Stochastic sensors inspired by biology

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Sensory systems use a variety of membrane-bound receptors, including responsive ion channels, to discriminate between a multitude of stimuli. Here we describe how engineered membrane pores can be used to make rapid and sensitive biosensors with potential applications that range from the detection of biological warfare agents to pharmaceutical screening. Notably, use of the engineered pores in stochastic sensing, a single-molecule detection technology, reveals the identity of an analyte as well as its concentration.

Channels and pores that respond directly to molecules or to physical stimuli are found within sensory systems. They include amiloride-sensitive Na^+ channels, which detect the taste of salt, and the vanilloid receptors, which transduce both noxious stimuli, such as elevated temperature, and the sensation of hot peppers. Stochastic sensing with protein pores does not attempt to mimic the molecular devices found in nature, rather the approach is inspired by nature both in the use of protein pores and in the design of binding sites for analytes. Advances made in the past few years allow detection of small cations and anions, organic molecules, proteins and DNA.

Stochastic sensing with single protein pores

The first example of a single-molecule experiment on an identified functional biomolecule was conducted over 30 years ago: the observation of current flow through a single ion-conducting channel formed by the peptide antibiotic gramicidin in a planar lipid bilayer[1]. Soon afterwards, it was shown that single-channel currents can be modulated by molecules, known as channel blockers, that bind reversibly within the lumen of the channel. This finding forms the basis for stochastic sensing with engineered pores[2,3].

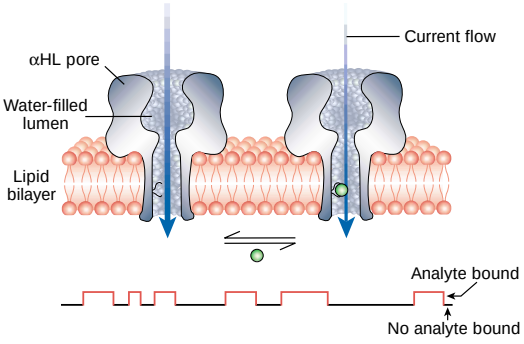
To understand stochastic sensing, the advantages of detection at the single-molecule level must be appreciated (Box 1). In the simplest case, the sensor element has two states — occupied (by analyte) and unoccupied — and a different output is associated with each. As well as revealing analyte concentration, stochastic sensing provides structure-specific information about an analyte, which is often sufficient for its identification.

The pores used for stochastic sensing are based on staphylococcal α-haemolysin (αHL). The pore formed by wild-type αHL consists of seven identical subunits arranged around a central axis. The transmembrane part of the lumen is a β-barrel with two antiparallel strands contributed by each subunit. The extramembraneous domain contains a large cavity that houses the transmembrane domain during the assembly process, but which is available for engineering in the assembled pore. Although channels and pores have been used for some time for analyte detection[4,5], protein engineering and single-molecule detection greatly enhance the potential of the technology[6,7]. The three-dimensional structure of the αHL pore is known at high resolution and, importantly, β-barrels are especially open to remodelling[8]. The large single-channel conductance (~1 nS in 1 M KCl) makes single-channel recording straightforward.

Engineering pores for a wide variety of analytes

Binding sites for analytes have been placed within the lumen of the αHL pore by a variety of means, and pores have also

Box 1
Stochastic sensing with engineered pores



A single engineered pore is placed in a planar lipid bilayer. In an applied potential, a current flows through the pore carried by the ions in salt solutions that bath both side of the bilayer. The pore contains a binding site for an analyte, represented by the green ball in the figure. Each time the analyte binds to the pore the current is modulated as illustrated in the trace. Hence, this technique monitors individual binding events. The frequency of occurrence of the events reveals the concentration of the analyte, whereas the current signature (the mean duration and amplitude of the events) reveals its identity.

In the case of a simple equilibrium, $\tau_{off} = 1/k_{off}$, where τ_{off} is the mean dwell time of the analyte and k_{off} is the dissociation rate constant, and $\tau_{on} = 1/k_{on}[A]$, where τ_{on} is the mean time between binding events, k_{on} is the association rate constant and $[A]$ is the analyte concentration

In this case, stochastic sensing provides useful kinetic data that are difficult to obtain by other techniques. In other cases, the binding kinetics are more complex, but this can be useful by providing highly distinctive current signatures. In practice, it is not necessary to understand the details of the kinetic behaviour to allow the identification and quantification of analytes.

been engineered so that binding of an analyte in the external solution produces a reorganization within the lumen. Consequently, the current passing through a single pore responds to each binding event. Although this kind of stochastic sensing does not occur in nature, the binding sites used are based upon natural examples.

Detection of ionic species

Engineered versions of the αHL pore have been used to detect divalent metal ions (M(II))[6,9]. In the best studied case, residues with side chains projecting into the lumen were replaced with four histidines to form the mutant subunit 4H. This was incorporated into a heteromeric pore comprising six wild-type subunits and one 4H subunit ($WT_6 4H_1$). The resulting pore contained a single M(II)-binding site resembling that of carbonic anhydrase. Single-channel recordings showed that $WT_6 4H_1$ responds to nanomolar concentrations of Zn(II) (Fig. 1a). Single-molecule detection also enables mixtures of ions to be analysed — more than one analyte can bind to the M(II)-binding site of $WT_6 4H_1$, but because binding is mutually exclusive, only one M(II) is bound and detected at any given moment, and the identity of each M(II) is revealed by its characteristic signature (Fig. 1a). The approach can be applied to other charged analytes. For example, αHL pores that detect anions have been prepared recently by replacement of residues within the β-barrel by mutagenesis (S. Cheley and L. Gu, unpublished results).

Comparison of multianalyte detection by the olfactory system

The mammalian olfactory system also uses 'crossreactive' receptors, each species of which binds a different set of analytes. The receptors differ in their affinities for analytes, such that the sensor array of the olfactory epithelium, which contains hundreds of receptor species each confined to a distinct set of cells, is capable of identifying thousands of individual odours. By contrast, analytes are identified in stochastic sensing from their current signatures. Combining the two ways of distinguishing signals from crossreactive receptors, by using arrays of stochastic sensing elements, could provide a powerful technology for analyte detection.

Because sensor elements capable of binding a variety of analytes can be used in stochastic sensing, the required genetic engineering. is simplified. By contrast, specific receptor sites are necessary where protein-based biosensors are used with outputs derived from ensemble properties. The application of crossreactive sensor elements might be extended to the notion of sensing groups of analytes. Stochastic sensors that reliably detect a class of molecules such as organophosphorus agents would be of considerable interest.

Families of genes encode receptors in the olfactory system. By comparison, large numbers of related responsive pores can be generated in a combinatorial approach. In the case of M(II), residues with side chains capable of coordination, such as histidine, aspartate, glutamate and cysteine, can be patterned in hundreds of ways on the interior surface of a β-barrel, and wild-type and mutant subunits can be arranged within the heptamer in numerous combinations and permutations[6].

Detection of organic molecules

A similar approach might be applied to the analysis of organic molecules[10]. Although it is more difficult to design binding sites in this case, it is possible to place non-covalent adapters in the lumen of the αHL pore for periods sufficiently long to observe host–guest interactions[10]. For example, when β-cyclodextrin is lodged in the pore, it remains capable of binding the same organic molecules that it binds

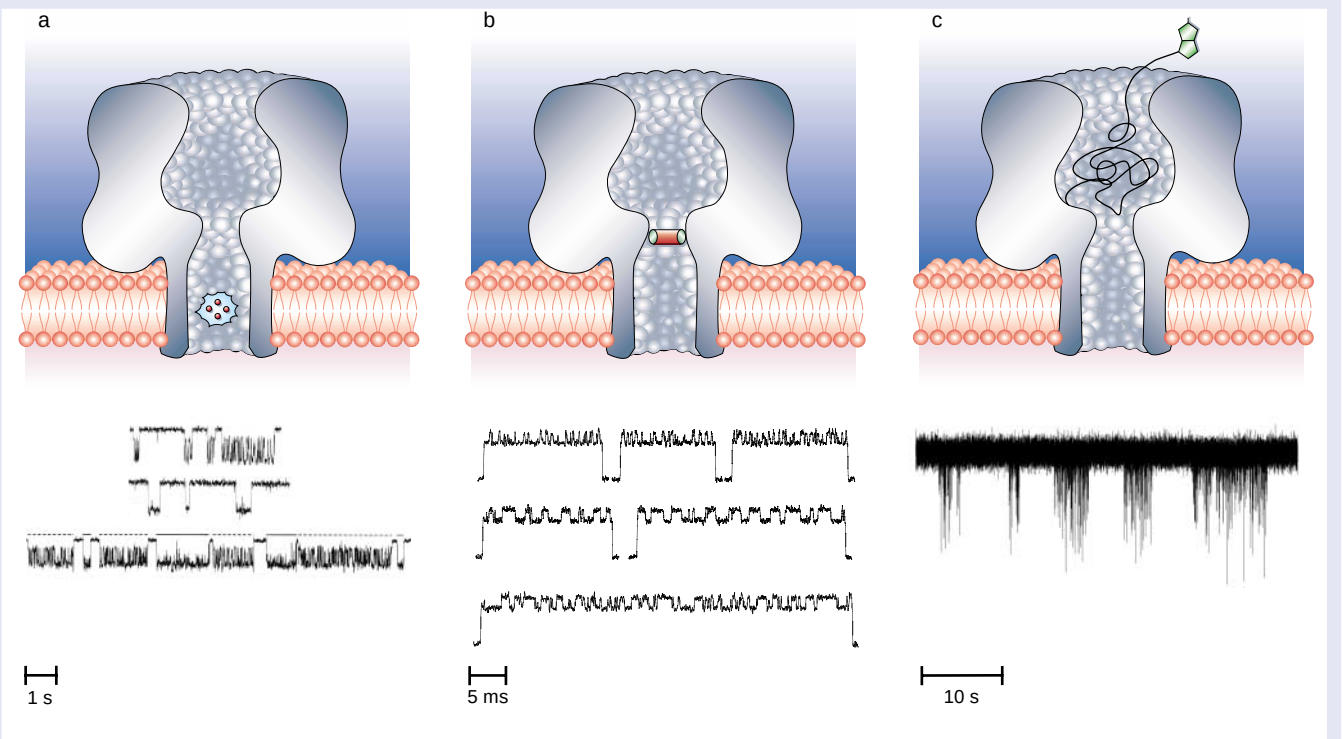


Figure 1 Detection of a variety of analytes by stochastic sensing. **a**, Metal ions[6,9]. The sensor element is an αHL pore with a genetically engineered binding site for divalent metal cations within the lumen. The site comprises four histidines on one of the seven subunits. Various metal ions are coordinated by the engineered side chains, which project into the transmembrane β-barrel. Each metal ion binds with different kinetics and gives slightly different extents of channel block, so that analysis of extended current traces allows the identification and quantification of metal ions in mixtures. Upper trace, Zn^{2+} alone; middle trace, Co^{2+}; lower trace, mixture of Zn^{2+} (0.23 μM) and Co^{2+} (4.7 μM). **b**, Organic molecules[10]. The pore contains a non-covalent β-cyclodextrin adapter, which is capable of carrying out host-guest chemistry while lodged in the lumen. Upper trace, promethazine; middle trace, imipramine; lower trace, mixture of promethazine (100 μM) and imipramine (100 μM). **c**, Proteins[21]. The pore contains a single poly(ethylene glycol) chain of relative molecular mass 3,400. One end of the chain is attached covalently within the cavity of the αHL pore, the other end carries a ligand for the target protein. The trace shows the response of a pore carrying a biotin ligand to a mutant streptavidin (14.5 nM) of weakened affinity.

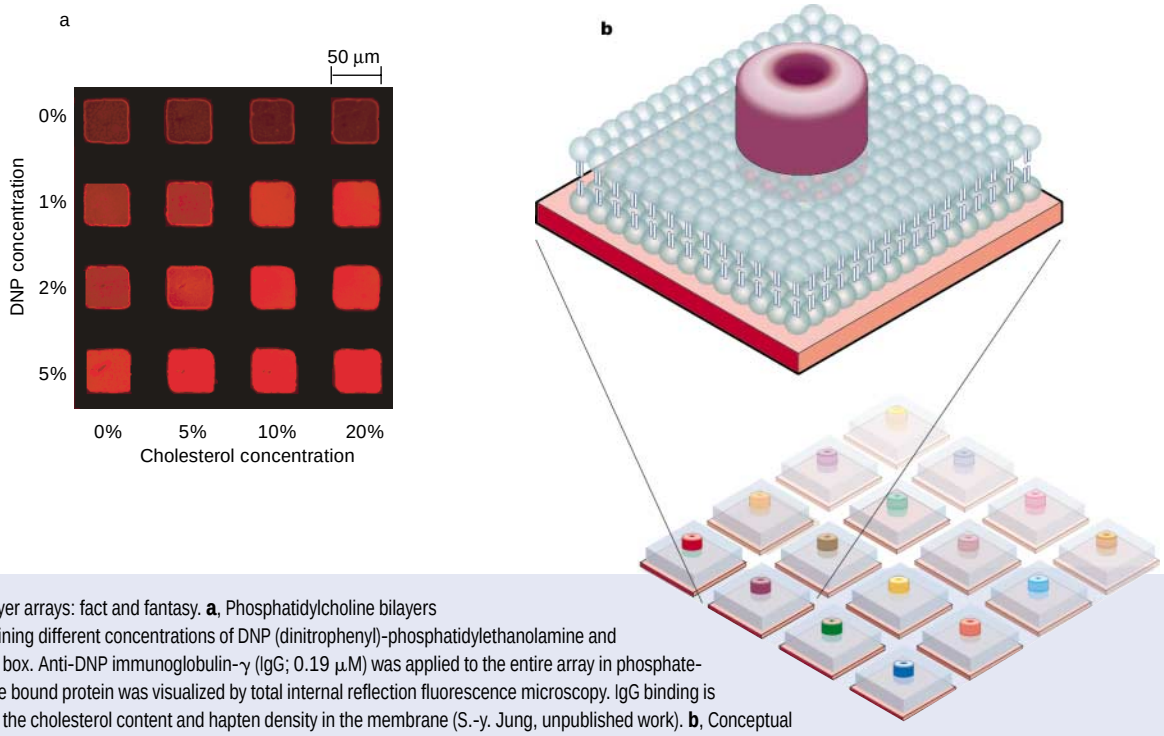


Figure 2 Lipid bilayer arrays: fact and fantasy. **a**, Phosphatidylcholine bilayers (4 × 4 array) containing different concentrations of DNP (dinitrophenyl)-phosphatidylethanolamine and cholesterol in each box. Anti-DNP immunoglobulin-γ (IgG; 0.19 μM) was applied to the entire array in phosphate-buffered saline. The bound protein was visualized by total internal reflection fluorescence microscopy. IgG binding is dependent on both the cholesterol content and hapten density in the membrane (S.-y. Jung, unpublished work). **b**, Conceptual rendition of a stochastic sensor array. Each box contains a different, single, engineered αHL pore. The bilayers are arrayed over a system of electrodes.

when free in solution; these binding events cause current fluctuations that permit the quantification and identification of the molecules[10] (Fig. 1b). Multiple molecules can be detected concurrently with the same cyclodextrin[10], and the system is highly flexible and combinatorial. Additional adapters can be used (for example, cyclic peptides[11]) and the pore can be engineered to accommodate one, or even two, adapters for far longer periods than the wild-type protein[12].

Detection of macromolecules

Single-stranded RNA or DNA molecules can pass through the wild-type αHL pore in an elongated conformation[13–17]. The transit time and extent of current block reveal information about the length of the nucleic acid and its base composition[13,15,16]. In certain cases, in a step beyond what would be offered by a molecular Coulter counter[3,18], the magnitude of the conductance changes within each blocking event communicates additional details about nucleic acid structure, such as the composition of stretches of ~50 bases[14] or the presence of mismatches in DNA hairpins[19].

Protein engineering of the αHL pore greatly increases the possibilities for stochastic sensing of macromolecules. When single-stranded oligonucleotides are covalently attached within the large cavity of the pore, sequence-specific duplex formation can be detected[20]. But protein analytes are too large to fit inside the pore and a means to couple external binding with events in the lumen is required. This was accomplished by anchoring one end of a polymer covalently inside the lumen and attaching biotin to the other end. The motion of the polymer within the pore was altered when streptavidin or an antibody captured the biotin in the external medium, producing a change in the single-channel conductance (Fig. 1c)[21]. The approach might be modified for use with a wide variety of protein ligands.

Advantages of stochastic sensing

Stochastic sensing is highly sensitive, the response is rapid and reversible (allowing real-time monitoring of analytes) and the dynamic range is wide. In addition to detecting both the concentration and identity of an analyte, several analytes can be quantified concurrently by a single sensor element. The sensor element need not be highly selective, as each analyte produces a characteristic signature. This has the advantage of easing the demands for protein engineering, as well as preventing confusion arising from signals from analytes that are structurally similar to the target molecule. Fouling of the sensor element cannot give a false reading, as the signal would not be characteristic of an analyte. Further advantages include no loss of signal-to-noise at low analyte concentrations (permitting the detection of rare events), a 'digital' output for facile electronic interfacing, self-calibration, reagentless operation, and the potential for nanoscale miniaturization.

Fabricating durable and practical devices

Improved apertures

One disadvantage of the current generation of stochastic sensors is their lack of durability, which confines their use to the laboratory. Several crucial advances will be required to make a practicable device. Present platforms use αHL in lipid bilayers suspended across apertures in a polymer film such as Teflon. The apertures are typically a few hundred microns in diameter, and the bilayers often rupture after a few hours of use. Several strategies could be implemented to remedy this problem. Nanoscale apertures (10 nm to 1 μm) might limit the magnitude of bilayer undulations, which probably are a source of breakage[22]. The current revolution in lithography should make it possible to limit the hole size to an area slightly bigger than the protein pore itself. This would not only increase membrane durability, but also allow apertures to be designed that accommodate only a single protein pore, thereby facilitating the manufacturability of stochastic devices. Recent work with larger microfabricated apertures in silicon-based substrates shows promise[23]. The smallest apertures reported so far that have been covered with bilayers are ~30 nm in diameter, but more work needs to be done to lower the capacitive noise in these systems[24]. It might even be possible to dispense with a

bilayer and use a hole into which engineered pores fit snugly. For example, gold-coated nanotubules can be created by plating etched particle tracks in polycarbonate filters. In this case, the surface of the nanotubule and the pore might be tailored for fit and compatibility[3].

Bilayers on solid supports

Another approach to rugged sensor design could involve the incorporation of αHL into bilayers on solid supports[25,26]. When the support is a conducting substrate, an alternating potential can be applied to allow impedance measurements. But impedance spectroscopy of tethered supported bilayers treated with αHL suggests that the protein does not fully penetrate the membrane, so improvements will be needed to obtain fully conducting pores[27]. Although sensors based on supported bilayers have been made with several other channel-forming peptides and proteins[28–31], single-channel recordings — the prerequisite for stochastic sensing — have not yet been obtained. In this regard, a major challenge is the production of tightly sealed bilayers to reduce leakage currents to the levels found in unsupported membranes. Most likely, this will require the reduction of substrate roughness and the elimination of edge effects. Through a combination of improved substrates, new membrane chemistry and faster electronics, single-channel measurements with supported bilayers at millisecond time resolution should be achieved in the near future[32].

Bilayers on porous supports

Porous supports might provide a middle ground between apertures and solid supports. They would reinforce the bilayer and at the same time provide access for aqueous ions to the entrance of a pore. Possibilities include nanochannel glass arrays[33] and coated polycarbonate membranes[34]. Bacterial S layers are especially promising as supports. S layers are porous, two-dimensional crystals of a single protein that envelope many species of bacteria. Single-channel currents have been observed from αHL incorporated into bilayers supported by S layers[35,36]. Peptide antibiotics and an anion channel have been incorporated into bilayers on agarose-coated glass and single-channel currents detected[37].

Sensor arrays

The use of conducting planar supports not only has the advantage of durability, but also provides a convenient environment for patterning arrays of membranes. Ultimately, individual electrodes in the array would act as transducers for each sensing element. Lithographic patterning of very small membrane sectors may also help eliminate problems with current leakage — as the size of the bilayer elements is shrunk, a larger fraction of them will be positioned over defect-free portions of the surface. Significant progress has been made on the fabrication of supported bilayer arrays[38]. For example, small arrays have been made in which individual 'boxes' have different lipid compositions (Fig. 2a)[39] and a means by which each box can be contacted by a different aqueous phase has been devised[40]. Nevertheless, considerably more progress will be needed to produce arrays of single pores that are individually electrically addressed (Fig. 2b).

In the future, the platforms described above will be combined with microfluidics to create lab-on-a-chip technology[41]. Fabrication of linear microfluidic arrays in which both the lipid bilayers and aqueous media can be varied has already been achieved[42]. Arrays of sensor elements will be patterned into a substrate connected to a system of microchannels through which complex mixtures of analytes can be delivered. The array would constitute a sophisticated electronic 'nose' or 'tongue' consisting of single-molecule sensors from which a stochastic response pattern would be read. Data processing approaches such as computational neural networks have been used for other types of sensor arrays[43] and are likely to be effective with stochastic systems. The detection devices would be powerful enough to tackle broad classes of chemical and biological analytes with millisecond time resolution and ultra-low concentration limits. Marrying the emerging technology of stochastic sensing to microfluidics affords the additional benefits of high throughput and very small sample volumes. Of course, in this case, miniaturization will not be limited by the size of the sensor elements as a single αHL pore is only ~10 nm in diameter.

Prospects and alternative implementation

Stochastic sensing platforms should find use in diverse applications from the detection of biological warfare agents to the testing of groundwater for heavy-metal contaminants. One of the most powerful potential applications may be in pharmaceutical screening based on proteomics, where fast time response, trace analyte detection and high throughput are required[44]. A significant characteristic of the technique is that the analytes, whether they be small or large molecules, need not be tagged or labelled for detection[44]. Also of note is that stochastic sensing reveals quantitative kinetic information (on and off rates) about the interaction of an analyte with its binding site. Indeed, stochastic sensing can operate under equilibrium conditions with very fast time resolution (hundreds of microseconds) without the complications arising from crowded chip surfaces such as unwanted polyvalency and rebinding. By using single αHL pores decorated with multiple ligands in a predetermined pattern, it should be possible to examine directly the multivalent attachment of proteins[45] in a far cleaner way than is presently possible. And stochastic sensing has exciting potential applications in basic science. For example, analytes might be detected inside cells by a variation of patch-cramming[7,46] in which a responsive pore is incorporated into a patch pipette that is inserted into the cell under examination.

Although engineered protein pores hold sway for the present, stochastic sensing might be executed in other ways. Nanoscale alternatives to protein-based pores are being sought. Tubules with diameters of less than 2 nm can be obtained by electroplating pores of larger diameter[47], although they have not yet been isolated as single entities. However, single carbon nanotubes, 150 nm in internal diameter, have been characterized[48], and further reduction in diameter should be possible. In a recent breakthrough, apertures as small as 2 nm in diameter have been made in silicon nitride, and the transport of double-stranded DNA through a 5-nm version has been detected[49]. Eventually, it might be possible to manipulate the chemistry of these entities as readily as proteins.

Alternatives to ion channel-based stochastic sensing include single-molecule fluorescence[50,51] and force detection[52,53]. If practicable implementation can be found, data from fluorescence-based stochastic sensing would be handled in the same way as that obtained through electrical measurements[51]. Exploiting these newer technologies may open up a wider field of potential analyte targets, because not all binding sites are readily incorporated into protein pores.

The acquired wisdom of nature has made several contributions to stochastic sensing: the use of protein pores, the design of binding sites, the sensing of unmodified analytes and, potentially, the use of sensor arrays. Yet, like the best of biomimetics, the development of stochastic sensing has not blindly followed nature but it has evolved in its own way[54]. With more exploration to be done, the final embodiment of this powerful technology cannot yet be foreseen. □

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