Monitoring the Transmembrane Proton Gradient Generated by Cytochrome $bo_3$ in Tethered Bilayer Lipid Membranes Using SEIRA Spectroscopy

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1. Synthesis of WK3SH

Figure SI 1 Scheme of the synthesis of the surface active tether molecules [WK3S]₂, WK3OAc, and WK3SH.

Dihydrocholesterol (dhChol), triethylene glycol di(p-toluenesulfonate) (TEGTs₂), potassium thioacetate (98%), and sodium hydride (98%, dry) were obtained from Sigma Aldrich. Sodium hydride was stored and handled inside an argon filled glove-box. Dry tetrahydrofuran was taken from an MS-Braun solvent purification system and anhydrous dimethylformamide (DMF anhydrous, 99.8%) was purchased from Aldrich and used as received. Reactions were monitored by thin layer chromatography (TLC) using Merck TLC Silica gel 60 F₂₅₄. Products were detected using a UV/Vis lamp (254 nm) and a vanillin-sulfuric acid staining reagent. Residual DMF was removed before staining by slight heating of the TLC plate with a heat gun (50 °C). Column chromatography was performed on Merck Silica Gel 60 (230-400 mesh). Nuclear magnetic resonance spectroscopy (¹H-NMR, ¹³C-NMR) spectra were recorded at room temperature using a Jeol ECX 400 or a Joel Eclipse 500. Two dimensional spectra (COSY, HMQC, HMBC) were recorded on a Joel Eclipse 500 or Bruker AV 700. Data were processed with Delta NMR Processing and Control Software (Version: 4.3.6 [Windows_NT]). The chemical shifts (d) are reported in parts per million (ppm) relative to tetramethylsilane. The spectra are referenced against the internal solvent (CDCl₃, δ¹H= 7.26 ppm). Data is reported as follows: s= singlet, d= doublet, t= triplet, q= quartet, quint= quintet and m= multiplet. High resolution ESI-MS spectra were recorded on an Agilent 6210 ESI-TOF, Agilent Technologies. The applied charge is reported as positive (+) or negative (−). The spray charge was set to 4 kV. The defractor charge is given in Volt (V). Data is reported in mass to charge (m/z). ATR-IR spectra for the characterization of the synthesized compounds were recorded on a Nicolet Avatar 320 FT-IR in the range 4000–500 cm⁻¹ on a diamond optical window. The absorption bands are given in wave numbers (cm⁻¹), intensities are reported as follows: s= strong, m= medium, w = weak.
Remark: A conceivable alternative approach, i.e. tosylation of dhChol and substitution by deprotonated oligoethylene glycol, was not pursued in order to maintain the configuration of the steroid C-O bond and, thus, a uniform orientation on the surface. Disubstitution of TEGTs₂ was not observed, possibly because dhChol tends to aggregate, which reduces its nucleophilicity. However, base-triggered elimination of the hydroxyl groups to cholestene was an often observed side reaction, which needed to be suppressed. For example, DMF exclusively led to the formation of elimination products, in contrast to pyridine, in which no conversion was observed at all.

**WK3OTs**

**Triethylene glycol cholestanyl ether toluenesulfonate** [2-(2-(2-(((3S,8R,9S,10S,13R,14S,17R)-10,13-dimethyl-17-((R)-6-methylheptan-2-yl)hexadecahydro-1H-cyclopenta[a]phenanthren-3-yl)oxy)ethoxy)ethoxy)ethyl 4-methylbenzenesulfonate]

![Chemical Structure](image)

In a glove box, a Schlenk flask was charged with dry NaH (95%, 73.4 mg, 2.91 mmol). Outside the glove box, 5α-cholestan-3β-ol (284 mg, 0.730 mmol) was added in a counter stream of argon. The flask with the powders was evacuated for 10 min, re-filled with argon and equipped with a bubbler from which a tube was leading directly into the air exhaust to discharge developing hydrogen. The following steps were performed while a permanent weak stream of argon through the apparatus was maintained. While cooling the flask in a brine/ice bath, dry THF (20 mL) was added and the resulting suspension was stirred for 15 min at -5 to 0 °C before the ice bath was removed and the suspension warmed up to room temperature for 30 min. Triethylene glycol-di-(p-toluenesulfonate) (3.00 g, 6.54 mmol) and anhydrous DMF (20 mL) were added and the reaction mixture was stirred at 90 °C (oil-bath) for 2 h. After the reaction was finished, the solvents were removed in high vacuum (40 °C oil bath). The oily residues were dissolved in ethylacetate which was also removed in high vacuum to yield a solid substance mixture which was dissolved under normal atmosphere in DCM and adsorbed on a small amount of silica gel. The product **WK3OTs** (246 mg, 0.356 mmol, 48%) was isolated by column chromatography (SiO₂, 4 × 25 cm, eluent mixture ethylacetate : hexane (400 mL 10:90; 500 mL 20:80; 400 mL 25:75; 200 mL 30:70; 600 mL 35:65; 800 mL 100:0). The reactant triethylene glycol-di-(p-toluenesulfonate) (1.34 g, 45% of original amount) was isolated as well and was pure enough for recycling.

**¹H-NMR** (500 MHz, CDCl₃): δ = 0.56-0.61 (m, 1H), 0.63 (s, 3H, Chol-CH₃), 0.77 (s, 3H, Chol-CH₃), 0.85 (dd, J = 2.4, 6.5 Hz, 6H, Chol-CH₂), 0.88 (d, J = 6.5 Hz, 3H, Chol-CH₃), 0.91-1.85 (m, 28H, Chol), 1.95 (td, J = 3.1, 12.3 Hz, 1H, Chol), 2.43 (s, 3H, Ts-CH₃), 3.18-3.25 (m, 1H, Chol-CHO[[-C₃H₄O-]₃]), 3.56-3.60 (m, 8H, [-C₂H₂O-]₃), 3.67 (t, J = 4.8 Hz, 2H, [-C₂H₂O-]₃), 4.14 (t, J = 4.8 Hz, 2H, [-C₂H₂O-]₃), 7.33 (d, J = 8.2 Hz, 2H, Ts), 7.79 (d, J = 8.2 Hz, 2H, Ts), 13C-NMR (100.6 MHz, CDCl₃): δ = 12.2 (Chol-CH₃), 12.4 (Chol-CH₃), 18.8 (Chol-CH₃), 21.3, 21.7 (Ts-CH₃), 22.7 (Chol-CH₃), 22.9 (Chol-CH₃), 23.9, 24.3, 28.1, 28.3, 28.4, 28.9, 32.2, 34.8, 35.6, 35.8, 35.9, 36.2, 37.1, 39.6, 40.2, 42.7, 45.0, 54.5, 56.4, 56.6, 67.2([-C₂H₄O-]₃), 68.8 ([-C₂H₄O-]₃), 69.4 ([-C₂H₄O-]₃), 70.5 ([-C₂H₄O-]₃), 70.6 ([-C₂H₄O-]₃), 71.1, 79.2 (Chol-
CHO), 128.1 (Ts-C_H), 129.9 (Ts-C_H), 133.0 (Ts-C_H), 144.9 (TsC_H). Using HMQC. IR (solid): ʋ = 2954 (m), 2928 (m), 2909 (m), 2890 (m), 2862 (m), 2850 (m)1596 (w), 1465 (m), 1450 (w), 1438 (w), 1416 (w), 1396 (w), 1381 (w), 1356 (s), 1303 (w), 1289 (w), 1262 (w), 1249 (w), 1233 (w), 1218 (w), 1210 (w), 1189 (m), 1175 (s), 1149 (m), 1132 (m), 1116 (m), 1099 (s), 1090 (s), 1075 (m), 1043 (m), 1016 (m), 985 (w), 978 (w), 952 (m), 943 (m), 919 (s), 884 (m), 862 (w), 846 (w), 833 (w), 819 (m), 805 (m), 780 (s), 732 (w), 706 (w) cm\(^{-1}\). HRMS (+ESI, 400V): m/z (%) = 697.4493 (100) [M+Na]\(^+\) (calc. 697.4472), 713.4220 (9) [M+K]+ (calc. 713.4212). R_f (SiO\(_2\), ethylacetate : hexane = 30 : 70) = 0.28.

[WK3S]\(_2\)

1,18-bis(((3S,8R,9S,10S,13R,14S,17R)-10,13-dimethyl-17-((R)-6-methylheptan-2-yl)hexadecahydro-1H-cyclopenta[a]phenanthren-3-yl)oxy)-3,6,13,16-tetraoxa-9,10-dithiaoctadecane

[WK3OTs] (50 mg, 0.074 mmol) and NaSH (contains approx. 30% H\(_2\)O, 12.5 mg, 0.222 mmol, corresponds to 2.1 equiv. NaSH) were given into a Schlenk flask which was evacuated for 10 min and re-filled with argon. Dry THF (5 mL) and anhydrous DMF (5 mL) were added and the reaction mixture was heated to 50 °C (oil bath) for 15 min before the solvents were removed in high vacuum at 40 °C (oil bath). The oily residue was dissolved in DCM and absorbed on a small amount of silica gel. The DCM was removed by rotary evaporation and the silica gel mixture was given on a silica gel column to purify the reaction mixture by column chromatography (silica gel, 2.5 × 10 cm, ethylacetate. hexane: 20:80 (200 mL), 25:75 (200 mL), 30:70 (200 mL)). The product [WK3-S]\(_2\) (32 mg, 0.026 mmol, 72%) was obtained as colorless oil.

\(^1\)H-NMR (500 MHz, CDCl\(_3\)): δ = 0.56-0.61 (m, 1H), 0.63 (s, 3H, Chol-CH\(_3\)), 0.77 (s, 3H, Chol-CH\(_3\)), 0.84 (dd, J = 2.0, 6.8 Hz, 6H, Chol-CH\(_3\)), 0.88 (d, J = 6.5 Hz, 3H, Chol-CH\(_3\)), 0.91-1.86 (m, 28H, Chol), 1.94 (d, J = 12.6 Hz, 1H, Chol), 2.79-2.97 (m, 2H, [-C\(_2\)H\(_2\)O\(_{-1}\)]), 3.20-3.26 (m, 1H, Chol-CHO[-C\(_2\)H\(_2\)O\(_{-1}\)]), 3.57-3.67 (m, 9H, [-C\(_2\)H\(_2\)O\(_{-1}\)]), 3.72 (t, J = 6.8 Hz, 1H, [-C\(_2\)H\(_2\)O\(_{-1}\)]). Using COSY. \(^{13}\)C-NMR (100.6 MHz, CDCl\(_3\)): δ = 12.2 (Chol-CHO), 12.4 (Chol-CHO), 18.8 (Chol-CHO), 21.3, 22.7 (Chol-CHO), 22.9 (Chol-CHO), 23.9, 24.3, 28.1, 28.3, 28.4, 28.9 ([C\(_2\)H\(_2\)O\(_{-1}\)]), 32.2, 34.9, 35.6, 35.8, 35.9, 36.2, 37.0, 38.5, 39.6, 40.1, 42.7, 45.0, 54.5, 56.3, 56.6, 67.3([C\(_2\)H\(_2\)O\(_{-1}\)]), 69.8 ([C\(_2\)H\(_2\)O\(_{-1}\)]), 70.5 ([C\(_2\)H\(_2\)O\(_{-1}\)]), 70.6 ([C\(_2\)H\(_2\)O\(_{-1}\)]), 71.1 ([C\(_2\)H\(_2\)O\(_{-1}\)]), 79.2 (Chol-CHO). Using HMQC. IR (solid): ʋ = 2961 (m), 2952 (m), 2926 (m), 2899 (m), 286 (m), 2846 (m), 1465 (m), 1445 (m), 1380 (m), 1364 (m), 1350 (m), 1332 (w), 1295 (w), 1260 (m), 1236 (w), 1211 (w), 1189 (w), 1170 (w), 1132 (m), 1103 (w), 1093 (s), 1029 (s), 955 (m), 930 (m), 905 (m), 877 (m), 841 (m), 799 (m), 732 (w), 705 (w), 665 (w) cm\(^{-1}\). HRMS (+ESI, 400V): m/z (%) = 1093.8328 (100) [M+Na]\(^+\) (calc. 1093.82620). R_f (SiO\(_2\), ethylacetate : hexane = 50 : 50) = 0.68.
**WK3SAc**

S-(2-(2-(2-(((3S,8R,9S,10S,13S,14S,17R)-10,13-dimethyl-17-((R)-6-methylheptan-2-yl)hexadecahydro-1H-cyclopenta[a]phenanthren-3-yl)oxy)ethoxy)ethoxy)ethyl) ethanethioate

**WK3OTs** (150 mg, 0.222 mmol) and potassium thioacetate were given into a Schlenk flask. The flask was evacuated for 5 min in high vacuum and re-filled with argon. A mixture of dry THF (15 mL) and anhydrous DMF (15 mL) was added and the resulting solution was stirred for 2 h at 50 °C (oil bath). After cooling down to room temperature DCM (30 mL) was added under normal atmosphere and the mixture was washed with 40 mL of water (after this first washing cycle, the organic phase appeared as an inhomogeneous mixture of organic phase and water while the aqueous phase appeared to be a clear solution). The aqueous phase was extracted with DCM (2 × 20 mL) and the combined organic suspensions were washed with brine (2 × 40 mL). Afterwards, the organic phase was dried over Na$_2$SO$_4$, filtered and the volatile compounds were removed by rotary evaporation. The resulting brown oil was dried over night in high vacuum to yield the product **WK3SAc** (123 mg, 0.212 mmol, 95%).

**$^1$H-NMR** (400 MHz, CDCl$_3$): $\delta = 0.55$-0.60 (m, 1H), 0.62 (s, 3H, Chol-CH$_3$), 0.77 (s, 3H, Chol-CH$_3$), 0.84 (dd, J= 1.9, 6.7 Hz, 6H, Chol-CH$_3$), 0.88 (d, J= 6.7 Hz, 3H, Chol-CH$_3$), 0.92-1.87 (m, 28H, Chol), 1.87-1.97 (m, 1H, Chol), 2.31 (s, 3H, S-CO-CH$_3$), 3.08 (t, J= 6.5 Hz, 1H, [-C$_2$H$_4$O-$\bar{\text{I}}$]), 3.19-3.27 (m, 1H, Chol-CHO[-C$_2$H$_4$O-$\bar{\text{I}}$]), 3.57-3.64 (m, 10H, [-C$_2$H$_4$O-$\bar{\text{I}}$]). $^{13}$C-NMR (100.6 MHz, CDCl$_3$): $\delta = 12.1$ (Chol-CH$_3$), 12.4 (Chol-CH$_3$), 18.7 (Chol-CH$_3$), 21.3, 22.7 (Chol-CH$_3$), 22.9 (Chol-CH$_3$), 23.9, 24.3, 28.1, 28.3, 28.4, 28.9 ([-C$_2$H$_4$O-$\bar{\text{I}}$]), 30.6 (S-CO-CH$_3$), 32.4, 34.8, 35.6, 35.8, 35.9, 36.2, 37.1, 39.6, 40.2, 42.7, 45.0, 54.5, 56.3, 56.6, 67.3([-C$_2$H$_4$O-$\bar{\text{I}}$]), 69.8 ([-C$_2$H$_4$O-$\bar{\text{I}}$]), 70.4 ([-C$_2$H$_4$O-$\bar{\text{I}}$]), 70.6 ([-C$_2$H$_4$O-$\bar{\text{I}}$]), 71.1 ([C$_2$H$_4$O-$\bar{\text{I}}$]), 79.2 (Chol-CHO), 195.6 (S-CO-CH$_3$). Using HMQC. IR (solid): $\tilde{\nu} = 2927$ (s), 2863 (m), 2849 (m), 1692 (s), 1465 (w), 1443 (w), 1414 (w), 1380 (w), 1352 (w), 1332 (w), 1294 (w), 1250 (w), 1213 (w), 1170 (w), 1129 (s), 1105 (s), 1031 (m), 1006 (w), 952 (m), 930 (w), 905 (w), 881 (w), 798 (w), 760 (w), 733 (w), 683 (w), 659 (w) cm$^{-1}$. HRMS (+ESI, 200V): m/z (%) = 601.4319 (100) [M+Na]$^+$ (calc. 601.42610). $R_f$ (SiO$_2$, ethylacetate : hexane = 50 : 50) = 0.68.

**WK3-SH**

2-(2-(2-(((3S,8R,9S,10S,13S,14S,17R)-10,13-dimethyl-17-((R)-6-methylheptan-2-yl)hexadecahydro-1H-cyclopenta[a]phenanthren-3-yl)oxy)ethoxy)ethoxy)ethyl) ethanethiol

**WK3SAc** (324 mg, 0.560 mmol) was dissolved in methanol (20 mL) and methanolic HCl (1.2 M, 20 mL). The solution was degassed by a stream of argon for 20 min. The argon stream was removed; the flask equipped with a reflux condenser and the reaction mixture was stirred at 90°C
(oil bath) for 3 h. After cooling down to room temperature, DCM (40 mL) was added and the resulting solution was washed with water (40 mL). The organic layer was extracted with DCM (2×30 mL) and the combined organic layers were washed with sat. NaHCO₃ (40 mL), water (40 mL) and brine (40 mL). The organic phase was dried over Na₂SO₄, filtered and the volatile compounds were removed by rotary evaporation. After drying over night in high vacuum, the product WK3-SH (266 mg, 0.495 mmol, 88%) was obtained as a thick, light yellow paste.

On the TLC plate, the starting material and the product have the same Rₜ value (eluent:ethylacetate:hexane = 50 : 50). However, under UV-light only the starting material shows a dark spot, so it indicates full conversion of starting material in the reaction mixture.

\[ ^1H-NMR \ (500 \text{ MHz}, \text{CDCl}_3) : \delta = 0.56-0.60 \text{ (m, 1H)}, 0.63 \text{ (s, 3H, Chol-CH}_3)_2, 0.77 \text{ (s, 3H, Chol-CH}_3)_3, 0.84 \text{ (dd, } J= 2.4, 6.5 \text{ Hz, 6H, Chol-CH}_3)_2, 0.88 \text{ (d, } J= 6.8 \text{ Hz, 3H, Chol-CH}_3)_3, 0.91-1.87 \text{ (m, 28H, Chol)}, 1.94 \text{ (d, } J= 12.6 \text{ Hz, 1H, Chol}, 2.66-2.71 \text{ (m, 2H, [-C}_2\text{H}_4\text{O}_3)_2], 3.20-3.26 \text{ (m, 1H, Chol-CHO[-C}_2\text{H}_4\text{O}_3)_3], 3.57-3.67 \text{ (m, 9H, [-C}_2\text{H}_4\text{O}_3)_3].} \]

\[ ^{13}C-NMR \ (100.6 \text{ MHz, CDCl}_3) : \delta = 12.2 \text{ (Chol-CH}_3)_2, 12.4 \text{ (Chol-CH}_3)_3, 18.8 \text{ (Chol-CH}_3)_2, 21.3, 22.7 \text{ (Chol-CH}_3)_2, 22.8 \text{ (Chol-CH}_3)_3, 23.9, 24.3, 24.4, 28.1, 28.3, 28.4, 29.0([-C}_2\text{H}_4\text{O}_3)_3], 32.2, 34.8, 35.6, 35.8, 35.9, 36.2, 37.1, 39.6, 40.1, 45.0, 54.5, 56.4, 56.6, 67.3([-C}_2\text{H}_4\text{O}_3)_3], 70.3 \text{ ([-C}_2\text{H}_4\text{O}_3)_3], 70.6 \text{ ([-C}_2\text{H}_4\text{O}_3)_3], 71.9 \text{ ([-C}_2\text{H}_4\text{O}_3)_3], 79.2 \text{ (Chol-CHO). Using HMQC. IR (solid): } \tilde{\nu} = 2949 \text{ (m), 2925 (s), 2901 (m), 2863 (m), 2848 (m), 1465 (m), 1443 (m), 1415 (w), 1380 (w), 1212 (w), 1193 (w), 1172 (w), 1131 (s), 1105 (s), 1092 (s), 1057 (m), 1031 (m), 977 (m), 954 (m), 931 (m), 903 (w), 880 (w), 840 (w), 820 (w), 800 (w), 732 (w), 667 (w) \text{ cm}^{-1}. \]

HRMS (+ESI, 400V): m/z (%) = 559.4236 (100) [M+Na]⁺ (calc. 559.41554), 575.3910 (20) [M+K]⁺ (calc. 575.38947), Rₜ (SiO₂, ethylacetate : hexane = 50 : 50) = 0.68.

2. Formation of SAMs of WK3-derivatives

The adsorption kinetics play a crucial role to control the composition of mixed SAMs. Figure SI 2 (right) shows the time-dependent increase of the ν(C-O) of 6MH (1059 cm⁻¹, Figure SI 2 left), WK3SH, (WK3S)₂, and WK3SAc (Figure 2 in the manuscript). Whereas 6MH and WK3SH can be fit using a monoexponential function with time constants of (0.9 ± 0.1) min and (1.8 ± 0.1) min, respectively, which is in line with an unhindered direct adsorption, the other two molecules show a biexponential time course with a slow and a fast time constant of (0.7 ± 0.1) min and (110 ± 4) min for (WK3S)₂, and (11 ± 1) min and (73 ± 3) min for WK3SAc. In the case of (WK3S)₂, the fast time constant is in line with the direct adsorption as for WK3SH (ca. twice as fast since one disulfide contains two WK3SH molecules), while the slow process can be associated with a sterically hindered binding due to already adsorbed molecules. A similar process might apply for WK3SAc; the direct binding occurs in a slower manner due to slower kinetics of the reaction of the thioacetate group with the gold surface.[1]

Due to the monophasic and fast kinetic of WK3SH (such as the one of 6MH with a similar time constant), this thiol compound is most suitable for the formation of mixed WK3SH/6MH SAMs with controllable compositions. (WK3S)₂ and WK3SAc, however, can be employed when the tBLM has to be constructed on a pure lipid tether SAM.
Due to the hydrophilic TEG-linker of WK3SH and the hydrophobic C6-chain of 6MH, a mixture of both surface-active molecules spontaneously produces phase-separated SAMs on Au electrodes.\[^2\] As we demonstrated previously, one can estimate the composition of such a phase-separated surface using the capacitances of the pure SAMs.\[^3\] In Figure SI 3 the frequency-weighted Cole-Cole plots of the EIS data of a WK3SH and a 6MH SAM are shown and yield specific capacitances of \( C_{WK3SH} = 0.85 \pm 0.05 \, \mu \text{F cm}^{-2} \) and \( C_{6MH} = 3.55 \pm 0.15 \, \mu \text{F cm}^{-2} \). The composition is calculated based on the fraction-weighted average of these two capacitances that yield the overall capacitance of the mixed WK3SH/6MH SAM (\( x_i \) denotes the fractions of the constituents of the SAM, WK3SH and 6MH, on the Au surface):

\[
C_{WK3SH/6MH} = x_{WK3SH} \cdot C_{WK3SH} + x_{6MH} \cdot C_{6MH} \quad (1)
\]

\[
x_{WK3SH} = \frac{C_{WK3SH/6MH} - C_{WK3SH}}{C_{6MH} - C_{WK3SH}} = \frac{C_{WK3SH/6MH} - 0.85 \, \mu \text{F cm}^{-2}}{2.7 \, \mu \text{F cm}^{-2}} \quad (2)
\]

Figure SI 2 Left: SEIRA spectrum of a 6MH SAM taking a spectrum of 1-propanol as reference. Right: Time-dependent increase of the C-O vibration bands at 1059 cm\(^{-1}\) of 6MH and at 1113 cm\(^{-1}\) of the three derivatives WK3SH, (WK3S)\(_2\), and WK3SAc and their respective time constants from mono or biexponential fit to the data.
Figure SI 3 EIS data of a pure WK3SH SAM and a pure 6MH SAM. The capacitance was determined as explained in the manuscript using the radius of the first half-circle.

### 3. Phase-separation of WK3SH/6MH SAM

It was shown in previous work by Whitesides et al.\[^4\] that phase-separation of binary SAM mixtures can be observed by analyzing the ratio of both SAM molecules on the surface as a function of different ratios of both thiols in solution. Later, Jeuken et al.\[^2\] showed for mixed SAMs of CPEO3 and 6MH that this approach can be applied to the capacitances obtained from EIS experiments. Figure SI 4 shows a plot of the capacitance of the mixed SAM and the respective surface fraction of WK3SH (from equation 2) as a function of the concentration of WK3SH in solution. The fact that this graph shows a sigmoidal shape, which can be fitted with a Hill function, points at a cooperative binding process and is characteristic for phase-separated binary SAM.

![Figure SI 4 Capacitance values of mixed WK3SH/6MH SAMs as a function of the fraction of WK3SH in the mixed WK3SH and 6MH propanolic solution. The capacitances were determined from the Cole-Cole plots as indicated in the main text and the ratio of WK3SH on the surface was calculated using equation 2. The line represents a fit with the Hill function that describes a cooperative binding behavior: \( y = C_1 + (C_1 - C_2) \cdot x^n / (k^n + x^n) \) with \( C_1 = 3.47 \pm 0.17 \ \mu F cm^{-2}, C_2 = 0.72 \pm 0.15 \ \mu F cm^{-2}, n = 6.5 \pm 1.4, k = 54 \pm 2, \chi^2 = 6.1 \cdot 10^{-2}. \)
Figure SI 5 shows cyclic voltammograms (CVs) of the reductive desorption processes of pure WK3SH and 6MH SAM as well as of a mixed WK3SH/6MH SAM (as performed previously for CPEO3 and 6MH on template stripped electrodes previously). The CVs of the pure SAMs show distinct peaks at different potentials of -1.05 and -0.9 mV for WK3SH and 6MH, respectively. The CV of the mixed SAM shows an overlap of two peaks with desorption potentials close to the ones of the pure SAMs pointing at a phase-separated SAM in which the major fraction of WK3SH molecules are surrounded by other WK3SH molecules, whereas 6MH molecules are surrounded by 6MH molecules. Based on this data, that resemble the behavior of CPEO3 and 6MH on flat Au electrodes, WK3SH and 6MH form phase-separated SAMs on nanostructured Au electrodes for SEIRA at ratios relevant for this work.

![Cyclic voltammograms of the reductive desorption of a pure WK3SH SAM, a pure 6MH SAM, and a mixed WK3SH/6MH SAM assembled on the SEIRA Au electrode (at a scan rate of 5 mV/s; 0.5 M NaOH under constant purging with Ar). Inset shows the baseline corrected reduction peaks.](image)

Figure SI 5 Cyclic voltammograms of the reductive desorption of a pure WK3SH SAM, a pure 6MH SAM, and a mixed WK3SH/6MH SAM assembled on the SEIRA Au electrode (at a scan rate of 5 mV/s; 0.5 M NaOH under constant purging with Ar). Inset shows the baseline corrected reduction peaks.

4. Equivalent circuit

![Equivalent circuit used for the analysis of the impedance data.](image)

Figure SI 6 Equivalent circuit used for the analysis of the impedance data. The $R_{\text{Spacer}}C_{\text{Spacer}}$-element describes the electric properties of the „spacer“ molecules, i.e. the 6MH and the triethylene glycol-chain of WK3SH. The cholesteryl-headgroup region and the POPC-bilayer region is described by the $R_{\text{Bilayer}}C_{\text{Bilayer}}$-element. The constant-phase element (CPE) accounts for the non-ideal electrical behaviour of the system visible as a frequency-dependent tail at low frequencies in the impedance spectra, which can be rationalized based on a combination of the flexibility of the WK3SH SAM and tBLM, the roughness of the electrode surface or the interfacial
polarization known as the Maxwell-Wagner-Siller effect. The CPE used in this work is defined as $Z_{CPE} = \frac{1}{Q(i\omega)^\alpha}$. According to Jovic et al. it is possible to calculate the capacitance of electrolyte solutions from a Q value using the equation: $C = Q(\omega_{\text{max}})^{\alpha-1}$. Although here, the system is more complicated, $C_{tBLM}$ can be calculated from the sum of the 2 capacitances and the CPE in series:

$$\frac{1}{C_{tBLM}} = \frac{1}{C_{\text{Blayer}}} + \frac{1}{C_{\text{Spacer}}} + \frac{1}{C_{\text{CPE}}} = \frac{1}{C_{\text{Blayer}}} + \frac{1}{C_{\text{Spacer}}} + \frac{1}{Q(\omega_{\text{max}})^{\alpha-1}}$$

According to Jovic et al., $\omega_{\text{max}}$ is chosen as the frequency at the maximum of the imaginary part of the impedance in the half circle of the Nyquist plot.

Note:
In general, the values of $R_{\text{Spacer}}$, $C_{\text{Spacer}}$, and $Q$ are less suitable indicators for tBLM formation. Comparing tBLMs and cyt $bo_3$-tBLM, their different behavior can be a consequence of the composition of the proteoliposomes, produced by fusion with E. coli inner membranes, in contrast to pure POPC vesicles. Beside the presence of proteins, E. coli inner membranes contain a negative net charge and lipids of different fatty acids that may affect $C_{\text{Spacer}}$ and $Q$ due to lipid-SAM interactions and a changed flexibility of the bilayer.

5. pH-dependent Nernst equation

The Nernst equation for the quinone/quinol ($Q/H_2Q$) couple is given by (with $n = 2$):

$$E = E_0 + \frac{RT}{nF} \ln \left( \frac{c(Q)\cdot c(H^+)^2}{c(H_2Q)} \right) = E_0 + \frac{RT}{nF} \ln \left( \frac{c(Q)}{c(H_2Q)} \right) - \frac{RT \cdot \ln 10}{F} pH \quad (3)$$

The apparent pH-dependent redox potential of the quinone/quinol couple can be expressed as

$$E_{0,\text{App}} = E_0 - \frac{RT \cdot \ln 10}{F} pH \quad (4)$$

and thus allows to determine the pH change under turnover conditions:

$$\Delta pH = pH_{tBLM-cyt bo_3+DUQ} - pH_{tBLM+DUQ} = \frac{E_{0,\text{App},tBLM+DUQ} - E_{0,\text{App},tBLM-cyt bo_3+DUQ}}{RT \cdot \ln 10/F} \quad (5)$$

6. Kinetics of quinone/quinol oxidation/reduction in the tBLM

Performing the redox titration in the opposite direction, i.e. while observing the oxidation with -500 mV as the reference spectrum, one obtains a shifted apparent redox potential of -121 mV and $n = 0.8$ (Figure SI 7, left: blue data). For both redox titrations (the oxidation and the reduction, the latter is described in the main manuscript) the SEIRA spectra have been acquired after no further changes could be observed in the SEIRA spectra, i.e. after ca. 5 min (reduction) and 1 h (oxidation) of continuous application of the potentials (Figure SI 7 right). Thus, the shift of the redox potential by ca. + 80 mV does not reflect the kinetic hindrance, but the change in the local pH. According to the pH-dependent Nernst equation for the quinol/quinone couple, the shift of 78 mV from -199 mV...
to -121 mV corresponds to a pH shift of -1.3 (equation 5), which is in line with a more acidic submembrane aqueous reservoir during oxidation due to the deprotonation of the quinol at the bilayer/electrode interface \((\text{QH}_2 \rightleftharpoons \text{Q} + 2 \text{H}^+ + 2 \text{e}^-)\). The fact that this pH change is measurable and thus the pH of the submembrane region and the bulk solution were not equilibrated while performing the redox titration (i.e. to the bulk pH of 7.4) indicates that the tBLM provides a good resistive quality, so that the transmembrane proton gradient can be maintained.

Figure SI 7 Left: Plot of the potential-dependent intensities of the 1611 cm\(^{-1}\) difference band of DUQ from the potential dependent SEIRA spectra, representing the redox titration of the tBLM+DUQ system. The intensities of the reduction against a potential of +400 mV and refer to the left axis; the blue data result from the oxidation with -500 mV as reference and refer to the right axis. Lines are the results of a Nernst fit. Right: Time-dependent evolution of the intensity of the 1611 cm\(^{-1}\) band of quinones measured with potential-induced time-resolved SEIRA difference spectroscopy.

7. CVs of the tBLM+DUQ system with \(O_2\) and while purging with Ar

Figure SI 8 CV of the tBLM+DUQ system without (red) and with purging with Ar (black). There is no effect on the DUQ reduction peak, but only a slightly higher current of \(O_2\) reduction at the electrode.
8. Cyt bo$_3$ on pure 6MH SAM

To derive if the the amide I/amide II ratio reflects a well oriented arrangement of proteins, we performed a reference experiment, in which we immobilized the cyt $bo_3$-rich *E. coli* inner membrane fragments electrostatically directly onto a pure 6MH SAM (Figure SI 9; incubation at concentration of 0.5 mg mL$^{-1}$ for 3 h). Both the cyt bo$_3$ spectrum in tBLM (subtracted by the tBLM spectrum to remove the contribution of the negative water band at ca. 1645 cm$^{-1}$; see Figure 3 from the main manuscript) and the cyt bo$_3$ spectrum immobilized on a 6MH SAM show comparable amide I/amide II ratios of ca. 1.2.

![Figure SI 9 SEIRA spectra of cyt bo$_3$ form E. coli inner membranes incorporated into tBLMs and immobilized electrostatically directly onto a pure 6MH SAM.](image)

9. Cyt bo$_3$ orientation and its effect on the net proton pumping

Cyt bo$_3$ pumps in total 4 protons from the cytoplasmic (N-side) to the periplasmic side (P-side) under turn-over condition per one water molecule that is produced.$^{[9]}$ 2 protons are directly translocated from the N-side to the P-side, 2 protons from the N-side are consumed by the chemical reduction to water, and 2 protons are released to the P-side by the quinol that transfers 2 electrons to heme b. In addition, in the presented tBLM system, the quinones are reduced by the electrode and thus each quinone removes 2 protons from the sub-membrane reservoir. Because the cyt bo$_3$ is likely to be present in both orientations, net proton transport due to these reactions will partially cancel each, depending on the exact ratio of the enzyme (Figure SI 10):

**N-side facing the electrode:** In total, 6 protons are removed from the sub-membrane reservoir; 2 protons are consumed by the quinone reduction, 2 protons are pumped across the membrane, and 2 protons are consumed by the reduction of 0.5 O$_2$.

**P-side facing the electrode:** The sub-membrane reservoir is acidified by 2 protons that are translocated across the membrane. The “chemistry protons” are removed from the bulk solution and
the quinone/quinol pool takes up 2 protons, but releases them again to the sub-membrane reservoir; thus these two processes do not affect the pH submembrane space.

Therefore, unless the cyt bo₃ preferentially orientates with its P-side facing the electrode, which is unlikely, the combined electrochemical reduction of the quinone pool and the proton transport of cyt bo₃ results in an alkalization of the submembrane reservoir. For instance, in the case of a 1:1 mixture, the directly translocated protons will cancel each and the alkalization occurs due to 2 protons ascribed to the reduction of a quinone molecule and 2 protons that are consumed by the cyt bo₃ in the chemical reaction to H₂O.

![Scheme of the net proton flux by the cyt bo₃/ubiquinol couple and its dependence on the orientation of cyt bo₃ in the tBLM (number of protons per water molecule produced or quinone reduced under turn-over conditions).](image)

**Figure SI 10** Scheme of the net proton flux by the cyt bo₃/ubiquinol couple and its dependence on the orientation of cyt bo₃ in the tBLM (number of protons per water molecule produced or quinone reduced under turn-over conditions).

**References**


