REAL-TIME MOLECULAR DETECTORS USING GRAMICIDIN ION CHANNEL NANO-BIOSENSORS

Vikram Krishnamurthy, Kai Yiu Luk

University of British Columbia
Dept of Elec. and Comp. Engin.
Vancouver, V6T 1Z4. Canada.

Bruce Cornell

AMBRI Pty Ltd.
Chatswood, N.S.W. 2067.
Australia.

Don Martin

University of Technology Sydney
Sydney, N.S.W. 2007.
Australia.

ABSTRACT

This paper deals with the experimental construction, stochastic modeling and statistical signal processing of a novel biosensor comprising of biological ion channels. Such nano-scale biosensors are built by incorporating dimeric Gramicidin ion channels into the bilayer membranes of giant unilamellar liposomes and then excising small patches of the membrane loaded with ion channels. We show that target molecules affect the statistics of the gating mechanism of the dimeric Gramicidin ion channels and present statistical verifications on the adequacy of a Hidden Markov Model for modeling of the biosensor. A likelihood ratio test is then devised to detect the presence of target molecules. To test the sensitivity of this model we conducted patch-clamp experiments with and without the Methylbenzthonium Chloride compound. The real-time detection algorithm was able to accurately detect the presence of the compound from alterations in the patch-clamp recordings. This algorithm provides the sensitive detection system for ongoing development of lipid-based nano-sensors.

***Index Terms*—** Biomedical transducers, membrane ion channels, Gramicidin, estimation, maximum likelihood detection

1. INTRODUCTION

Ion channels are protein macromolecules commonly found in biological cell membranes that form water filled nanotubes, typically a few Angstrom units in radius. In biological systems, ion channels selectively regulate the flow of ions into and out of a cell. By exploiting the selective conductivity of ion channels in the presence of target molecules, biosensors are developed to detect molecular species of interest across a wide range of applications. These include medical diagnostics, environmental monitoring and general bio-hazard detection. In particular, a novel biosenor, which incorporated monomeric Gramicidin ion channels into a tethered lipid bilayer membrane and exploited the changes in the association and disassociation probabilities of the Gramicidin dimers, was published by a coauthor of this paper in *Nature*. [1]

This paper deals with the construction, modeling and statistical signal processing associated with another ion channel based biosensor. In a giant lipid vesicle, covalent dimeric Gramicidin ion channels are incorporated by codispersion with the vesicle forming lipids. The gating mechanism in this biosensor is thought to arise from the random movement of excess lipid lenses in the liposome that diffuse over the membrane surface and block the conducting channels. We verify statistically that our Hidden Markov Model, which takes into account of the 1/f noise in the biosensor's response, is an adequate model for the measured currents and can be used to derive important biological characteristics of these dimeric bis-gA ion channels. Using Bayesian signal processing methods on the output current of the biosensor, a likelihood ratio hypothesis test is devised to detect the presence of the target molecules. In the presence of the target molecules, the stochastic model of the output current of the biosensor changes. By detecting this model change in real time, we show that the biosensor can be used in real-time target molecule detection.

2. EXPERIMENTAL CONSTRUCTION OF THE MODEL BIOMIMETIC BIS-GA ION CHANNEL BIOSENSOR

The biosensor considered in this paper was constructed by incorporating bis-gA ion channels into the lipid bilayer membrane of giant unilamellar liposomes and then excising small patches (1 μm in diameter) of the lipid membrane using a patch-clamp micropipette. Figure 1 shows a fluorescence image of the optical section through the diameter of the biosensor. The solutions and chemicals used for the biosensor included bL-alpha-phosphatidylcholine (PC) from soybean, cholesterol, chloroform, sucrose, glucose, sodium chloride (NaCl), potassium chloride (KCl) and 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES).

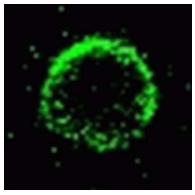

Fig. 1. Fluorescence image of biosensor's horizontal optical section. The Gramicidin channels are labeled using FITC and identified by the green color.

Giant unilamellar liposomes were prepared using our recent protocol [2] that was modified from a standard hydration procedure [3, 4, 5] with some modifications. In brief, a completely dried lipid film containing 100 μL of 10 mg·mL^{-1} PC with 10% (w/w) or 40%(w/w) cholesterol in chloroform was prepared in a glass test tube as described earlier. Rehydration of the lipid was later made at 45°C by the addition of a small amount of pure water (5 μL) to the tube for a few minutes (prehydration) followed by the addition of 5 mL of an aqueous solution of 0.1 M or 0.2 M sucrose. The tube was incubated at 45°C for 2-3 h. After gentle rocking overnight at room

temperature, the lipid film dispersed uniformly in the solution and a white cloud was floating in the middle of the solution, which contained giant liposomes. The obtained liposomes were around 20 m in diameter and were stable up to four days at 4°C.
Patch-clamp electrophysiology was used to record the ionic currents from the biosensor that resulted from permeation of ions through the bis-gA ion channels incorporated in the lipid membrane patches that were excised from the giant unilamellar liposomes. To perform the patch-clamp recordings, the liposomes were allowed to settle on the bottom of a recording chamber, which was either a 35 mm plastic tissue-culture dish or a purpose-built recording bath fitted with the glass cover-slip (Warner, RC13). Patch-clamp pipettes with a tip-opening between 0.9 and 1.5 m were fabricated from special capillary glass tubing (G75-1511) using an automated puller (Sutter, P97). The patch-clamp pipette was used to excise patches of lipid membrane from the giant unilamellar liposomes. The Gramicidin channel currents from these membrane patches were amplified and filtered at 1 kHz (4-pole Bessel) using an Axopatch 200B amplifier (Axon Instruments) and sampled on-line at 10 kHz.

3. STOCHASTIC MODELING AND STATISTICAL VERIFICATION OF BIOSENSOR RESPONSE

Suppose a patch clamp experiment is conducted with $(N-1)$ Gramicidin channels in the biosensor. At each discrete time instant k, each Gramicidin channel can be either in the "open" or "closed" state and each open channel conducts a fixed current. Thus the total current due to all $(N-1)$ ion channels at any given time can take on one of N possible levels $\{\mu_1, \ldots, \mu_N\}$ and can be modeled as a N-state Markov Chain. Write $\mu = (\mu_1, \ldots, \mu_N)$. Let X_k denote the total channel current at discrete time k. Let

$$a_{ij} = P(X_k = \mu_j | X_{k-1} = \mu_i),\ i, j \in \{1, \ldots, N\} \quad (1)$$

denote the transition probabilities of the Markov chain. Also let

$$\pi_0(i) = P(X_1 = \mu_i), i \in \{1, \ldots, N\} \quad (2)$$

denote the initial distribution of the Markov Chain. Write $A = [a_{ij}]_{N\times N}$ and $\pi_0 = [\pi_0(i)]_{N\times 1}$. The measured current from the biosensor is a distorted version of the signal X_k. The distortion arises from thermal noise, the anti-aliasing effect from sampling and an open channel noise with its power proportional to the inverse of frequency. Thus it is also known as 1/f noise and is discussed in other studies of Gramicidin ion channels. [6, 7]. Figure 2 shows the power spectral density of a typical sequence of biosensor recordings and it can be seen that the power spectrum decreases at a rate of -10 dB/dec at low frequencies and has a sharp cutoff at approximately 1 kHz. We model the noise as a Gaussian process modulated by a AR-filter $H(z)$. Let $Y(z)$ denote the frequency response of the noisy observation and $W(z)$ the frequency response of a white Gaussian process:

$$Y(z) = \frac{X(z) + W(z)}{H(z)} \quad (3)$$

$$H(z) = 1 + h_1 z^{-1} + \ldots + h_M z^{-M} \quad (4)$$

Write $\mathbf{h} = (1, h_1, \ldots, h_M)^T$. It is convenient to model the noise corrupting the state of the biosensor as state dependent noise - that is the noise variance at any given time instant is dependent on the state of the biosensor at that time instant. Let σ_i^2 be the variance of state i, $i = (1, \ldots, N)$. Write $\sigma^2 = \left(\sigma_1^2, \ldots, \sigma_N^2\right)$. As a result, the observations can be formulated as a hidden Markov model sequence. Let $\lambda = \left(A, \pi, \mu, \sigma^2, \mathbf{h}\right)$ be the HMM that characterizes the output measured current from the biosensor.

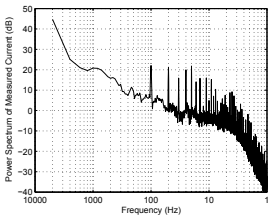

Fig. 2. Power spectral density of biosensor's response clearly shows the 1/f open channel noise and the anti-aliasing effect in the samples.

4. CHARACTERIZING BIOSENSOR RESPONSE TO TARGET MOLECULES

In this section we discuss estimation techniques to extract the model parameters of the biosensor's response. Statistical tests are introduced to verify the goodness-of-fit of the model. Applying the estimation algorithm in this section, the biosensor's response to known analytes can be completely characterized by the model parameters and the detection is reduced to a classification problem.

4.1. Parameter Estimation

Given an observation sequence $\{Y_k\}$, we define $L_k(\lambda)$ as the log-likelihood of our HMM λ at discrete time k. The estimation of the model λ involves processing $\{Y_k\}$ through a HMM maximum likelihood estimator (MLE). The system in (4) can be rewritten as:

$$\mathbf{h}^T \mathbf{Y_k} = X_k + W_k \quad (5)$$

where $\mathbf{Y_k} = \{Y_k, Y_{k-1}, \ldots, Y_{k-M}\}$. This formulation is analogous to a standard HMM, except that the observation sequence is FIR-filtered. The Expectation Maximization (EM) algorithm is an iterative procedure that solves for local maximum of the likelihood function. The E-step evaluates the log-likelihood, which is defined as:

$$L_k(\lambda) = \sum_{t=1}^{k}\sum_{i=1}^{N} \gamma_t(i) \log\left(\frac{1}{\sqrt{2\pi\sigma_i^2}} \exp\left(\frac{-(\mathbf{h}^T\mathbf{Y_k} - \mu_i)^2}{2\sigma_i^2}\right)\right) + \sum_{t=1}^{k}\sum_{i=1}^{N}\sum_{j=1}^{N} \zeta_t(i,j) \log(a_{ij}) \quad (6)$$

and the M-step maximizes the log-likelihood with respect to the model parameter by computing the first-order derivative of the log-likelihood function. For details about the EM estimation algorithm, please see [8, 9, 10].

4.2. Statistical Verification

Statistical verifications plays a key role in the stochastic modeling of the biosensor's response. The MLE defined earlier estimates the most likely model for a fixed topology, but we need to statistically verify the adequacy of the estimated model by analyzing the autocorrelations of the residuals, which are generated via a HMM one-step

predictor:

$$e_{k|k-1} = Y_k - \sum_{i=1}^{N}\sum_{j=1}^{N} a_{ij}\alpha_{k-1}(i)\mu_j - \sum_{n=1}^{M} h_n Y_{k-n} \quad (7)$$

where α_k is the forward variable defined in [10]. The residuals of an adequately fitted model should be uncorrelated and the autocorrelation should approach 0 as $T \to \infty$. Rather than examining the autocorrelation at each lag, the Ljung-Box Q-statistic, defined in (8), computes the cumulative sum of autocorrelations at the first L lags and is used as a portmanteau lack of fit test for model adequacy.

$$Q = N(N+2)\sum_{l=1}^{L} \frac{r_l^2}{(N-l)} \quad (8)$$

where r_l^2 is the autocorrelation of the residual at lag l. It is shown in [11] that for an adequate model, the Q-statistics of the residual is approximately distributed as $\chi^2(L)$.

5. ANALYTE DETECTION USING MLR TEST

Given a sequence of response observed in unknown condition, the detection problem involves the identification of the condition that most likely contributes to the biosensor's response. This is a model classification problem and can be solved by comparing the likelihood of each known model. We devise a likelihood ratio hypothesis test that detects the presence of an analyte. Let $Y = (Y_1, \ldots, Y_T)$ be a sequence of observed response of the biosensor. Let θ_1 be the condition with no analyte present and θ_2 be the condition with analyte present. For $i = 1, 2$, let λ_i be the estimated model for θ_i. At each time point k, the sequence Y behaves according to model λ_1 or λ_2.

5.1. Filtered Likelihood

For each estimated model λ_i, the log-likelihood at each time point L_k can be computed from (6). To make the detection more robust to nonstationary disturbances and outliers in the measurements, we apply a geometric moving-average filter to the log-likelihood. Let ρ be the forgetting factor, $0 < \rho < 1$. Define the filtered likelihood at time k:

$$S_k(\lambda) = \begin{cases} L_1(\lambda) & \text{for } k = 1 \\ (1-\rho)S_{k-1} + \rho L_k(\lambda) & \text{for } 2 \le k \le T \end{cases} \quad (9)$$

The filtered likelihood is a weighted sum of the likelihood of the entire sequence $\{Y_1, \ldots, Y_k\}$, with higher weights on the recent observations.

5.2. Likelihood-Ratio Test

The likelihood-ratio test is formulated as a hypothesis test and rewritten as:

$$H_0 : Y_k \sim \lambda_1 \text{ versus } H_1 : Y_k \sim \lambda_2 \quad (10)$$

where H_0 is the null hypothesis that no analyte is present and H_1 is the alternative hypothesis that analyte is present. Assume a uniform prior on the hypotheses, it can be shown that the optimal decision rule δ is:

$$\delta = \begin{cases} 0 & \text{if } \dfrac{P(Y_k|\lambda_1)}{P(Y_k|\lambda_2)} > 1 \\ 1 & \text{if } \dfrac{P(Y_k|\lambda_1)}{P(Y_k|\lambda_2)} < 1 \end{cases} \quad (11)$$

or equivalently,

$$\delta = \begin{cases} 0 & \text{if } \Delta S_k > 0 \\ 1 & \text{if } \Delta S_k < 0 \end{cases} \quad (12)$$

where δ is the index of the accepted hypothesis and $\Delta S_k = S_k(\lambda_1) - S_k(\lambda_2)$ is the difference in filtered likelihood.

6. EXPERIMENTAL RESULTS

Here we report on the performance of the detection algorithm and biosensor on actual experimental data. We recorded output from the model biosensor by measuring the activity of the bis-gA ion channels incorporated into the small lipid membrane patches that were excised from the unilamellar giant liposomes. Bis-gA ion channels were incorporated into the unilamellar giant liposomes at a concentration of 1/100 from a 66nM stock solution. We used 0.5 M KCl solution in the recording pipette with the microparticles suspended in a 0.5 M NaCl solution.

6.1. Model Estimation and Verification of Biosensor Current

Figure 3 shows a short sequence of observed currents. The sequence is fitted to our model with two-state HMM with an AR-12 filter. The model parameters are estimated with the MLE and listed in Table 6.1. The most likely conductance level sequence, extracted from the HMM procedure, is plotted in Figure 4. Residuals of the model are

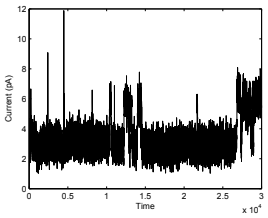

Fig. 3. Time sequence of biosensor recordings

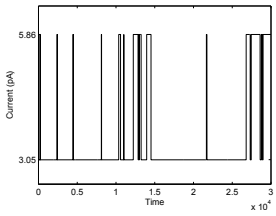

Fig. 4. Maximum likelihood estimate of individual conductance level

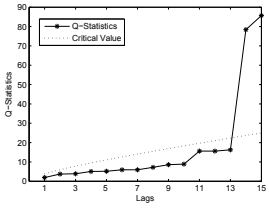

Fig. 5. Q-statistics of residuals vs critical value of Ljung-Box test

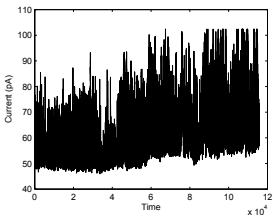

Fig. 6. Biosensor response to the addition of MBC

generated using the HMM one-step predictor. The Q-statistics of the Ljung-Box test are computed for the first 15 lags. Figure 5 shows that for the first 13 lags, the Q-statistics of the residuals are below the critical values of the chi-square distribution at 0.05 significance level. In other words, our model is adequate in modeling the linear dependencies that exist in the biosensor's response.

Transition Probabilities		Conductance Level (pA)	Variance $(pA)^2$
0.9964	0.0036	5.831	0.513
0.0006	0.9994	3.057	0.371

Table 1. Estimated parameters of sequence in Figure 3.

6.2. Real-Time Detection of Analyte

In this section we illustrate the performance of the algorithm in detecting the presence of an analyte in real-time. Patch-clamp experiments are conducted with and without Methylbenzthonium Chloride (MBC) in the bath solution. The compound MBC, shown in Figure 7, is synthesised by AMBRI Pty Ltd to inhibit the conduction of ions in the bis-gA ion channels. Using the MLE, the detection program is trained offline to model the biosensor's response. To simulate the addition of MBC into the bath solution, we merge together a sequence recorded with no analyte with a sequence recorded with MBC. The change point occurs at $k = 57793$. From the time series plot of the merged sequence in Figure 6, it is extremely difficult to the change point where the MBC compound is added.

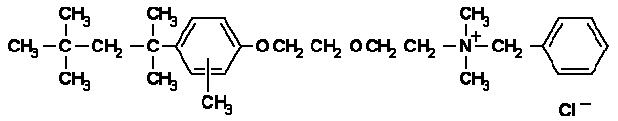

Fig. 7. Chemical structure of Methylbenzthonium Chloride (MBC)

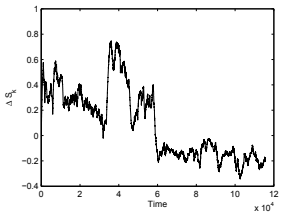

Fig. 8. Filtered likelihood ΔS_k for λ_1 and λ_2

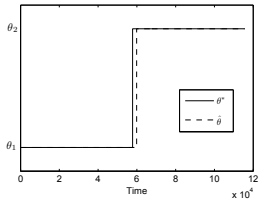

Fig. 9. Detection trace δ is computed from the filtered likelihood ΔS_k.

Using the parameters in the estimated models λ_1 and λ_2, The filtered likelihoods $S_k(\lambda_1)$ and $S_k(\lambda_2)$ are computed with the forgetting factor $\rho = 0.005$ and the difference, $\Delta S_k = S_k(\lambda_1) - S_k(\lambda_2)$, is plotted in Figure 8. When comparing the detection trace with the actual experimental condition in Figure 9, The detection trace indicates a switch in the most likely model from λ_1 to λ_2 at $k = 59865$, approximately 0.2 seconds after the change point.

7. CONCLUSION

This paper has described the stochastic modeling and detection algorithm of an ion channel based nano-biosensor. The biosensor comprises dimeric Gramicidin ion channels incorporated into small patches of bilayer membranes that were excised from giant unilamellar liposomes. We showed and verified statistically the stochastic modeling of the gating mechanism, caused by random movement of excess lipid lenses in the liposome, and the 1/f noise in the patch-clamp recordings. Based on the HMM, we devised a likelihood ratio test to detect target molecules. Our experimental results show that the biosensor together with the likelihood ratio test results in a highly sensitive detector that can detect the presence of MBC, a compound known to inhibit the permeation of ions through the bis-gA ion channels. This detection algorithm provides a sensitive means of detecting alterations in the ion channel activity, and allows further development of this lipid-membrane based biosensor that utilizes ion channels as the readout for binding of analytes to the biosensor.

8. REFERENCES

[1] B. A. Cornell, V. L. Braach-Maksvytis, L. G. King, P. D. Osman, B. Raguse, L. Wieczorek, and R.J. Pace., "A biosensor that uses ion-channel switches," *Nature*, vol. 387, pp. 580–583, 1997.

[2] I. L. di Maio, D. Carl, P. Langehanenberg, S. M. Valenzuela, A. R. Battle, S. al Khazally, M. Killingsworth, B. Kemper, G. von Bally, and D. K. Martin, "Structural properties of liposomes from digital holographic microscopy," *Proc of SPIE*, vol. 6036, pp. 60361R, January 2006.

[3] H. Itoh K. Akashi, H. Miyata and K. Kinosita, "Formation of giant liposomes promoted by divalent cations: Critical role of electrostatic repulsion," *Biophysical Journal*, vol. 74, pp. 2973–2982, June 1998.

[4] H. Itoh K. Akashi, H. Miyata and K. Kinosita, "Preparation of giant liposomes in physiological conditions and their characterization under an optical microscope," *Biophysical Journal*, vol. 71, pp. 3242–3250, December 1996.

[5] T. Tanaka Y. Yamashita, M. Oka and M. Yamazaki, "A new method for the preparation of giant liposomes in high salt concentrations and growth of protein microcrystals in them," *Biochimica Et Biophysica Acta-Biomembranes*, vol. 1561, pp. 129–134, April 2002.

[6] R. Sauve and E. Bamberg, "l/f noise in black lipid membranes induced by ionic channels formed by chemically dimerized gramicidin a," *Journal of Membrane Biology*, vol. 43, pp. 317–333, November 1978.

[7] R. Sauve and G. Szabo, "Interpretation of 1/f fluctuations in ion conducting membranes," *Journal of Theoretical Biology*, vol. 113, pp. 501–516, April 1985.

[8] V. Krishnamurthy S. H. Chung and J. B. Moore, "Adaptive processing techniques based on hidden markov models for characterising very small channel currents buried in noise and deterministic interferences," *Proc. Phil. Trans. Roy. Soc. Lond. B*, vol. 334, pp. 357–384, 1991.

[9] M. M. Laird A. P. Dempster and D. B. Rubin, "Maximum likelihood estimation from incomplete data via the em algorithm," *Journal of the Royal Statistical Society B*, vol. 39, pp. 1–38, 1977.

[10] L. R. Rabiner, "A tutorial on hidden markov models and selected applications in speech recognition," *Proc. IEEE*, vol. 77, pp. 257–285, 1989.

[11] G. E. P. Box and D. A. Pierce, "Distribution of residual autocorrelations in autoregressive-integrated moving average time series models," *Journal of American Statistical Association*, vol. 65, pp. 1509–1526, December 1970.