Tethered-bilayer lipid membranes as a support for membrane-active peptides
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Abstract
An immunosensing device, comprising a lipid membrane incorporating ion channels tethered to the surface of a gold electrode, has been reported (Cornell, Braach-Maksvytis, King, Osman, Raguse, Wieczorek and Pace (1997) Nature (London) 387, 580–583). The present article describes key steps in the assembly of the device and provides further evidence for its proposed sensing mechanism.

Introduction
The tethering of molecular analogues of biological membranes to solid surfaces has been used in a variety of biomimetic systems [1–3]. Because of the simplicity of sulphur–gold tethering chemistry [4–6], thiol- and disulphide-labelled compounds have been the basis of most of these studies [7–9]. In particular, we have reported a generic immunosensing device [10] in which the polypeptide ion channel gramicidin A was assembled into a tethered lipid membrane and coupled to an antibody targeting a compound of diagnostic interest. The device operates as an ion channel switch (ICS®) in which the binding of the target molecule shifts the conformation of the gramicidin channels from predominantly conducting dimers to non-conducting monomers. Applying a small alternating potential between the gold substrate and a reference electrode in the test solution generates a charge at the gold surface and causes electrons to flow in an external circuit. The device provides a rapid, quantitative measure of the concentration of the target compound in the test solution. The test solution can be any electrolyte containing biological fluid, including blood, serum, urine or saliva. The specificity of the response is dependent on the specificity of the receptor and is equivalent to a conventional sandwich immunoassay. The receptors most extensively studied have been antibody Fab fragments; however, the approach is generic and has been demonstrated using oligonucleotide probes, heavy metal chelates and cell-surface receptors. Here we describe the fabrication process of the device and provide evidence for its proposed detection mechanism.

Materials
Representative examples of the materials from which the device is assembled are shown in Figure 1. The synthesis of most of the compounds has been described elsewhere [11–15]. They are divided into two families, one that is tethered to the gold surface and one that is physically absorbed to the surface but free to diffuse in the two-dimensional plane of the membrane (see Figure 1).

In species 1–3 in Figure 1, attachment of the membrane to the gold substrate is via a disulphide moiety. The use of this disulphide compound both brings a tether for the membrane lipids and introduces a benzyl spacer group that lowers the two-dimensional packing density of the assembled membrane. The lower packing density facilitates the entry of ions into the space between the membrane and the gold surface. This space is termed the reservoir.

A further series of tethering compounds are shown in Figure 2. Compounds such as 7, termed the C11 series, possess both the sulphur-attachment chemistry and an additional binding energy generated by the Van der Waals attraction of the C11 sequences for each other and for the gold surface. In addition, these compounds protect the surface of the gold from the electrolyte solution, lowering the surface charge and minimizing deleterious electrochemical effects. The reduction in surface charge also significantly alters the reservoir performance.

In compounds 7 and 8, all-ether reservoir linkers are substituted for the succinate groups of 14 (Figure 1). This eliminates instabilities that can arise from the hydrolysis of ester groups and significantly extends the storage lifetime of the membrane. The all-ether reservoir also beneficially alters the reservoir properties.

The area per lipid molecule is determined by a balance of contributions from at least three regions within the membrane, the sulphur–gold interface, the spacer molecules and the interaction of the hydrocarbon chains within the body of the membrane. The packaging density of the membrane also beneficially alters the reservoir properties.

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membrane. The area per lipid can range from 20 to 150 Å² depending upon the bulk and position of the dominant packing constraint. Membrane-spanning lipids such as 2 and half-membrane-spanning lipids such as 1, 5 and 6 are mixed in different ratios to adjust the membrane packing. Packing at various levels within the membrane may also be adjusted by the series shown in species 9 and by the spacers 10a and 10b shown in Figure 3.

The phytanyl lipid chains used here are commonly found in thermophilic archaebacteria.

**Figure 1**

**Chemical structures of representative compounds from which the tethered membrane is formed**

Compounds 1–3 depict tethered species and 4–6 the mobile species. A minor component of 2 and all of 4 are linked to R₂, an aminocaproyl-linked biotin. The concentration of 1 is 350 μM and that of 5 is 2 mM. A typical mole ratio of 1:2(R₁):2(R₂):3 is 40000:400:1:1 and of 5:6:4 is 28000:12000:1.

**Tethered species**

1. ![Structural diagram](image1)
2. ![Structural diagram](image2)
3. ![Structural diagram](image3)

**Mobile species**

4. ![Structural diagram](image4)
5. ![Structural diagram](image5)
6. ![Structural diagram](image6)

Where for both the mobile and tethered species:

\[
R_1 = H \\
R_2 = \text{amine}
\]

**Figure 2**

**Additional tethered compounds**

In 7 a C₉₃ alkane provides more secure tethering, shields the substrate from electrolysis and improves the reservoir properties. In compounds 7 and 8 the succinate groups are replaced with all-ether linkers, extending the storage lifetime and improving the reservoir properties. Compound 9 permits adjustment of the packing constraints within the hydrocarbon region of the membrane.

7. ![Structural diagram](image7)
8. ![Structural diagram](image8)
9. ![Structural diagram](image9)
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They are stable at high temperatures and the bulkiness of the methyl substituents eliminates temperature dependencies in the membrane disorder around 20–30 °C.

**Gramicidin A**
Many analogues of gramicidin A have been synthesized. Each is tested for single-channel conduction to ascertain the conductivity per channel. Most modifications have only a minor effect on conduction, but modifications too near the channel entrance can eliminate conduction.

**Antibody fragments**
Whole antibodies are enzymically cleaved at the hinge region and biotinylated. Attachment of these fragments to the membrane is usually performed using streptavidin–biotin complexes. Other linker chemistries include covalent coupling and a novel multi-nitriloacetic acid organometallic coupling chemistry.

**Assembly**
The details of the membrane assembly are shown below in Figure 4 and described in the figure caption.

**Results**

**Ellipsometry**
Ellipsometry permits a measure of the membrane thickness. Direct calibration using alkane thiols from C₉ to C₁₈ yields a thickness of 4 nm. The mobile outer layer contributes 2 nm and is measured by the change in thickness on rinsing off the layer with ethanol.

**Impedance spectroscopy**
Membrane thickness can also be determined using the membrane capacitance derived from modelling the impedance spectrum over a swept frequency range of typically 0.1–1000 Hz. Both phase and modulus are measured and fitted. Simple RC (R, resistor; C, capacitor) networks provide a fit with < 2% residual. A measured capacitance of 0.5 μF/cm² is obtained. Assuming a relative dielectric constant of 2.2 for the membrane chains again estimates the membrane thickness at approx. 4 nm.

**Figure 3**
Spacer groups used to vary the two-dimensional packing within the membrane

![Spacer groups](image)

**Figure 4**
Membrane assembly

A fresh gold surface is exposed to an ethanol solution of the tethering species 1–3 for 10 min. This produces the inner and part of the outer leaflet of the membrane. Following an alcohol rinse, a second ethanol solution brings the mobile elements of the membrane, species 4 and 5. Rinsing with water causes a lipid bilayer structure to form spontaneously. Some of the lipids span the membrane, whereas the remainder are mobile within the two-dimensional plane of the membrane. Antibody fragments are then added in the aqueous solution.

![Membrane assembly](image)
**Gramicidin titration**

The membrane conduction arising from the gramicidin may also be obtained from modelling the impedance spectrum. The conduction is found to be dependent on the insertion of gramicidin into the inner, outer or both layers.

**Reservoir capacity**

In a conductive membrane the swept-frequency measure of impedance causes the apparent capacitance to switch from a value dominated by the membrane to one dominated by the Helmholtz capacitance. The magnitude of this effect depends on the ionic capacity of the reservoir. Many factors influence the reservoir capacity. Particularly significant is the composition of the reservoir linkers [16]. The composition of the linker also influences the voltage dependency of the apparent membrane conductance.

**Non-specific binding**

Ellipsometry has been used to determine the level of non-specific binding. Non-specific binding appears to be below the level of detection on compound 5 and related phosphatidylcholine membranes, although hydroxy-terminated lipids or methylene groups can cause substantial non-specific binding.

**Modelling**

A computer model has been devised of the function of the biosensor. It specifies the three-dimensional capture rates of the analyte on to the membrane-tethered receptors, the two-dimensional monomer–dimer kinetics of the gramicidin ion channel and the two-dimensional cross-coupling kinetics of the receptor–analyte–receptor interaction on the membrane surface.

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**Figure 5**

Steps in the detection mechanism

The binding of analyte to the antibody fragments causes the conformation of gramicidin A to shift from conductive dimers to non-conductive monomers. This causes a loss of conduction of ions across the membrane. A competitive assay has also been devised in which the analyte causes the population of channel dimers to increase. Scale: 1 cm on the figure is approximately equivalent to 9 nm.

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**Figure 6**

Experimental responses

Left-hand panel: current decrease following target addition. Upper curve, negative control; middle curve: response to the addition of β-human chorionic gonadotrophin at the indicated concentrations; lower curve: differential response, subtracting the upper from the middle curve. The measured maximum rates of change of current (admittance) immediately following the sample addition. The rate of change of current (admittance) is normalized to the value, measured at the time of sample addition. Right-hand panel: titration obtained from the initial slope of current decrease; units on the vertical axis are x 10³S.
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These parameters, which can be determined separately using other techniques along with estimates of the two-dimensional density of the membrane components, provide a prediction of the sensor performance. The steps in the detection mechanism are shown in Figure 5. Experimental responses are shown in Figure 6.

Discussion

Sensitivity
Sensitivity is dependent on the antibody ‘on rates’ and affinity. For an antibody with $10^4$ M$^{-1}$ s$^{-1}$ ‘on rate’ and an affinity $>1$ nM$^{-1}$, detection to sub-picomolar levels is possible in $<5$ min.

Stability and variability
The stability of the tethered membrane has been enhanced by replacing ester with ether linkers. A test of the sealed membrane was successfully extended beyond 3 months when fully hydrated. It has also been shown that the sensing function returns on re-hydrating a fully assembled sensor. Long-term storage will be of dried sensors.

Depending on the concentration range, coefficients of variation for the sensor performance are in the range 6–15%.

Chip arrays
A major advantage of this approach is the potential for miniaturization. A silicon chip array is under development. An optical technique permits specific receptors to be tethered to chosen sites within the array.

Optical patterning
The patterning of the electrode arrays is by optical masks that permit selective deprotection of biotin sites attached to either the membrane lipid or the ion channels in the membrane.

References

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Membrane-disordering effects of β-amyloid peptides

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Abstract

β-Amyloid (Aβ) protein is the major constituent of senile plaques and cerebrovascular deposits characteristic of Alzheimer’s disease (AD). The causal relationship between Aβ and AD-specific lesions like neurodegeneration and atrophy is still not known. The present article summarizes our studies indicating that rather low concentrations of Aβ significantly alter the fluidity of cell membranes and subcellular fractions from different tissues and different species including humans, as a possible initial step of its biological effects. Using different fluorescent probes our data show clearly that Aβ peptides specifically disturb the acyl-chain layer of cell membranes in a very distinct

Key words: Alzheimer-β amyloid, cholesterol, fluidity, membrane. Abbreviations used: Aβ, β-amyloid; AD, Alzheimer’s disease; DPH, 1,6-diphenylhexa-1,2,3-triene; TMA-DPH, trimethylamino-DPH; SPM, synaptosomal plasma membranes.

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