The Assembly and Use of Tethered Bilayer Lipid Membranes (tBLMs)

Charles Cranfield[1,2,4], Sonia Carne[3], Boris Martinac[1,2] and Bruce Cornell[3]

[1]Victor Chang Cardiac Research Institute, Darlinghurst, NSW 2010, Australia

[2]St Vincent's Clinical School, University of New South Wales,
Sydney, NSW 2052, Australia

[3]SDx Tethered Membranes Pty Ltd, Unit 6, 30-32 Barcoo St, Roseville, NSW 2069, Australia

[4]School of Life Sciences, University of Technology Sydney

Running title: Tethered Bilayer Lipid Membranes

[#]Corresponding author: Dr Charles Cranfield

University of Technology Sydney,

PO Box 123

Ultimo 2007, NSW, Australia

(TEL) +61-2-9514-4034

(e-mail) charles.cranfield@uts.edu.au

SUMMARY

Because they are firmly held in place, tethered bilayer lipid membranes (tBLMs) are considerably more robust than supported lipid bilayers such as black lipid membranes (BLMs) [1]. Here we describe the procedures required to assemble and test tethered lipid bilayers that can incorporate various lipid species, peptides, and ion channel proteins.

Key words: Tethered Bilayer Lipid Membranes, AC Impedance Spectroscopy, Ion Channels, Lipid Bilayers.

1. Introduction

Tethered membranes consist of a metal electrode, typically gold, to which tethering moieties are anchored preferably via benzyl disulfide groups (**Note 1**). The tethers incorporate into one leaflet of a subsequently self-assembled lipid bilayer, thus tethering the bilayer to the metal surface [1] (Fig.1). Interspersed with the tethers are *spacer* molecules that provide a *scaffolding* for the tether molecules. When the amphiphilic tethering molecules are interspersed with polar short-chained *spacer* molecules a volume between the metal surface and the bilayer is formed that creates an aqueous reservoir for ions crossing the membrane. Because they can be firmly held in place with a chemical attachment to the metal, tethered lipid bilayers are far more robust than solvent based BLMs. The formation of tBLMs is also a more predictable and controllable process than forming untethered BLMs. tBLMs remain intact for months, unlike untethered BLMs which typically have lifetimes of the order of minutes to hours.

The process of depositing smooth (< 1.5 nm) ultra-pure (99.9995%) gold onto polymeric substrates with no contaminating intermediate metal layers involves considerable process development. The resulting gold patterned electrodes are, however, ideally suited to the preparation of reproducible, well-sealed tethered membranes. The tethering chemistry is coated from an ethanol solution onto the gold surface immediately following gold deposition. This protocol describes the techniques required to use pre-prepared gold electrodes supplied from the company *SDx Tethered Membranes Pty Ltd* (SDx), the unique supplier in the world of such a tBLM platform. Gold patterned, chemically coated electrodes (as 25 mm x 75 mm slides (Fig. 2A)) are shipped to the user in hermitically sealed foil packages in ethanol solution. This format is chosen in order to provide the user the flexibility of forming tBLMs comprising their choice of lipid. The coated electrodes may also be stored at 4°C for > 1 year. Different tether : spacer densities are offered in order to accommodate protein or peptide components of molecular weights from 1– 350 kD within the membrane.

2. Materials

2.1. Electrode selection

Ready-made SDx electrodes comprise a close-packed array 2-4 nm strands of ethylene glycol that act as *spacers* and 4 nm strands of ethylene glycol terminated with a C20 phytanyl that act as membrane *tethers*. Typically electrodes are provided that have 10% tether molecules and 90% spacer molecules. Electrodes with this ratio of tethers to spacers have been designated as *T10 electrodes*. T10 electrodes create tBLMs that are very stable with little membrane leakage, and with the ability to incorporate up to 40 kD of the membrane bound fraction of proteins and peptides. Although T1 electrodes provide greater capacity in the tBLM for molecular weights up to 350 kD to penetrate the bilayer molecules, the resulting membrane is less stable. The length of the spacer molecules can also be altered. Hydrophilic spacers of hydroxyl-terminated lipid chains can stretch to the inner leaflet of the lipid bilayer membrane, or can extend only half way to the inner leaflet (as depicted in Fig. 1) in order to create additional space between the gold tethering electrode and the tethered bilayer in order to accommodate protein loops extending beyond the membrane surface at the inner leaflet.

2.2 Cartridge preparation kit

The SDx six-channel measurement electrodes are assembled into a flow cell cartridge (Fig 4A) which, in turn, plugs into the *SDx tethaPod*™ conductance and capacitance reader (Fig 4B). A cartridge preparation kit is supplied by SDx which consists of:

- individually packaged electrodes pre-coated with tethering chemistry (Fig. 2A)
- a flow cell cartridge top on which is coated the gold counter electrode (Fig. 2B)
- an alignment jig for use when attaching the electrode to the flow-cell cartridge (Fig. 2C)
- a silicon rubber pressure pad used when attaching the electrode to the flow cell cartridge (Fig. 2D)
- an aluminium pressure plate used when attaching the electrode to the flow cell cartridge (Fig. 2E)
- a pressure clamp also used when attaching the electrode to the flow cell cartridge (Fig. 3B)

2.3 Solutions

- Also supplied by SDx is a standard membrane forming lipid mixture that has been optimised to achieve the best electrical seal which comprises 3mM ethanolic solution of a 70% : 30% mix of diether diphytanyl (C16) phosphatidyl choline : diether diphatanyl (C16) hydroxyl (mixture designated *AM199* by SDx). Alternative lipid combinations may be employed provided they are soluble in ethanol at 3mM concentrations at room temperature (**Note 2**).
- Preferred electrolyte solution such as Phosphate Buffered Saline (PBS) (**Note 3**).

3. Methods

3.1 Preparing cartridges

1. Remove six-channel electrode from its sealed foil package using tweezers. Care should be taken not to touch the six gold regions that will form the gold tethered membrane. The side of the electrode where" SDX" appears inverted is the *up-side* upon which the gold has been deposited (Fig. 2A). Allow 2 – 3 min for any residual ethanol to evaporate (**Note 4**).
2. Place electrode into alignment jig so that the inverted "SDX" on the slide overlays the inverted "SDX" on the alignment jig (Figure 2B). This will ensure the gold electrodes are correctly oriented.
3. Peel the thin plastic protective cover from the underside of the flow cell cartridge, ***taking care to leave the 0.1 mm flow cell laminate and adhesive layer in place.***
4. Place the flow cell cartridge over the alignment jig with the adhesive laminate facing the electrode (Fig. 3A). The flow cell cartridge should be aligned such that the numbers 1-6 on the cartridge align with the 1-6 on the alignment jig. Insert the short end of the flow cell cartridge nearest to well 6 into the matching slot in the alignment jig and lower the cartridge onto the electrode.
5. Press the silicon rubber pressure pad into the flow cell cartridge top. Then position the aluminium pressure plate over the assembly and insert into the pressure clamp.
6. Compress by ¾ of a turn of the knob and leave for at least one minute (Fig 3B).
7. Gently remove the assembly from the pressure clamp. Remove the aluminium pressure plate and silicon pressure pad taking care to prevent flow cell cartridge separating from the electrode.

8. By gently lifting from the underside of the electrode the electrode-flow cell cartridge assembly can be removed intact from the alignment jig. The remaining exposed gold surfaces are not critical to bilayer formation. The functional gold tethering electrodes are protected within the flow cell cartridge assembly (Fig 4A).
9. Once the cartridge is made it is important to attach a bilayer lipid membrane to the tethers as soon as practicable (within 1-2 minutes; See **Note 4**).

3.2 Creating a tethered bilayer lipid membrane using solvent exchange

When forming a tBLM a solvent exchange method is employed as it permits the formation of tBLMs at low tethering ratios well beyond that possible employing liposomal fusion.

1. To well 1 add 8 μL AM199 or desired ethanolic lipid formulation via the circular opening in the cartridge top.
2. Wait 10 sec and add 8 μL lipid solution to the 2nd well, and wait a further 10 sec before making additions to each of the subsequent 4 wells. A delay of 10 sec provides a convenient operational delay to permit each well to be incubated for same period of time.
3. Let each ethanolic solution of lipid incubate within the wells for exactly 2 minutes then rinse with 100 μL of PBS (or desired buffer solution, see **Note 5**) taking care not to introduce air bubbles into the flow cell chamber as this will damage membrane formation. Alternative lipid mixtures may require optimisation of their concentration and assembly times to achieve the highest possible membrane seal.
4. Rinse with at least 3 x 100 μL aliquots of the PBS. After the first wash with PBS time is no longer a critical factor. From the reservoir side of the flow cell cartridge remove 100 μL of the wash through solution and then repeat rinses to ensure the removal of any residual ethanol and lipids. Following formation, the assembled tethered bilayer must remain totally covered with aqueous solutions to prevent membrane disassociation (**Note 6**).

3.3 Testing the bilayer using AC impedance spectroscopy

The conductance and capacitance of the tethered membrane may be measured by inserting the assembled electrode within the flow cell cartridge into a tethaPod™ reader. The reader simplifies the interpretation of the AC impedance spectrum and provides a measure of

membrane conductance and capacitance. Typical conduction values for a freshly formed membrane using AM199 in PBS are 0.35 ±0.15 µS and capacitance values of 18 ±2 nF at room temperature. The conductance is proportional to the ion flux through the membrane and the capacitance is *inversely* proportional to the membrane thickness. A significant additional measure using a tethaPod™ is the *Goodness of Fit* (GOF). This indicates the quality of match between the experimental data and a model of the tethered membrane. GOF values of less than 0.1 indicate a good match of the data to this simple model, and suggest that the membrane is homogenous.

3.4 Incorporation of proteins and peptides

3.4.1 Spontaneous inserting proteins and peptides

Peptides and proteins such as *alamethicin*, *α-hemolysin*, *gramicidin A* and *valinomycin* will spontaneously insert into pre-formed tBLMs [2-4,1]. The kinetics of their insertion can be measured by the time dependency of the increase in conduction. Although there are no established protocols for inserting ionophores into tBLMs the following guidelines should be considered:

1. Peptides dissolved in ethanol/buffer or methanol/buffer mixtures should be less than 10% ethanol or methanol and be thoroughly rinsed to remove any residual solvent.
2. In order to obtain quantitative kinetics it is necessary to employ a controlled flow rate for the introduction of the peptide to the tBLM. This can be achieved using a syringe pump that couples to the flow cell cartridge (Fig 5).
3. In order to model the *kinetics* of the conduction increase following peptide or protein insertion, important considerations include:
    - the temperature of the solutions being added are equivalent to the temperature in the tBLM.
    - the aggregation state of the spontaneous inserting peptide or protein in the aqueous buffer. The form of the concentration versus conduction relationship will report on the aggregation state of the protein or peptide in the membrane, with a non-linearity suggesting that a multimeric form of the peptide or protein is required for conduction.
4. Insertion of proteins and peptides may depend on the need for specific charged lipids being present in the tBLM.

5. The conduction of some peptides, such *gramicidin A*, are selective for cations over anions and so applying a fixed negative potential bias to the tethering gold electrode will enhance conduction. The ability to apply such a bias is available on the SDx TethaPod™ [1].
6. The insertion of peptides such as *alamethicin* is catalysed by the application of a transmembrane potential which can be applied to the tBLM [3]

3.4.2 Non spontaneous insertion of proteins and peptides

For membrane intrinsic proteins and peptides, their insertion occurs at the time of tBLM formation through their inclusion as a detergent micelle in the hydrating, membrane forming rinse step (see Section 3.2, Step 3 above). When selecting detergents to form micelles the general rule is to use *high aggregation number* detergents such as Brij 58, Cymal 5 or DDM. **Table 1** lists the upper tolerable concentration levels for AM199 T10 tBLMs for a list of common detergents.

4.0 Notes

1. The use of disulphides rather than thiols results in a greater stability of the coating solutions.
2. Lipids that are insoluble in ethanol may be dispersed in ethanol/methanol mixtures. Care should be taken not to use solvents, such as chloroform, which will degrade the polycarbonate cartridge.
3. In order to measure conductance and capacitance, a standard electrolyte concentration of 135 mM is assumed by the fitting model of the tethaPod™. Significant variation from this concentration will alter the accuracy of the membrane conduction and capacitance values reported by the tethaPod™.
4. Drying of the electrodes will result in adsorption of the tethering chemistry to the gold preventing its incorporation into the subsequently formed lipid bilayer. For this reason electrodes should not be left exposed left to dry for longer than 2-3 min. Small droplets of residual ethanol on the electrodes will not effect the subsequent assembly process.

5. A series of impedance spectroscopy measurements have demonstrated that 2 minutes incubation in the lipid ethanolic solution of AM199 produces the best sealed tBLMs at room temperature.
6. Following formation of the membrane an adhesive seal can be applied across the cartridge opening to prevent evaporation and drying of the sample in the flow-cell chamber.

Acknowledgments

This work was supported by the Australian Research Council, the National Health and Medical Research Council of Australia (Grant 1047980). We declare that Bruce Cornell is a shareholder, and Sonia Carne is an employee, of *SDx Tethered Membranes Pty Ltd.*

References

1. Cornell BA, Braach-Maksvytis VLB, King LG, Osman PDJ, Raguse B, Wieczorek L, Pace RJ (1997) A biosensor that uses ion-channel switches. Nature 387 (6633):580-583.

2. Vockenroth IK, Atanasova PP, Jenkins ATA, Köper I (2008) Incorporation of α-hemolysin in different tethered bilayer lipid membrane architectures. Langmuir 24 (2):496-502

3. Yin P, Burns CJ, Osman PD, Cornell BA (2003) A tethered bilayer sensor containing alamethicin channels and its detection of amiloride based inhibitors. Biosensors and Bioelectronics 18 (4):389-397

4. Raguse B, Braach-Maksvytis V, Cornell BA, King LG, Osman PD, Pace RJ, Wieczorek L (1998) Tethered lipid bilayer membranes: formation and ionic reservoir characterization. Langmuir 14 (3):648-659

Fig 1. Tethered bilayer lipid membrane (tBLM) schematic.

Fig 2. 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.

Fig 3. ***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.

Fig 4 ***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.

Fig 5. An example of a syringe pump attachment to control flow across the tBLMs.

Table 1: Aggregation Numbers as reported by Sigma Aldrich.

Detergent	**Aggregation Number**	**Concentration µM**	**Concentration % w/v**
Brij 58	70	1	0.0001
Tween 20	not reported	4	0.0005
Triton X100	140	15.5	0.001
DDM	98	50	0.0025
Cymal 5	47	400	0.02

Figure 1

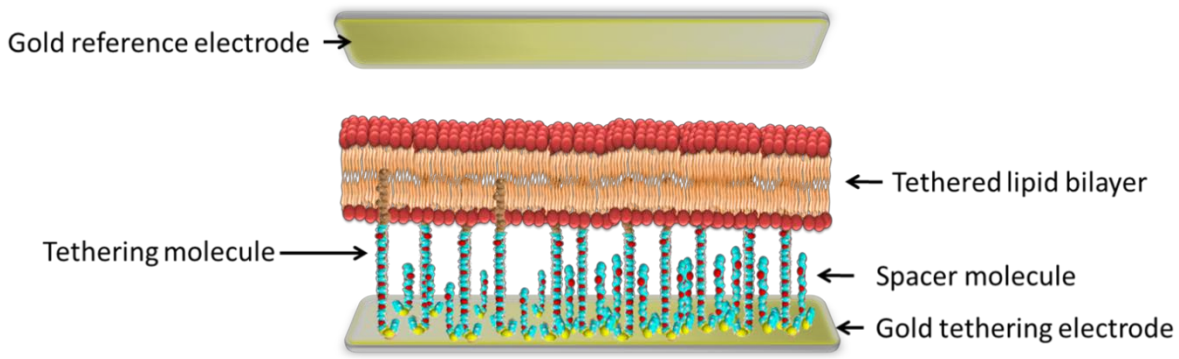

Figure 2

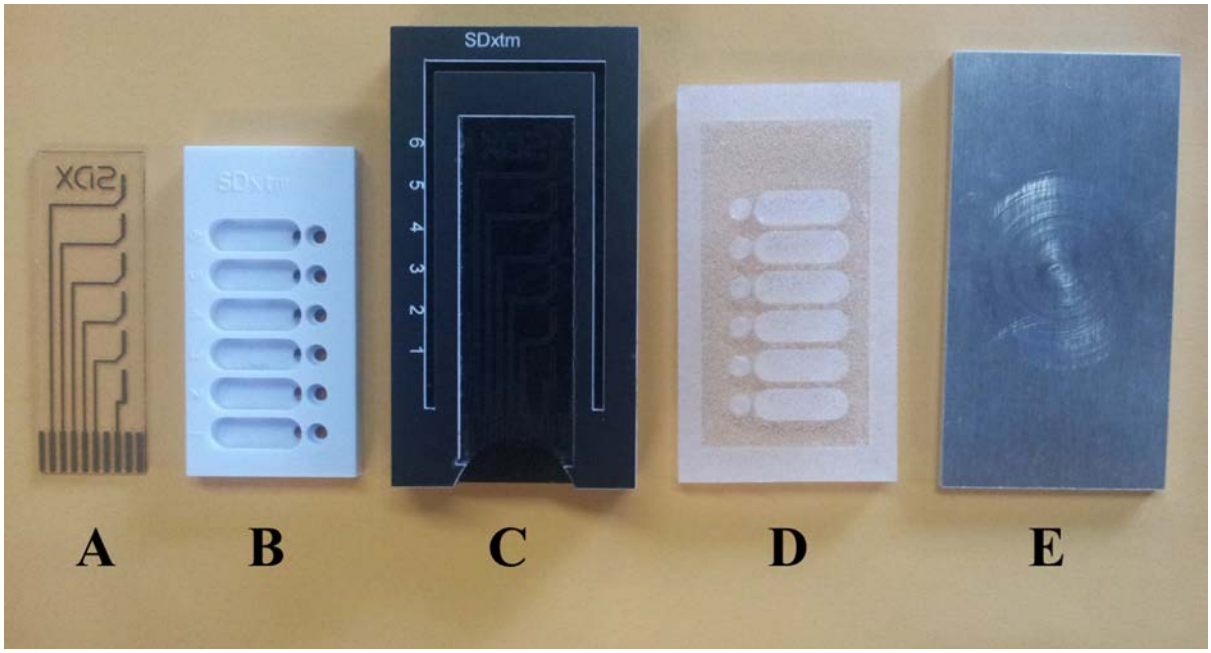

Figure 3

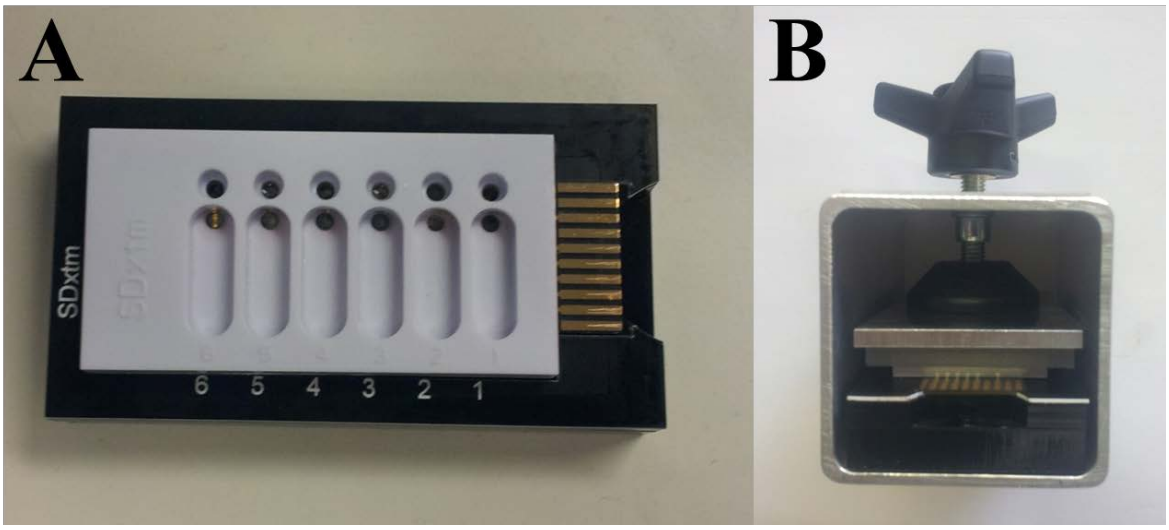

Figure 4

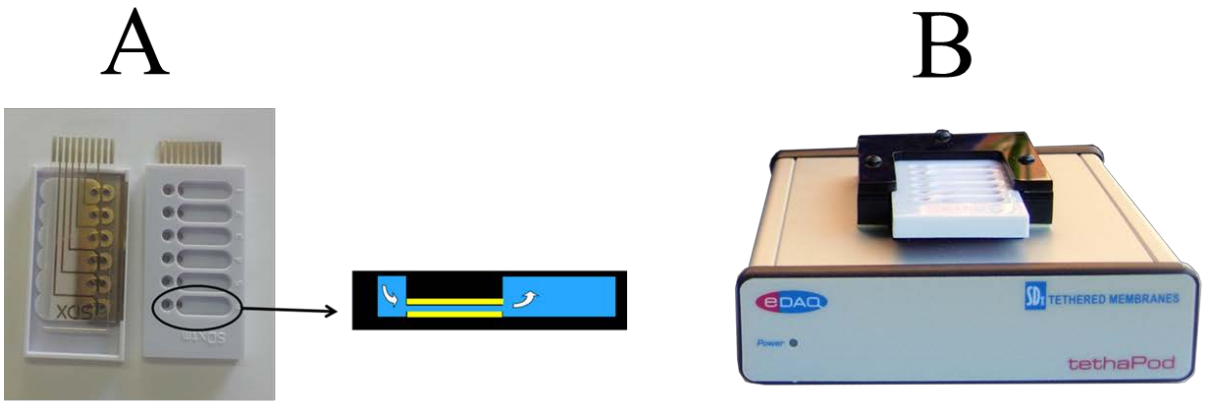

Figure 5