Langmuir-Schaefer deposition to create an asymmetrical lipopolysaccharide sparsely-tethered lipid bilayer.

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SUMMARY

Because they are firmly anchored to a noble metal substrate, tethered bilayer lipid membranes (tBLMs) are considerably more robust than supported lipid bilayers such as black lipid membranes (BLMs) [1]. The challenge to rapidly create asymmetrical tBLMs that include a lipopolysaccharide outer leaflet for bacterial model membrane research can be overcome by use of a Langmuir-Schaefer deposition protocol. Here we describe the procedures required to assemble and test asymmetric lipopolysaccharide (LPS) tethered lipid bilayers.

Key words: Tethered Bilayer Lipid Membranes, AC Impedance Spectroscopy, Langmuir-Schaefer deposition, Asymmetric Bilayers.

1. Introduction

Tethered membranes consist of a metal electrode, typically gold, to which lipid membrane tethering moieties are anchored [1]. The tethers can incorporate into one leaflet of a subsequently self-assembled lipid bilayer if using a solvent exchange technique [2], thus tethering the bilayer to the metal surface., tethered lipid bilayers are far more robust than solvent-based BLMs because they can be firmly held in place with a chemical attachment to the metal. tBLMs formation is also a more controllable and predictable process than forming unanchored BLMs. Unlike untethered BLMs which typically have lifetimes of the order of minutes to hours, tBLMs can remain intact for months at a time.

Depositing smooth (< 1.5 nm) ultra-pure (99.9995%) gold onto polymeric substrates with no contaminating intermediate metal layers does require process development. However, the resulting gold patterned electrodes are especially suited to reproducible, well-sealed tethered membranes. Immediately following gold deposition, coating of the tethering chemistries is from an ethanol solution directly onto the gold surface. This protocol describes the techniques required to use such pre-prepared tether-coated gold electrodes supplied from the company *SDx Tethered Membranes Pty Ltd* (SDx). These chemically coated electrodes (as 25 mm x 75 mm slides are shipped to the user in ethanol solution in hermetically sealed foil packages. The user is then provided the flexibility of forming tBLMs comprising their choice of lipids. These tether-coated electrodes may be stored at 4°C for > 1 year before use.

In order to create asymmetrical tethered bilayers that resemble the microbial membranes of Gram negative bacteria, liposome deposition techniques can be used [3], but the formation of these tBLMs is an 18 hour process, not including the time required for vesicle preparation. Here we describe, using a Langmuir-Schaefer deposition technique similar to that used for creating asymmetrical bilayers directly onto flat substrates [4,5], an alternative preparation method for an asymmetrical LPS tethered bilayer (**Figure 1**). This bacterial membrane model can be ready for use in less than two hours.

2. Materials

2.1. Electrode selection

Ready-made SDx electrodes comprising of 100% close-packed array 2-4 nm strands of ethylene glycol molecules terminated with a C20 phytanyl will act as the inner membrane leaflet for the tBLM. A thin layer of laminate with cavities over the six tethering electrodes is applied to each slide (**Figure 2**). *Be careful not to touch the tethering chemistries on the electrodes where the tBLMs are to be formed.*

2.2 Cartridge preparation kit

The SDx six-channel measurement electrodes can be assembled into a flow cell cartridge (**Figure 3A**) which then plugs into the *SDx tethaPod*™ swept-frequency electrical impedance spectroscopy reader (**Figure 3B**). Cartridge preparation kits are supplied by SDx Tethered Membrane Pty Ltd and consists of:

1. electrodes pre-coated with tethering chemistry (**Figure 3A**)
2. a flow cell cartridge top which incorporates a coated gold counter electrode (**Figure 3B**)

3. the alignment jig for attaching the electrode to the flow-cell cartridge (**Figure 3C**)
4. a silicon rubber pressure pad for attaching the electrode to the flow cell cartridge (**Figure. 3D**)
5. an aluminium pressure plate for attaching the electrode to the flow cell cartridge (**Figure 3E**)
6. a pressure clamp for attaching the electrode to the flow cell cartridge (**Figure 4B**)

2.3 Langmuir-Blodgett trough

A Langmuir-Blodgett trough is a Teflon lined vessel with barrier arms for controlling the surface pressure. The main elements of a Langmuir-Blodgett trough are (**Figure 5**):

1. A Teflon lined trough filled with a liquid substrate that the monolayer will be deposited onto;
2. Barrier arms (also usually made of Teflon) which will open and close to increase or decrease the monolayer area and thus change the area per molecule and surface pressure accordingly;
3. A Wilhelmy plate (usually made from filter paper or platinum) connected to an electrobalance for measuring surface pressure.
4. A dipping arm for raising or lowering a solid substrate for a Blodgett or Schaefer dip.

Some advanced troughs may include a laser alignment system to ensure that solid substrates are aligned perfectly parallel with the liquid surface for Langmuir-Schaefer deposition.

A Langmuir-Blodgett deposition is where the solid substrate is raised or lowered vertically through the monolayer on the liquid subphase. Whereas a Langmuir-Schaefer

deposition is where the solid substrate is pushed horizontally through the monolayer (**Figure 1**).

2.4 Solutions

1. The preferred subphase solution for the Langmuir-Blodget trough is 5 mM $CaCl_2$ in deionized water (dH_2O) when depositing LPS (**Note 1**).
2. For AC electrical impedance spectroscopy measures, this solution can be exchanged with 100 mM NaCl in milliQ H_2O at a pH of less than 7.5. An appropriate buffer, such as tris-buffer can be used to maintain a constant pH.
3. Lipopolysacharride (rough strains) from *Escherichia coli* J5 Rc mutant dissolved in a mixture of 60 % chloroform, 39% methanol and 1% H_2O. *Use of a fume cabinet and appropriate personal protective equipment is strongly advised when dealing with chloroform solutions.*

3. Methods

3.1 Preparing Langmuir-Trough

1. Because of the use of chloroform containing solutions, we strongly recommend the Langmuir-Blodgett trough be situated in an appropriate fume cabinet or well-ventilated area.
2. Ensure empty tough is clean of any residual surfactants and lipids by wiping surfaces with Kimwipes^{TM} or other suitable laboratory tissue and 100% chloroform solution. Allow chloroform to evaporate.
3. Fill the trough with 5mM $CaCl_2$ in H_2O solution.
4. Expand barrier and then do an isotherm reading to ensure that trough is clean of any contaminants (confirmed by a surface pressure of <0.5 mN m in the fully closed

position). If the liquid surface is not clean, then surface contaminants can be removed by the suction of the top of the liquid layer using a suitable syringe connected to a vacuum pump and solvent trap. If the surface is particularly unclean, then empty trough and repeat the chloroform cleaning process.

5. With barrier(s) positioned to approximately half-open, using a 25-100 μL glass syringe, place LPS solution, dropwise, onto the surface of the $CaCl_2$ solution in multiple locations. Pressure readings via the Wilhelmy plate will begin to significantly increase when LPS surface saturation is reached. Then open the barriers and add further LPS if needed. Perform an isotherm measurement to ensure sufficient LPS surface saturation (**Note 2**). The isotherm process can be repeated a number of times to ensure that a stable monolayer has been formed (**Figure 6A**).

3.2 Langmuir-Shaefer Deposition.

1. To reduce free-liquid surface vibration, switch off any ventilation fan of the safety cabinet surrounding the Langmuir-Blodgett trough.
2. Attach laminate covered electrode slide with tethering chemistries to dipping platform with the electrodes facing down (**Figure 1**). Electrodes must be positioned ~ 5° from parallel to the LPS monolayer normal and *not perpendicular* (**Note 3**).
3. Close the barriers until the desired pressure for the LS dip is reached (generally over 30 mN m^{-1} works best) and hold at that pressure (**Figure 6B**).
4. At a slow rate (eg 2 mm / min) gradually dip electrodes through the surface LPS layer and into the aqueous subphase – *do not withdraw the slide as it needs to remain in an aquous environment.* A slight jump in surface pressure will be observed which should recover to approximately the starting pressure when the electrode makes contact with the monolayer (**Figure 6B**).

5. Keeping the electrode slide submerged in aqueous phase of the Langmuir trough, remove it from the dipping mechanism and raise the dipping mechanism.
6. Keeping it submerged, rotate the electrode slide such that the laminate is now facing the surface of the aqueous phase, and raise it gently, ensuring there is a bubble of aqueous solution covering each tBLM well. Proceed to cartridge preparation as rapidly as possible so as to prevent evaporation of the the aqueous layer surrounding the tBLMs.

3.3 Preparing cartridges

1. Place electrode slide into alignment jig so that the inverted "SDX" on the slide overlays the inverted "SDX" on the alignment jig (Figure 3B). This is to ensure the gold electrodes are oriented correctly.
2. Peel thin plastic protective cover from the underside of the flow cell cartridge. *Take care to leave the 0.1 mm flow cell laminate and adhesive layer in place*.
3. Position the flow cell cartridge over the alignment jig with the adhesive laminate facing the electrode (**Figure. 4A**). The numbers 1-6 on the cartridge should align with the 1-6 on the alignment jig. Introduce the short end of the flow cell cartridge nearest to well 6 into the matching slot in the alignment jig and then lower the cartridge onto the electrode.
4. Press the silicon rubber pressure pad onto the flow cell cartridge. Then lay the aluminium pressure plate over the assembly and insert into the pressure clamp.
5. Compress by ¾ of a turn of the knob and leave for 30 seconds (**Figure 4B**).
6. Gently remove the assembly from the pressure clamp and remove the aluminium pressure plate and silicon pressure pad. Take care to prevent flow cell cartridge separating from the electrode.

7. The electrode-flow cell cartridge assembly can be removed intact from the alignment jig by gently lifting from the underside of the electrode. The remaining exposed gold surfaces are not critical to bilayer formation. The functional gold tethering electrodes are protected within the flow cell cartridge assembly (**Figure 7A**).

3.4 Testing the bilayer using AC impedance spectroscopy

By inserting the assembled electrode within the flow cell cartridge into a tethaPod™ reader, the conductance and capacitance of the LPS – tethered membrane may be measured. A visual analysis of the Bode plots will determine whether an LPS layer has been formed (**Figure 8**). By measuring impedance and phase characteristics between 0.1 and 1000 Hz using AC impedance spectroscopy, the successful formation of a lipid bilayer will typically have a *phase angle minima* below ~50°. The corresponding frequency at the phase minima, for a well-sealed membrane, should ideally be below ~50 Hz.

4. Notes

1. The presence of calcium ions in the subphase solution is important as it has been shown that Ca^{2+} stabilises the LPS monolayer which results in being able to achieve higher surface pressures [4].
2. The LPS monolayer film needs to be compressed to just below the collapse point. The surface pressure of the film should increase from 0 mN/m to ~35 mN/m, see **Figure 6B**. The film can then held at constant pressure by means of a feedback mechanism that adjusts the trough area to maintain the pressure. It is essential that there is sufficient access for the electrode slide substrate to enter the dipping well.
3. When positioned parallel, the propensity for air-bubbles to be trapped in the laminate wells is greatly increased, preventing outer-leaflet assembly.

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Figure 1. Langmuir-Shaefer deposition graphic. Tether molecules attached to a gold electode can be dipped into a layer of compressed lipopolysaccharide molecules on the surface of the aqueous phase of a Langmuir-Blodgett trough.

Figure 2. Laminate covered electrode slide.

Figure 3 Components required to form a tBLM flow cell. ***A,*** Electrodes pre-coated with tethering chemistry ***B,*** A flow cell cartridge top ***C,*** Alignment jig for use when attaching the electrode to the flow cell cartridge ***D,*** Silicon rubber pressure pad used when attaching the electrode to the flow cell cartridge ***E,*** Aluminium pressure plate used when attaching the electrode to the flow cell cartridge.

Figure 4. ***A,*** the flow cell cartridge attached to the electrode slide on the alignment jig. ***B*** The pressure clamp used when attaching the electrode to the flow cell cartridge.

Figure 5. Photograph of a Langmuir-Blodgett trough set-up **A,** top view, **B,** side view. Legend to numbered labels: 1) Teflon barrier arm, 2) dipping arm, 3) electrosensor for Wilhelmy plate, 4) laser scanning arm (not used in this work), 5) liquid surface with monolayer deposited.

Figure 6. A, Pressure-area isotherms of a monolayer of Rc-LPS from *E. coli* at the air-water interface. The isotherm is repeated in four cycles. **B,** Pressure-area-time graph of the LS

dipping process. The red cure is surface pressure and the blue curve is barrier area. As the surface pressure increases to the target pressure for the LS dip the barrier area decreases. The arrow at approximately 1100 seconds indicates the point where the solid substrate makes contact with the LPS monolayer and is characterised with a slight pressure jump which recovers to the original pressure indicating a good LS dip.

Figure 7. ***A,*** assembled flow cell cartridge with electrodes. The flow cell cartridge provides the counter electrode which is overlayed onto the 6 tethering electrodes with a 0.1 mm gap for perfusion of reagents and buffer solutions. ***B,*** assembled flow cell cartridge fitted into a tethaPod™ AC impedance reader.

Figure 8. ***A,*** Bode plots of a newly formed asymmetric lipopolysaccharide tethered bilayer. The phase minima between 0.1 and 1000 Hz indicates the successful creation of the bilayer. **B,** demonstration of Bode plot where the bilayer failed to form correctly. In this instance, there is no recognisable phase minima below ~50°.