Detection of Organophosphates Using an Acetyl Cholinesterase (AChE) Coated Microcantilever

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ABSTRACT

A microcantilever modified with a layer of acetyl cholinesterase (AChE) responding to paraoxon is reported in this work. Acetyl cholinesterase was immobilized on a microcantilever through a cross linker to a monolayer on gold surface of the microcantilever. The microcantilever underwent a maximum of 7 nm bending due to the inhibition of AChE by paraoxon that slightly changed the conformation of AChE.

Key Words: Microcantilever; Acetyl cholinesterase (AChE); Organophosphate detection; Adsorption-induced stress; Self-assembled monolayers (SAMs).

INTRODUCTION

The majority of nerve agents belong to a class of compounds known as the organophosphates, which are among the most toxic chemical substances. The nerve agents interfere with the action of the nervous system. Their primary mode of action is inhibition of acetyl cholinesterase (AChE), which results in acetylcholine (ACh) accumulation in...
synaptic junctions, and produces an initial stimulation followed by prevention of cholinergic neurotransmission.\(^1\) Early detection of organophosphates neurotoxins is critical for national security against terrorism activity, including alarm for the chemical warfare attack, protection of our water resources and food supplies, and monitoring of detoxification processes, etc. Accordingly, there are considerable interests in the development of reliable devices for the sensitive detection of organophosphates.

Recently, microcantilevers have been emerging as an outstanding platform for sensors with on-chip electronic circuitry and extreme sensitivity.\(^2\) Microcantilevers can be mass fabricated by microlithographic methods. Such a small cantilever, hanging off the edge of a support piece, deflects readily under nano-Newton forces due to the adsorption of analyte molecules on the microcantilever surface. When a chemical/biological specific coating is applied on one side of cantilever surface, it absorbs chemical or biological species, selectively, and results in microcantilever bending. The bending of the cantilever can be sensitively detected by using a number of techniques, such as optical reflection from microcantilever beams. Microcantilevers provide a perfect platform for the development of chemical and biological sensors with a number of advantages, including high sensitivity and label-free detection. Furthermore, an array of microcantilevers can be readily utilized for the accurate measurement of specific chemicals or a number of chemical species. A number of chemical and physical sensors based on the cantilever platform have been demonstrated recently.\(^2\) In an effort to develop microcantilever based nerve agent sensors, we report here the development of AChE-coated microcantilever sensor for detection of the organophosphates. Paraoxon was used in this work.

**EXPERIMENTAL**

We used commercially available silicon microcantilevers (Veeco Instruments, CA) in our experiments (Fig. 1). The dimensions of the V-shaped silicon microcantilevers were 180 µm in length, 25 µm in leg width, and 1 µm in thickness. One side of these cantilevers was covered with a thin layer of chromium (3 nm) followed by a 20-nm layer of gold, both deposited by e-beam evaporation. In these experiments, all the solutions were prepared with a 0.01 M sodium phosphate buffer solution at pH 7.2. The pH of the phosphate buffer solution was adjusted by changing the ratio of 0.01 M of NaH₂PO₃ and Na₂HPO₃ stock solutions.

Microcantilever modification was completed in three steps (Fig. 1) according to known surface conjugation chemistry.\(^1\) First, a self-assembled monolayer (SAM) of 2-aminoethanethiol was formed on the gold film by immersing the cantilever into a 5 x 10^-3 M solution in a 0.01 M phosphate buffer for 12 hr, followed by rinsing the microcantilever in H₂O. Secondly, the cross-linker, glutaraldehyde, was linked to the amino groups of 2-aminoethanethiol SAM by immersing the microcantilever into a glutaraldehyde solution (2.5% wt) for 12 hr. At last, the enzyme, AChE, was immobilized on the microcantilever by cross linking its amino groups with the glutaraldehyde-activated surface. This was realized by incubating the microcantilever in an AChE solution (1 mg/mL) for 24 hr.

The deflection experiments were performed in a quartz flow glass cell (Digital Instruments, CA), such as that used in atomic force microscopy. The V-shape microcantilever was placed in the flow cell and equilibrated with phosphate buffer solution.
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Figure 1: A SEM picture of a silicon microcantilever and the modification procedure of an AChE covered microcantilever.
(0.01 M with pH 7.2), which was circulated through the cell using a syringe pump. A schematic diagram of the apparatus used in this study is shown in Sch. 1. Since a change in the flow rate induces noise in the cantilever bending signal due to turbulence, a constant flow rate of 4 mL/hr was maintained during the entire experiment. Experimental solutions containing different concentrations of paraoxon were injected directly into the slowly flowing fluid stream via a low-pressure injection port/sample loop arrangement with a loop volume of 2 mL. This arrangement allowed for continuous exposure of the cantilever to the desired solution without disturbing the flow cell or changing the flow rate. Since the volume of the glass cell, including the tubing, was only 0.3 mL, a relatively fast replacement of the liquid in contact with the cantilever was achieved. Microcantilever deflection measurements were determined using the optical beam deflection method. Briefly, the bending of the cantilever was measured by monitoring the position of a laser beam reflected from the cantilever onto a four-quadrant photodiode. In our experiment, the laser beam was reflected off the gold surface. We define “bending down” as cantilever bending toward the silicon side, while “bending up” refers to bending toward the gold side. For each measurement, a new cantilever was used.

RESULTS AND DISCUSSION

Enzyme Immobilization on the Microcantilevers

Surface modification is critical in developing a microcantilever chem/biosensor. It has been determined, that full surface coverage of molecular recognition agents on a microcantilever was required for maximum microcantilever deflection response. UV absorption was used to monitor the conjugation of AChE on the microcantilever surface as shown in Fig. 2. It was observed that the conjugation of AChE by the cantilever was complete after 10 hr, suggesting that the gold surface of the microcantilever was fully covered by the conjugated AChE enzyme. In our experiments, the cantilevers were incubated in an AChE solution 24 hr.

Microcantilever Deflection Due to Acetyl Cholinesterase Inhibition by Paraoxon

When a $10^{-4}$ M solution of paraoxon in phosphate buffer (pH 7.2) was introduced into the liquid cell to replace the buffer solution at a 4 mL/hr flow rate, the microcantilever deflection measurements were determined using the optical beam deflection method.
Figure 2. (a) The change of UV absorption of a 1 mg/mL AChE solution in 0.01 M phosphate buffer solution in a UV-transparent cuvet before and after exposure to a glutaraldehyde activated microcantilever at certain hours. The cuvet (BrandTech Scientific Inc.) is transparent between 220 and 900 nm. No UV absorption change was observed to an AChE solution in the cuvet in the presence of a bare gold coated microcantilever (figure not shown). This control experiment suggests that AChE does not adsorb onto the wall of cuvet and the gold surface of a microcantilever. (b) Changes in the UV absorption intensity at 278 nm of a 1 mg/mL AChE solution in 0.01 M phosphate buffer solution, to a glutaraldehyde activated microcantilever vs. the exposure time.
bent up and reached a maximum amplitude in about 30 min as shown in Fig. 3. The maximum amplitude of the microcantilever deflection was approximately 6 nm. It took ca. 30 min for the injected paraoxon solution to flow through the fluid cell, and at this time the phosphate buffer was circulated back into the fluid cell. When a phosphate buffer solution entered into the liquid cell to replace the paraoxon solution, the microcantilever stopped bending, but the microcantilever did not return to the initial position.

Many organophosphorus compounds inhibit the activity of AChE through phosphorylation of the serin group of an AChE, according to the following reaction.[12]

The inhibition of AChE by many organophosphorus compounds, including paraoxon, is irreversible. Bending of the microcantilever may result from a change in the conformation of AChE due to exposure to paraoxon. Change in conformation can result in the surface stress variation of the microcantilever. Since the AChE only slightly changes its conformation upon complexation with organophosphates,[13] the surface stress change on the microcantilever caused by possible AChE conformational change was very small. For a 6 nm deflection, the surface stress change was only 0.014 N/m according to the following equation.[14]

$$\Delta Z = \frac{3(1 - \nu)L^2}{Et^2} \delta \delta$$

where $\Delta Z$ is the observed deflection at the end of the cantilever, $\nu$ and $E$ are Poisson’s ratio

Figure 3. Bending response as a function of time, $t$, for a silicon microcantilever with AChE enzyme coated on its gold side after injection of $10^{-4}$ M paraoxon in 0.01 M phosphate buffer solution at pH 7.2. The microcantilever was pre-equilibrated in a 0.01 M phosphate buffer solution before injection of the paraoxon solution.
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(0.2152) and Young’s modulus (155.8 GPa) for the silicon substrate, respectively, \( t \) is the thickness of the cantilever (1 \( \mu \)m), \( L \) is the length of the cantilever (180 \( \mu \)m), and \( \delta \) is the differential stress on the cantilever.

Figure 4 shows the maximum deflection amplitudes of AChE coated microcantilevers vs. the concentration of paraoxon in the 0.01 M phosphate buffer solutions. The concentrations of paraoxon in the solutions were varied from a low concentration of \( 1 \times 10^{-8} \text{ M} \) to a high concentration of \( 1 \times 10^{-3} \text{ M} \), and the cantilever deflection changed from 0 to 7 nm. The microcantilever deflection increased as the concentration of paraoxon increased. The detect limit was \( 10^{-8} \text{ M} \). For each measurement, a 2.0-mL aliquot of paraoxon solution was switched into the fluid cell where the microcantilever was held. As the detection limit of the AChE-based biosensor is directly related to the capacity of the target chemical to inhibit AChE, the detection limit might be different for other organophosphorus compounds or other inhibitors.

We have demonstrated that the slightly conformational change of AChE caused by inhibition of organophosphates can be used to detect organophosphates by confining AChE on a microcantilever. The same concept can be used to develop other microcantilever biosensors by varying the enzyme. Because the bending amplitude of the microcantilever generated by the inhibition of AChE was relatively small, this microcantilever sensor will by no means be a real time sensor for field detection of organophosphates. However, significant microcantilever deflection can be achieved by changing the structure or materials of the cantilever. For instance, the predicted deflection of a 0.3-\( \mu \)m-thick silicon microcantilever under the same surface stress change can be 10 fold of that of the commercially available...
1-μm-thick microcantilever used in this work, according to Eq. (1). We are currently fabricating thin cantilevers that can be used to characterize small surface stress changes.

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