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Structure and Properties of Tethered Bilayer Lipid Membranes with Unsaturated Anchor Molecules

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Abstract

The self-assembled monolayers (SAMs) of the new lipidic anchor molecule HC18 [*Z*20-(*Z*-octadec-9-enyloxy)-3,6,9,12,15,18,22-heptaooxatetracont-31-ene-1-thiol], and mixed HC18/ β -mercaptoethanol (β ME) SAMs were studied by spectroscopic ellipsometry, contact angle measurements, reflection adsorption infrared spectroscopy (RAIRS), electrochemical impedance spectroscopy (EIS), and evaluated in tethered bilayer lipid membranes (tBLMs). Our data indicate that HC18, containing a double bond in the alkyl segments, forms highly disordered SAMs up to anchor/ β ME molar fraction ratios of 80/20 and result in tBLMs that exhibit higher lipid diffusion coefficients, relative to previous anchor compounds with saturated alkyl chains, as determined by fluorescence correlation spectroscopy. EIS data shows the HC18 tBLMs, completed by rapid solvent exchange (RSE) or vesicle fusion, form more easily than with saturated lipidic anchors, exhibit excellent electrical insulating properties indicating low defect densities, and readily incorporate the pore forming toxin, α -hemolysin. Neutron reflectivity measurements on HC18 tBLMs confirm the formation of complete tBLMs, even at low tether compositions and high ionic lipid compositions. Our data indicates HC18 results in tBLMs with improved physical properties for incorporation of integral membrane proteins (IMPs) and that 80% HC18 tBLMs appear to be optimal for practical applications such as biosensors where high electrical insulation and IMP/peptide reconstitution is imperative.

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Supporting Information

Electronic supplementary information (ESI) on additional contact angle data/analysis and neutron reflectivity data/additional fitting parameters is available free of charge via the Internet at <http://pubs.acs.org>.

Introduction

Many advances in our understanding of the physical properties, structure, and function of biomembranes have been achieved with membrane mimics such as solid-supported membranes (SSMs) and tethered bilayer lipid membranes (tBLMs). SSMs, first reported by Brian and McConnell, readily assembled on Si or glass substrates^{1,2}, exhibit limited stability with no control over the space between membrane and substrate that may impede the functional reconstitution of membrane proteins³ or lead to denaturation of the incorporated protein, through direct contact between extramembraneous protein domains and the underlying substrate.⁴

Bilayers tethered to a surface by hydrophilic segments or low molecular weight polymers^{5–14}, tBLMs, are an alternative to SSMs that exhibit improved stability and are being increasingly used as experimental platforms for studies of reconstituted membrane proteins^{15–23} and sensors^{10,24–26} that range from the detection of biological agents to pharmaceutical screening. In addition, tBLMs can directly address membrane-support proximity problems through tether/polymer design.

Within a broad range of structural possibilities, we have focused on tBLMs tethered to planar Au films via 1-mercapto(ethylene oxide)_n [HS(CH₂CH₂O)_nHS(EO)_n, *n* = 6 to 9; 1-mercapto(EO)_n] segments, laterally diluted on the Au surface by co-adsorption with β-mercaptoethanol (βME).^{7,27,28} These 1-mercapto(EO)_n oligomers are covalently linked to a dual-chain glycerol-based lipidic motif that intercalates into the bilayer leaflet proximal to the substrate, ligating the membrane to the solid surface. After an initial formation of a mixed self-assembled monolayer (SAM) of the tether lipid and βME, membrane completion is achieved either by precipitation of a dissolved phospholipid (lipid mixture) on the SAM by rapid solvent exchange (RSE)^{7,24} or by vesicle adsorption and fusion. Similar systems have been developed by others.^{29–31} The physical properties of the tBLM constructs can be controlled by the mixed SAM tether lipid/βME ratio, which, in turn, determines the rigidity of the proximal bilayer leaflet.^{7,32} SAMs of only tether lipids at the surface lead to tBLMs with excellent membrane resistances (see Table IV in Ref. 7); however, bilayer systems densely-packed with molecules anchored to the substrate may pose problems to the functional reconstitution of proteins.³³ On the other hand, tBLMs in which the tether lipids are diluted with βME show (a) membrane resistances only slightly reduced from that measured for fully-tethered membranes, (b) full fluidity of the distal phospholipid layers, and (c) only modestly reduced lipid diffusion in the proximal layers.³² These tBLMs have been shown to accommodate reconstituted proteins such as the exotoxin α-hemolysin (αHL) at high concentrations in a functional state.^{22,34,35}

In addition to the lateral dilution of the tether compound on the substrate, the physical properties of tBLM systems will depend on the untethered lipids and the tether compound, the hydrophobic alkyl and the hydrophilic tether segments. While unsaturated lipids, such as 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1,2-dioleoyl-*sn*-glycero-3-phospho-choline (DOPC) have been used in the completion of tBLMs²⁷, the effects of unsaturation in the tBLM tether compounds remained to be explored. In the current work, we characterize tBLMs using a new lipidic anchor molecule HC18 [(*Z*-20-(*Z*-octadec-9-enyloxy)-3,6,9,12,15,18,22-heptaooxatetracont-31-ene-1-thiol, (C₁₈ = oleoyl)] {Scheme 1}, containing one carbon-carbon double bond in each of the hydrophobic chains and demonstrate the formation of tBLMs with high electrical resistances and dynamic lateral diffusion, with high concentrations of anionic lipids, improved tBLM completion with vesicle fusion, and the reconstitution of αHL under conditions not observed with the saturated chain tethers WC14 [20-tetradecyloxy-3,6,9,12,15,18,22-heptaooxahexatricontane-1-thiol, C₁₄ (myrisoyl)]⁷ and FC16 [29-

hexadecyloxy-3,6,9,12,15,18,21,24,27,31-decaoxaheptatetracontan-1-thiol, C₁₆, palmitoyl]²⁷.

Materials and Methods³⁶

Hexa(ethylene oxide), oleyl alcohol (*Z*-octadec-9-enol, 85 %), and thiolacetic acid were purchased from Sigma-Aldrich Chemical Co. (Milwaukee, WI) and used without further purification. Tetrahydrofuran (THF) (Mallinckrodt, AR or North Strong Scientific, Phillipsburg, NJ) was distilled from calcium hydride immediately before use. All other solvents (AR grade) were used without purification and all reactions were carried out under nitrogen. The lipids POPC, DOPC, 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhyPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (sodium salt) (POPG), and 1-palmitoyl (d₃₁)-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC-d₃₁) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL) and used as received.

Materials Synthesis

The new lipidic anchor molecule HC18 was synthesized as generally outlined for WC14 (Scheme 1 in Ref. 7 and supplementary material) with the following modifications: (1) oleyl bromide, prepared from oleyl alcohol (chlorotrimethylsilane-LiBr/CH₃CN;³⁷ 83 %) was used in the conversion of IV to V, (2) the corresponding VII was a mesylate (ClSO₂CH₃/C₂H₅)₃N; 93 %), (3) conversion to HC18 from the corresponding VII mesylate was carried out in two steps (a) 1.5 eq. CH₃COS-Na⁺/MeOH, ≈ 90 %, and (b) .1 mol/L HCl/MeOH, reflux 8 h, ≈ 92 %.

Sample preparation

(A) SAM/mixed SAM preparation—For all substrates other than the neutron reflectometry measurements, NOCHROMIX[®]-cleaned silicon (100) wafers (Silicon, Inc., Boise, ID) were initially coated with chromium (≈ 2 nm) and then with Au (≈ 100 nm) by magnetron sputtering (Edwards Auto 306, UK). The freshly-coated Au wafers were immediately immersed in ethanolic solutions of either the pure tether compound (HC18, WC14, or FC16) or various mole fraction mixtures of HC18, WC14, or FC16 and βME (*c*_{total} = 0.2 mmol/L), expressed hereafter as x %, where x = mole fraction of the tether compound × 100 %). For the neutron reflectivity experiments, 76.2 mm (3.0 inch) diameter × 5 mm thick, phosphorous-doped silicon wafers (El-Cat, Inc., Waldwick, NJ) were sequentially coated with Cr (3 nm) and Au (15 nm) using a Denton Discovery 550 sputtering instrument (Denton Vacuum LLC, Moorestown). Film thickness uniformity was better than 3 % over the diameter of the wafer. Exposure of these films to ethanolic solutions of x % HC18 for ≈ 12h was identical to that just described.

(B) tBLM preparation—For tBLMs completed with RSE, lipid mixtures of the desired bilayer composition were dissolved in organic solvents at a concentration of 2.5 mg/mL. Zwitterionic lipid mixtures were dissolved in ethanol, mixtures containing anionic lipids were dissolved in a mixture of 95 % methanol, 4 % chloroform, and 1 % water by volume. SAM/mixed SAM-coated wafers were exposed to the lipid solution for 5 min and thereafter the organic lipid solution rapidly replaced by buffer (RSE method). Buffers for tBLMs prepared with RSE contained 10 mM Na₂HPO₄ and 100 mM NaCl adjusted to pH 7.4 using diluted HCl solution.

For tBLMs completed with vesicle fusion, vesicles were prepared from 2.5 mg lipid-dried films of the desired lipid composition. Lipids were suspended in 1 mL of buffer and sonicated until the solution became clear. The vesicles were extruded at least 13 times using a manual extruder (Avanti Polar Lipids, Inc., Alabaster, AL) equipped with two 100 nm

filters. For vesicle fusion, the vesicles were diluted to a final concentration of 1mg/ml and kept at room temperature. Silicon wafers coated with the mixed tether/ β ME SAMs were exposed to the vesicle solution for 1h and then rinsed with buffer. Buffers for the preparation and measurement of tBLMs completed by vesicle fusion were 50 mM K_2HPO_4 adjusted to pH 7.45 using HCl solution.

Electrochemical Impedance Spectroscopy (EIS)

EIS measurements were performed using a Solartron (Farnborough, United Kingdom) system (1287A potentiostat and 1260 frequency response analyzer) and using a Parstat 2273 (Princeton Applied Research, TN) with Power Suite software between 0.1 Hz and 100 kHz, with 10 logarithmically distributed measurement points per decade. Data were fitted using ZView software (Scribner Associates, Southern Pines, NC). The Au-coated wafers (20 mm $\times$ 40 mm) served as the working electrodes in a setup that allowed simultaneous EIS measurements in six separate electrochemical cells (volume, $V \approx 290 \mu\text{L}$) on each wafer, with the working surface area $A_{\text{eff}} \approx 0.32 \text{ cm}^2$ exposed to the solution. EIS data were normalized to the geometric surface area A_{g} . The roughness factor was ≈ 1.39 , estimated from the gold surface oxidation/oxide stripping charge. A saturated silver-silver chloride (Ag/AgCl/NaCl (aq., sat)) microelectrode (M-401F, Microelectrodes, Bedford, NH) was used as reference, which has the potential +196 mV vs. standard hydrogen electrode. The auxiliary electrode was a 0.25-mm-diameter platinum wire (99.99% purity, Aldrich) coiled around the barrel of the reference electrode. Measurements were carried out with 10 mV alternating current at 0 V bias versus the reference electrode in aerated solutions.

Spectroscopic Ellipsometry (SE)

SE data was collected on a Woollam M2000D ellipsometer (J. A. Woollam Co., Inc., Lincoln, NE) between 193 nm and 1000 nm aligned at a nominal incidence angle of $\approx 70^\circ$ from the surface normal. Calculations, including ellipsometric (optical) thickness and the exact incidence angle were determined using vendor supplied software ($WVASE^{\text{TM}}$). The reported SE thicknesses are the averages of at least four measurements on each sample wafer as a Cauchy layer built point-by-point on the optical constants of a clean, dry Au surface [successively treated with UV-ozone (15 min), water (15 min for complete removal of gold oxide layers), and finally dried (N_2)].

Contact Angle (CA) Measurements

Advancing water contact angles (CAs) were measured on a camera-based system (First 10 Angstroms, Portsmouth, VA) with vendor-supplied image capture and analysis software using the same substrates as those used in the SE measurements. The reported CAs are the average of three drops on each substrate (standard deviation $\pm 1^\circ$).

Reflection-Absorption IR Spectroscopy (RAIRS)

RAIRS spectra were recorded on FT-IR spectrometer Vertex 80v (Bruker, Inc., Leipzig, Germany) equipped with liquid nitrogen cooled MCT narrow band detector and horizontal reflection accessory. The spectral resolution was set at 4 cm^{-1} . Spectra were acquired by 400 scans at a grazing angle of 80° by using p -polarized light. The sample chamber and the spectrometer were evacuated during the measurements. The spectrum of a SAM of hexadecanethiol- d_{33} , $HS(CD_2)_{15}CD_3$, on Au was used as a reference. Parameters of the bands were determined by fitting the experimental contour to Gaussians using the GRAMS/AI 8.0 (Thermo Scientific) software.

Neutron Reflectivity (NR) Measurements and Data Analysis

NR measurements were performed at the NG1 and AND/R reflectometers at the NIST Center for Neutron Research.³⁸ Each tBLM sample was measured in three isotopically distinct solvent compositions of either D₂O-based buffer, H₂O-based buffer or a mixture of the both with a neutron scattering length density (nSLD), $\rho_n \approx 4 \times 10^{-6} \text{ \AA}^{-2}$, referred to as “CM4”. The stability of the tBLMs permits solvent composition exchanges *in situ* on the instrument, and therefore measurements on one physical sample, ensuring that the SiO_x/Cr/Au surface layers on the 76.2 mm (3 inch) Si wafers, which dominate the neutron interference patterns in the data, contribute in the same way to the overall data structure in subsequent measurements.

Because of the loss of phase information of the neutron scattering experiment, data evaluation proceeds by fitting the experimental results with models of the surface structure. We recently developed techniques²⁸ to evaluate bilayer structures at interfaces with composition-space models^{39,40} that parameterize the interface structure in terms of chemical compositions and connectivity and yield distributions of sub-molecular components across the interfacial region. Among other structural properties, this approach provides the density of lipid molecules within the bilayer of a tBLM, *i.e.*, the (average) area per phospholipid, A_0 . These results may be directly compared to lipid densities in vesicles membranes or fully hydrated multi-bilayer samples.^{41,42} In addition, the contrast-variation approach using isotopically distinct solvent compositions permits the determination of the bilayer completeness and the water distribution throughout the sample with high precision. Reflectivity curves were fitted using *ga_refl* software.⁴³ Confidence limits of the model parameter values were evaluated by Monte-Carlo resampling²⁷ of the data based upon the error bars of the individual data points and are reported as 68% confidence intervals.

Fluorescence Correlation Spectroscopy (FCS)

FCS spectra were obtained on tBLMs prepared with 5×10^{-3} mol % lissamine-rhodamine-DOPE (LR-DOPE) admixed to the DOPC solutions using a custom-built two-photon FCS system³² consisting of a Verdi 10 W continuous wave DPSS laser ($\lambda = 532 \text{ nm}$) that pumps a Mira 900F mode-locked titanium-sapphire laser (125 fs pulse-width, 76 MHz repetition rate, 1.8 W at $\lambda = 780 \text{ nm}$) from Coherent (Santa Clara, CA). The output wavelength from the Ti-sapphire laser can be tuned between 700 nm and 980 nm, and $\lambda = 840 \text{ nm}$ is used for exciting the lissamine-rhodamine labeled lipids. A neutral density (ND) filter attenuates the IR output to 5–10 mW before it enters the reflector turret of an Axiovert 200M inverted microscope (Carl Zeiss, Jena, Germany). The light then passes through a dichroic mirror (750dcspxr; Chroma Technologies, Rockingham, VT) and is focused using a $63 \times 1.2 \text{ NA}$ C-Apochromat water immersion lens (Carl Zeiss) corrected for the coverslip thickness (0.14 to 0.18) mm. The fluorescence is epi-collected, passed through a bandpass filter (et575/50m-2p, Chroma Technologies), focused via the microscope tube lens, re-collimated via an achromatically corrected doublet lens, split into two beams with a 50:50 beam splitter cube (Thorlabs, NJ) and focused via two achromatically corrected doublet lenses onto two separate avalanche photodiode detectors (SPCM-AQR14, Perkin-Elmer, Fremont, CA). The signal from the detectors is correlated in a hardware correlator (5000EPP; ALV, Langen, Germany).

Fluorescence autocorrelation spectra of labeled lipids diffusing in the plane of the tBLMs were acquired by scanning the interfacial films across the waist of the laser focus in ‘z-scans’ and analyzed to determine the lipid diffusion constants, D , as described previously.^{32,44}

Reconstitution of α HL

DPhyPC-completed 80 % and 20 % HC18- and WC14-tBLMs were monitored by EIS before and after exposure to α HL (140 nM) at room temperature for 60 min. The 80 % tBLMs were completed by vesicle fusion, while the 20 % compositions were completed by RSE as the vesicle-fused 20 % tBLMs were too defective to be detected by EIS.

Results

(SE and Advancing Contact angle (CA) of HC18/ β ME SAMs

Spectroscopic ellipsometric thickness (d) and advancing contact angle (CA) data of mixed HC18/ β ME SAMs as a function of increasing HC18 mole fraction are shown in Figure 1. As can be seen, the SE and CA data show parallel trends. From 0 % to 20 %, the d and CA values increase rapidly to ≈ 1.6 nm and $\approx 90^\circ$, respectively, leveling off thereafter. Comparison of the HC18 SE and CA data to that of WC14 (C_{14} , myrisoyl) is informative (Figure 1 and Table 1 in Ref. 7) The HC18 SAMs are thicker than the WC14 SAMs from 0 % to 60 % and thinner at all higher compositions, contrary to what might be expected due to the longer oleoyl chains in HC18. The CA data exhibit an analogous transition. The $CA_{(HC18)}$ are less than $CA_{(WC14)}$, at lower compositions, and greater than the $CA_{(WC14)}$, at higher compositions, with approximately equal values $\approx 30^\circ$ (see Figure S1 in the supplementary material section).

EIS of Tether/ β ME SAMs

EIS results of HC18/ β ME SAMs with direct comparison to those of WC14/ β ME and FC16/ β ME are shown in Figures 2A (30 %) and 2B (70 %). Figure 2A shows all SAMs exhibit nearly perfect semicircular Complex capacitance plots with the HC18/ β ME SAMs exhibiting slightly lower capacitance values. At 70 %, the EI spectra of the HC18 SAMs differ drastically from those of WC14 and FC16. While WC14 and FC16 produce low-capacitance SAMs of $\approx 1 \mu\text{F}/\text{cm}^2$, the HC18 SAMs exhibit about seven-fold higher capacitance values that do not differ significantly from those of the 30 % HC18 SAMs. EI spectra at 80 % HC18 SAM compositions were comparable to that for the 70 % HC18 SAMs (data not shown).

RAIRS spectra of tether/ β ME SAMs

Figure 3 shows the RAIRS spectral features of 20 % to 100 % SAMs in the C–H stretch region [Figures 3A (HC18), 3C (WC14), and 3E (FC16)] and in the mid-range region [Figures 3B (HC18), 3D (WC14), and 3F (FC16)]. In the C–H stretch region, bands at around $2928 \pm 1 \text{ cm}^{-1}$ and $2858 \pm 1 \text{ cm}^{-1}$ – assigned to the methylene asymmetric, ν_a , and symmetric, ν_s , vibrations, respectively⁴⁵ – dominate at the lower tether compositions, whereas the bands at $2966 \pm 1 \text{ cm}^{-1}$ and $2878 \pm 1 \text{ cm}^{-1}$ – assigned to the methyl asymmetric, ν_a (IP), and the symmetric, ν_s (FR) stretches, respectively⁴⁵ – become more prominent as the tether composition increases, for all three tether compounds. Noteworthy, the position of the methylene ν_a band is 2920 cm^{-1} , appearing at 2923 cm^{-1} for HC18 (Figure 3A), 2921 cm^{-1} for WC14 (Figure 3C), and 2920 cm^{-1} for FC16 (Figure 3E) in the 100 % SAMs (top spectra). In the mid-range region, [Figures 3B (HC18), 3D (WC14), and 3E (FC16)] an intense absorption band is found in the range from 1050 cm^{-1} to 1150 cm^{-1} , at all but the lowest of tether compositions, accompanied by higher wavenumber shoulders of variable intensity. Assigned as the EO asymmetric stretching vibration, $\nu_{as}(\text{C–O–C})$,^{46–48} the position of this band as an indicator of order in the EO segment^{47–53} will be discussed subsequently.

EIS of HC18 tBLMs

The electrochemical impedance (EI) spectral changes that occur upon RSE of an ethanolic DOPC solution with buffer in the presence of HC18 monolayers are consistent with the formation of bilayers on the anchor SAMs. Complex capacitance plots of the EI spectra as a function of the HC18 composition are shown in Figure 4. In all cases, the semicircular part of the EI spectra sharply decrease (compare Figures 4 A and B and Figure 2). After RSE, the high frequency semicircular arch now points to values spanning from ≈ 0.7 to $\approx 0.8 \mu\text{F}/\text{cm}^2$ depending on the tether/ β ME ratio (Figure 4A); more clearly exemplified in the expanded high frequency part of the EI spectra (Figure 4B). Taking into account a surface roughness factor of 1.39, such capacitance values are commensurate with the formation of a ≈ 3 nm thick dielectric layer with a relative dielectric constant of ≈ 2.0 to ≈ 2.3 over a Helmholtz layer with $\approx 8 \mu\text{F}/\text{cm}^2$ capacitance. This dielectric constant range is lower than that obtained for WC14 tBLMs RSE-completed with DOPC (≈ 2.9).²²

At all HC18 compositions, the high frequency (farthest left) parts of the complex capacitance spectra are similar. As the HC18 composition decreases, the plots begin to exhibit different characteristics as the frequency decreases. At 50 % HC18, the mid-frequency extremum of the spectrum no longer returns to the x-axis and the semicircular diameter is larger than that of the > 50 % HC18 tBLMs. At compositions < 50 % HC18, the mid-frequency extrema continue to “lift off” the x-axis (Figure 4B) and the spectra exhibit low frequency “tails” that increase in length and approximate additional features. The 20 % and 10 % spectra (Figure 4A) show the emergence of a third semicircular feature.

HC18 and WC14 tBLMs by Vesicle Fusion

We compared the propensities of the HC18 and the WC14 SAMs to form membranes by fusion with DOPC vesicles. Exposure of 20 % and 80 % SAMs of each tether to DOPC vesicle solutions gave EI spectra shown in Figure 5. For the 80 % SAMs, fusion resulted in the formation of a complete high frequency semicircular feature, i.e., mid-frequency minimum ($\text{Im } C \approx 0$), in the EI spectra. However, vesicle-fused 20 % HC18 and WC14 tBLMs exhibit EI spectra more closely resembling those of the corresponding SAMs (see Figure 2) showing only an emergence of a high frequency semicircular feature, indicating the inability to form intact tBLMs at this tether composition.

2D FCS of HC18, WC14, and FC16 tBLMs

The enhanced biological relevance of the 80 % HC18 tBLMs, alluded to in the RAIRS data showing increased flexibility of the SAMs (Figure 3), does not directly address lateral phospholipid mobility, i.e., fluidity. To this end, we measured 2D FCS on tBLMs formed with HC18, WC14 and FC16. Figure 6 displays representative spectra and Table 1 summarizes the lipid diffusion coefficients D calculated from such FCS results.³² The measurements reported here had fluorescent labels introduced into both membrane leaflets, presumably at a higher concentration in the distal membrane leaflet than in the proximal membrane leaflet, and the reported values are therefore a weighted average of the diffusive properties of these two monolayers. In an earlier, more detailed study³², we showed that the D value in the proximal leaflet of the WC14 tBLM is at least a factor 2 smaller than that in the distal leaflet. In this work, the HC18 tBLMs consistently showed compounded diffusion coefficients at $\approx 4 \mu\text{m}^2/\text{s}$, while D measured for WC14 and FC16 tBLMs did not exceed $2 \mu\text{m}^2/\text{s}$. This indicates that lipids supported by the unsaturated alkyl chain anchor HC18 show increased lateral mobility compared to the WC14 and FC16 tBLMs.

Reconstitution of α HL in High and Low Tether Composition HC18 and WC14 tBLMs

We investigated the reconstitution of α HL into DPhyPC-vesicle fusion-completed 80 % HC18 and WC14 tBLMs and RSE-completed 20 % HC18 and WC14 tBLMs. The reconstitution of α HL in tBLMs, readily apparent from characteristic changes in the EI spectra (Bode plots)^{22,24}, has been found to be difficult on high tether composition SAMs, as observed earlier with hybrid bilayer membrane (HBM) systems.³³ Exposure to α HL resulted in EI spectral changes (Figure 7, Bode plots) consistent with α HL incorporation in the 80% HC18 tBLMs (Figure 7A) as well as for the 20 % HC18 and WC14 tBLMs (data not shown; qualitatively similar to Figure 7A and that seen earlier²²) but not for the 80 % WC14 tBLMs (Figure 7B), which shows no changes.

Neutron Reflectivity (NR)

NR measurements of HC18 tBLMs focused on samples that formed well-structured bilayers on HC18/ β ME SAMs, for which tBLM formation failed on SAMs of WC14 (Ref. 7) and FC16 (Ref. 27). Samples include tBLMs with very low tether density (12 % HC18), a sample with a high proportion of anionic lipids of (40 % POPG), and a tBLM prepared using vesicle fusion (Table 2 and Figure 8) [See supplementary material for NR measurements and fitting parameters on additional HC18 tBLMs].

The completeness of all tBLMs exceeded 90%, demonstrating the ability of HC18 to promote bilayer formation under these conditions. Using the hydrocarbon thickness values of the outer lipid leaflet, areas per free lipid molecules in the outer lipid leaflet were calculated. For single-lipid tBLMs they are in agreement with published values for single-lipid stacked membranes at full hydration: $A_0 = 72.4 \text{ \AA}^2$ and $68.3 \text{ \AA}^2$ for DOPC and POPC, respectively⁴¹. The inner lipid leaflet consistently shows a larger hydrocarbon thickness than the outer lipid leaflet ranging from $16.1 \text{ \AA}$ to $17.2 \text{ \AA}$ for all measured tBLMs. This larger hydrocarbon thickness can at least partially be attributed to the ether linkage of the HC18 hydrocarbon chains to the glycerol group.⁵⁴ The hydrocarbon thickness of the inner lipid leaflet is independent from the HC18/ β ME ratio within the uncertainties of the measurement. The mole fraction of tether molecules in the inner lipid leaflet varies between 38% and 70%. The number of tether molecules per β ME at the surface is with 68% confidence in agreement with the ratio of the two molecules in the SAM-forming solutions (Row 9, Table 2) except for the samples prepared from a 12% HC18 solution, where the density of tether molecules per β ME at the surface is comparable to the samples made from 30% HC18 solutions.

The sub-membrane space of all measured samples, $d_{\text{tether}} = (10 \pm 2) \text{ \AA}$, is significantly thinner than those observed for WC14 and FC16 tBLMs, $(15 \pm 2) \text{ \AA}$ and $(18 \pm 3) \text{ \AA}$, respectively.^{7,27} The sub-membrane space contains the β ME molecules, the hydrated (EO)₆-glycerol segment of the HC18 molecule, and the lipid head groups of the free lipids in the inner lipid leaflet. The d_{tether} values are close to the current estimates for the thickness of an unperturbed phosphocholine head group layer of a lipid bilayer of $d_{\text{headgroup}} \approx 9.75 \text{ \AA}$ ²⁸. Therefore, the (EO)₆-glycerol chain of HC18 is highly disordered or exhibits a large average tilt with respect to the surface normal, a result that is in agreement with the RAIRS data (*vide supra*). The hydration of the sub-membrane space is lowest for the 80 % HC18 SAM with a fill factor $f_{\text{water}} = 7 \%$ of the total volume between the membrane and the substrate. 12 % and 30 % HC18 tBLMs showed larger hydrations, $f_{\text{water}} = 18\text{--}24 \%$, with no significant differences between the various samples.

Discussion

Characterization of the physico-chemical, spectroscopic and structural properties of HC18 in SAMs and completed tBLMs show this lipidic tether compound affects various aspects of molecular organization. HC18 contains a cis double bond in each of two oleoyl chains that can be expected to influence the orientation of the C₁₈ chain. Our SE, CA, RAIRS, and EIS data provide convincing evidence that the HC18 SAMs and tBLMs are consistently different from those of WC14 and FC16 with more disorder in the alkyl and EO segments.

SE and CA—The gradual increase in SE thickness for the HC18 SAMs from 0 to 100 % HC18 (Figure 1) suggests the HC18/ β ME surface concentrations correlate with the solution concentrations. The HC18/ β ME solution/surface concentration correlation is quantified further in the NR analysis (*vide infra*), albeit within relatively large uncertainties. Analogous SE increases were observed for 0 to 100 % WC14 and FC16 SAMs (Refs. 7 and 27, respectively), though the HC18 SAMs exhibit some differences at the higher composition end, i.e., for WC14 and FC16, SE thickness did not increase above 80 % tether whereas the 100 % HC18 SAM is significantly thicker than the 80 % and 90 % SAMs. This suggests a structural rearrangement/reorganization in the 100 % HC18 SAMs, which is strongly supported in the RAIRS data (Figure 3) and discussed subsequently.

At the higher compositions, the HC18 SAMs, with longer alkyl segments, are thinner and less hydrophobic (Figure 1) than the corresponding WC14 SAMs (Table 1 in Ref. 7), whose SE and CA values are consistent with an “upright” structure orienting the polymethylene chains along the substrate normal.⁴⁵ The CA_{100 % HC18 SAM} values are similar to those of disordered alkanethiol SAMs⁴⁵ and reflect greater exposure of the underlying methylenes to the probing liquid (water).

Interestingly, at the lower compositions the HC18 SAMs exhibit slightly higher hydrophobicity than the corresponding WC14 and FC16 SAMs [see Figure S1 (supplemental materials) for direct comparison as well as Table 1 in Ref. 7 and Table 1 in Ref. 27]. This suggests a more uniform surface coverage of HC18 molecules and greater coverage of the surface by the oleoyl chains. WC14 and FC16 can be expected to cluster, i.e., patches of aggregated molecules on the surface, to maximize hydrophobic interactions along the saturated alkyl chains. At low tether compositions, clusters of the saturated tethers would result in significant surface areas covered with the hydrophilic β ME, thereby resulting in lower CA values. A more uniform distribution of HC18 molecules would result in fewer/smaller patches of β ME and greater surface coverage by the oleoyl segments affording a higher surface energy, i.e., more hydrophobic (higher CA values).

RAIRS—Order in SAMs may be assessed from infrared spectroscopy data. The C–H stretch region, containing the C–H stretches for the alkyl chains and the EO segments, is dominated by the bands of the alkyl chains because each tether has two of them and it is well known that prominent bands in this region are absent for EO segments in all but the most ordered SAMs.⁵¹ The position of the methylene ν_a band in this region is a strong metric for order in the alkyl chains^{45,55,56} appearing at 2917 cm⁻¹ for highly ordered SAMs and shifting to higher wavenumbers with increasing gauche conformations along the chain, i.e., disorder. For the SAMs of all three tethers the methylene ν_a band is found at or above 2920 cm⁻¹ at all compositions [Figures 3A (HC18), 3C (WC14), and 3E (FC16)] and at 2923 cm⁻¹ (100 % HC18), 2921 cm⁻¹ (100 % WC14), and 2920 cm⁻¹ (100% FC16). Thus, although the SAMs of all three tethers are disordered, the 100 % FC16 SAMs are the most ordered, as might be expected for the tether with the longest saturated chains, and the HC18 SAMs the least ordered.

The prominence of the methylene ν_a band collapses at the higher tether compositions suggesting a change in the orientation, i.e., the cant angle, of the alkyl chains. Although observed for all three tether compounds, the transition is different for HC18 [80 % $\rightarrow$ 100 %] relative to the saturated analogues [40 % $\rightarrow$ 60 %]. A prominent methylene stretching ν_a band is indicative of an alkyl chain cant angle of $\approx 30^\circ$, as is the case for alkane thiols on Au, whereas attenuation of this band is indicative of a smaller cant angle ($< 20^\circ$), as is the case for alkane thiols on Ag.⁴⁵ As the tether composition increases, lateral interactions between tether compounds should increase and the alkyl segments, decoupled from the Au surface, should be able to adopt an orientation more along the substrate normal (smaller cant angle), i.e., an “upright” structure, consistent with the CA values (Figure 1). HC18, with a propensity for a more uniform surface coverage (CA data) and weaker nearest-neighbor interactions, can be expected to require a higher surface concentration for such a transition, as is observed.

The bands in the midrange region are principally those of the EO segment.^{49,50} For the 100 % SAMs the $\nu_{as}(C-O-C)$ at $(1129 \pm 2) \text{ cm}^{-1}$ (top spectra Figure 3B, 3D, and 3F) indicates a lack of order over any significant length scale.⁵³ The spectra of the mixed SAM compositions exhibit the $\nu_{as}(C-O-C)$ bands at lower wavenumbers, 1113 to 1119 cm^{-1} (lower spectra in Figure 3B, 3D, and 3F). Although this frequency range straddles 1118 cm^{-1} , suggestive of EO segments in an ordered 7/2 helix oriented normal to the substrate, other bands for this helical conformation⁵³ are not observed. In addition, the mixed SAM $\nu_{as}(C-O-C)$ bands are accompanied by significant higher wavenumber shoulders, another indication of disorder in the EO segment.⁵¹ It is noteworthy that, for all the mixed HC18 SAM compositions, the $\nu_{as}(C-O-C)$ bands are consistently observed at 6 to 10 cm^{-1} lower wavenumbers relative to the corresponding WC14 and FC16 mixed SAMs. While a full explanation of this difference is lacking at the present time it does suggest that the conformation of the EO segment in the HC18 films are different from the saturated tether compounds.

The 100 % FC16 SAMs, with the most order in the alkyl segment (Figure 3F), have some order in the EO segment. A discernible band at 1347 cm^{-1} (Figure 3F, top spectrum) is evident; however, it is attenuated compared to OEO SAMs that have the entire EO segment in the 7/2 helical conformation.⁵¹ This suggests that order in the alkyl segment may influence the order in the EO segment for this type of tether compound.

EIS—SAM capacitance (C_{SAM}) values and the dielectric constants obtained from them are sensitive measures of film composition, especially to the presence of dipolar molecules. C_{SAM} s values (Table 3) can be derived from the complex capacitance plots (Figure 2), which upon modeling yield the constant phase element (CPE) coefficient and exponent. The CPE coefficient, Q_{SAM} , which determines the impedance $Z_Q = (Q_{\text{SAM}})^{-1}(\dot{\omega})^{-\alpha}$, can be considered as the capacitance of the dielectric layer formed by the anchor molecules if $\alpha \rightarrow 1$, where i is the complex unit, $\omega = 2\pi f$, f is the frequency in Hz, and α is the CPE exponent. Fits to a $Q_{\text{SAM}}(R_{\text{sol}}C_{\text{stray}})$ model, written here using the Boukamp notation⁵⁷, show α to be close to 1 (Table 3), validate the approximate equality $Q_{\text{SAM}} [\mu\text{F}\cdot\text{s}^{(1-\alpha)} \text{ cm}^{-2}] \approx C_{\text{SAM}} [\mu\text{F}\cdot\text{cm}^{-2}]$, and give the C_{SAM} values, from which the dielectric constants (ϵ) are calculated. Taking into account the near ideal capacitive features of the EI spectra, and assuming a Helmholtz plane capacitor model of the interface, we may write for the capacitance ratio:

$$\frac{C_{\text{SAM,HC18}}}{C_{\text{SAM,WC14}}} = \frac{\epsilon_{\text{HC18}}}{\epsilon_{\text{WC14}}} \times \frac{d_{\text{WC14}}}{d_{\text{HC18}}} \quad (1)$$

Using the C_{SAM} values for the 70 % HC18 and WC14 SAMs and the SE thickness values of ≈ 2.3 nm for HC18 (Figure 1) and ≈ 2.8 nm for WC14 (Ref. 7), one obtains $\epsilon_{\text{HC18}} \approx 5.6 \times \epsilon_{\text{WC14}}$. A similar comparison of the 70 % HC18 and FC16 SAMs [$d = 4.5$ nm (Ref. 27)] gives approximately the same result, $\epsilon_{\text{HC18}} \approx 3.5 \times \epsilon_{\text{FC16}}$. The higher ϵ_{HC18} s indicate an increased concentration of dipolar molecules, presumably water, in the oleoyl chains of the HC18 SAMs as compared to the myristoyl and the palmitoyl chains in the WC14 and FC16 SAMs, respectively, and is evidence of increased disorder in the higher tether concentration HC18 SAMs, consistent with our SE, CA, and IR data, and likely the basis of the increased FCS diffusion coefficients (Table 1).

A different situation was observed for the 30 % SAMs, where one obtains $\epsilon_{\text{HC18}} \approx \epsilon_{\text{WC14}}$ and $\epsilon_{\text{HC18}} \approx 0.7 \times \epsilon_{\text{FC16}}$ – using eq. (1), the calculated C_{SAM} values (Table 3), and SE thickness values of $d \approx 1.8$ nm for HC18 (Figure 1), ≈ 1.6 nm for WC14 (Ref. 7) and ≈ 2.1 nm for FC16 (Ref. 27), respectively. These estimates suggest the dielectric environments are essentially independent of the tether at this concentration, presumably due to a dominance of the solvent molecules at the interphase.

The evolution of EI spectral features for the DOPC-completed HC18 tBLMs at different tether compositions (Figure 4) may be rationalized utilizing a recent theoretical framework.⁵⁸ A defect-free tBLM should exhibit a plot in the complex capacitance plane in which all spectral points are confined to a perfect semicircle spanning from $\text{Re } C \rightarrow 0$ ($f \rightarrow \infty$) to $\text{Re } C \rightarrow C_{\text{tBLM}}$ ($f \rightarrow 0$), where: $C_{\text{tBLM}} = 1/(1/C_m + 1/C_H)$, C_m is the bilayer membrane capacitance and C_H is the Helmholtz capacitance. Defects in tBLMs give rise to low frequency features in the EI spectra such as lines, semicircles, or combinations thereof.¹² Several physical factors are responsible for the development of these features, most importantly the defect density (N_{def}). Other parameters are defect size (r_0), the thickness of the submembrane layer (d_{sub}) that separates the bilayer and the solid support, specific resistance (ρ) of the submembrane layer, and C_H . The low frequency “tails” seen in Figure 4A, elongating with HC18 concentration decrease attest for the increasing number of defects in tBLMs and/or decreasing specific resistance of the submembrane layer.⁵⁸ Submembrane resistance decrease is expected from NR data indicating water (electrolyte) molar fraction increase from 7 % to (18–24) % with tether percentage decrease from 80 % to (12–30) %. Perturbed or overlapping double or triple semicircular features, as seen for 10 % HC18 DOPC tBLMs in Figure 4A, are indicative of tBLM systems with significantly different lateral defect densities or when polydisperse defect sizes coexist.¹² The EIS features in Figure 4 demonstrate an inverse relationship between the defectiveness and tether density in the tBLMs.

Vesicle fusion occurs differently on the HC18 and the WC14 SAMs (Figure 5). At low WC14 concentration (20 %) the EIS spectra after interaction with DOPC vesicles (blue open diamonds) exhibits marginal differences from that of the as-prepared 20% WC14 SAMs [see Figure 5, Ref. 7 (20 % WC14) and Figure 2A (30 % WC14)] with only a small portion of the semicircular patch visible at the high frequency end. In contrast, for 20 % HC18, the spectrum after interaction with the DOPC vesicles (red open circle) exhibits over half of the high frequency semicircular feature, indicating that less defective tBLMs may be accomplished via the vesicle fusion process. At high concentrations (80%) both SAMs form tBLMs via vesicle fusion, as it is seen from the near perfect high frequency semicircular part of the complex capacitance plots [Figure 5 (filled red circles and blue diamonds)].

Such high tether density tBLMs, which are approaching the earlier HBM systems, may not exhibit enough fluidity to functionally reconstitute membrane proteins.³³ We estimate the 2D diffusion coefficients, D ($\mu\text{m}^2/\text{s}$), of fluorescently labeled DOPC in our high tether density tBLMs (Table 1) from FCS data, such as that shown for HC18 (Figure 6). DOPC

exhibits a significantly higher 2D diffusion coefficient relative to the saturated WC14 and FC16 ($D_{\text{HC18 tBLMs}} \approx 2 D_{\text{WC14 tBLMs}}$ and $D_{\text{FC16 tBLMs}}$).

The biological relevance of the increased fluidity of the high tether density (80 %) DPhyPC vesicle-fused HC18 tBLMs was demonstrated by the reconstitution of α HL (Figure 7A). The property of the reconstitution of α HL and, by extension, other integral membrane proteins (IMPs), over a broader range of tether density (20 % to 80 %) that includes the more electrically insulating high tether density compositions increases the potential usefulness of HC18-based tBLMs in biosensor applications.

NR data shows that the sub-membrane reservoir in the HC18 tBLMs is thinner, and holds less water, than the WC14 tBLMs [$d_{\text{sub,WC14}} = 1.5 \pm 0.2$ nm (Ref. 7) vs $d_{\text{ether,HC18}} = 1.0 \pm 0.2$ nm (Table 2)], despite identical EO segments. This has consequences for the electrical properties of the membrane, as the specific resistance of the sub-membrane space affects the total impedance of a defect.^{58,59} This contribution increases with the specific resistance and becomes dominant for large water-filled defects. Our results, as well as data published earlier by others,⁵⁹ point to a higher specific resistance of an electrolyte confined in the sub-membrane space as compared to that of the same electrolyte in the bulk. This is the consequence of reduced ion mobility and differences in the dielectric environment in the confined space and in the bulk.⁵⁹ Confinement of the EO segments to the smaller sub-membrane volume in the HC18 tBLMs localizes the EO segments closer to the solid surface. This is consistent with the observed small capacitances of HC18 SAMs and, as recent theoretical work predicts,⁵⁸ results in higher resistances of the HC18 tBLMs, despite their lower hydrophobic membrane thickness.

The more disordered HC18/ β ME SAMs (SE, CA, and RAIRS data) support the formation of electrically insulating, low defect tBLMs at < 20 % tether compositions (EIS data), lower than WC14 and FC16 (30 % tether).^{7,27} However, at all tether compositions, the HC18 tBLMs consistently show better electrical parameters than those of WC14 and FC16 tBLMs (EIS data). Importantly, the HC18 tBLMs are readily completed by vesicle fusion over a broad range of tether composition, affording the distinct advantages for incorporation of IMPs and alleviating restrictions in the RSE method, i.e., ethanol insoluble lipids.

Conclusions

The new tether lipid HC18 [*Z*20-(*Z*-octadec-9-enyloxy)-3,6,9,12,15,18,22-heptaoxatetracont-31-ene-1-thiol] with double bonds in its alkyl segments allows the formation of complete tBLMs on pre-formed HC18/ β ME SAMs by RSE or vesicle fusion. SE, CA, RAIRS, and EIS data lead to a consistent picture of more disorder in the HC18 SAMs that leads to enhanced bilayer fluidity in the tBLMs. The resulting membrane mimics exhibit biologically relevant fluidity and readily reconstitute the pore-forming toxin α HL at tether mole fractions up to 80%, not possible with WC14 and FC16 tBLMs containing saturated alkyl segments. In addition, HC18 affords the formation of stable and complete tBLMs with high anionic lipid content (e.g., 40% POPG/POPC). We demonstrate that the 80 % HC18 tBLMs appear to be optimal for practical applications such as biosensor applications where high electrical insulation and the ability for protein (peptide) reconstitution is an imperative and believe the HC18 tBLMs are an interesting model system for biological membranes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

SE thickness (black squares) and advancing CAs (white squares) of HC18/ β ME SAMs from 0–100 % HC18. The CA value a 100 % β ME SAM is $< 10 \pm 5^\circ$ (Ref. 7).

Figure 2. EIS spectra of HC18, WC14, and FC16 SAMs: (A) 30 % SAMs, (B) 70 % SAMs. Data was obtained on the same-batch SAM-coated, Au-sputtered Si wafers precluding any spectral differences due to surface roughness or other preparation parameters.

Figure 3. RAIRS spectra of the C-H and mid-range regions: (A) and (B) for HC18/ β ME SAMs, respectively, (C) and (D) for WC14 SAMs, respectively, and (E) and (F) for FC16 SAMs, respectively, at tether compositions between 20 % and 100 %.

Figure 4.

EI spectra of DOPC-completed HC18 tBLMs from 10 %–100 % HC18. (A) Full spectra in the frequency range from 0.1 to 65000 Hz, (B) high frequency part of the spectra shown in pane A. Arrows indicate frequency points on EI spectra. Data normalized to a geometric surface area. Experimentally determined roughness factor of the electrodes is 1.39.

Figure 5.

EI spectra of DOPC vesicle fusion on 20 % and 80 % WC14 and HC18 SAMs. Spectra normalized to geometric surface area. Experimentally determined roughness factor of the electrodes is 1.39.

Figure 6.
A representative FCS curve measured on DOPC-completed HC18 tBLM

Figure 7.

EI Bode spectra of tBLMs at 80 % tether content, completed with DPhyPC by vesicle fusion, before incubation (open diamonds) and after incubation (filled squares) with 140 nM α HL for 60 min. (A) HC18 tBLM and (B) on WC14 tBLM.

Figure 8.

Volume occupancy profiles of sub-molecular fragments of HC18-tethered tBLMs derived from NR measurements. (A) DOPC-completed tBLM on 12 % HC18 SAM (B): tBLM completed with a lipid mixture of high anionic content, 60/40 POPC/POPG. The panels show material distributions normalized to the average areas per free phospholipid in the tBLM (left axis), and normalized to 1 (volume fraction, right axis). Colored, solid lines visualize the fractional in-plane filling of space by different molecular moieties as indicated. Dashed lines show the sum profiles of the organic components of the tBLM, with the implication that the distance between the full space (solid black line) and the dashed line indicates space filled with buffer. See the supplemental information for a complete set of volume occupancy profiles for all measured samples.

Table 1

Lipid diffusivities in 70 % tether tBLMs.

Tether	2D diffusion coefficient, D ($\mu\text{m}^2/\text{s}$)
HC18	4.03 ± 0.21 (n = 6)
FC16	1.6 ± 0.16 (n = 5)
WC14	1.9 ± 0.18 (n = 6)

Table 2

Fit parameters and derived properties obtained from the neutron data analysis.^a

Parameter	DOPC HC18/ β ME = 12/88 RSE	DOPC HC18/ β ME = 80/20 RSE	DOPC HC18/ β ME = 30/70 vesicle fusion	POPC-d ₃₁ HC18/ β ME = 12/88 RSE	POPC/POPG = 60/40 HC18/ β ME = 30/70 RSE
Best-fit χ^2	1.21	2.40	2.86	1.32	1.38
Derived properties					
Area per lipid in the outer bilayer leaflet, $A_0/\text{\AA}^2$	73.3 _{-5.7} ^{+7.9}	76.5 _{-10.7} ^{+10.1}	70.7 _{-1.4} ^{+1.4}	68.2 _{-2.0} ^{+2.1}	97.3 _{-4.3} ^{+5.5}
Water fraction in sub- membrane space f_{water}	0.19 _{-0.04} ^{+0.04}	0.07 _{-0.06} ^{+0.06}	0.21 _{-0.02} ^{+0.02}	0.24 _{-0.02} ^{+0.02}	0.18 _{-0.03} ^{+0.03}
Membrane fit parameters					
Tether length, $d_{tether}/\text{\AA}$	9.4 _{-0.7} ^{+1.1}	8.2 _{-1.4} ^{+1.4}	11.3 _{-0.2} ^{+0.2}	13.2 _{-0.4} ^{+0.4}	11.4 _{-0.6} ^{+0.5}
Surface-proximal hydrocarbon chain length $d_{lipid}/\text{\AA}$	16.7 _{-1.1} ^{+1.1}	17.3 _{-1.4} ^{+1.0}	16.1 _{-0.3} ^{+0.4}	17.0 _{-0.6} ^{+0.7}	17.0 _{-0.5} ^{+0.6}
Surface-distal hydrocarbon chain length $d_{lipid}/\text{\AA}$	13.3 _{-1.3} ^{+1.2}	12.7 _{-1.5} ^{+2.1}	13.8 _{-0.3} ^{+0.3}	13.6 _{-0.4} ^{+0.4}	9.5 _{-0.6} ^{+0.5}
Molar fraction of tether in inner lipid leaflet, m_{tether}	0.38 _{-0.21} ^{+0.31}	0.60 _{-0.46} ^{+0.23}	0.53 _{-0.18} ^{+0.22}	0.70 _{-0.02} ^{+0.02}	0.64 _{-0.20} ^{+0.22}
Tether surface density, $P_{tether}/\text{molecules per } 100 \text{ \AA}^2$	0.59 _{-0.32} ^{+0.47}	0.97 _{-0.72} ^{+0.37}	0.85 _{-0.28} ^{+0.34}	1.27 _{-0.03} ^{+0.03}	1.11 _{-0.30} ^{+0.35}
Number of tether per β ME, n_{β ME	0.53 _{-0.33} ^{+2.80}	2.0 _{-1.6} ^{+8.0}	0.42 _{-0.18} ^{+0.35}	0.59 _{-0.06} ^{+0.13}	0.63 _{-0.26} ^{+0.48}
Completeness of bilayer, $v_{bilayer}$	0.92 _{-0.01} ^{+0.02}	0.93 _{-0.02} ^{+0.02}	1.00 _{-0.01} ^{+0.00}	0.99 _{-0.01} ^{+0.01}	1.00 _{-0.02} ^{+0.00}

^aConfidence limits (~ 68 %) were obtained using a Monte-Carlo resampling technique.²⁷ For a complete list of fit parameters see the supplemental materials.

Table 3

Comparison of the electrical parameters of SAMs.

Tether	30 % SAM		70 % SAM	
	$C_{\text{SAM}}^a, \mu\text{F}/\text{cm}^2$	α	$C_{\text{SAM}}^a, \mu\text{F}/\text{cm}^2$	α
HC18 ^b	6.56±0.20	0.996±0.001	6.23±0.20	0.996±0.001
WC14 ^c	7.18±0.21	0.996±0.001	0.92±0.03	0.997±0.001
FC16 ^d	7.56±0.19	0.996±0.0004	0.90±0.04	0.993±0.002

^aEIS data was fitted to a $QSAM(R_s C_{\text{stray}})$ model in the frequency interval from 25 to 65000 Hz. The approximate equality $QSAM \approx CSAM$ was assumed due to a closeness of α to 1. Presented $CSAM$ values are normalized to a geometric surface area. To obtain $CSAM$ values normalized to a real surface area divide by the roughness coefficient 1.39.

^b Average of 14 samples for 30% SAM and average of 13 samples for 70 % SAM.

^c Average of 10 samples for 30 % SAM and average of 5 samples for 70 % SAM.

^d Average of 12 samples for 30% SAM and average of 6 samples for 70 % SAM.