Soft matter biosensors: stochastic and deterministic membrane sensing

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Abstract | The concept of bilayer lipid membrane sensing is elaborated. Disposable bilayer lipid membrane sensors permit both stochastic and deterministic sensing regimes. Detection of cyano-bacterial toxins in waters by means of a stochastic sensing (ion channel induction) reveals a signature for a particular toxin type. Flexoelectricity of membranes provides a way of deterministic sensing. Detection of environmental pollution of waters by heavy metal ions (Cd^{++} and Hg^{++}) is thereby rendered possible.

1. Introduction

Bilayer lipid membranes are self-assembled aggregates of amphiphilic lipid molecules in water. Amphiphilic (biphilic) molecules consist of two distinct parts: a polar, often ionic, head and a nonpolar, often hydrocarbon, tail. These two parts are named hydrophilic and hydrophobic respectively.

Amphiphilic molecules in water solutions tend to self-assemble, forming above a certain concentration 3D lyotropic liquid crystals, named also lyotropic mesophases. For this process lyotropic mesogens with high enough molecular weight and well expressed molecular asymmetry are necessary. A very common group of lyotropic mesogens comprises soaps and detergents. Another group of lyotropic mesogens is provided by lipid molecules. On Figure 1 one can see a single, 2D lipid bilayer that is a prototype for a biological membrane.

Nowadays is well known that lyotropic liquid crystal state is the most convenient condensed state for the living matter as it combines molecular order and disorder in a very special manner, intermediate between the fully disordered isotropic fluid state (which is dead), and the complete order of solid state (which is dead again).

Biomembranes are built up by lipid molecules according to the general principles of lyotropic liquid crystals.[1] Celebrated "fluid lipid–globular protein mosaic model"[2,3] implies that lipids are organized in a liquid crystalline bilayer in which integral proteins are embedded (Figure 2). Membranes with high protein concentration, where lipids do not form a continuous bilayer but rather patch the spaces between proteins, while proteins themselves are arranged in a double tiered pattern, are also well known (cf. "structure-function unitization model of biomembranes".[4] In both cases, though, the principles of liquid crystal physics[5] (viz., long range translational disorder vs. long range orientational order of constituent flexible lipid chains or rod-like protein molecules) are valid.

Biological lipid membranes separate the interior of a cell from the outside world. Sub-cellular compartments are also separated by lipid membranes. The amphiphatic nature of the lipids assembles these molecules in such a way that a large hydrophobic barrier is created. The hydrophobic barrier prevents the flow of charged molecules across the membrane. However, to allow for a facilitated passage of ions or molecules from one side of the compartment to the other the lipid membrane is spanned by particular proteins adapted perfectly to the hydrophobic lipid environment. Some examples of membrane proteins are: ATPases, G-protein

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Figure 1: Scheme of lipid molecule (a) and lipid bilayer formed in water (b).

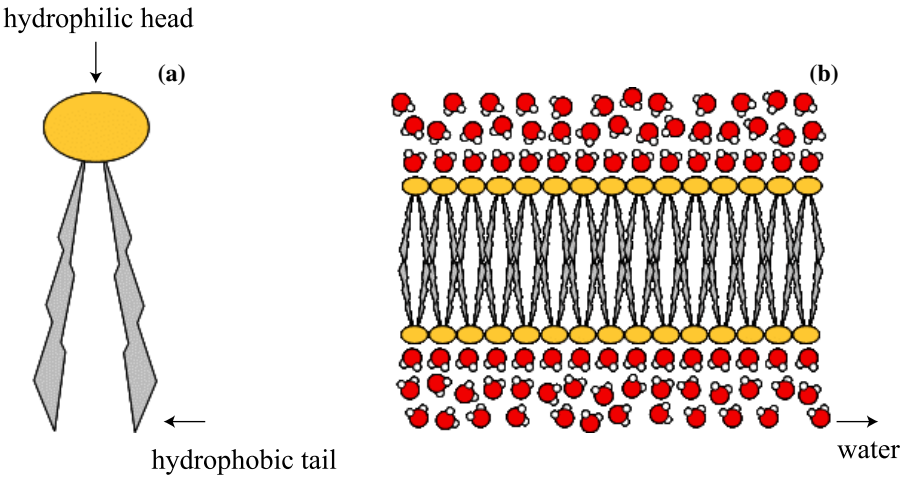
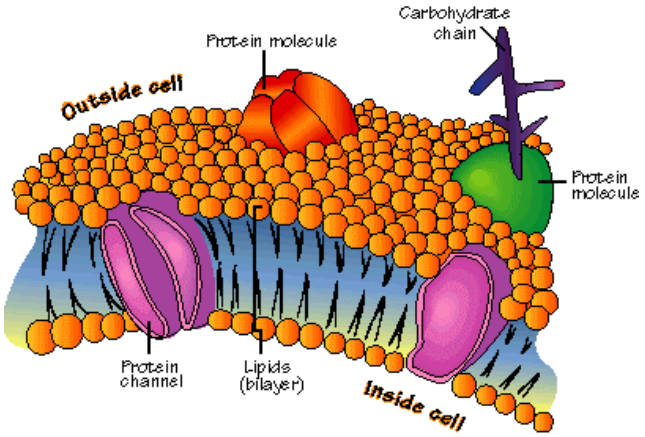


Figure 2: Diagram of a cell membrane. The proteins are embedded inside the lipid bilayer



coupled receptors, light harvesting membrane proteins, ion channels.

Biological membranes offer by far the most selective and specific sensors *in vivo*. Containing many diverse peptides in lipid environment, they ensure on molecular level the specific features required for sensing.[6] Applying a biomimetic approach we may hope to match the membrane excellent selectivity and specificity *in vitro*.

Ion channels are essential for many cellular functions, including electrical excitability, synaptic transmission, hormone release, intracellular Ca^{2+} signaling, salt/water balance, fluid secretion, cell volume regulation. In a broad sense, ion channels can be classified as voltage-gated, ligand-gated, intracellular messenger- gated, or constitutively active.[7] Ion channels, in addition being gated by their primary activator (or inhibitor), are also substantially modulated by phosphorylation, dephosphorylation, accessory proteins, ion concentrations, pH, interactions with scaffolding and/or cytoskeletal proteins, and other "secondary" effectors. Thus, cellular regulation of ion channel activity can be extremely complex.[8]

With the availability of the patch-clamp technique, the presence of ion channels has been

Figure 3: Design principles of biosensors: (a) Biocatalyst— converts the analyte into product. (b) Transducer—detects the occurrence of the reaction and converts it into an electrical signal. (c) Amplifier—amplifies the usually tiny signal to a useable level. (d) Microprocessor—signal is digitised and stored for further processing, e.g. integration, derivatisation, etc. e) Display - usually need a real-time display of the analyte concentration. In membrane sensors (a) and (b) are usually integrated. (Deterministic models describe the behavior on the basis of some physical law. In practice, a totally deterministic relationship is unlikely due to unpredictable factors. Where the influence of several unknown factors is sizable, exact prediction is not possible, but it may be possible to predict to within a known confidence interval—or to predict the probability that a particular value will be observed at a particular time. This is called a stochastic (or probabilistic) process.)

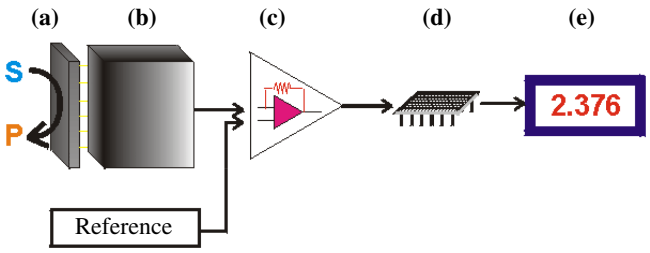
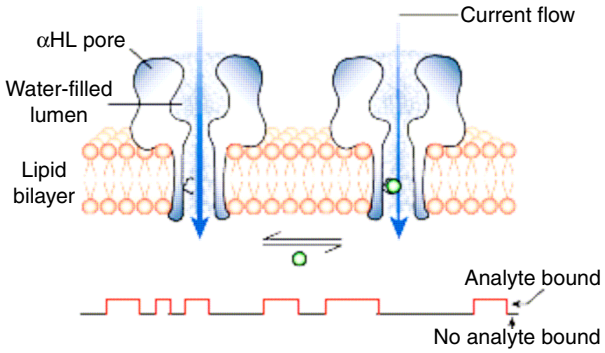


Figure 4: A single engineered pore, placed in a planar lipid bilayer. Under applied potential, a current flows through the pore carried by ions in salt solutions bathing both sides of the bilayer. The pore contains a binding site for an analyte represented by the green ball in the figure. Each time the analyte binds to the pore the current is modulated as illustrated in the trace.



established in all sorts of biomembranes.[9] A patch clamp method was originally developed for the investigation of single ion channels. To this aim, small patches of native membranes were sealed at the tips of glass micropipettes. The patch clamp method is well described in the literature. With the development of the tipdip technique formation of model phospholipid membranes on patch clamp pipettes from lipid monolayers also became possible. Thus, the method became available for reconstituted channel studies as well.

Many investigations on the potential use of ion channels in biosensor devices have been carried out for the last 2 decades.

Stochastic sensors have been the subject of considerable recent interest as a result of their ability for reconfigurable, rapid, multi-analyte detection as well as their potential for sequencing DNA. These sensors are created by placing a nanometre-sized pore in an insulating membrane and measuring the ionic transport through the pore in the presence of the molecules of interest. The magnitude, duration, and rates of occurrence of the resultant current blockades allow rapid determination of analyte concentration as well as discrimination between similar molecular species.[10]

Stochastic sensing has several advantages over conventional sensing including high sensitivity, a rapid and continiuos response, and wide dynamic range.[11,12]

Scheme of action of a stochastic sensor, based on a single pore placed in a planar lipid bilayer is given on Figure 4.

Figure 5: Scheme of lipid bilayer formation at the tip of patch pipette on left and corresponding current recordings on right.

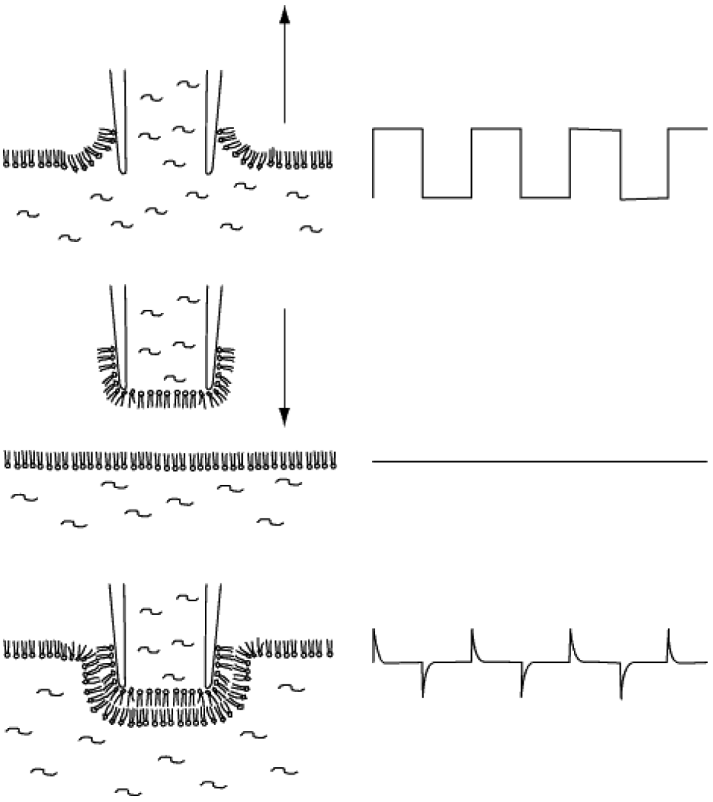


Figure 6: Two types of aggregation of defect-forming molecules (peptides) in lipid bilayers. A. channel; an aggregate of cylindrical molecules featuring transverse biphilic asymmetry (hydrophilic portions hatched in surface view illustrated below); B. pore; an aggregate of wedge-shaped molecules featuring longitudinal biphilic asymmetry.

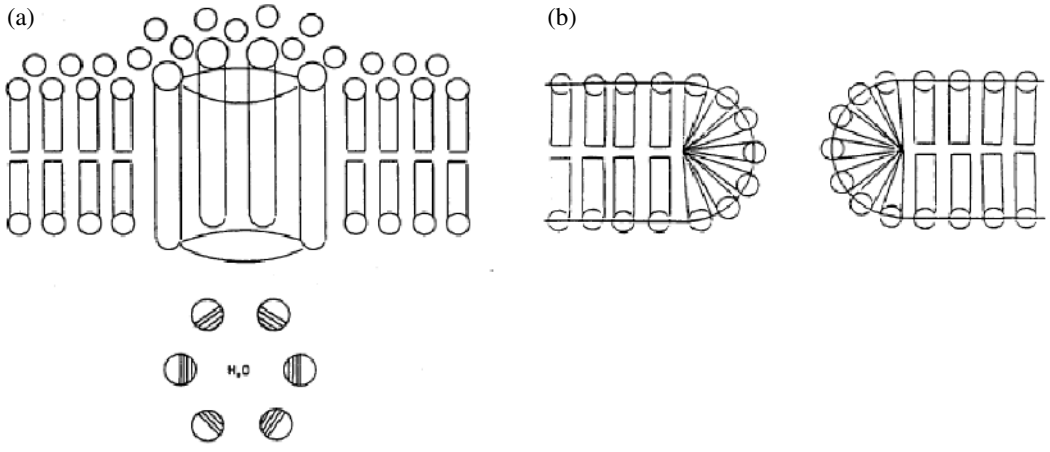
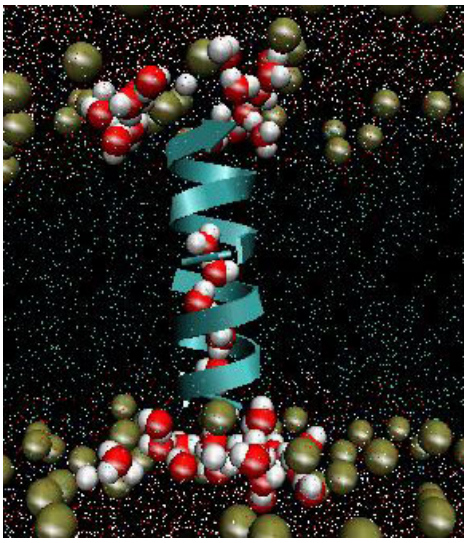


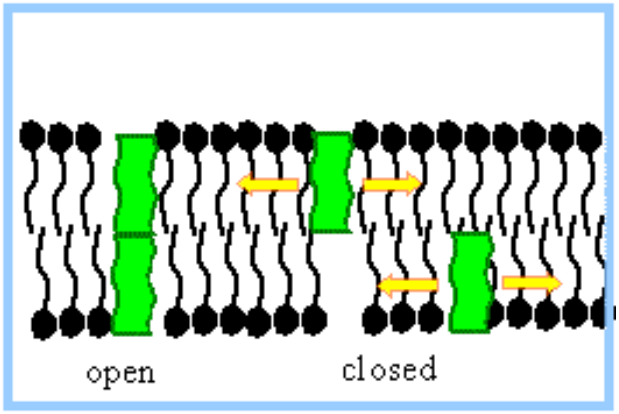
Figure 7: Molecular model of Gram.



A membrane has a number of mechanical degrees of freedom: area stretching, thickness compression, shear deformation, chain tilting, and, notably, curvature deformation. The last one, curvature, is just a liquid crystal degree of freedom since membrane curvature is equivalent to a splay of lipid chains (under the condition that chains remain parallel to the local normal in each point of the curved bilayer).

Flexoelectricity is a fundamental property of biomolecular layers relating their mechanical and electrical degrees of freedom. It is closely related to mechanosensitivity, electromotility and mechanotransduction, basic properties of living systems (see our general reviews[13,14]). Understanding the mechanism of mechanosensitivity would open the way for construction of artificial bioelectronic mechanosensors.[15–17]

Figure 8: Scheme of Gram ion channel.



2. Lipid bilayer membranes containing ion channels

In our experiments lipid bilayers (membranes) were self-assembled at the tips of glass patch pipettes using the method of Coronado and Latorre[18] from lipid monolayers formed by spreading the membrane-forming solution onto the surface of electrolyte solutions contained in Petri dishes (see Figure 5). This technique is named tip-dip patch clamp and it was used in all our experiments with model membranes.

Membranes were tested both with voltage ramps from −100 to +100 mV and with current recording at constant voltage. The data were analyzed using current-voltage surfaces software.[19]

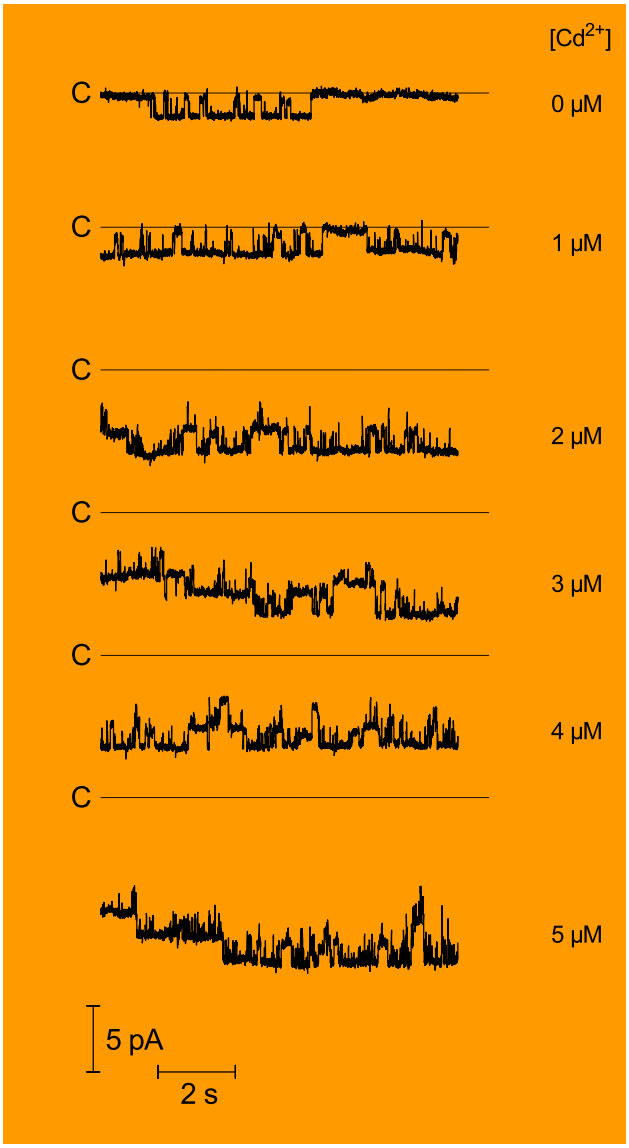
2.1. *Interaction of lipid bilayers containing channel-forming peptides with cadmium and mercury ions*

Let us consider lipid/peptide model membranes with multimeric ion channels formed by aggregation of peptide monomers. Depending on the biphilic asymmetry and shape asymmetry[20–22] two limiting types of peptide aggregates could be expected (Figure 6): cylindrical and semi-toroidal. The former are likely to be formed by roughly cylindric peptides with hydrophilic groups located on one side of the cylinder only (e.g. alamethicin, δ-toxin etc.)(Figure 6a). The latter are to be expected for edge-shaped peptides with hydrophilic groups located on the thicker end of the wedge (Figure 6b). We will designate the first type as a channel and the second type as a pore. Intermediate types of aggregates are also possible.

Heavy metals (Hg, Cd etc.) are known human toxicants, with the nervous system as a major target.[23]What is not clear is how some of these metals act on the nervous system. They have many potential targets, with membrane-bound signaling proteins, such as ion channels, being particularly vulnerable. Heavy metals may also act on the lipid components of the membranes of nerve cells, thereby, adversely influencing the excitable properties of these cells.

In the present investigation two channel-forming peptides were used: gramicidin (Gram)[25] and alamethicin (Alm)[26]. Lipid membranes were self-assembled at the tips of patch pipettes using the tip-dip patch clamp method of Coronado and Latorre.[18] Aqueous solutions of $HgCl_2$ and $CdCl_2$ were added to the Petri dish to final concentrations in the micromolar range.

Figure 9: Records of Gram channel currents with added Cd^{2+} in the range 0–5 μM (bath 100 mM KCl, pH 7.0; −80 mV holding potential). Closed current level is denoted by C.



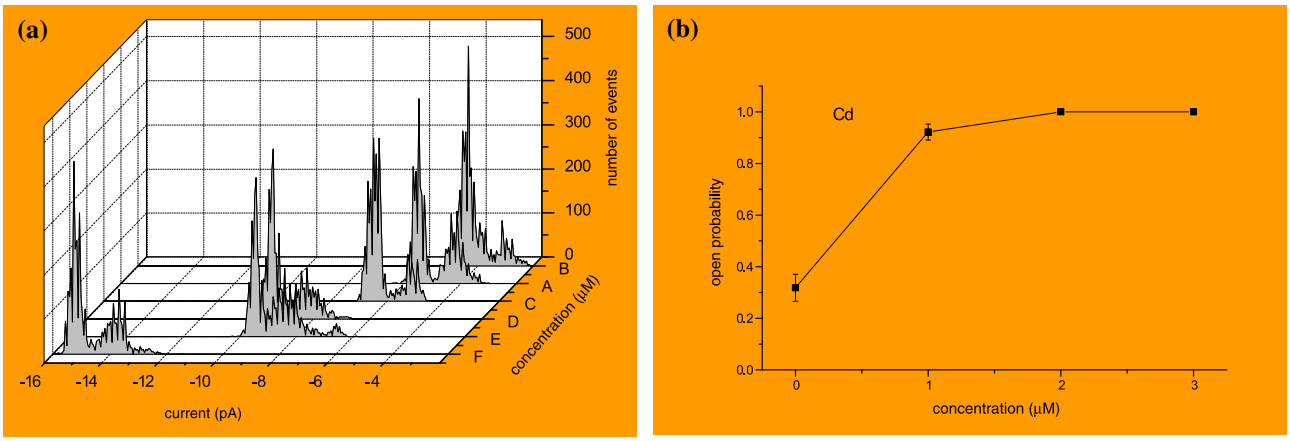
2.1.1. *Hg $^{2+}$ and Cd $^{2+}$ with Gramicidin D ion channels*

Gramicidin is a heterogeneous mixture of six antibiotic compounds, gramicidins A, B and C, making up 80%, 6%, and 14% respectively all of which are obtained from the soil bacterial species Bacillus brevis and called collectively gramicidin D. Gramicidin D are linear peptides; that is chains made up of 15 amino acids.[4] The chain assembles inside of the hydrophobic interior of the cellular lipid bilayer to form a β-helix. The helix itself is not long enough to span the membrane but it dimerizes to form the elongated channel needed to span the whole membrane.

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.[24]

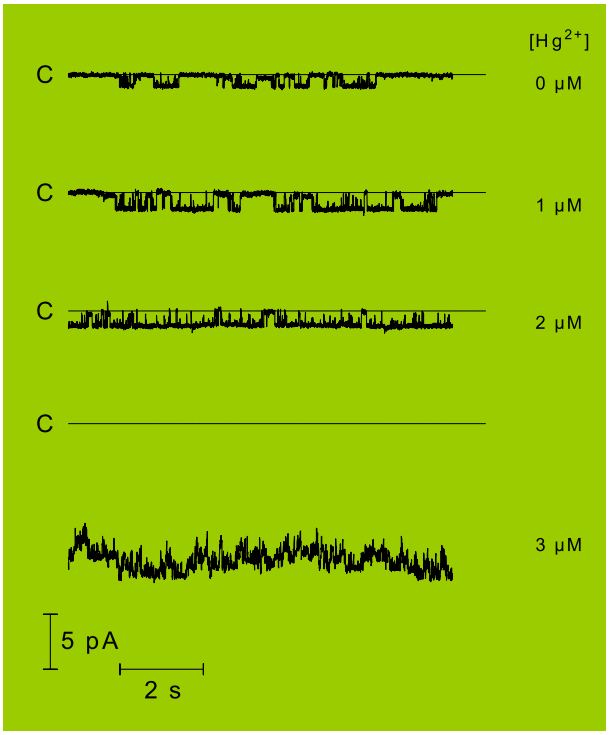
The effects of the heavy metals ions Cd^{2+} and Hg^{2+} on channels formed by gramicidin D in lipid bilayers have been investigated.[25] Cadmium and mercury increase the open probability of gramicidin

Figure 10: Concentration-dependent cadmium ion effects on gramicidin channels in Soybean lecithin membrane ($CdSO_4$ stock solution added to bath in the indicated final concentrations). (a) Current histograms of channel records, (b). Gram channel open probability vs. Cd concentration. Data are averaged area ratios from the Gaussian fit of current histograms of 5 independent membranes.



D channels. The conductance of these peptide channels is influenced by the heavy metals in a non-monotonic concentration-dependent manner.

Figure 11: Records of Gram single channel currents with added Hg^{2+} in the range 0–3 μM (bath 100 mM KCl, pH 7.0; −80 mV holding potential). Closed current level is denoted by C.



In the absence of the heavy metal salt, channel openings were alternated with relatively long closings. Cd caused a marked increase in open channel probability, resulting in simultaneous openings of channels (Figure 9 and Figure10).

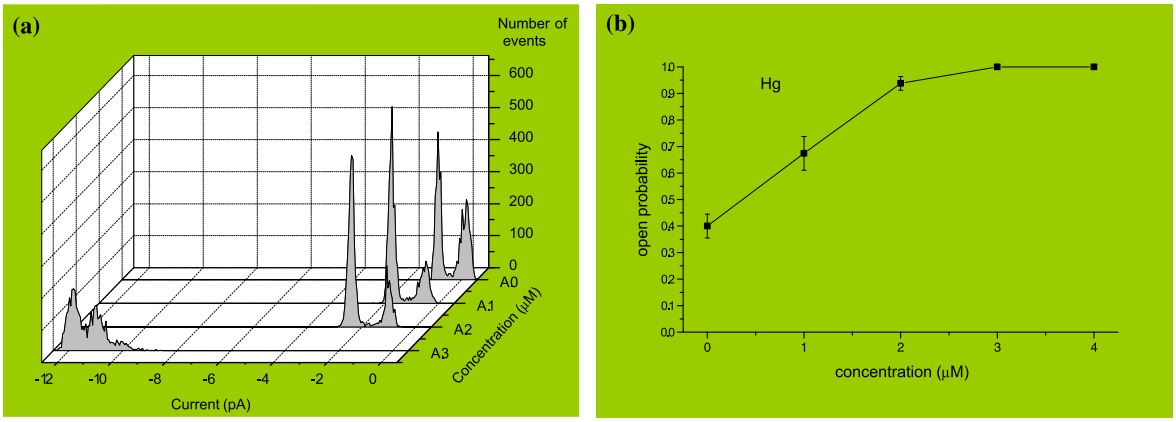
Figure 11 shows current traces representing the effect of Hg^{2+} on openings and closings of Gram channels. An abrupt increase of total current at 3 μM is also typical for the rest of investigated membranes (Figure 12). This may be due to a leak current of $HgCl^{+}$ ions through the lipid bilayer. The halogen salts of Hg are known to readily penetrate pure lipid bilayers as halogenated ions $HgHal^{+}$ [27]. This is due to the stepwise dissociation of these salts, where the dissociation constant of the first step to halogenated ion is much larger than the second one to complete dissociation.

2.1.2. *Hg^{2+} and Cd^{2+} with Alamethicin ion channels*

Alamethicin (Alm) is a peptide antibiotic produced by Trichoderma viride. It contains the non-proteinogenic amino acid 1-amino isobutyric acid (Aib), which strongly induces helical peptide structures (Figure 13). The peptide sequence is: Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Glu-Gln-Phl (Ac = acetyl, Phl = phenylalaninol).

In cell membranes Alm forms voltage dependent ion channels by aggregation of several molecules into a "bundle" or "barrel" structure which traverses the lipid bilayer. Single Alm channel recordings show multiple conductance levels (see Figure 14).

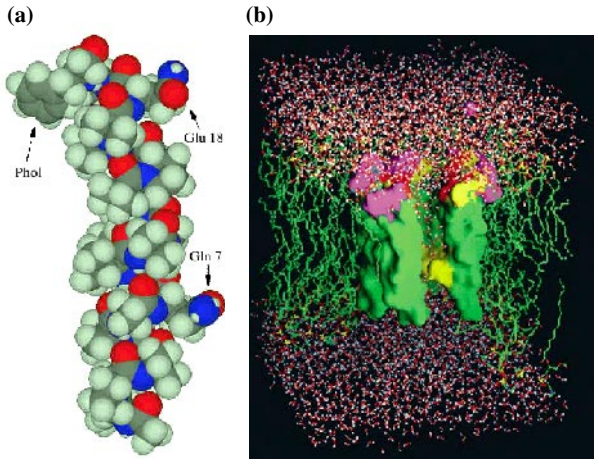
Figure 12: Concentration-dependent mercury ion effects on gramicidin single channel in Soy lecithin membrane ($HgCl_2$ stock solution added to bath in the indicated final concentrations): (a) Current histograms of channel records (b). Gram channel open probability vs. $HgCl_2$ concentration. Data are averaged area ratios from the Gaussian fit of current histograms of 5 independent membranes.



Our finding of Cd-induced inactivation of Alm could favourably be compared with the Ca-induced inactivation of the same channel found earlier.[28,29]

Hg^{2+} caused a monotonic increase in the open probability of Alm channels (Figure 16). Channel current histograms of a membrane at several $HgCl_2$ concentrations are given on Figure 16(a), while Figure 16(b) presents the second Alm conducting level opening probability of 4 independent membrane preparations at various holding potentials as a function of $HgCl_2$ concentration.

Figure 13: Alamethicin: (a) Crystal structure according Fox and Richards, (b) Model of alamethicin ion channel.



It is well-known that Alm ion channels display conductance asymmetry when Alm is added to the bath only: channels appear only at negative membrane potentials[30,31] (Figure 17a) Figure 17 shows another Hg effect on Alm channel opening: symmetrisation of the I–V curve (Figure 17b); channel openings appear also at positive pipette potentials).

Present investigation aimed to model Cd^{2+} and Hg^{2+} effects on the two components of a native membrane: the lipid matrix and the ion channels. This would then provide a clue for their neurobiological effect on native ion channels in neural and muscle membranes. It is known that the behavior of Gram and Alm channels depends heavily on the membrane environment in which they are formed. Membrane fluidity affects the Gramicidin-A dimer dissociation rate so that in more fluid membranes the channel lifetime is reduced.[33,34] A similar observation was made for Alm where transition rates between different conducting states were found dependent on the membrane fluidity. Our results demonstrate that ion channels are influenced in a specific way: Gram is activated by both ions, while Alm in activated by mercury, but deactivated by cadmium. Control experiments (not shown) evidence that SL lipid membranes without channels are also sensitive to these heavy metal ions at some critical concentrations (around 5 and 50 μM), and tend to develop metastable lipid pores that could lead to increased leak current and, eventually, electric breakdown.[32]

The results of this investigation suggest that the different effects of Cd^{2+} and Hg^{2+} could be used to discriminate between these two ions.

Figure 14: Current recording of a single Alamethicin channel in a patch membrane of soy lecithin, 0.5 M NaCl, pH7, 80 mV. From our experiments [26]is evident that Cd^{2+} inhibits Alm channel activity. The monotonic character of this inhibition is evident in Figure 15(a), which shows Alm channel histograms for concentrations ranging from 0 to 80 μM. The effect of Cd^{2+}concentration on p_o of the second Alm conductance level is shown in Figure15(b) for 4 independent membrane preparations at various holding potentials. Apart from the curve at 190 mV all other curves demonstrate a monotonic inhibition of open probability.

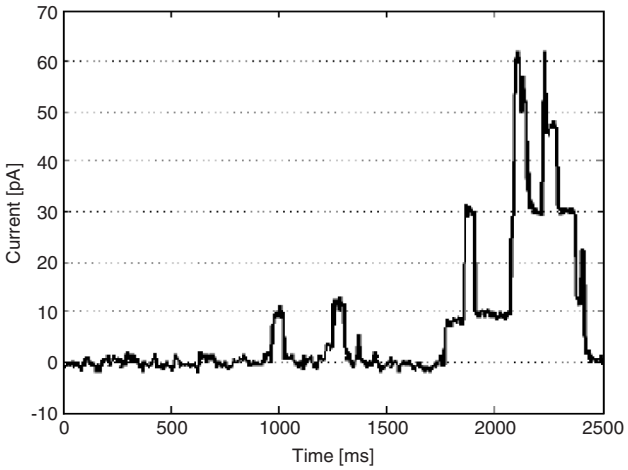
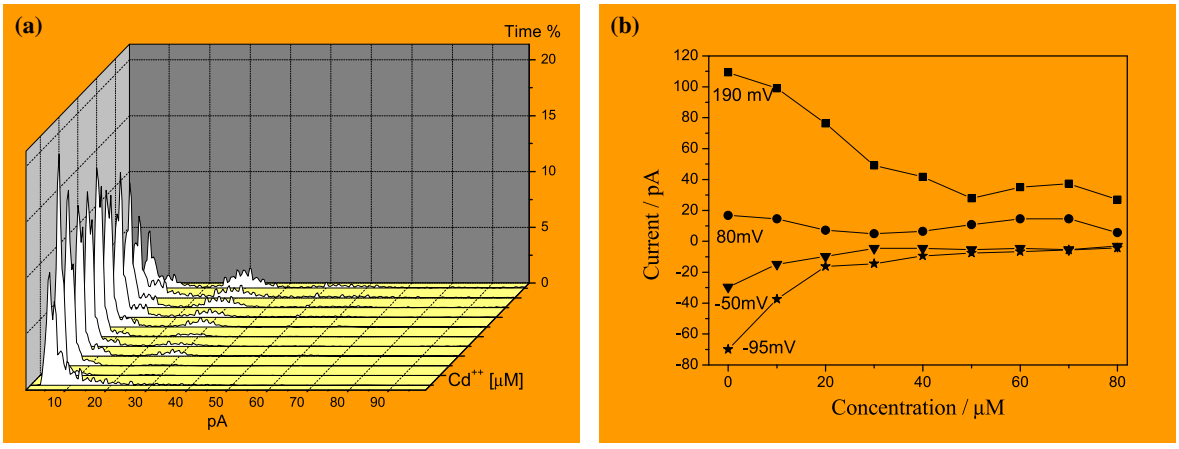


Figure 15: Effects of cadmium ions on alamethicin channels in Soy lecithin membrane ($CdCl_2$ stock solution added to bath in the indicated final concentrations): (a) Current histograms of channel records, (b) Time-averaged current for 4 individual membranes at the indicated holding potentials.



2.2. *Membrane sensing of cyanotoxins*

Microcystins and nodularins are the most widespread cyanotoxins. They can be found in cyanobacterial blooms ranging from freshwater bodies to oceans. They can be very toxic for plants and animals including humans. The presence of cyanobacterial toxins in drinking water supplies poses a serious problem to water treatment facilities, since not all technical procedures are able to effectively remove these toxins to below acceptable levels.

2.2.1. *Microcystin-LR*

Microcystins are a group of cyclic heptapeptide (7 amino acids) hepatotoxins (liver toxins), containing special β-amino acid ADDA (*(all-S,all-E)*-3-Amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-diene acid) (Figure 18). There

Figure 16: Effects of Hg ions on Alm channels ($HgCl_2$ stock solution added to bath in the indicated final concentrations): (a) Current histograms of channel records at holding potential −70 mV. (b) Time-averaged current for 4 individual membranes at the indicated holding potentials (concentration range 0–80 μM; bath 500 mM NaCl, pH 7).

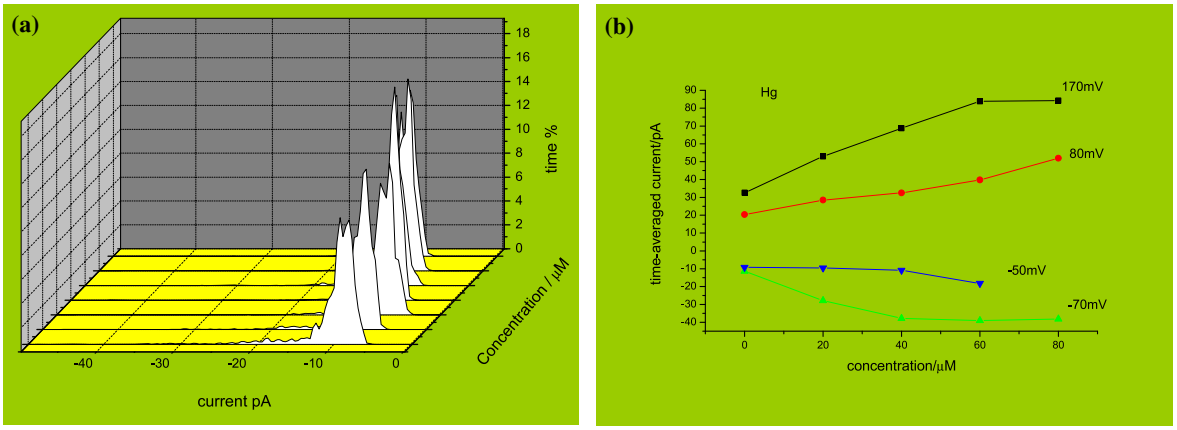
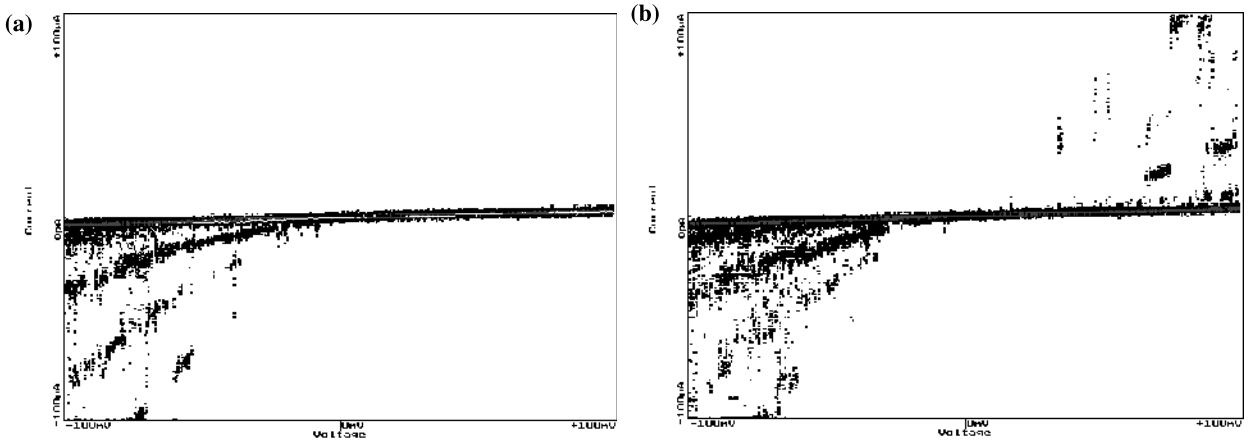


Figure 17: Current-voltage surfaces for alamethicin channels. Symmetrisation of the I–V curve: (a). Alm channels' I–V surfaces in a patch clamped membrane after adding Alm to the bath. (b) I–V surfaces of the same membrane after $HgCl_2$ addition to bath.



have been approximately 60 different microcystins identified to date. The most common and potently toxic is Microcystin-LR (MC-LR).

The cycle contains hydrophilic aminoacids and serves as a polar head, while the residue of the nontypical aminoacid ADDA is hydrophobic and serves as nonpolar tail. The overall molecular structure thus features a wedge-like steric asymmetry that is a prerequisite for self-assembling in multimers having the structure of an inverted toroidal pore of the structure shown on Figure 6B.

Lipid bilayers of diphytanoyl lecithin (DPhL) in which a cyanobacterial toxin, Microcystin-LR (MC-LR) was incorporated from the ambient solutions, were found to be a convenient model for toxin sensing.[35] These model membrane formed on the tips of glass micropipettes were investigated using path-clamp methodology. Emplacement of MC-LR from the bathing solution was enhanced by transmembrane voltage. MC-LR pores could be recorded over a wide voltage range, their opening probability being first increased and than reduced at high membrane potential. The pores exhibited several open pore conductance levels. Presumably, these pores feature the structure displayed on Figure 6B. Ion gradient experiments established that MC-LR pores are cation selective, but discriminate only weakly between K^+ and Na^+.

Figure 18: Molecular structure of Microcystin.

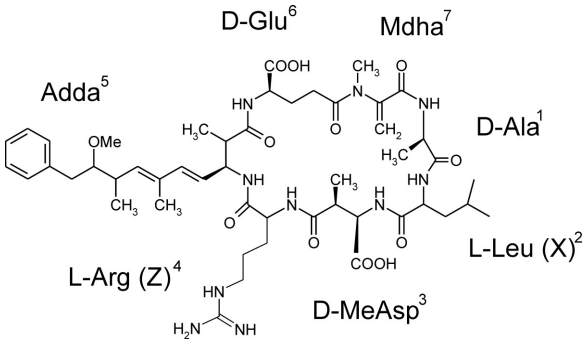


Figure 19: Molecular structure of Nodularin.

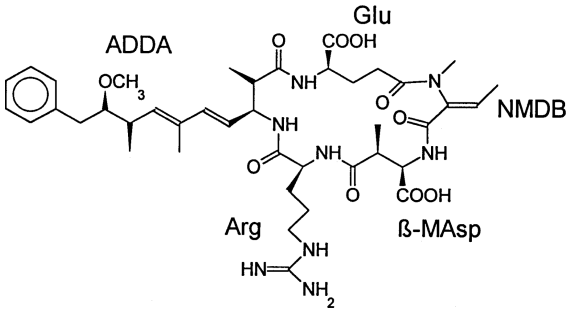


Figure 20: Current-voltage surfaces: (a) of NODLN containing bilayer: (b) of Microcystin-LR containing bilayer. Insets: individual current ramps before processing. These currents were obtained as responses to slow voltage ramps (−100 mV to 100 mV) with a triangular (a) or sawtooth (b) shape. The corresponding (I–V) pairs were binned is pixels over the I-V plane, and isoprobability lines were drawn by the dedicated software through the pixels with the same probability of the I–V pairs, illustrating the cross-sections of the probability surface projected over the I–V plane.

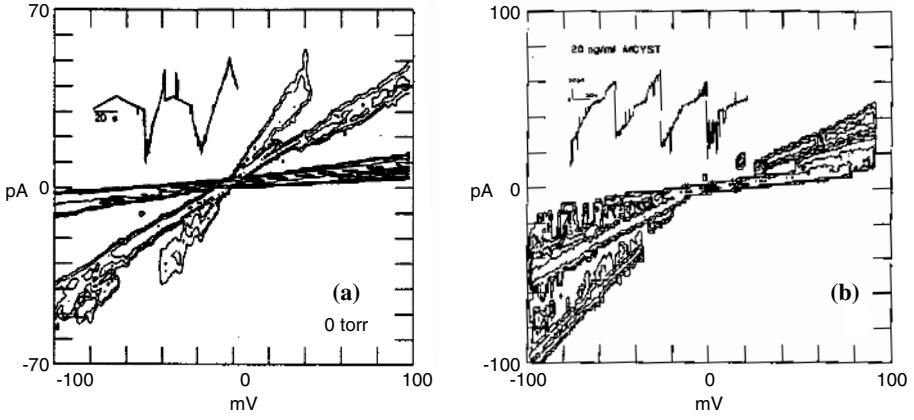
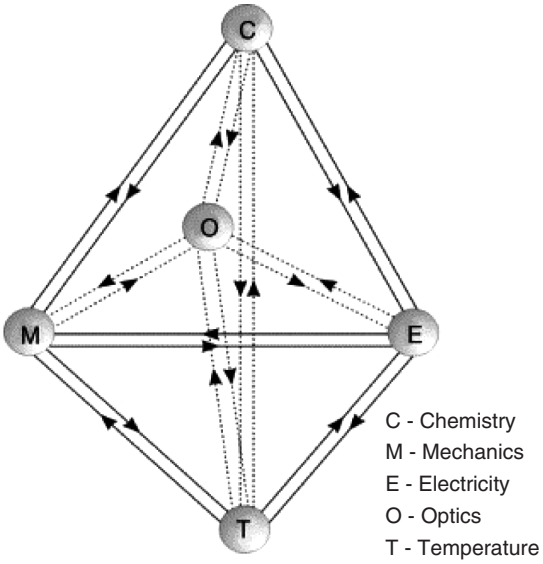


Figure 21: Possible mechanisms of energy interconversion in flexible, electro-, photo-, chemi- and thermo-active membranes. Effects involving 2 degrees of freedom of the membrane system can be marked by two letters (e.g., ME and EM, the direct and converse flexoeffect) and those involving 3 degrees of freedom by three letters (e.g., OME, opto-mechano-electric effect, viz. photo flexoelectric effect).



The toxin containing membranes are subjected to slow voltage ramps and the current responses are patch-clamp amplified, recorded and processed by a current-voltage software to provide an averaged picture of all conductance states of a toxin-induced pore (Figure 20B)

2.2.2. *Nodularin*

Nodularin (NODLN) is a cyclic pentapeptide hepatotoxin (Figure 19). It contains two aminoacids less in his cycle. Interestingly, both NODLN and MCYST contain the unusual aminoacid ADDA.

Lipid bilayers of Diphytanoil lecithin (DPhL), in which NODLN was incorporated from the bathing solution, were also investigated[36]. It was found that NODLN also induces conductive pores in membranes. These pores also exhibit many open conductance states. They are also of the type displayed on Figure 6B. However, since the NODLN is a pentapeptide, while MCYST is a heptapeptide, the steric asymmetry of the two molecules differ. Consequently, the number of toxin monomers necessary to complete an inverted pore (cf. Figure 6B) is different (higher for NODLN), and the conductances of the various open states (obtainable by recruiting a few more monomers) should be different, too. Correspondingly, the current-voltage surfaces look quite different, thus providing a signature of a particular toxin type.

The results described above suggest that a lipid membrane containing multimers of pore-forming toxin molecules provides a convenient way to detect the toxins and to discriminate between them.

3. Flexoelectricity

Flexoelectricity is a phenomenon of curvature-induced electric polarization of liquid crystal membrane, in which the molecules of the membrane are uniaxially oriented. Flexoelectricity is a liquid crystal analogue of piezoelectricity. Instead of a translational mechanical degree of freedom of a membrane in the case of piezoelectricity (i.e., area stretching, thickness compression, etc.), flexoelectricity involves an orientational degree of freedom, the membrane curvature. Flexoelectricity stands for a reciprocal relationship between electric and mechanical degree of freedom of a membrane, i.e., curvature-induced membrane polarization (direct flexoeffect) or voltage-induced membrane curvature (converse flexoeffect). The theory of this phenomenon was considered in several papers of ours[1,37–40] Many native membranes (e.g. mitochondria, chloroplasts, erythrocytes, pseudopodia, muscle membrane etc.) are known to exhibit changes of curvature. This implies that flexoelectricity is a fundamental biological phenomenon and may be a mechanism for coupling the mechanical and electrical degrees of freedom of a biological membrane (Figure 21)[41]

Figure 22: Time dependence of the 1st harmonic of the flexoelectric current of Membranes treated with Cd^{2+} at 410 Hz.

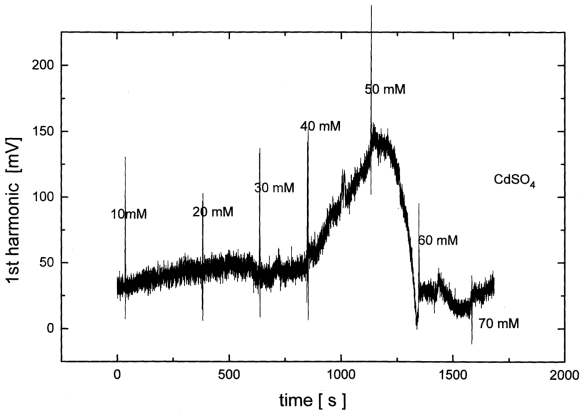
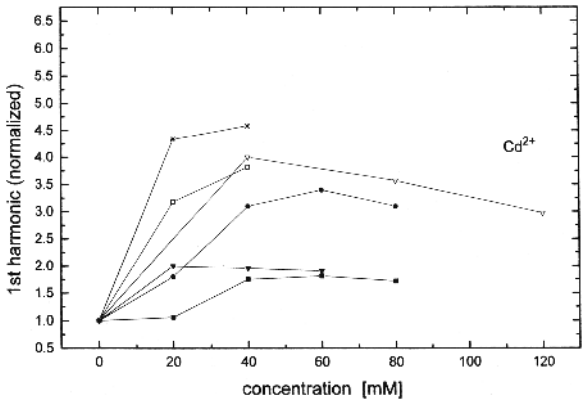


Figure 23: Normalized 1st harmonic of flexoelectric current of several membranes treated with Cd^{2+} vs. the concentration of Cd^{2+}, 410 Hz.



Flexoelectricity enables membrane structures to function like soft micro- and nano-machines, sensors and actuators, thus providing important input to molecular electronics applications.

3.1. *Flexoelectricity of lipid bilayers treated with Cd^{2+} and Hg^{2+}*

In this investigation[16] the use of patches of membranes as flexoelectric sensors detecting the presence of heavy metal ions in aqueous solutions was reported. The idea of using the flexoelectric properties of artificial membranes to sense chemical species is based on the concept that the flexoelectric response of an oscillating membrane is strongly dependent on the adsorption of charged or dipolar species at the membrane surface.[42]

Soy Lecithin membranes was prepared using patch-clamp technique. An oscillating pressure for inducing membranes curvature oscillation was provided by a piezo-sounder.

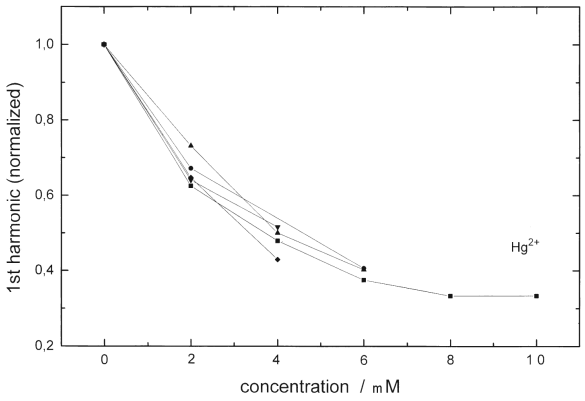
In this investigation was found that Cd^{2+} initially enhanced the flexoelectric response but at higher concentrations the response was reduced in amplitude (Figure 22 and Figure 23).

Flexoelectric response of membranes treated with Hg^{2+} decreased monotonically with Hg^{2+} increasing concentrations and trends to saturate above 5 mM (Figure 24).

The opposite effects at low concentrations of Cd^{2+} and Hg^{2+} on the flexoelectric response could be used to discriminate between their salts.

In conclusion, possible application of flexoelectric membranes as sensors for ion and dipolar species follows from the great sensitivity of flexoresponse to such adsorbed molecules. First prototypes of such sensors using black lipid

Figure 24: Normalized 1st harmonic of flexoelectric current of several membranes versus the concentration of Cd^{2+}, 410 Hz.



membranes[16] or patch-clamped bilayers[43] have already been demonstrated.

4. Conclusion

A specific feature of the membrane sensors described in this review is their disposable character. That means the sensor is prepared before the measurement, used to detect the species, and then discarded. The life time of lipid bilayers is normally limited to one laboratory day. A longer term membrane sensor could still be prepared, if needed, by stabilizing lipid bilayers by some chemical means: cross linking of polymerizable lipids, polyamines[44], etc. Individual acts of pore sensing are stochastic, while time averaged values of pore currents are deterministic. Deterministic is also the flexoelectric sensing using lipid membranes. Pursuing the biomimetic approach we could match the excellent membrane selectivity and sensitivity *in vitro*.

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