Available online at www.sciencedirect.com
ScienceDirect
Biosensors and Bioelectronics 22 (2006) 605–612
BIOSENSORS & BIOELECTRONICS
www.elsevier.com/locate/bios

Acetylcholinesterase–ISFET based system for the detection of acetylcholine and acetylcholinesterase inhibitors

Aviad Hai [a], Deborah Ben-Haim [b], Nina Korbakov [b], Ariel Cohen [a], Joseph Shappir [c], Ruthi Oren [a], Micha E. Spira [a,*], Shlomo Yitzchaik [b,**]

[a] *Department of Neurobiology, The Life Sciences Institute, The Hebrew University of Jerusalem, Jerusalem 91904, Israel*
[b] *Department of Inorganic and Analytical Chemistry, The Hebrew University of Jerusalem, Jerusalem 91904, Israel*
[c] *School of Engineering, The Hebrew University of Jerusalem, Jerusalem 91904, Israel*

Received 13 November 2005; received in revised form 21 January 2006; accepted 24 January 2006
Available online 10 March 2006

Abstract

A bioelectronic hybrid system for the detection of acetylcholine esterase (AChE) catalytic activity was assembled by way of immobilizing the enzyme to the gate surface of an ion-sensitive field-effect transistor (ISFET). Photometric methods used to characterize bonded enzyme and linker layers on silicon substrates confirm the existence of a stable amino-cyanurate containing AChE monolayer. The transduction of the enzyme-functionalized ISFET, in ionic solutions, is detected in response to application of acetylcholine (ACh). Recorded sensitivity of the modified ISFET to ACh has reached levels of up to 10^{-5} M. The Michaelis–Menten constant of the immobilized AChE is only moderately altered. Nevertheless, the maximum reaction velocity is reduced by over an order of magnitude. The ISFET response time to bath or ionophoretic application of ACh from a micropipette was in the range of a second. The catalytic activity of the immobilized AChE is inhibited in a reversible manner by eserine, a competitive inhibitor of AChE. We conclude that the immobilized enzyme maintains its pharmacological properties, and thus the described bioelectronic hybrid can serve as a detector for reagents that inhibit AChE activity.

Keywords: Biosensors; Acetylcholine; Acetylcholine antagonists; Acetylcholine esterase; *Aplysia*

1. Introduction

The integration of biologically active molecules with electronic transducers has emerged as an elegant and effective way of creating high fidelity systems for detection of a wide range of biological activities (Anderson et al., 2000). Such systems are designed to serve as translators of molecular events into electrical signals. The aim of such bio-electronic hybrid systems is to specifically recognize a substrate and to produce electrical signals that are proportional to the substrate concentration. The high specificity of biomolecules such as enzymes, receptors and antibodies allows for the assembly of a reaction-specific biosensor system that can be used for a wide range of applications. These include medical applications such as the detection of blood glucose levels (Ito et al., 1992), the quantification of molecules that serve as cancer indicators (Nagy et al., 2002), the identification of specific venomous toxins (Selvanayagam et al., 2002) and others. Furthermore, such biosensor systems can be used in various environmental applications, such as the detection of pollutants including pesticides (Martorell et al., 1997) and cyanide (Volotovsky and Kim, 1998).

The ion-sensitive field-effect transistor (ISFET) offers a technology by which the normal metal-oxide-silicon field-effect transistor (MOSFET) gate electrode is replaced by an ion-sensitive surface with the ability to detect ion concentrations in solution (Bergveld, 1970, 1972). ISFET technology offers fast response time ranging from tens of milliseconds to several minutes, depending on several parameters such as the substrate affinity to the receptor, its concentration and the solution used in the analysis. In comparison with traditional techniques for the detection of biochemical agents in solution, ISFET technology has proven to have high quality performance, specifically in

* Corresponding author. Tel.: +972 2 6585091; fax: +972 2 5637033.
** Co-corresponding author. Tel.: +972 2 6586971; fax: +972 2 6585319.
E-mail addresses: spira@cc.huji.ac.il (M.E. Spira), sy@cc.huji.ac.il (S. Yitzchaik).

0956-5663/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.bios.2006.01.028

the detection and quantification of enzyme–substrate reactions (Kharitonov et al., 2000).

Earlier enzyme-based sensor systems such as film based sensors and enzyme-electrodes make use of the immobilization of a thick enzyme polymer layer (~10–200 μm). Such systems are commonly used in medical applications such as real-time blood monitoring and analysis (Lauks, 1998) but offer very slow response time (>0.5 h). This is mainly due to long diffusion time of substrate/product through the thick polymer layer. In contrast, emerging technologies of covalent immobilization of enzyme-monolayer (~50–200 nm) onto the gate surface of ISFETs (Gorchkov et al., 1997) lack the diffusion barrier and thus offer better response time.

The construction of a fast-response biosensor system for the detection of acetylcholine (ACh) and acetylcholine esterase (AChE) inhibitors has been demonstrated previously using various methods of detection. One example is the creation of an Au-nanoparticle-CdS-AChE layer on Au-coated glass electrodes (Pardo-Yissar et al., 2003). This system makes use of the biocatalyzed hydrolysis of acetylthiocholine to acetic acid and thiocholine. Thiocholine acts as an electron donor for the generation of a photocurrent in the system which is blocked in the presence of AChE inhibitors. Another system makes use of a specific immobilization pattern of AChE across gold electrodes using dendrimeric linking molecules (Snejdarkova et al., 2004). These and other systems (Hart et al., 1997; Deo et al., 2005) can be applied in the detection of pesticides and "nerve gas" (Trojanowicz, 2002). In vivo AChE resides in the synaptic cleft between presynaptic cholinergic neurons and their post synaptic counterparts, and catalyses the hydrolysis of ACh. ACh is a ubiquitous neurotransmitter, found in the peripheral and central nervous systems. We report here on the development of a biosensor for the detection of ACh and AChE inhibitors.

2. Materials and methods

2.1. Reagents

AChE (Sigma, from human source) solution was prepared by adding 1 ml PBS (pH 7.4) to glass vial containing 0.17 mg AChE in powder form (stocked at 4 °C). Acetylcholine chloride (ACh, Sigma), acetylthiocholine iodide (ATChI, Sigma), eserine (Physostigmine, Sigma), carbamylcholine-chloride (Carbachol, Sigma), 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB, Sigma) and cyanuric-chloride (CyC, Aldrich), dry dichloromethane, isopropanol and acetone (J.T. Baker) were used.

Electronic measurements were taken in standard phosphate buffer solution (0.1 M Na_2HPO_4, 0.1 M NaH_2PO_4, pH 7.4) and phosphate buffer saline (PBS) solution (0.1 M KH_2PO_4, 0.1 M NaOH, pH 7.4), both at room temperature. Triple distilled water (TDW, $R = 18.3\ m\Omega/cm^2$) used in the preparation and dilution of all solutions.

Intracellular recordings performed at room temperature in artificial sea water (ASW) composed of NaCl 460 mM, KCl 10 mM, $CaCl_2$ 10 mM, $MgCl_2$ 55 mM, HEPES [*N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid), Sigma] 10 mM at pH 7.6, using 2 M KCl micropipette ($R = 3\ m\Omega$). Iontophoresis experiments performed using 1 M ACh micropipette (in ASW or PBS).

2.2. Modification of the ISFET gate

ISFETs used in the present study were purchased from G.C. Fiaccabrino's Lab, Institute of Microtechnology, University of Neuchatel, Switzerland.

ISFETs were rinsed using isopropanol and dried with argon. Linker molecule CyC is used for the covalent linkage between the Al_2O_3 surface of the ISFET and AChE. A solution of 0.1 M CyC in dry and freshly distilled dichloromethane was prepared and then applied to the alumina (Al_2O_3) containing gate surface of the ISFET. ISFETs were consequently dried with argon, heated at 70 °C for 15 min in a vacuum oven, rinsed with dry dichloromethane and dried with a gentle stream of argon. 0.1 M AChE (in PBS) was applied onto the CyC-modified gate surface and left for 1 h at room temperature in a sealed container, and then rinsed with PBS.

2.3. Electronic measurements

AChE–ISFET hybrid system was immersed in PBS or phosphate buffer solution (1 ml) as a background electrolyte for the measurements. A standard Ag/AgCl electrode was used as the reference electrode. The change in potential between the ISFET gate and source electrodes ΔV_{gs} (mV) reflecting the change in potential between the bulk solution and the source was measured as a response to application of reagents, while potential between drain and source electrodes (V_{ds}) was held constant at operation potential 0.1 V. Initial gate–source voltage was set to 0.45 V. A calibration pulse (1 mV) was given for all measurements and used to determine the effective recorded signal amplitude.

ISFETs were rinsed three times in PBS in between measurements of dose application. Addition of ACh, carbamylcholine into the bath was performed distally from the ISFET using Gilson pipettors.

2.4. Surface chemistry analysis

Glass, quartz (Chemglass) and n-Si ⟨1 0 0⟩ (Virginia Semiconductors) substrates were used in order to characterize the ISFET gate surface following the chemical modifications. The substrates' surface was activated by using the cleaning procedure detailed below to increase the hydroxyl groups' number density. The substrates were cleaned by sonication in soapy water at 60 °C for 30 min, washed three times with TDW, and then immersed in piranha solution (H_2SO_4/H_2O_2, 70:30, v/v) at 90 °C for 1 h. The substrates were then rinsed three times with TDW and then the RCA procedure was employed. This involved sonicating the substrates in ammonium hydroxide solution ($H_2O/H_2O_2/NH_3$, 5:1:1/4, v/v/v) for 30 min followed by washing three times with TDW, rinsing with pure acetone and drying in a clean convection oven for 10 min at 110 °C.

The glass and quartz substrates for the kinetic experiments were treated using a similar procedure. The substrates were first rinsed three times in isopropanol and then transferred in UVOCS

cleaning instrument for 30 min, oxidation is accomplished by the combination of ultraviolet light and ozone. The RCA procedure was then employed.

Variable angle spectroscopic ellipsometry (VASE) measurements were carried out on a VB-200 ellipsometer (Woollam Co.) in order to analyze SiO_2, CyC and AChE layers bonded to the silicon surface. Enzyme activity in solution was analyzed using Karnovski and Roots dyeing technique (Karnovsky and Roots, 1964). UV–vis spectra were acquired using a Schimadzu spectrophotometer UV–3101PC on quartz or glass substrates. Contact angle measurements were performed using Si with a Ramé-Hart (model 100) goniometer.

Bonding techniques for silicon and glass surfaces comprised of two different self-assembly procedures. First procedure used is assembly of CyC layer in solution based self-assembly process using Schlenk techniques. Freshly cleaned glass, quartz and/or silicon substrates were immersed in a 0.05 M solution of CyC in dry dichloromethane for 2 h 30 at room temperature and then washed with copious amounts of dry dichloromethane, followed by 1 min sonication in pure acetone in order to remove any excess of CyC.

Second procedure used for bonding of CyC layer is the solid state reaction conducted under topotactic self-assembly (Yitzchaik and Marks, 1996) conditions. Freshly cleaned glass, quartz and/or silicon were covered by 0.1 M solution of CyC in dichloromethane, spin-coated at 4000 rpm for 30 s and then cured at 70 °C for 10 min. The substrates were washed with copious amounts of dichloromethane, followed by a short sonication in pure acetone. AChE solution (10 μl) was applied on the substrate surface, which was covered by a polycarbonate dish. After 1 h, the substrates were washed fluently with PBS.

2.5. Cell cultures and electrophysiology

Identified MCn neurons from the metacerebral ganglia of *Aplysia californica* were isolated and maintained in culture using well established protocols (for details see Spira et al., 1996). Conventional intracellular recording with single glass microelectrodes were used. The microelectrodes were pulled from 1.5/1.02 mm borosilicate glass tubes with filaments and filled with 2 M KCl, the microelectrode tip was inserted into the cell body, to initiate stimulation and recording.

3. Results

3.1. Immobilization of AChE onto the ISFET gate surface

Acetylcholine esterase was immobilized onto the ISFET gate surface using cyanuric-chloride (CyC) as short aromatic coupling molecule (Fig. 1). When incubated at 70 °C in vacuum, one (or two) of the three cyanuric-chloride groups condenses with the free hydroxyl group(s) on the aluminum oxide gate surface by eliminating HCl and forming covalent ether bond (Fig. 1A). Condensation reaction between an amine group of an arbitrary lysin residue of the enzyme with a free cyanuric-chloride group of the immobilized CyC was done at room temperature (Fig. 1B). This condensation reaction is also accompanied by eliminating HCl and forming secondary amine bond, covalent bond, with the enzyme.

The formation of a CyC monolayer was verified by contact angle measurements, UV–vis spectroscopy (Fig. 2A) and spectroscopic ellipsometry measurements (Fig. 2B) on glass, quartz and silicon substrates. Specifically, the contact angle of clean surface increased from 15° to 77° upon coupling. The UV spectrum shows a characteristic absorbance peak of CyC at 230 nm although the optical density obtained is low (Fig. 2A). Spectroscopic ellipsometry measurements (Fig. 2B) clearly indicated the formation of a monolayer with a thickness ca. 6.7 Å obtained from the coupling layer and therefore verified CyC adsorption onto the surface.

The bonding of AChE was monitored by variable angle spectroscopic ellipsometry measurements (Fig. 2C). The silicon substrate modified with a CyC monolayer was covered with the AChE solution (solution based reaction) for 30 min, then washed with PBS solution and then air-dried. The thickness of the AChE layer was calculated to be 30 Å, as is derived from a measurement in three different angles (72°, 75°, 78°; Fig. 2C). From the dimensions of the enzyme (6.5 nm × 6.0 nm × 4.5 nm),

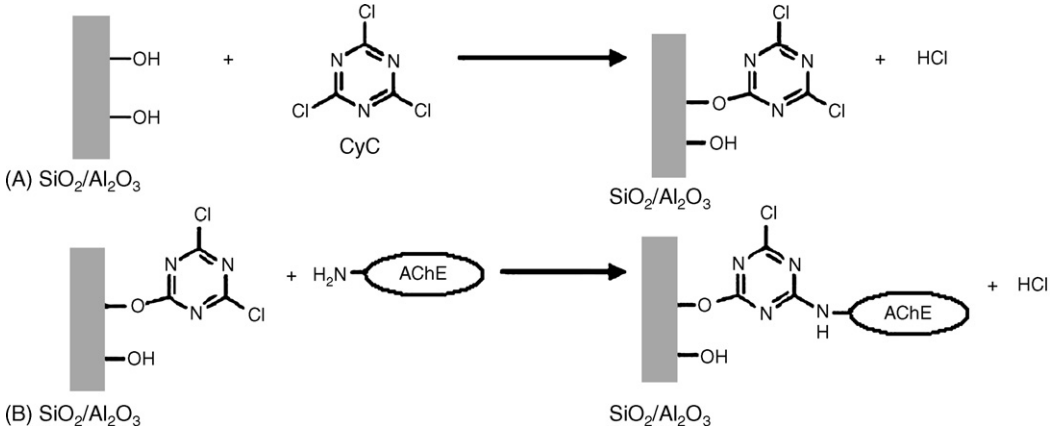

Fig. 1. (A) Covalent linkage between CyC and the hydroxide layer of ISFET gate surface and (B) immobilization of AChE onto CyC monolayer.

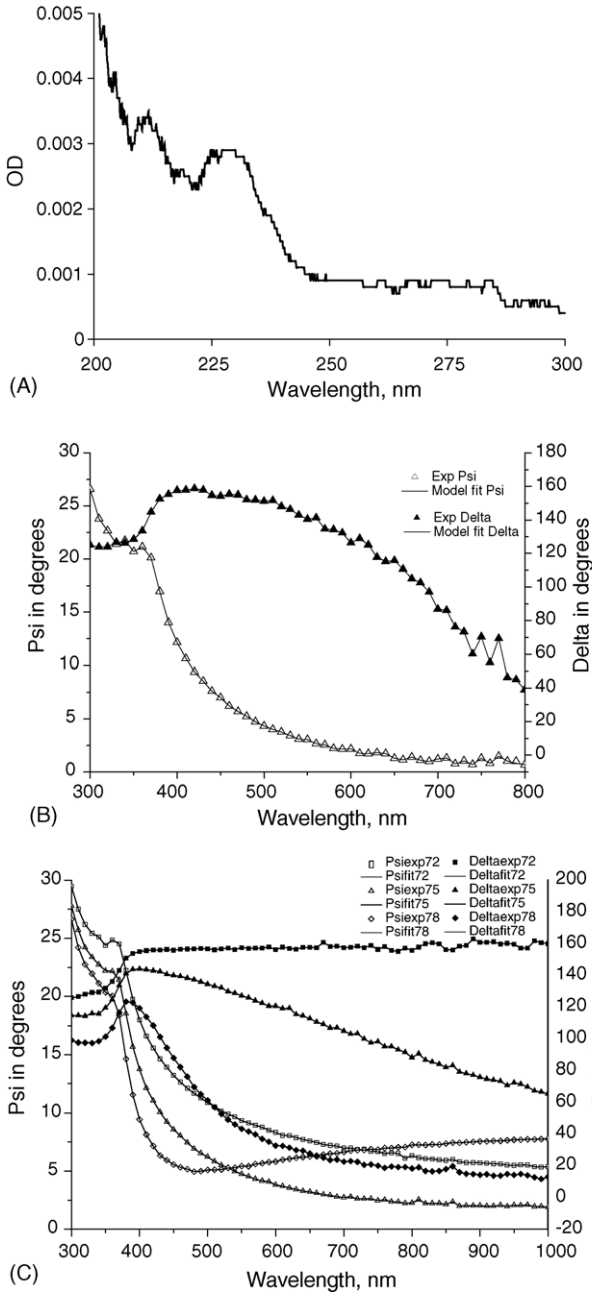

Fig. 2. Surface characterization of CyC and AChE monolayers. (A) UV–vis absorption spectrum of the CyC monolayer, (B) spectroscopic ellipsometry measurements and fitting of the CyC monolayer and (C) variable angle spectroscopic ellipsometry measurements and fitting of the AChE monolayer.

the coverage of the enzyme onto the surface is approximately 50%.

In order to determine the preservation of enzyme catalytic activity, kinetic analysis of free enzyme catalytic activity in solution (PB, pH 7.4) was compared to catalytic activity of the immobilized enzyme on silicon. Thiocholine, a hydrolysis product of ATChI (an ACh homologue) reacts with DTNB to give the yellow colored TNB anion. TNB concentration was monitored photometrically at 412 nm in situ in real-time. The slopes of the kinetic curves for each substrate concentration were converted into the reaction rates and data was fitted with Michaelis–Menten

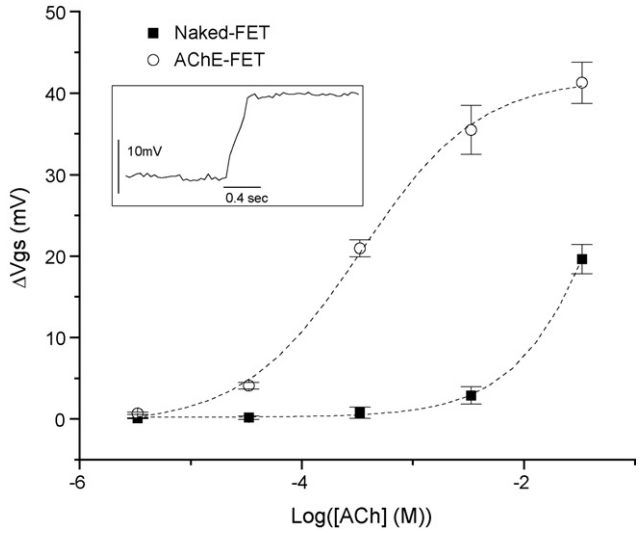

Fig. 3. Acetylcholine dose–response curve. Shown are the steady state voltages generated by the AChE–ISFETs to varying doses of ACh. Functionalized AChE–ISFET (circles), control (naked ISFETs) (squares). In both cases, ACh was applied into a 1 ml PBS in which a FET was bathed. The AChE–ISFET sensor shows fast response time of ~0.5 s to a dose of 3.3×10^{-5} M ACh (shown in box). Vertical bars represent standard error of the mean.

equation for first-order enzyme kinetic system. V_{max} and K_m were deduced from Lineweaver–Burk equation. It was observed that K_m values for the free (2.4×10^{-3}) and the immobilized (2.7×10^{-3}) AChE are similar. This implies that the catalytic activity of the enzyme is unchanged when bound to the silicon surface. The maximum rate (V_{max}) is an order of magnitude lower for the immobilized enzyme (1.73×10^{-7}) in comparison with the free enzyme (2.29×10^{-6}), which can be explained by a diffusion barrier for the substrate in and out sterically hindered catalytic sites of the surface bound enzyme, due to spatial constraints, as a fraction of the enzyme molecules are bound to the silicon such that the active site faces the silicon.

3.2. Sensing of ACh by functionalized ISFET

Change in voltage across the ISFET gate and source electrodes (ΔV_{gs}) was measured in response to the bath application of ACh (0.5 ml of varying concentrations of ACh). The resulting dose–response curve is sigmoid-shaped and corresponds to normal enzyme kinetics (Fig. 3). The lowest concentration detectable thus far is 10^{-6} M generating (in the series of seven experiments depicted in Fig. 3) a steady state voltage of 0.695 ± 0.136 mV across the ISFET, while saturation of response is reached at approximately 5×10^{-2} M generating a steady state voltage of 41.279 ± 2.54 mV ($n = 7$) (Fig. 3, empty circles). In contrast, control ISFETs that have not undergone the AChE bonding process displayed a change in steady state voltage of 0.101 ± 0.0465 mV in response to the application of 10^{-6} M ACh and a small response of 2.9 ± 1.07 mV to the application of 5×10^{-3} M acetylcholine ($n = 2$) (Fig. 3, filled squares). The responses generated by nonfunctionalized ISFETs to concentrations higher than 5×10^{-3} M ($\Delta V_{gs} = 19.626 \pm 1.817$ mV in response to 5×10^{-2} M ACh)

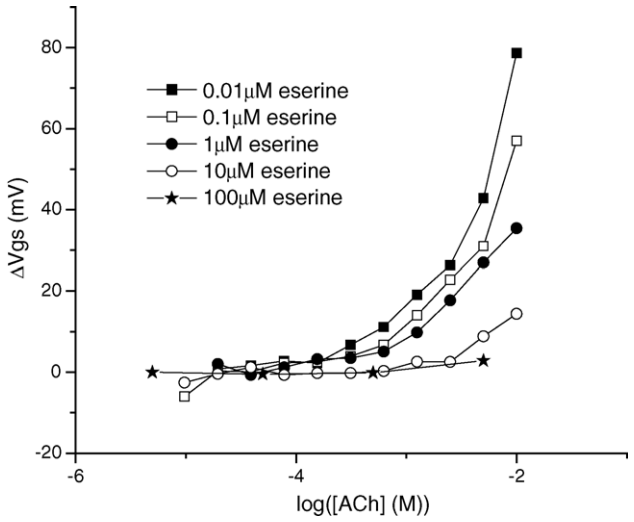

Fig. 4. AChE inhibition by eserine is detected by AChE–ISFET. Increasing doses of ACh were applied in the presence of varying concentrations of the competitive inhibitor eserine. The sensor is able to detect levels of AChE inhibition within the range of 0.1–100 μM.

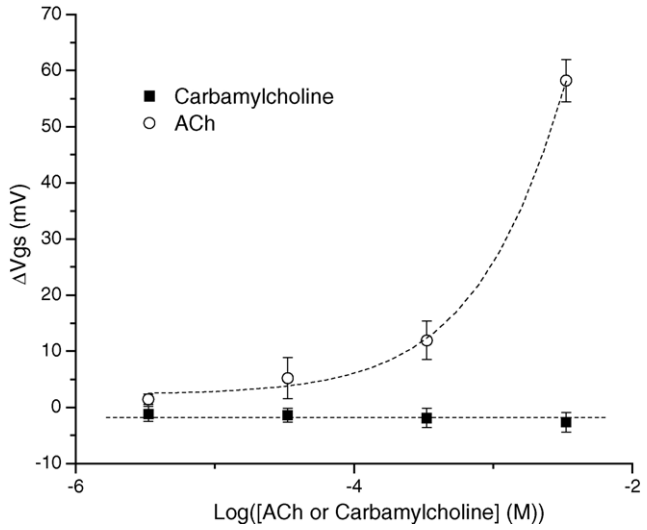

Fig. 5. Application of carbamylcholine onto an AChE–ISFET does not produce a response in comparison to the application of ACh. Carbamylcholine (filled squares) does not induce a response in contrast to the full-scale response evoked by the application of ACh (empty circles). Vertical bars represent standard error of the mean.

may be attributed to either spontaneous hydrolysis of ACh or to the increase in ionic strength of the solution. Application of control PBS dose (0.5 ml) into the bathing solution did not yield any response. Fast response time of as low as 0.049 mV/ms is evident at concentrations of 10^{-6} to 10^{-3} M (Fig. 3, inside box), while at concentrations near saturation; steady state voltage on the FET's gate electrode is reached after 30–60 s. While the average threshold sensitivity to ACh was 10^{-5} M, in few devices we have been able to detect 10^{-6} M ACh (not shown).

3.3. Detection of AChE inhibitors by the AChE–ISFET sensor

AChE ability to hydrolyze ACh is inhibited by different types of pharmacological reagents. Among them is eserine, a competitive and reversible inhibitor of AChE (Changeux et al., 1968). Characterization of the system's response to ACh in the presence of eserine indicates that the immobilized enzyme maintains its pharmacological properties and thus can serve as a detector for reagents that inhibit AChE activity (Fig. 4).

Application of eserine at concentrations ranging between 0.01 and 100 μM onto an AChE–ISFET device did not induce any significant voltage changes across the ISFET ($n = 5$, not shown). The inhibition of the enzyme activity by eserine application was demonstrated as follows. Increasing doses of ACh were applied to AChE–ISFETs that were bathing in a solution that contain different concentrations of eserine while monitoring the change in voltage across the ISFET's gate and source electrodes. Eserine was washed from the solution between each series of ACh application. Each experiment thus consisted of a series of 50–60 points of recording at different eserine and ACh concentrations. The experiment depicted by Fig. 4 (and three others of the same type) demonstrated that the immobilized AChE maintains its pharmacological properties. In all four experiments, complete inhibition of the AChE activity was observed at a concentration of 100 μM eserine, as the ISFET response reaches values similar to reference samples in which no enzyme is bonded to the ISFET. Response in the presence of 0.01 μM eserine resembled the response without inhibitor, thus the sensor is able to detect eserine concentration at a range of 0.1–100 μM.

ISFET's responsiveness shows a distinct recovery after wash of eserine, which corresponds to the reversible nature of eserine inhibition. This demonstrates the effectiveness of the system, and shows that resulting AChE–ISFET response is indeed due to specific interactions of the substrate and the enzyme.

3.4. Mechanism of ACh detection

The ACh-induced changes in ISFET transduction could result from the increased acidity of the solution by the hydrolysis of ACh to choline, acetate and a proton or as a result of changes in enzyme conformation and charge dispersal, which in turn change the voltage across the ISFET sensing gate.

To differentiate between these possibilities we examined whether carbamylcholine—a non-hydrolysable AChR agonist induces a change across the AChE–ISFET sensing gate. We found that the AChE–ISFET does not detect carbamylcholine and responds to carbamylcholine application in an identical manner to the response of a naked ISFET to ACh ($n = 4$, Fig. 5). As carbamylcholine does not undergo hydrolysis by AChE we conclude that the sensing mechanism of the AChE–ISFET is related to the hydrolysis of ACh and solution acidification in the vicinity of the gate surface.

3.5. Towards the assembly of a neuron–FET synapse

One possible application of the above described AChE–ISFET hybrid is the formation of a neuron-FET "artificial

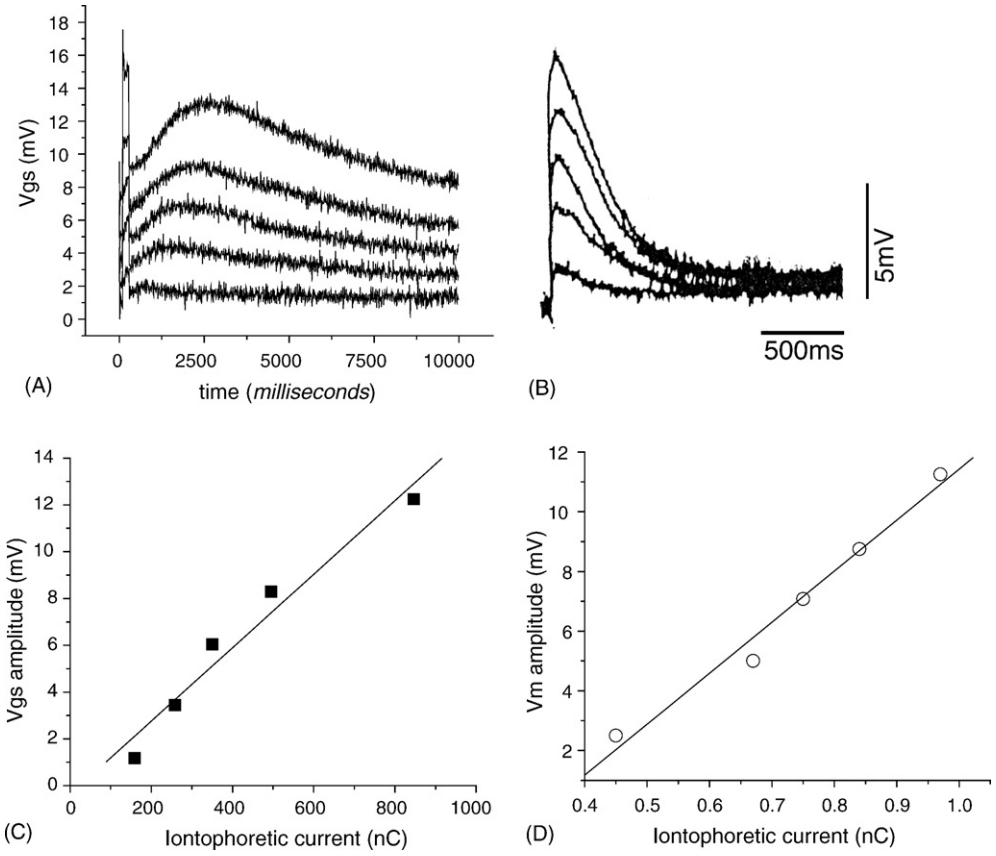

Fig. 6. Comparison of the responses to ACh iontophoresis onto an ISFET (A and C) and a cultured *Aplysia* neuron (B and D). (A) ACh iontophoresis onto AChE–ISFETs. ACh micropipette was brought in close proximity (∼5 μm) to the sensing gate surface of AChE functionalized ISFETs. Negative square current pulses of varying amplitudes were applied, resulting in the release of ACh, and a change in the ISFET gate voltage. Positive pulses did not elicit any response. Shown are response to 158.8, 258.2, 350.6, 495.3 and 846.6 nC, respectively. Iontophoresis experiments using naked ISFETs resulted in little or no response. (B) ACh iontophoresis onto a cultured metacerebral neuron. Shown are response to 0.45, 0.67, 0.75, 0.84 and 0.97 nC. (C) The relation between the response of an ISFET (Vgs) and the ionophoretic charge (nC) and (D) the voltage generated by the neuron in response to ionophoretic pulses (nC). Note that the sensitivity of the neuron is orders of magnitude better than that of the functionalized ISFET both in terms of rise time and threshold for detection.

synapse". To begin to explore this possibility we compared the sensitivity of the AChE–ISFET hybrid to that of AChR expressed by cultured *Aplysia* neurons. To that end, ACh was applied iontophoretically to the AChE–ISFET hybrid (Fig. 6A) and to a cultured neuron (Fig. 6B). Iontophoresis was performed from a glass micropipette loaded with 1 M ACh. The tip of the pipette was then brought within distance of approximately 5 μm from the gate surface of the ISFET. A constant holding positive DC current was applied to prevent leakage of ACh from the micropipette. For the experiment, negative square current pulses (180 ms long) were applied into the micropipette with increasing amplitudes. This led to release of ACh. In contrast to negative pulses that resulted in the release of ACh and the ISFET's response, positive pulses did not result in a similar, nor reverted response. This demonstrates the ISFET ability to respond specifically to ACh and not to background currents in solution, as well as demonstrates its ability to detect small and local ACh release. Iontophoresis experiments using naked ISFETs resulted in little or no response, which demonstrates the response to be a result of the existence and reactivity of AChE on the ISFET surface. The comparison between the responses of a cultured *Aplysia* neuron (Fig. 6D) and an ISFET (Fig. 6C) reveals that the ISFET response curve slope is of 4.69 mV/nC, and that of the neuron is 18.56 mV/nC. Using Coulomb's law, we estimated that $\sim 10^{12}$ ACh molecules are to be iontophorised from the pipette tip in order to produce a 1 mV output signal by the ISFET. Assuming that only 0.1–1% of the iontophorised molecules (ejected from a pipette tip, located ∼5 μm from the gate surface) collided with the enzyme and undergo hydrolysis, 10^9–10^{10} ACh molecules are required to generate sufficient number of protons and thus a transient sensor's response. Pioneering studies of the frog neuromuscular junction revealed that a miniature potential (of 0.5–1 mV), generated by the spontaneous release of ACh content from a single presynaptic vesicle, corresponds to the release of 10,000–47,000 molecules into the confined volume of the synaptic cleft (Kuffler and Yoshikami, 1975; Wagner et al., 1978). Thus, the coupling efficiency (number of ACh molecules/voltage generated by the ISFET or post synaptic cell) is five orders of magnitude better in the neuromuscular junction than that of the AChE based hybrid system. This is probably due to the fact that whereas in the post synaptic membrane, the formation of the AChR-ACh complex leads to opening of ion channels and shift of the membrane potential, the formation of the AChE–ACh complex on the ISFET generates

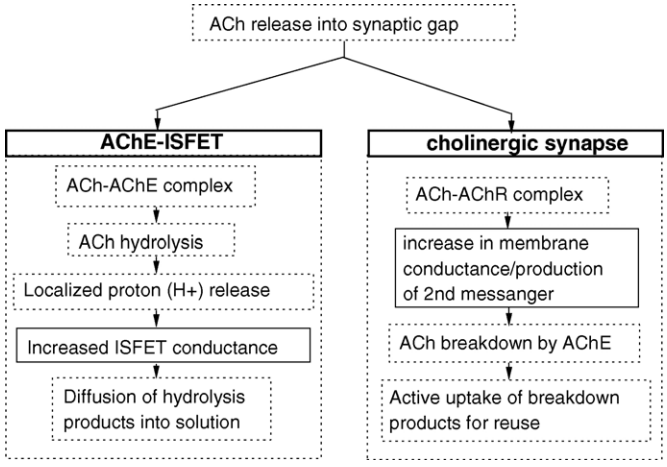

Fig. 7. Proposed model for AChE–ISFET sensing mechanism in comparison to the detection of ACh release in a cholinergic synapse.

protons that tend to diffuse away from the gate surface down the concentration gradient into the bathing solution. Nevertheless, in view of the confined volume formed in between cultured neurons and the gate surface (Fromherz, 2003) it is reasonable to assume that evoked release of neurotransmitter, which can involve the almost simultaneous release of the ACh content of tens to hundreds of vesicles, may be detected by an AChE–ISFET hybrid device (see Section 4).

4. Discussion

The main finding of the present study is that the use of cyanuric-chloride to link AChE to the gate surface of ISFET enables the construction of a relatively fast-responding biosensor system for the detection of ACh and AChE inhibitors.

By way of comparing the ability of the hybrid device to detect ACh and carbamylcholine we conclude that the AChE–ISFET sensing mechanism operates by hydrolysis of acetylcholine to acetic acid and choline and the acidification of the solution in close proximity to the gate electrode (Fig. 7). Charge dispersal within the enzyme due to ACh binding does not appear to contribute to the sensing events.

Our study reveals that the Michaelis–Menten constant, K_m, is only moderately altered by the covalent anchoring of the enzyme to the device surface. Nevertheless, the maximum reaction velocity, V_{max}, is reduced by over an order of magnitude (factor of ~13). The apparent reduction in V_{max} most likely reflects the time required to build up a local proton concentration at the gate surface. Thus, further optimization of the density of AChE molecules on the gate surface (the ratio between free and bound aluminum hydroxide groups) may improve the detector quality.

Apart from the potential use of the hybrid system to detect inhibitors of AChE, it is interesting to note that this hybrid system is potentially suitable for real-time detection of ACh release from cholinergic neurons grown on to the gate surface. In neuromuscular junction, ACh released into the synaptic cleft reaches the 10^{-3} M level (Kuffler and Yoshikami, 1975), two orders of magnitude higher than the detection level of the device. In view of the sensitivity of the AChE–ISFET system, we expect that in a neuron-FET hybrid, evoked release of ACh would rapidly elevate the ACh concentration within the confined volume of the junction and therefore lead to the a transient elevation of the free proton concentration. This in turn would lead to increased FET gate–source potential. The hydrolytic products are then expected to diffuse away from the junctional point into the bulk solution, and the ISFET response recovers (Fig. 7). The use of AChE as the anchored biocatalyst "receptor" molecule offers a form of self-reactivating reaction in which the ACh is hydrolyzed and thus allows for another reaction cycle to occur. In contrast, if AChR would have been used the rate at which the AChR-ACh complex would dissociate, and the ACh undergo spontaneous hydrolysis would not allow for repetitive measurements of transmitter release.

It should be noted that the use of AChR would have to rely on molecular conformation and charge dispersal across the gate surface that would in turn affect voltage across the ISFET sensing gate electrode. While theoretically allosteric effects and fast conformational changes may cause detectable events in the electrochemical vicinity of the gate electrode, this mode of detection was not yet implemented. Nevertheless, ISFET response to structural changes in protein, as opposed to changes in H^+ concentration would eliminate the dependency of sensor responsiveness on the composition of the solution and its ionic strength and will allow for operation in physiological solutions.

5. Conclusions

We conclude that the protocol described above to covalently immobilize AChE to the gate surface of an ISFET enabled to preserve the enzyme's catalytic and pharmacological activity. Thus the AChE–ISFET hybrid can serve as a fast and sensitive detector for ACh and reagents that inhibit AChE. Nevertheless, the sensitivity and response time of cultured neurons to ACh application is significantly better than that of the sensor in its current state. Future optimization of the AChE–ISFET hybrid is required before it can be used as an artificial post synaptic sensing element.

Acknowledgments

This study was supported by a grant from the Future and Emerging Technologies arm of the 5th IST Programme (No. IST-1999-29091). Parts of the study were carried out at the Charles E. Smith Family Laboratory for Collaborative Research in Psychobiology. The Neuro-Electronic research group of M.E.S., J.S. and S.Y. was established in 2000 by funding from the Israel Ministry of Science. Funding of this project was refused by the Israel Science Foundation. M.E. Spira is the Levi DeViali Professor in neurobiology.

References

Anderson, J.L., Coury Jr., L.A., Leddy, J., 2000. Anal. Chem. 72 (18), 4497–4520.
Bergveld, P., 1970. IEEE Trans. Biomed. Eng. 17 (1), 70–71.
Bergveld, P., 1972. IEEE Trans. Biomed. Eng. 19 (5), 342–351.

Changeux, J., Leuzinger, W., Huchet, M., 1968. FEBS Lett. 2 (2), 77–80.
Deo, R.P., Wang, J., Block, I., Mulchandani, A., Joshi, K.A., Trojanowicz, M., Scholz, F., Chen, W., Lin, Y., 2005. Anal. Chim. Acta 530 (2), 185–189.
Fromherz, P., 2003. Phys. E: Low-dimensional Syst. Nanostruct. 16 (1), 24–34.
Gorchkov, D.V., Soldatkin, A.P., Poyard, S., Jaffrezic-Renault, N., Martelet, C., 1997. Mater. Sci. Eng. C 5, 23–28.
Hart, A.L., Collier, W.A., Janssen, D., 1997. Biosens. Bioelectron. 12 (7), 645–654.
Ito, N., Saito, A., Kuriyama, T., Kimura, J., Arai, T., Kikuchi, M., Kayashima, S., Nagata, N., 1992. Front Med. Biol. Eng. 4 (1), 35–45.
Karnovsky, M.J., Roots, L., 1964. J. Histochem. Cytochem. 12, 219.
Kharitonov, A.B., Zayats, M., Lichtenstein, A., Katz, E., Willner, I., 2000. Sens. Actuators B 70, 222–231.
Kuffler, S.W., Yoshikami, D., 1975. J. Physiol. 251 (2), 465–482.
Lauks, I., 1998. Acc. Chem. Res. 31, 317–324.
Martorell, D., Cespedes, F., Martinez-Faregas, E., Alegret, S., 1997. Anal. Chim. Acta 337, 305–313.
Nagy, L., Nagy, G., Gyurcsanyi, R.E., Neuman, M.R., Lindner, E., 2002. J. Biochem. Biophys. Methods 53 (1–3), 165–175.
Pardo-Yissar, V., Katz, E., Wasserman, J., Willner, I., 2003. J. Am. Chem. Soc. 125 (3), 622–623.
Selvanayagam, Z.E., Neuzil, P., Gopalakrishnakone, P., Sridhar, U., Singh, M., Ho, L.C., 2002. Biosens. Bioelectron. 17 (9), 821–826.
Snejdarkova, M., Svobodova, L., Evtugyn, G., Budnikov, H., Karyakin, A., Nikolelis, D.P., Hianik, T., 2004. Anal. Chim. Acta 514 (1), 79–88.
Spira, M.E., Dormann, A., Ashery, U., Gabso, M., Gitler, D., Benbassat, D., Oren, R., Ziv, N.E., 1996. J. Neurosci. Methods 69 (1), 91–102.
Trojanowicz, M., 2002. Electroanalysis 14 (19–20), 1311–1328.
Volotovsky, V., Kim, N., 1998. Biosens. Bioelectron. 13 (9), 1029–1033.
Wagner, J.A., Carlson, S.S., Kelly, R.B., 1978. Biochemistry 17 (7), 1199–1206.
Yitzchaik, S., Marks, T.J., 1996. Acc. Chem. Res. 29, 197–202.