gently mixed, and the fluorescence emission spectra were measured with λ_{ex} 340 nm. The obtained spectra were normalized at 375 nm, and fractional excimer emission intensities at 470 nm (I_{470} / I_{375}) were plotted as a function of polymer concentration and subjected to Hill analysis (Figure S1).

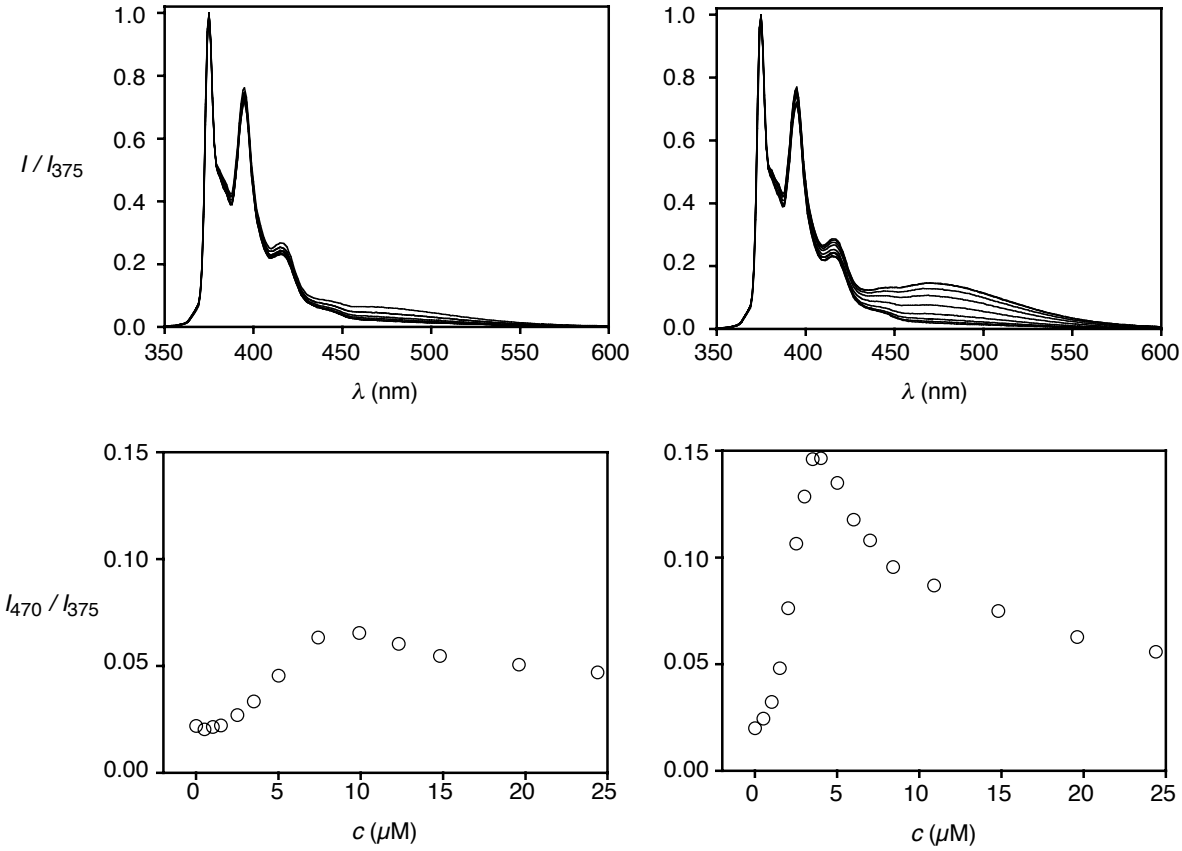


Figure S1. (Top) Fluorescence emission spectra of pyrenebutyrate (120 μM) in water (10 mM $Na_mH_nPO_4$, pH 7.4) in the presence of **N14** (left) and **N41** (right); excitation at 340 nm. (Bottom) Relative excimer emission intensity (I_{470} / I_{375}) as a function of **N14** (left) and **N41** (right) concentration.

6. Dependence on Membrane Fluidity (DPPC-LUVs ⊃ CF)

Preparation of DPPC-LUVs ⊃ CF.[S5] A thin lipid film was prepared by evaporating a solution of 25 mg DPPC in 1 ml MeOH/$CHCl_3$ (1:1) on a rotary evaporator (40 °C) and then *in vacuo* overnight. After hydration (> 30 min) with 1.0 ml buffer (10 mM Tris, 10 mM NaCl, 50 mM CF, pH 7.5) at 60 °C (which is well above the phase transition temperature)[S6] the resulting suspension was subjected to >5 freeze-thaw cycles (liquid N_2, 60 °C water bath). The extruder was assembled and placed on top of a hot plate to warm up to 60 °C, which was controlled by measuring the temperature inside the heating block. The vesicle suspension was >15 times extruded through a polycarbonate membrane (pore size 100 nm) and extravesicular components were subsequently removed by size exclusion chromatography (Sephadex G-50, Sigma-Aldrich) with 10 mM Tris, 107 mM NaCl, pH 7.5. Final conditions: ~2.5 mM DPPC; inside: 10 mM Tris, 10 mM NaCl, 50 mM CF, pH 7.5; outside: 10 mM Tris, 107 mM NaCl, pH 7.5.

PGON Activity in DPPC-LUVs ⊃ CF.[S3,S5] 25 or 50 μl DPPC-LUVs ⊃ CF were added to 1975 or 1950 μl gently stirred, thermostated buffer (10 mM Tris, 107 mM NaCl, pH 7.5) in a disposable plastic cuvette. The mixture was equilibrated for >10 min and temperatures were assumed to be the same as that of the circulating water bath. Fluorescence intensity was monitored during addition of 20 μl PGON14 stock solution (50 μM in DMSO, 0.5 μM final concentration) at t = 0 min, and addition of 40 μl 1.2 % (aq.) triton X-100 at the end of every experiment. Normalization of fluorescence time traces and conversion into transmembrane activity Y was performed as described above for EYPC-LUVs ⊃ CF.

7. Dependence on Surface Potentials (EYPC/EYPG-LUVs ⊃ CF)

Preparation of EYPC/EYPG-LUVs ⊃ CF. Thin lipid film were prepared by evaporating a 1 ml MeOH/$CHCl_3$ (1:1) solution of 25 mg lipids in molar ratios 10:0, 9:1, 7:3, and 1:1 of EYPC and EYPG on a rotary evaporator (40 °C) and then *in vacuo* overnight. After hydration (> 30 min) with 1.0 ml buffer (10 mM Tris, 10 mM NaCl, 50 mM CF, pH 7.5), the resulting suspension was subjected to >5 freeze-thaw cycles (liquid N_2, 40 °C water bath), and >15 times extruded through a polycarbonate membrane (pore size 100 nm). Extravesicular components were removed by size exclusion chromatography (Sephadex G-50, Sigma-

Aldrich) with 10 mM Tris, 107 mM NaCl, pH 7.5. Final conditions: ~2.5 mM EYPC; inside: 10 mM Tris, 10 mM NaCl, 50 mM CF, pH 7.5; outside: 10 mM Tris, 107 mM NaCl, pH 7.5.

PGON Activity in EYPC/EYPG-LUVs ⊃ CF. 25 μl of each lipid preparation were added to 1975 μl gently stirred, thermostated buffer (10 mM Tris, 107 mM NaCl, pH 7.5) in a disposable plastic cuvette. The time-dependent change in fluorescence intensity I_t (λ_{exc} = 492 nm, λ_{em} = 517 nm) was monitored during the addition of PGON14 (20 μl stock solution in DMSO, 1 nM - 100 μM final concentration) at t = 0 min, and addition of 40 μl 1.2 % (aq.) triton X-100 at the end of every experiment. Time courses of I_t were normalized to fractional intensities I_f and analyzed using equations S1 and S2 as described above.

8. Dependence on Membrane Potentials (EYPC-LUVs ⊃ ANTS/DPX)

Preparation of EYPC-LUVs ⊃ ANTS/DPX.[S7] A thin lipid film was prepared by evaporating a solution of 25 mg EYPC in 1 ml MeOH/$CHCl_3$ (1:1) on a rotary evaporator and then *in vacuo* overnight. After hydration (> 30 min) with 1.0 ml buffer (10 mM Hepes, 20 mM KCl, 12.5 mM ANTS, 45 mM DPX, pH 7.5), the resulting suspension was subjected to >5 freeze-thaw cycles (liquid N_2, 40 °C water bath), and >15 times extruded through a polycarbonate membrane (pore size 100 nm). Extravesicular components were removed by size exclusion chromatography (Sephadex G-50, Sigma-Aldrich) with 10 mM Hepes, 100 mM NaCl, pH 7.5. Final conditions: ~2.5 mM EYPC; inside: 10 mM Hepes, 20 mM KCl, 12.5 mM ANTS, 45 mM DPX, pH 7.5; outside: 10 mM Hepes, 100 mM NaCl, pH 7.5.

Membrane Polarization.[S7] x μl K^+-buffer (10 mM Hepes, 100 mM KCl, pH 7.5), 20 μl safranin O (6 μM in H_2O) and 50 μl EYPC-LUVs ⊃ ANTS/DPX were added to 1950-x μl gently stirred, thermostated Na^+-buffer (10 mM Hepes, 100 mM KCl, pH 7.5) in a disposable plastic cuvette. By assuming no K^+ in the Na^+-buffer, the resulting membrane potential ψ can be calculated according to the Nernst equation S5

$$\psi\,[\mathrm{mV}] = 59 \times \log([K_{out}]/[K_{in}]) \qquad \text{(S5)},$$

where $[K_{out}]$ and $[K_{in}]$ are the potassium concentrations inside and outside of the vesicle. During the addition of 20 μl valinomycin stock solution (60 μM in DMSO), the emission of safranin O (λ_{exc} = 522 nm, λ_{em} = 581 nm) was monitored, which gradually increased according

to the expected built-up of membrane potential until a plateau was reached. Lysis was initiated by addition of 20 μl melittin stock solution (200 μM in H_2O). The calibration curve was obtained by plotting the averaged safranin O fluorescence emission intensity of three measurements *versus* the calculated membrane potential. A linear correlation was found between −60 mV and −140 mV.

PGON Activity in EYPC/EYPG-LUVs ⊃ CF.[S6] x μl K^+-buffer (10 mM Hepes, 100 mM KCl, pH 7.5), 20 μl safranin O (6 μM in H_2O) and 50 μl EYPC-LUVs ⊃ ANTS/DPX were added to 1950-x μl gently stirred, thermostated Na^+-buffer (10 mM Hepes, 100 mM KCl, pH 7.5) in a disposable plastic cuvette. Simultaneously recording the emission of safranin O (λ_{exc} = 522 nm, λ_{em} = 581 nm) and ANTS (λ_{exc} = 353 nm, λ_{em} = 510 nm) gave the applied membrane potential after addition of 20 μl valinomycin stock solution (60 μM in DMSO) by comparing the safranin O emission intensity with the calibration curve and the transmembrane activity of PGON14 after addition of 20 μl PGON14 stock solution (0.1 mM, 0.3 mM, and 1 mM in DMSO, i.e. 1, 3, and 10 μM final concentration) and 20 μl melittin stock solution (200 μM in H_2O) by normalizing and analyzing the ANTS emission intensity using equations S1 and S2 as described above.

9. Lactate Sensing[S3]

Detection of Enzyme Activity. 20 μl of 50 mM (L)-lactate in H_2O (adjusted to pH 6.5 with NaOH), 10 μl of 22000 U/ml catalase in H_2O, and 10 μl lactate oxidase (0, 10, and 50 U/ml in H_2O) were added to 160 μl 50 mM Na_2HPO_4 (pH 6.5) at 37 °C. Final concentrations: 5 mM (L)-lactate; 1100 U/ml catalase; 0, 0.5, and 2.5 U/ml lactate oxidase. Aliquots (5 μl) of the reaction mixture were withdrawn after defined time intervals and diluted with 20 μl 0.4 M NaOAc/HOAc, pH 4.5. 25 μl of CB hydrazide stock solution (1 mM) in DMSO was added and the reaction mixture was incubated for 30 min at 50 °C. 20 μl aliquots were subjected to fluorescence analysis as described above (*cf.* paragraph 5, equation S3).

Sensor Calibration. x (0.1 to 30) μl of 100 mM (L)-lactate in H_2O (adjusted to pH 6.5 with NaOH), 5 μl of 22000 U/ml catalase in H_2O, and 5 μl lactate oxidase (50 U/ml in H_2O) were mixed with 90-x μl 50 mM Na_2HPO_4 (pH 6.5) at 37 °C for 60 min (**concentration of lactate in the reaction mixture c_{rx}** = 0.1 - 30 mM). Aliquots (5 μl) of the reaction mixture were withdrawn after defined time intervals and diluted with 20 μl 0.4 M NaOAc/HOAc, pH

4.5. 25 μl of CB hydrazide stock solution (1 mM) in DMSO was added and the reaction mixture was incubated for 30 min at 50 °C. 20 μl aliquots were withdrawn, subjected to fluorescence analysis and converted into fractional activity Y_f as described above (*cf.* paragraph 4, equation S3). Hill analysis was carried out in analogy to equation S4 with equation S6 to give the concentration in the enzymatic reaction mixture which *finally* leads to 50% inactivation of membrane transport ($IC_{50,rx}$).

$$Y_f = Y_{\infty,f} + (Y_{0,f} - Y_{\infty,f}) / \{1 + c_{rx} / IC_{50,rx})^n\} \quad \text{(S6)},$$

The obtained c_{rx} = 1.8 ± 0.3 mM can be related to the IC_{50} by considering 10x dilution during derivatization and 100x dilution during transduction. This gives an IC_{50} = 1.8 ± 0.3 μM which is in excellent agreement with the IC_{50} = 1.5 ± 0.1 μM of pyruvate.

Determination of Lactate Content in Sour Milk. Sour milk (Valflora M-Dessert, Migros, CH) was centrifuged twice for 2 min at 14500 rpm. The upper, white phase was removed, and the lower, clear phase (milk serum) was filtered through glass wool. The filtered serum (source phase) was dialyzed against 1 ml 50 mM KH_2PO_4, pH 6.5 (receiving phase) for 3 h (cut off = 5 kDa), followed by the replacement of the source phase with fresh serum, and further dialysis for 16 h (total: 1.33fold dilution). The pH of the receiving phase was carefully controlled and remained constant during dialysis. Subsequently, x μl (**initial volume V_i**) of the receiving phase, 5 μl of 22000 U/ml catalase in H_2O, and 5 μl lactate oxidase (50 U/ml in H_2O) were mixed with 90-x μl 50 mM Na_2HPO_4 (pH 6.5) at 37 °C for 60 min (**total volume V_0** = 100 μl). Aliquots (5 μl) of the reaction mixture were withdrawn after defined time intervals and diluted with 20 μl 0.4 M NaOAc/HOAc, pH 4.5. 25 μl of CB hydrazide stock solution (1 mM) in DMSO was added and the reaction mixture was incubated for 30 min at 50 °C. 20 μl aliquots were withdrawn, subjected to fluorescence analysis and converted into fractional activity Y_f as described above (*cf.* paragraph 4, equation S3). Hill analysis was carried out in analogy to equation S4 and S6 with equation S7 to give the effective volume of milk serum which finally leads to 50% inactivation of membrane transport (V_{50}).

$$Y_f = Y_{\infty,f} + (Y_{0,f} - Y_{\infty,f}) / \{1 + V_i / V_{50})^n\} \quad \text{(S7)},$$

The obtained V_{50} = 3.3 ± 0.3 μl can be converted into the lactate concentration in the receiving phase c_{rec} using equation S8

$$c_{rec} = (V_0 \times IC_{50,rx}) / V_{50} \qquad \text{(S8)},$$

which gives c_{rec} = 54.5 mM. Considering the 1.33fold dilution during dialysis, one obtains a concentration of 72.7 mM (L)-lactate in the milk serum, which is in excellent agreement with expectations from the literature.[S3,S8] Blockage of membrane transport caused by the receiving phase treated in the same manner, but without exposing it to lactate oxidase, was not observed (*cf.* Supporting Online Material of ref. S3).

10. References

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