

Making lipid membranes even tougher

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Biosensors based on lipid membranes promise an inexpensive and versatile platform for application in many fields of molecular sensing. An extensive review of the applications for tethered membranes was reported in the July 2006 MRS Bulletin [A.N. Parikh and J.T. Groves, Materials science of supported lipid membranes. *MRS Bull.* **31**(8), 507 (2006)]. The commercial use to which tethered lipid membranes have been applied has been limited by their stability under long-term storage. This report describes a novel membrane construct that is stable at room temperature for months, eliminates the mobile lipid phase present in lipid bilayers, and is robust against detergents under conditions that would destroy a lipid bilayer.

I. INTRODUCTION

A recent review¹ described the opportunities and applications for the interdisciplinary field of tethered lipid membranes. Biosensors based on tethered lipid membranes are of particular interest and are under development by many groups.^{2–13} These biosensors use a variety of approaches to modulate the admittance of ion channels incorporated into the membrane and promise an inexpensive versatile platform for applications across the field of point-of-care, time-critical diagnostics. Advantages of this approach include avoiding the optical interference effects arising from biological samples, which affect nephelometry, fluorescence, or similar optical measurement techniques. Membrane-based biosensors may be miniaturized and addressed to permit multiple measures from a single test sample and provide an objective, direct electrical output.

A problem limiting the commercial applications of membrane-based biosensors has been the short storage lifetime of these devices, which, even under refrigeration, has been only 3–6 months.^{1,13} The market requirement for a widely applicable technology platform is stability of six to twelve months at room temperature. This report describes a novel, tethered monolayer membrane incorporating a gated ion channel that has been successfully stored at room temperature for up to three months and is free of any untethered mobile lipid.

II. MATERIALS AND METHODS

A. Membrane structure

In earlier reports, we demonstrated a functioning biosensor based on a modified form of the ion channel,

gramicidin A.^{14–18} Modulation of the two-dimensional monomer–dimer reaction kinetics of the gramicidin channels was used as a means to measure the binding kinetics of analyte–protein interactions at the membrane surface. The change in conduction as a result of this interaction can be directly related to the concentration of the target analyte. Figure 1 shows the structure of both the bilayer membrane and the novel monolayer membrane presented here.

B. Membrane composition

As seen in Fig. 1, the bilayer consists of seven synthetic organic compounds and the monolayer of three compounds. These are shown in more detail in Fig. 2. In the bilayer, four of the compounds (2{a}–2{d}) are tethered through sulphur–gold bonds formed by immersion of a gold electrode in an ethanolic solution and three (2{e}–2{g}) are mobile and are self-assembled by a subsequent immersion in water. In the monolayer, all three compounds are tethered to the electrode. The three elements of the monolayer membrane address the three functions it is required to perform. Compound 2(b) provides structural integrity and a barrier against the passage of ions across the membrane. Compound 2(c) is the receptor attachment element to which antibodies or haptens may be attached, and compound 2(h) is the ion channel conduction element. The gramicidin channel 2(h), in the monolayer consists of one tethered monomer. A key issue determining the monolayer composition is to match the lipid spacing at the tethering sulfur–gold interface with the need for a close lipid packing in the membrane. A conflicting need is to provide sufficient flexibility to allow the tethered ion channel to renature into a conducting conformation. The principal determinant in establishing the area per molecule is the benzyl terminated disulphide groups. The ethylene glycol hydrophilic spacers between the membrane and the gold surface have a

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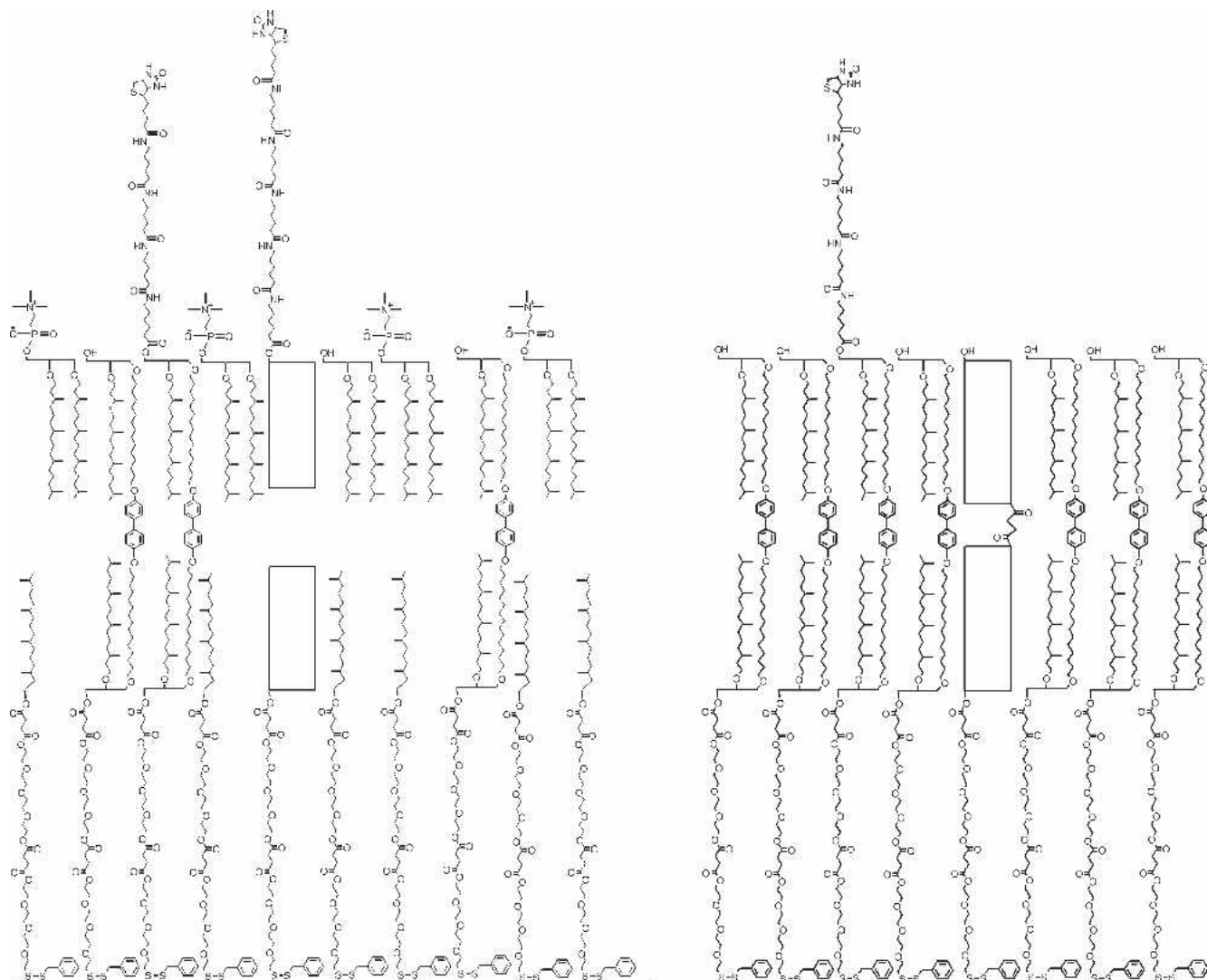


FIG. 1. (a) Bilayer (LHs) and (b) monolayer (RHs) membrane constructs. More details of the seven bilayer components and three monolayer components are given in Fig. 2.

smaller cross-sectional area, and it is assumed they are not the limiting factor in determining the area per lipid. The two lipid components of the monolayer are the same as the tethered species in the bilayer. The new element 2(h) in the monolayer is bis-gramicidin. No half-membrane spanning elements are used. This construct eliminates a problem encountered in bilayer membranes that, on storage at room temperature, the mobile half membrane spanning lipids are free to self-associate and form modified structures with other non-lipid elements degrading the performance of the sensor.

C. Monolayer assembly

Freshly sputtered gold (Unaxis-Pyramet, Zurich, Switzerland) was deposited onto clean polycarbonate slides. Gold (0.9995%) purity was sputtered to a depth of 100 nm directly onto the polycarbonate. Sets of seven

slides were incubated in 80 mL volumes of ethanolic monolayer solutions for 30 min. Monolayer solution concentrations were all 105 μM benzyl-disulphide in ethanol. The benzyl-disulphide is common to all species. The relative concentrations of the three were in the ratio 2(b):2(c):2(h) as 100:1:1. The slides were then rinsed three times with ethanol before being transferred into MilliQ water and stored at 20 $^{\circ}\text{C}$ until used. Storage at room temperature (RT; 20 $^{\circ}\text{C}$) was successful for up to and, in an on-going trial, beyond 3 months without loss of performance. Coating solutions have been reused ten times before replacement.

D. Biochemistry assembly

Following the assembly of the monolayer membrane, 10 μL 80 nM streptavidin was added to 20 μL phosphate-buffered saline (PBS) containing 0.1% Tween-20

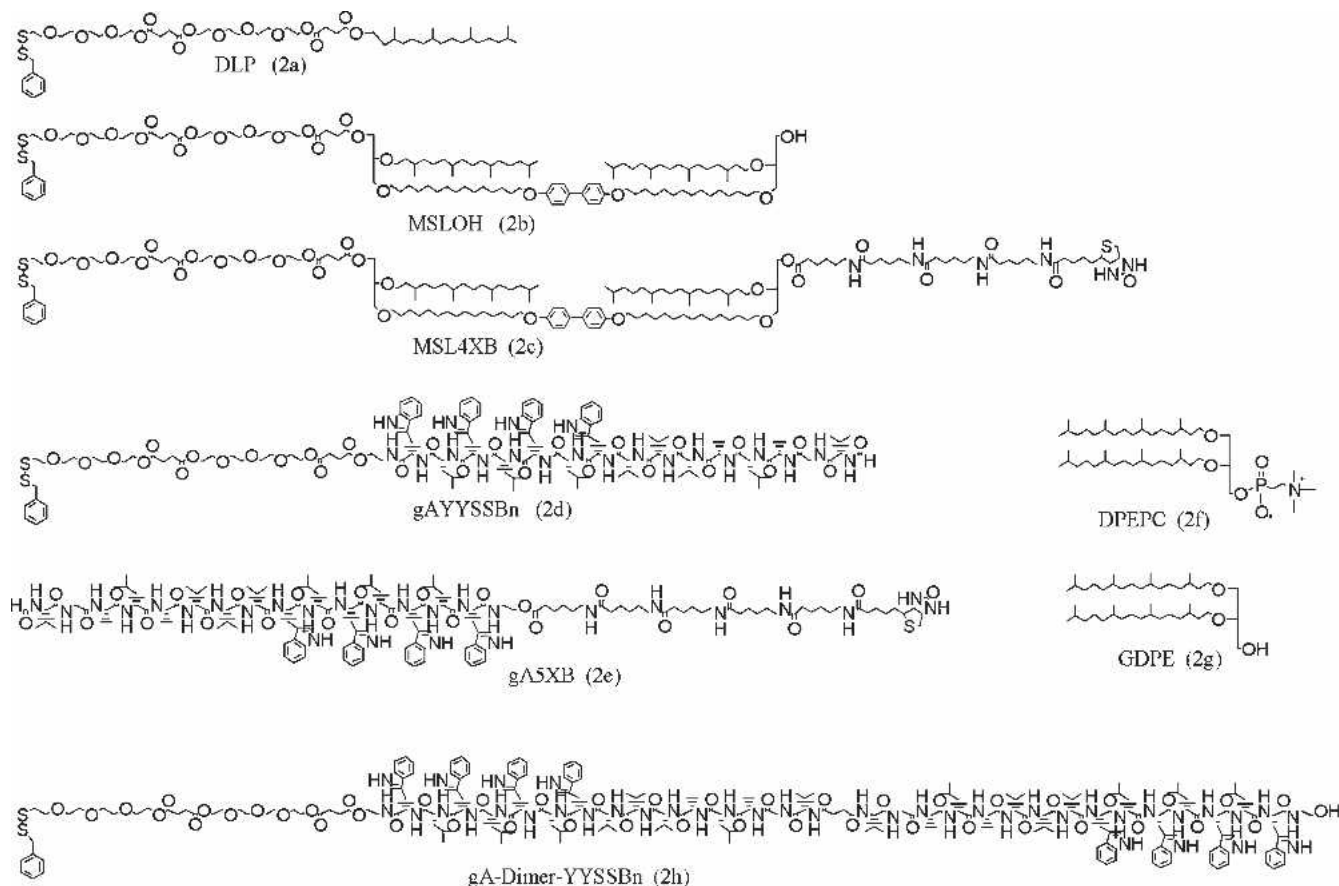


FIG. 2. Compounds used to prepare either bilayer or monolayer membranes. See the text for details.

(PBST) in each well. Following incubation for 5 min, each well was rinsed with PBST and 10 μ L 15 nM biotinylated antigen-binding fragment of an IgG (Fab-anti hCG, ME106) were added and incubated for a further 5 min before it was again washed with PBST. The Fab fragment is complementary to the epitope targeted by the monoclonal antibody (MAb) on the beads, as discussed in Sec. III.

E. Monolayer stability

Figure 3 shows the membrane admittance over 51 days for 16 measures during storage at 20 $^{\circ}$ C. Following a 2–3 day equilibration period at 20 $^{\circ}$ C, the bis-gramicidin conduction plateaued at $50,000 \pm 10,000$ nS. By contrast, in the absence of bis-gramicidin there was no significant conduction. This is seen in Fig. 3 as the barely visible bars on the time axis at times beyond 30 days. These measures have continued for beyond three months and have remained unaltered (data not shown). Other membrane measures such as the admittance at 1 kHz, which reports on the membrane thickness (inversely related to the membrane capacitance), and the minimum phase value, which reports on the ratio of the membrane capacitance to the interfacial capacitance of the saline–gold interface, remained unaltered over the same period (data not shown).

This indicates a stable membrane ~ 4 nm thick possessing ion conducting pathways arising from the gramicidin ion channel and a negligible leakage current through the membrane.

F. Channel blockers

Many species are known to block the conduction of Gramicidin.¹⁹ These include calcium salts, gadolinium salts, and quaternary ammonium components, such as tetra-methyl ammonium (TMA), tetra-ethyl ammonium (TEA), guanidinium, and benzylalkonium chloride. Methyl-benzethonium chloride (MBC) is used here to demonstrate a sensor based on the tethered membrane. MBC may be detected well below 100 nM in PBST solution. When it is attached to a macromolecule or coated onto a polymer bead, further improvements in detection sensitivities may be achieved with rapid response rates of approximately 250 s at room temperature. Figure 4 shows a titration of the Gating Ratio against the concentration of MBC in PBST.

G. Assembly of the sensor

1. Plastic disposable

Figure 5 shows an example of a transduction mechanism that allows the monolayer membrane to be used as

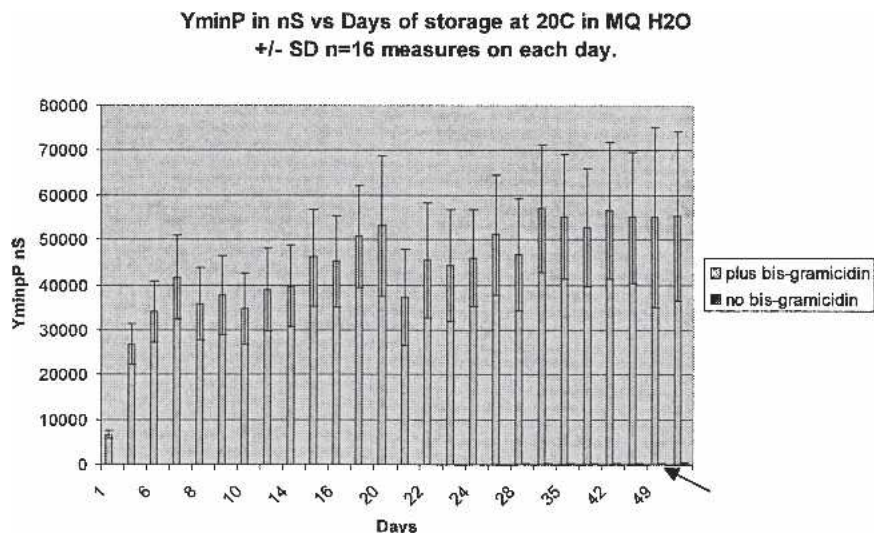


FIG. 3. Plot of admittance at minimum phase (YminP) of membranes containing bis-gramicidin or no bis-gramicidin stored at 20 °C for 51 days. The conductance from the membranes not containing bis-gramicidin (marked with an arrow) is small as to be barely visible.

an immunoassay. Figure 6 shows the disposable test electrode. Six 2-mm-diameter, 50-nm-thick gold sensor pads were sputtered through a 100 μm magnetically tethered stainless steel shadow mask onto a 1 mm × 25 mm × 75 mm polycarbonate microscope slide. The 2-mm-diameter measurement electrodes (×6 tracks) are at the lower end of the sample well opening, and the common return electrode (×3 tracks wide) is at the upper end. The sample is well defined by a 17 mm × 55 mm × 1 mm thick silicone rubber sheet fastened to the slide by 100 μm double-sided tape. The sample volume per well was 20 μL. To achieve a low impedance return path for the return electrode, its surface was exposed to a Ag plating electrode at 5 V for 10 s in PBS prior to the measurement.

2. Electrical reader

The gold tracks were patterned to plug into a 9-pin, 1-mm spacing standard printed circuit board connector attached to a low-cost admittance meter.

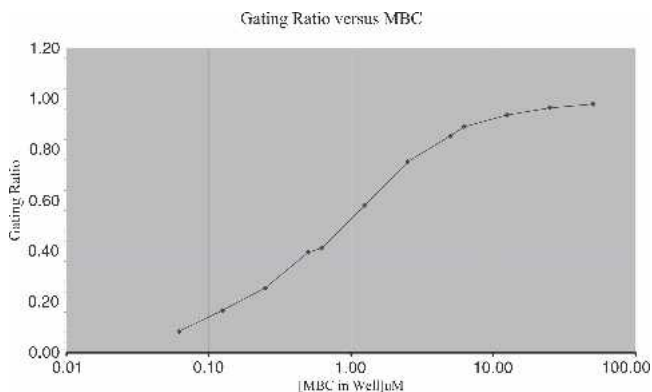


FIG. 4. Gating ratio = $(\text{admittance}_{\text{initial}} - \text{admittance}_{\text{final}}) / (\text{admittance}_{\text{final}})$ titrated for a range of MBC concentrations in 0.1% Tween in PBS.

3. Labeled beads

Magnetic beads, 1 μm diameter with tosylate leaving groups, were supplied by Dynal Biotech (Oslo, Norway). Following the manufacturer’s recommended procedure, these were coated with 0.67 mM channel blocker, MBC, and 26.8 μM MAb (HCG03) {25:1 mol ratio}. Titrating the MBC/Antibody ratio from 1:1 to 2000:1 molar ratio yielded an optimal ratio in the range 25:1–50:1. At the lower MBC concentrations, the magnitude of the MBC blocking becomes less, while at higher concentrations of MBC, the effectiveness of the antibody is reduced (data not shown).

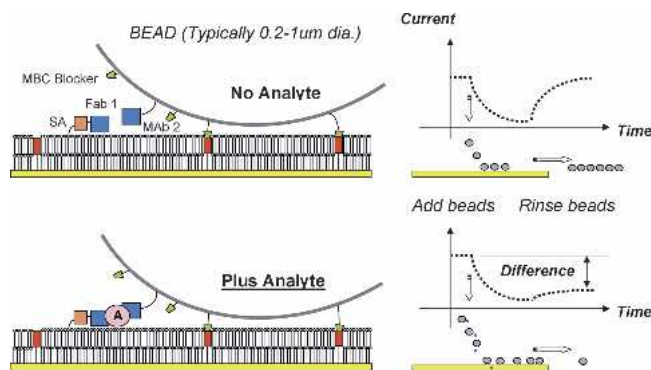


FIG. 5. Example of a sensing mechanism using the gated ion channel monolayer in a sensor. A small quantity of MBC/MAB coated beads is applied to the monolayer membrane. This reduces the conductance by typically >50% from an admittance of approximately $(1/100 \text{ kOhms}) = 10 \text{ μMho}$ for a 2-mm-diameter electrode. A variety of approaches may be used to scavenge the beads from the surface of the electrode and return the admittance to the original value. These include simple gravity, flow, or magnetic scavenging. If the beads have become attached to the electrode via binding of the analyte from solution and antibodies on the electrode surface and on the bead, the admittance remains low in proportion to the number of retained beads.

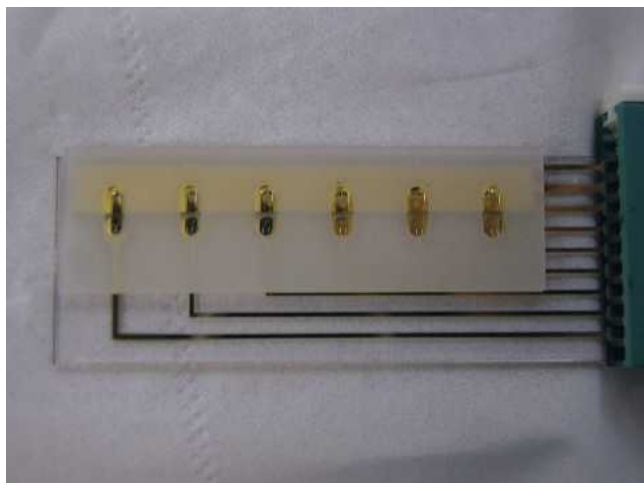


FIG. 6. Disposable sensor electrode. The three wells to the left had beads added and were incubated with PBST; the three to the right had beads added that had been incubated with 300 mIU/ml hCG. Note the rusty color evident in the three right-hand wells resulting from the retained beads.

III. RESULTS AND DISCUSSION

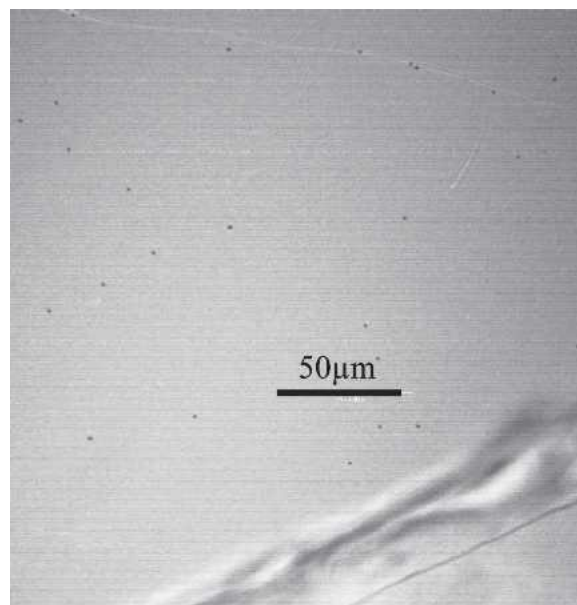
Admittance measures from a monolayer membrane as MBC and antibody coated beads are contacted and removed from the membrane surface are shown in Fig. 7. To a 20- μ L well, 2 μ L of the bead solution is added, and the admittance was observed to change from ~ 10 μ mMho to ~ 6 μ mMho in less than 1 min. After 2–3 min, the beads were magnetically scavenged from the surface, causing the admittance to return toward its original value. Many variations on the approach are possible, ranging from capturing beads from a flowing liquid stream in a flow cell to concentrating molecular complexes of antibodies and channel blockers at the membrane surface. The novel monolayer membrane demonstrated here has a number of beneficial properties.

A. Objective electrical output

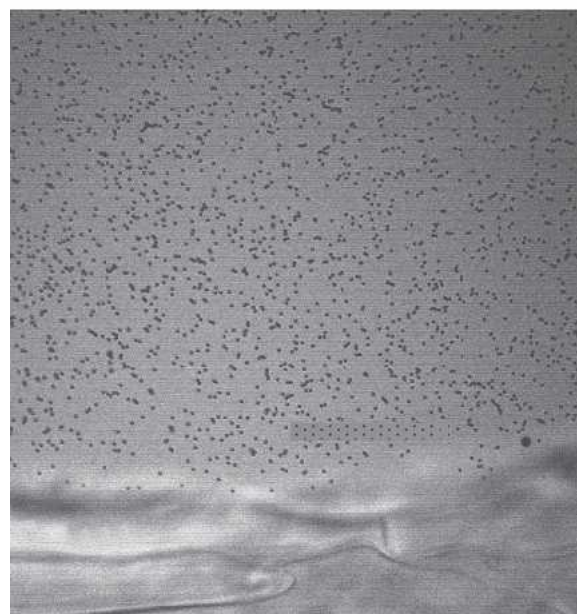
A defining difference between the present approach and that of the majority of immunoassays is that the output is directly electrical over an easily accessible impedance range of 100–200 k Ω , requiring only inexpensive electronics to read. A direct electrical output based on this signal provides an objective output rather than the subjective visual interpretation required by many optical assays.

B. Stability

The conduction and responsiveness of the monolayer membrane has been shown to be stable in excess of 3 months either dry or wet at 20 $^{\circ}$ C, providing a basis for a platform transduction approach to many immunoassays. A further consequence of using a monolayer as



(a)



(b)

FIG. 7. (a) Optical microscopy image of the sensor surface with no binding sites for the bead attachment. This background count arises from non-specific bead binding from adventitious attachment of the bead via Van der Waals and other dispersive forces. (b) Optical microscopy image of the bead bound specifically to the membrane surface.

the transduction element is the resistance to detergents. The results shown in Fig. 8 were obtained with phosphate buffered saline plus 0.1% Tween (PBST). This permits the analysis of samples without the need to remove detergents that have been added to maximize the availability of protein analytes. At these levels of Tween, a standard bilayer would be dispersed by the surfactant.

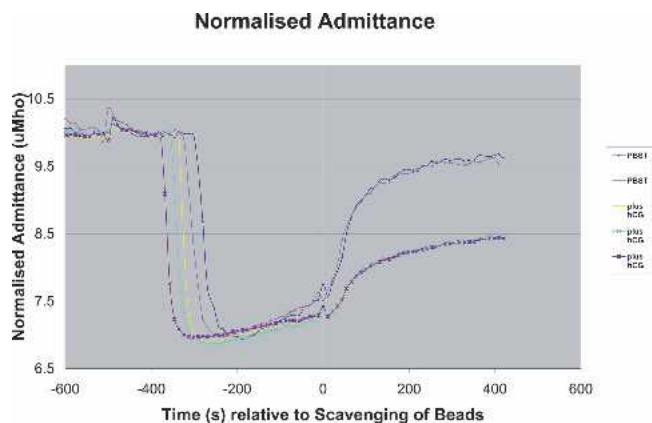


FIG. 8. Admittance changes following the addition of 1 μL of MBC/MAb bead to the electrode and its subsequent magnetic scavenging. The retention of beads by immunoreactions at the membrane surface retains the low admittance in proportion to the analyte concentration.

C. Sensitivity

The ability of the antibody-coated beads to capture and concentrate analyte from solution volumes of 100 μL to 1 mL results in a low detection threshold. Assuming a 1 μm bead is used, with a 2-mm-diameter electrode, a total coverage of the electrode with beads is achieved with 5×10^6 beads each capturing up to 5×10^4 analyte molecules. If this is captured and concentrated from a 100 μL volume, an analyte limited sensitivity of $\sim\text{nM}$ is obtained. Using a smaller electrode to read the beads results in a proportionately lower concentration; i.e., a 100- μm -diameter electrode yields an analyte limitation threshold of $\sim\text{pM}$ under the same conditions. Mass transport and diffusion limitations are minimal due to the use of beads to capture analyte prior to the addition of the beads to the sample well. “On rates” in the sample well are dominated by the bead arrival and do not affect sensitivity or response times.

D. Arrays and multiple tests in a single sample

The use of the monolayer membrane construct described here permits an array of electrodes to be prepared within a single sample well, with each electrode coated with a different capture antibody. A mixed bead population composed of mixed antibody coatings permits multiple measures from a single sample addition.

IV. CONCLUSION

Tethered lipid membranes represent a novel family of materials that permit many properties seen in biological systems to be mimicked in more stable synthetic compounds. This report describes an example of extending

the applications for these materials into areas requiring greater stability and longer term storage.

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