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

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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

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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