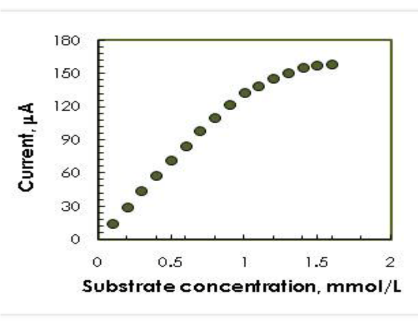
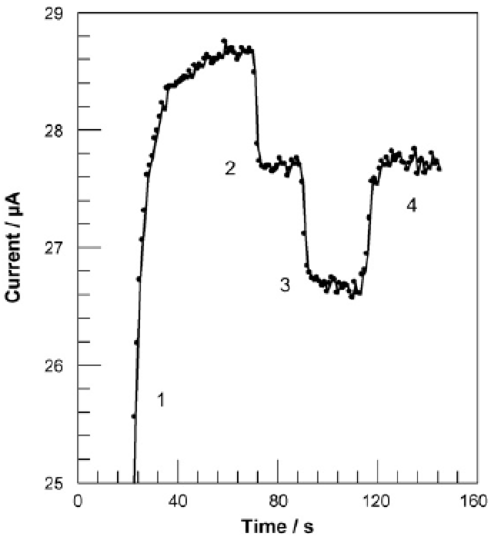
UNIVERSIDAD AUTÓNOMA DE BAJA CALIFORNIA

INSTITUTO DE INGENIERÍA

MAESTRÍA Y DOCTORADO EN CIENCIAS E INGENIERÍA

Caracterización y aplicación de biosensores para el monitoreo de contaminantes ambientales





Tesis que para obtener el grado de:
DOCTOR EN CIENCIAS

Presenta
MARCELA OVALLE MARROQUIN

Director: Dra. MARGARITA STOYTCHEVA
Codirector: Dr. ROUMEN ZLATEV

Mexicali, B. C. **Diciembre de 2009**

AGRADECIMIENTOS

A través de estas líneas, quisiera expresar mi más sincero reconocimiento y agradecimiento a todas aquellas personas, Instituciones u Organismos que han hecho posible la realización de esta Tesis Doctoral en especial a:

Al Consejo de Ciencia y Tecnología (CONACYT) y a la UABC por su apoyo económico para la realización de mis estudios doctorales y que junto con el CNRS me permitieron realizar la estancia en la Universidad Joseph Fourier (Grenoble, Francia) y unirme por un corto tiempo al grupo de trabajo del Dr. Cosnier.

Al Instituto de Ingeniería de la UABC en la dirección del Dr. Benjamín Valdez que me recibió en su programa doctoral durante 4 años y ha jugado un papel fundamental en el desarrollo de esta Tesis, siguiendo muy de cerca mi trabajo desde sus comienzos. Gracias por estar ahí siempre que he necesitado ayuda y por todos esos consejos y sugerencias que han permitido la consecución de esta Tesis Doctoral.

Al mencionar el Instituto de Ingeniería debo agradecer a la Dra. Mónica Carrillo, Dra. Sara Ojeda, las secretarias (Cruz, Norma y Lolita) y en especial a Daniel Sauceda porque pude contar con ellos cuando los necesité como profesionales y como amigos.

Debo agradecer también a los sinodales: Dr. Alejandro Martinez, Dra. Gisela Montero, Dr. Benjamin Valdez y Dra. Monica Carrillo, por revisar mi tesis y por las valiosas aportaciones que me hicieron para mejorar la misma.

Al Dr. Roumen Zlatev, codirector de esta investigación doctoral, por sus indicaciones, amabilidad, paciencia y buen humor que ha tenido conmigo.

A mi familia: mis papas, hermano y abuela, porque a pesar de no estar presentes físicamente, siempre han estado pendientes de mí y desde la distancia siempre me han dado ánimo, apoyo y alegría lo que me ha dado la fortaleza para seguir adelante.

A Andrea, el Che, Diana y a mis perros por animarme, acompañarme, alegrarme, aguantarme, soportarme y porque han hecho que mi vida en Mexicali sea feliz.

A mi hermana y a mi cuñado porque por ellos estoy acá, porque han sido mi figura paterna en México y mi ejemplo a seguir.

Deliberadamente he dejado para el final a la Directora de este Trabajo, la Dra. Margarita Stoytcheva, a quien le debo, en definitiva, todo lo que he aprendido durante su realización y que siempre ha estado dispuesta a escucharme, orientarme y corregirme, además porque he tenido la oportunidad no solo de aprender sino también de disfrutar del trabajo a su lado. Gracias por todo ello en especial por haber sido una estupenda amiga.

A todos mi mayor reconocimiento y gratitud.

CONTENIDO

LISTADO DE FIGURAS

LISTADO DE TABLAS

Resumen

El trabajo desarrollado en la presente tesis doctoral consiste en la construcción, caracterización y aplicación de sensores electroquímicos enzimáticos, bacterianos e híbridos para la monitorización de compuestos de interés medioambiental que proponen un nuevo enfoque para la detección y cuantificación de contaminantes ambientales. Se evalúan los parámetros cinéticos de las reacciones enzimáticas usando métodos electroquímicos apropiados, así como las características analíticas de las determinaciones según las recomendaciones de la IUPAC. Se demuestra que estos tipos de biosensores pueden ser una alternativa simple, económica y rápida a los métodos estándares de cuantificación de pesticidas organofosforados OP, metales pesados y fluoruros, los cuales podrían ser usados para el control en línea y en el campo.

Los biosensores y los métodos desarrollados, así como los resultados y discusión de los mismos son presentados en los artículos de los anexos A, B, C y D.

Palabras Clave – Pesticidas organofosforados, metales pesados, fluoruros, *A. globiformis*, *L. Ferrooxidans*, acetilcolinesterasa, biosensores electroquímicos.

Capítulo 1:

INTRODUCCIÓN

1 INTRODUCCIÓN

1.1 Planteamiento del problema

El desarrollo económico y tecnológico que se ha observado en las últimas décadas se ha logrado entre otras razones, debido a un uso indiscriminado de los recursos naturales, en consecuencia se observa una gran pérdida en la biodiversidad y un aumento de las zonas desérticas y de la contaminación. Debido a lo anterior se está presentando una disminución a la tolerancia de la contaminación del ambiente, lo cual hace que se desarrollen proyectos como el programa frontera 2012 que busca atender los problemas ambientales de la región fronteriza entre Estados Unidos y México. Todo esto conduce al desarrollo de nuevas herramientas de mediciones que den respuestas rápidas y selectivas de productos químicos peligrosos que son liberados en el ambiente, especialmente en agua.

Es claro, que aunque las herramientas clásicas para la medición como la cromatografía de gases con detector de flama, captura de electrones o espectro de masas de estos compuestos dan resultados exactos en concentraciones muy bajas, son dispositivos complejos y su uso puede producir retrasos que se deben principalmente al transporte de muestras al laboratorio para el análisis, por lo que no son convenientes para mediciones rápidas, en campo y en línea, además son costosos y consumidores de tiempo. Tal retraso no es compatible con las necesidades de la intervención rápida, como por ejemplo cuando se ponen en peligro los recursos del agua potable.

Una alternativa propicia a las técnicas convencionales es el uso de biosensores, esta instrumentación es complementaria a los grandes y complejos equipos de control analítico. Estos dispositivos poseen una serie de características que los hacen adecuados para una gran variedad de aplicaciones, poseen pequeñas dimensiones, son simples, robustos, de bajo costo, son fácilmente integrables en sistemas automáticos de análisis y, además, nos brindan la posibilidad de disponer de información fiable y en tiempo real.

1.2 Objetivos

1.2.1 Objetivo General

Desarrollar, caracterizar y aplicar sensores electroquímicos enzimáticos, bacterianos e híbridos para la determinación de pesticidas organofosforados, metales pesados y fluoruros.

1.2.2 Objetivos específicos

1. Diseñar nuevos biosensores electroquímicos integrando:
    - fracción de membrana de cerebro de rata como fuente de la enzima acetilcolinesterasa y un electrodo de carbono;
    - la cepa bacteriana *Leptospirillum ferrooxidans* o la cepa bacteriana *Arthrobacter globiformis* y una sonda de oxígeno tipo Clark modificada;
2. Caracterizar los transductores electroquímicos (la sonda de oxígeno tipo Clark modificada: determinación del tiempo de respuesta del transductor; evaluación de los parámetros de transporte de la membrana);
3. Desarrollar y monitorear el crecimiento del cultivo bacteriano;
4. Inmovilizar el elemento de reconocimiento biológico aplicando técnicas originales;
5. Definir los parámetros operacionales de los biosensores en diversas condiciones experimentales (pH, temperatura, concentraciones, etc.);
6. Evaluar los parámetros cinéticos de las reacciones enzimáticas;

7. Caracterizar los biosensores de acuerdo a las recomendaciones de la IUPAC;

1.3 Hipótesis

La determinación de la concentración de pesticidas organofosforados, metales pesados y fluoruros con sensores amperométricos usando bacterias y enzimas como elemento de reconocimiento biológico es un método simple, sensible y rentable.

Capítulo 2:

MARCO TEÓRICO

2 MARCO TEÓRICO

2.1 Contaminación ambiental en México

El rápido desarrollo económico y social que ha tenido México en las últimas décadas se ha visto reflejado no sólo en el aumento del producto interno bruto del país sino que además en una degradación ambiental significativa [EIA report, 2004], debido más que todo a un inadecuado control de la contaminación debido a la industrialización y a la creciente producción de alimentos y fibra.

Se saben de casos como el de la comunidad agrícola de la Villa Juárez (estado de Durango, México), donde se realizaron estudios en vegetales (tomate, maíz, habas, col, lechuga, chile y zahína) que demostraron que la frecuencia de los pesticidas usados durante los años recientes era 49 % de OP, fungicidas 19 %, herbicidas 6.3 %, carbamatos 5 %, piretroides 5 %, pesticidas biológicos 3.8 %, pesticidas órganoclorados 1.3 %, y otros 10.6 %. En cuanto al paratión metílico, los metamidofos, el endosulfan, el dimetoato y el diazinón se reporta que han sido usados con mucha frecuencia [Sánchez-Peña et al., 2004]. Se estimó que el uso de OP era de 4.600.000 kilogramos por año para las regiones agrícolas que rodean el golfo de México [Boettger, S., 2001]. Otros casos que demuestran el excesivo uso así como la carencia del sistema de supervisión y control de estas sustancias para el control de plagas, son los que se han reportado en ciudades como Ensenada donde se encontraron niveles bajos de lindano, clorpirifos, diazinón y malatión a través del sistema de recolección de aguas residuales [Status report, 2003] o el de Mexicali en el Río Nuevo, considerado como uno de los ríos más sucios del hemisferio occidental [Pauw, T., 1994].

Otro importante foco de contaminación es debido a la presencia de metales pesados como Fe, Cd, Cu, As y del ion fluoruro en aguas y suelos, éste último ha sido reportado por Wyatt et al., donde indica la presencia de una alta concentración de fluoruro en las redes de distribución de agua de la ciudad de Hermosillo; sobre la presencia de metales pesados, Gomez-Alvarez et al., han reportado concentraciones importantes de metales pesados como Cd, Cu, Fe, Mn, Pb y Zn.

2.1.1 Pesticidas organofosforados

El gran desarrollo de los pesticidas organofosforados (OP) comenzó en 1944, cuando Schrader sintetizó el paratión. Los pesticidas organofosforados, cuya fórmula general se presenta en la figura 1, son ésteres, aminas o derivados tioles del ácido fosfórico o fosfónico. Las agrupaciones R_1 y R_2 pueden ser álcalis o arilos y X son un grupo hidrolizable alifático, aromático o heterocíclico. En la tabla 1 se observan las fórmulas químicas de los principales pesticidas organofosforados.. En general, son compuestos liposolubles y debido a esto son capaces de absorberse en los caparazones de los insectos [Lauwerys, 1994].

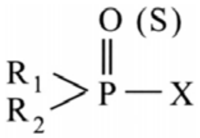


Figura 1. Fórmula general de los pesticidas organofosforados

La acción de los pesticidas organofosforados sobre los insectos y los mamíferos se sitúa en el sistema nervioso. Estas sustancias tóxicas inhiben la acetilcolinesterasa (AChE), implicando una acumulación de acetilcolina en los tejidos nerviosos, la acumulación de la acetilcolina debida a la inhibición de la AChE pueden tener graves consecuencias incluso la muerte.

El uso de estos pesticidas se han popularizado en los últimos 20 años, se conoce que de los 95 pesticidas más usados en México, 45 son de carácter organofosforados [Waliszewski, 1992], así mismo, los casos reportados por intoxicación por OP se ha incrementado en el país.

Tabla 1. Fórmulas químicas de principales pesticidas organofosforados

Nombre común	Nombre IUPAC	Formula química
Malatión	dietil 2-dimetoxifosfinotiolsulfanilbutanedioato	$C_{10}H_{19}O_6PS_2$
Paration	dietoxi-(4-nitrofenoxi)-sulfanilideno-λ^5-fosfano	$C_{10}H_{14}NO_5PS$
Clorofos	2,2,2-tricloro-1-dimetoxifosforiletanol	$C_4H_8Cl_3O_4P$
Dimetoato	2-dimetoxifosfinotiolsulfanil-N-metilacetamida	$C_5H_{12}NO_3PS_2$
Diazinon	dietoxi-(6-metil-2-propan-2-pirimidin-4-oxi-sulfanilideno-λ^5-fosfano	$C_{12}H_{21}N_2O_3PS$

Los métodos para la determinación de OP que se utilizan actualmente pueden ser muy exactos como el método potenciométrico de Michel o el espectrofotométrico de Ellman, el punto crítico de estos métodos son en el momento de la toma de muestras así como el almacenamiento y transporte antes de llegar al laboratorio. Existen métodos menos exactos como el método colorimétrico de Edson o el de Alcholest los cuales permiten un monitoreo aunque poco exacto, rápido y sin la necesidad de poseer grandes infraestructuras [Fonseca, 2007].

2.1.2 Fluoruros

Durante años se ha considerado benéfica la ingesta de flúor a bajas concentraciones (≤1 mg/L) debido a la capacidad de prevenir las caries dentales de este compuesto, es por esto, que se adiciona flúor en productos de consumo cotidiano como la pasta dental o el agua potable. A pesar de lo anterior, el consumo prolongado de productos con altos contenidos de flúor puede dar lugar a la fluorosis de dientes y huesos, y si la concentración es muy alta, puede provocar serios daños a la salud, tales como fragilidad de los huesos, e incluso rigidez total y deformación ósea [WHO, 1984].

La normatividad mexicana establece como concentración máxima permisible 1.5 mg/L de flúor en el agua de abastecimiento público (NOM -127-SSA1-1994) y 0.7 mg/L como máximo permisible para agua embotellada (NOM-041-SSA1-1993). En México se han realizado diferentes estudios sobre casos de fluorosis en distintas zonas del país; es el caso del estudio realizado por Loyola-Rodríguez et al. en San Luis Potosí donde encontró un 78% de los niños estudiados con dentición temporal, relacionado con el alto consumo de bebidas carbonatadas elaboradas con agua de la región. Alarcón et al. presentó el caso de la ciudad de Durango en donde casi el 84 % de los pozos que suministran el agua potable a los habitantes del Valle de Guadiana excedían los límites máximos de concentración de fluoruro establecidos tanto en regulaciones mexicanas como en internacionales.

Existen diferentes métodos que son aplicados en la medición del flúor como, por ejemplo [WPCF, 1989], el método colorimétrico con alizarina, que consiste en la reacción del flúor presente en la muestra, previamente acidificada con nitrato de torio, para formar un complejo que se colorea con el indicador rojo de alizarina; luego, la determinación se realiza por comparación del color obtenido en la muestra frente a un estándar de flúor de concentración conocida. Otro método muy usado es el método por ión selectivo, el cual se basa en la medición del potencial de una solución que contiene iones fluoruro, cuando se sumerge dentro de ella un electrodo específico para fluoruro y uno de referencia, creándose una corriente eléctrica entre la muestra y la solución interna del electrodo de ión selectivo, cuyo potencial será la medida de la concentración de fluoruro. Otros métodos para determinar flúor son: el método por cromatografía líquida de alta resolución (HPLC) con detector ultravioleta y el método indirecto por espectrometría de emisión de plasma con espectrometría de masas (ICP-MS).

2.1.3 Metales Pesados

Los metales pesados son introducidos al ambiente como resultado de erupciones volcánicas, lixiviados de suelos y rocas, además de actividades antropogénicas como la agricultura, desechos industriales, mineros, metalúrgicos y domésticos, todo esto hace que la contaminación por metales pesados sea un problema no solo ambiental sino que también de salud pública [Bautista, F, 1999].

Se sabe que el territorio mexicano posee una vasta región volcánica rica en minerales y por ese motivo la explotación de recursos juega un papel importante en la economía del país. Esta industria es considerada como una de las principales productoras de contaminación por metales pesados debido a la gran carga que tienen de estos compuestos sus desechos.

Son pocos los estudios que se han hecho en México sobre este tipo de contaminación, uno de los casos más reportados es el del Río San Pedro ubicado en el estado de Sonora, el cual recibe descargas directas de los residuos de la actividad minera localizada en la región, Gómez-Álvarez et al., encontró metales pesados Cd, Cu, Fe, Ni, Mn, Pb y Zn en el agua superficial del río. Se conoció de otro caso de contaminación por metales pesados cuando, en el 2008, la Cámara de Diputados obligó a la empresa Química Central de México, SA de CV, a retirar las 400 mil toneladas de residuos de cromo que habían arrojado al suelo y agua de los Pueblos del Rincón, Guanajuato.

Los métodos clásicos para determinar los metales en muestras ambientales son: análisis por fluorescencia de rayos X, espectrometría de emisión atómica de plasma, absorción atómica con flama, espectrometría de absorción atómica con horno de grafito (GFAAS). Todos ellos son aprobados por la Agencia de Protección Ambiental (EPA), el Instituto Nacional de Salud y Seguridad Ocupacional (NIOSH), la Asociación de Químicos Analíticos Oficiales (AOAC) y la American Public Health Association (APHA). Estas técnicas proporcionan datos sobre la cantidad total del metal, pero no permiten distinguir entre los diferentes estados de oxidación del mismo. La especiación de los estados de oxidación requiere un pretratamiento complejo de las muestras como quelación, precipitación, extracción o cromatografía. Por lo tanto, los análisis son costosos, consumidores de tiempo y requieren el uso de laboratorios especializados. Por otra parte, la concentración de los metales totales no siempre está directamente relacionado con la toxicidad de la muestra ya que depende de la concentración del metal que se encuentra biológicamente disponible. De hecho, la biodisponibilidad de metales definida como la forma iónica soluble del metal determina sus efectos fisiológicos y tóxicos.

El uso de biosensores para análisis cuantitativo de metales pesados ofrece una alternativa a las pruebas de toxicidad y a los métodos analíticos convencionales. Los biosensores permiten una rápida determinación de la concentración de iones metálicos mediante el uso de un equipo de bajo costo, portátil y compacto, que ofrece la misma sensibilidad y precisión de las técnicas de rutina.

2.2 Biosensores: definición y clasificación

Durante muchos años fue difícil definir el término “biosensor” y en muchas ocasiones se utilizó de forma inadecuada, es por ello que la IUPAC estandarizó las definiciones y protocolos. En este reporte técnico se define biosensor como “Un dispositivo que transforma información química mediadas por enzimas aisladas, inmunosistemas, tejidos, organelos o células enteras en una señal analíticamente útil” [Thevenot, 2001].

Un biosensor está compuesto básicamente por dos partes: un elemento de reconocimiento, capaz de interaccionar selectivamente con la especie de interés, con el resultado de un cambio físico o químico del sistema cuya intensidad estará relacionada con la concentración de la especie a analizar y un componente instrumental, integrado fundamentalmente por el transductor que puede ser de tipo *óptico, eléctrico, térmico, másico, acústico o magnético,* el cual transforma la interacción química en una señal analítica susceptible de ser medida (Figura 2).

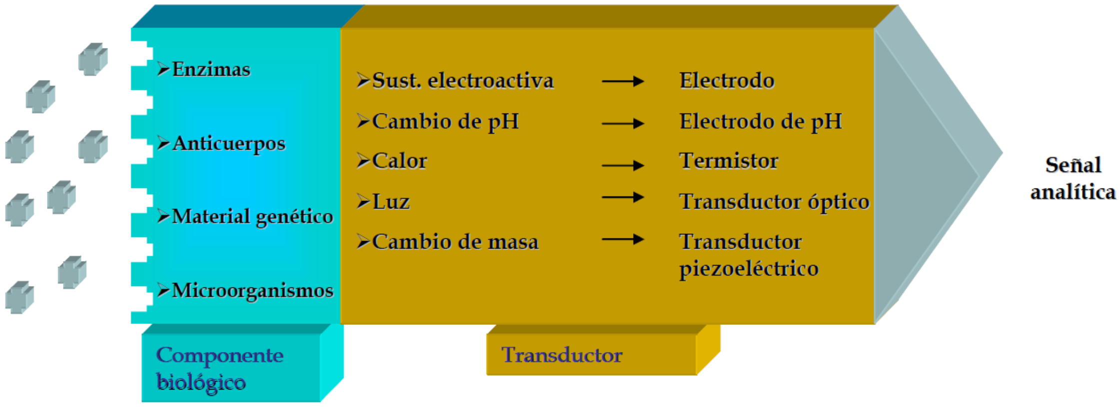


Figura 2. Diagrama del funcionamiento de un biosensor

Debido a la gran variedad de biosensores que se han desarrollado y que se desarrollarán, ha sido necesario clasificarlos bajo criterios como el tipo de receptor, la metodología utilizada para inmovilizar este receptor o el tipo de transductor empleado. En la tabla 1 se muestra la clasificación según este último criterio [B. Eggins, 1996].

Tabla 2. Clasificación de los biosensores según su transductor

Tipos de transductores	**Descripción**
Fotométricos	Transforman los cambios producidos en una señal óptica por la interacción de un analito con el receptor. La luz puede atravesar, reflejarse o emitirse del material analizado.
Electroquímicos	Pueden ser amperométricos, potenciométricos o semiconductores en donde la señal transformada se debe a una interacción electroquímica entre el analito y el electrodo.
Piezoeléctricos	Dispositivos que transforman un cambio de masa que se da sobre el electrodo modificado con materiales con propiedades piezoeléctricas.
Térmicos	Se usan termopares o termistores los cuales son capaces de medir el cambio de entalpía de una reacción.

2.2.1 Transductores amperométricos

Se puede decir que de todos los transductores que existen en la actualidad, los que más se han desarrollado y utilizado son los transductores electroquímicos, esto se debe a que son económicos, de fácil mantenimiento, manejo y miniaturización, además, poseen un amplio intervalo de linealidad y tiempos de respuesta muy cortos [Reviejo et al., 2000].

En el marco de biosensores electroquímicos, quizá los más prometedores sean los amperométricos, los cuales monitorizan las corrientes faradaicas resultantes de intercambios electrónicos entre un sistema biológico y un electrodo a un potencial apropiado. Por lo que respecta a su clasificación, puede realizarse teniendo en cuenta el sistema de reconocimiento biológico que emplee, es decir si usa enzimas, microorganismos, tejidos o inmunosensores.

El principio de estos dispositivos se basa en la aplicación de un potencial constante a un electrodo de trabajo (electrodo de Pt-, Au- o C-) respecto a un electrodo de referencia, en la mayoría de los casos es necesario usar un electrodo auxiliar si las corrientes son bajas. La medición resultante de la oxidación o reducción de especies electroactivas sobre la superficie del electrodo son registradas en el equipo [Thevenot, 2001].

2.2.2 Elementos de reconocimiento biológico: Microorganismos

Durante la última década, el uso de microorganismos como elemento de reconocimiento en los biosensores ha ido en ascenso debido a las amplias ventajas que éstos presentan frente a otros elementos usados [Yulei et al. 2006]. Su popularidad se debe a la capacidad que posee de metabolizar una amplia gama de compuestos químicos ya que tienen las enzimas en su microambiente natural que catalizan mediante su metabolismo los compuestos de interés.

Se han desarrollado biosensores microbianos usando transductores de tipo electroquímico y óptico, principalmente. Los biosensores electroquímicos reportados se clasifican en amperométricos, potenciométricos y conductímetros; los primeros son construidos principalmente con electrodos de oxígeno de Clark, o que usan el principio de éste, el cual consiste en medir una corriente que es proporcional a la velocidad de consumo de oxígeno por el electrodo. En cuanto a los electrodos potenciométricos se usan generalmente electrodos de iones selectivos que son recubiertos con microorganismos que al consumir el analito se produce un cambio en el potencial como resultado de la acumulación de iones [D'Souza, 2001].

En el caso de los biosensores ópticos, estos dispositivos aprovechan la capacidad lumínica natural o inducida de los microorganismos donde la concentración del compuesto de interés puede ser cuantitativamente medido por la intensidad de luz producida [Koenig, A., 1996].

2.2.3 Elementos de reconocimiento biológico: Enzimas

El uso de enzimas es tal vez el elemento de reconocimiento más estudiado y conocido. Químicamente las enzimas son proteínas, las cuales son capaces de catalizar reacciones químicas de manera específica y selectiva sin consumirse.

En resumen, cuando una enzima (E) se une químicamente con un substrato (S) formando un complejo *enzima-substrato* que luego se rompe para dar origen al producto (P) y liberar la enzima sin consumirse. Lo anterior es representado en el siguiente esquema:

$$\mathrm{E} + \mathrm{S} \rightleftharpoons \mathrm{E\text{-}S} \rightarrow \mathrm{E} + \mathrm{P}$$

Desde 1913 es bien conocido el comportamiento de las reacciones enzimáticas gracias a que Michaelis-Menten desarrollaron la teoría cinética de las enzimas donde expresa la velocidad de reacción catalizada enzimáticamente y se resume con la ecuación:

$$V_0 = \frac{V_{\max}[S]}{K_m + [S]}$$

donde V_0 es la velocidad inicial de la reacción, V_{max} es la velocidad máxima, K_m es la constante de Michaelis y Menten y $[S]$ es la concentración de substrato.

En ocasiones, la actividad enzimática es reducida debido a la presencia de moléculas llamadas inhibidores que se unen a la enzima obstaculizando la catálisis de la reacción correspondiente. Esta inhibición puede ser irreversible o reversible y dependerá de la unión que realicen los inhibidores con la enzima: si realizan un enlace covalente y la estructura química de la enzima es modificada la inhibición será irreversible, mientras que en el caso de los inhibidores reversibles se unen a la enzima de forma no covalente, este tipo de inhibición puede presentarse de tres formas: 1) **competitiva** el inhibidor se relaciona estructuralmente con el sustrato compitiendo por el centro activo de la enzima; 2) **no competitiva** se realiza en un sitio diferente al centro activo; finalmente, 3) **acompetitiva** el inhibidor reacciona con el complejo enzima-substrato [Halvor N., 1980].

Las enzimas son clasificadas según el tipo de reacción que catalizan:

- **Hidrolasas**, catalizan reacciones por hidrolisis.
- **Liasas**, rompen enlaces por mecanismos distintos a la hidrólisis o la oxidación. Las decarboxilasas y aldolasas son ejemplos de liasas.
- **Oxidoreductasas**, catalizan reacciones de oxidoreducción y se les llama también deshidrogenasas.
- **Ligasas**, unen moléculas utilizando energía proveniente del ATP. También son conocidas como sintetasas.
- **Transferasas**, transfieren grupos funcionales de un compuesto a otro.
- **Isomerasas**, catalizan reacciones de interconversión de isómeros.

Capítulo 3:

MATERIALES Y MÉTODOS

3 MATERIALES Y MÉTODOS

3.1 Reactivos y equipos

Los reactivos, obtenidos de Sigma, Merck y Chemapol (Checoslovaquia) son de grado analítico y fueron usados sin ningún pretratamiento de purificación. Las soluciones de yoduro de acetiltiocolina fueron preparadas e inmediatamente conservadas a 4ºC. Se utilizaron soluciones tampón de fosfato (pH 6-8), glicilglicina (pH 9-11) y glicina (pH 9-11).

Como fuente de acetilcolinesterasa se usó una fracción de cerebro de rata. Para extraerla, los cerebros de las ratas fueron recolectados inmediatamente después de la muerte y se homogenizaron en una solución amortiguadora Tris congelada a pH 7.4. El tejido resultante se centrifugó a 3000 rpm y 4°C por 15 minutos. Se extrajo el sobrenadante y se centrifugó a 16000 rpm por 15 minutos. El sobrenadante se removió nuevamente y se conservó en congelación hasta su uso.

Se usó una unidad amperométrica PRG-DEL Tacussel, para los sensores basados en acetilcolinesterasa la cual estaba acoplada a un sistema de adquisición de datos. Las investigaciones se llevaron a cabo en una celda de electrólisis convencional (5 cm^3 de volumen), a una temperatura de 26°C, pH 7 (solución amortiguadora Britton-Robinson), con una velocidad de rotación del electrodo de trabajo enzimático de 1000 rpm. Se usó Pt como electrodo auxiliar y Ag, AgCl/KCl (sat.) como referencia.

Las bacterias *Arthrobacter globiformis* NBIMCC 2369 se obtuvieron del *National Bank of Industrial Microorganisms and Cell Cultures* (Bulgaria). Por otro lado, el cultivo bacteriano de *Leptospirillum ferrooxidans* se adquirió *de la German Collection of Microorganisms and Cell Cultures* (DSMZ).

Para la preparación de las membranas bacterianas del biosensor, se usaron filtros de celulosa Sartorius con un tamaño de poro de 0.15 μm.

Se usó el espectrofotómetro Stasar III (Gilford, USA) para la medición de densidad óptica del cultivo bacteriano y para las determinaciones fotométricas de Fe^{2+}. Un analizador de pH/ion Corning Model 350 (Corning Inc., USA), se usó para la medición del pH y monitoreo del potencial redox. Una placa giratoria modelo 1010 DT de Unimax (Heidolf, Alemania) y un regulador de temperatura modelo 64R (Brookfield, E.E.U.U.) fueron utilizados para el desarrollo del cultivo bacteriano.

Para los biosensores basados en bacterias, se usó como transductor, una sonda de oxígeno de Clark a la que se le realizó una modificación distribuyendo uniformemente microcátodos de oro en todo el frente del dispositivo (Zlatev et al. 2006); este fue conectado a un detector amperométrico modelo LC-4B (BAS, USA) que envía la señal del puerto paralelo a una computadora con un software especialmente creado para la polarización del electrodo y el registro de la respuesta del sensor. Para suprimir el ruido generado entre la corriente al convertidor del voltaje y el convertidor analógico a digital (ADC) se conectó una unidad desarrollada especialmente la cual permite suprimir el ruido alrededor de 50 veces, obteniéndose una señal que no presenta ningún tipo de distorsión o degradación del tiempo de respuesta.

A esta sonda de oxígeno de Clark se le integró una celda de acrílico de 29 cm^3 de volumen. Todos los volúmenes se midieron con jeringuillas Hamilton y pipetas automáticas de precisión de 10 μl, 25 μl y 50 μl.

3.2 Cultivo Bacteriano y medio de cultivo

Para que la bacteria *Arthrobacter Globiformis* presentase especificidad para la colina, fue necesario desarrollarla de acuerdo al método descrito por Ikuta et al., este método consiste en cultivar las bacterias en un medio que contenga:

- ⊕ 1% de cloruro de colina,
- ⊕ 0.5% de extracto de levadura,
- ⊕ 0.5% de KCl, 0.1% de K_2HPO_4 y
- ⊕ 0.05% de $MgSO_4.7H_2O$,

Con un pH de 7 y a 30°C de temperatura.

El crecimiento bacteriano fue supervisado midiendo la densidad óptica (OD) de la suspensión formada por el cultivo bacteriano a 320 nm. La densidad óptica se relacionó con el número de las células viables establecidas por el conteo de unidades formadoras de colonias (UFC). Antes de alcanzar el inicio de la fase de latencia (6 horas de cultivo), la suspensión bacteriana se filtró y centrifugó, para obtener una masa de células concentradas. Lo obtenido se suspendió de nuevo en agua desionizada y se conservó en refrigeración hasta su uso.

En el caso de las bacterias *Leptospirillum Ferrooxidans,* se desarrollan en el medio DSM 882, que contiene:

Solución A:

- ⊕ $(NH_4)_2SO_4$ – 132 mg,
- ⊕ $MgCl_2.6H_2O$ – 53 mg,

- ⊕ KH_2PO_4 – 27 mg,
- ⊕ $CaCl_2.2H_2O$ – 147 mg
- ⊕ agua destilada - 950 ml; pH 1.8;

Solución B:

- ⊕ $FeSO_4.7H_2O$ – 20 g
- ⊕ H_2SO_4, 0.25 N – 50 ml; pH 1.2;

Solución C:

- ⊕ $MnCl_2.2H_2O$ – 62 mg;
- ⊕ $ZnCl_2$ – 68 mg,
- ⊕ $CoCl_2.6H_2O$ – 64 mg,
- ⊕ H_3BO_3 – 31 mg,
- ⊕ Na_2MoO_4 – 10 mg,
- ⊕ $CuCl_2.2H_2O$ – 67 mg,
- ⊕ agua destilada - 1000 ml; pH 1.8.

A la mezcla de las soluciones A y B, se añade 1 ml de la solución C.

Las bacterias *L. Ferrooxidans* se desarrollaron en un reactor de 0.5 L con una temperatura controlada de 30°C y una velocidad de agitación de 100 rpm. De esta forma fue asegurada una oxigenación moderada del cultivo bacteriano. Se aplicó el método espectrofotométrico de orto-fentantrolina el cual permite medir la concentración del ion Fe^{2+} al formar un complejo anaranjado que absorbe la luz a 505 nm. Paralelamente se aplicó el método potenciométrico basado en la medida del potencial redox E, el cual, depende de la relación $[Fe^{3+}]/[Fe^{2+}]$ y de acuerdo a la ecuación de Peters-Nernst:

$$E = E^o + (RT/F)ln\ ([Fe^{3+}] / [Fe^{2+}])$$

donde E^o es el potencial redox estándar, R es la constante de los gases, T es la temperatura, F es la constante de Faraday, $[Fe^{3+}]$ y $[Fe^{2+}]$ son las concentraciones de los iones en equilibrio. Cuando el cultivo bacteriano alcanzó su fase de latencia (el 90% de los iones ferrosos oxidados), se suspendió el desarrollo de las bacterias y se centrifugó el cultivo. La masa bacteriana concentrada fue lavada y suspendida de nuevo en agua desionizada.

3.3 Preparación del sensor basado en acetilcolinesterasa

El electrodo enzimático de trabajo consistió en un disco giratorio con un diámetro de 6 mm, fabricado con grafito espectralmente puro (Ringsdorf Werke, Alemania). Antes de inmovilizar la enzima sobre el electrodo, la superficie del mismo se trató preliminarmente con un pulido, desengrasado con alcohol y limpiado con ultrasonido; una vez hecho esto se procedió a inmovilizar la enzima acetilcolinesterasa la cual se realizó por adsorción, siguiendo el siguiente protocolo:

1. Se inyectaron 30 µL del sobrenadante que contiene la enzima sobre la superficie del electrodo de trabajo;
2. Se mantuvo por 30 minutos el volumen adicionado sobre el electrodo a temperatura ambiente;
3. Se limpió la superficie del electrodo durante 5 minutos en la solución amortiguadora a una velocidad de rotación del electrodo de 1000 rpm, para eliminar las trazas de enzima que no se fijaron adecuadamente.

3.4 Preparación del sensor basado en cultivo bacteriano

El biosensor desarrollado para las mediciones de OP y metales pesados consistió en un electrodo de oxígeno de Clark como transductor y una membrana bacteriana como elemento biológico de detección. La obtención de la membrana bacteriana se logró mediante la filtración de las células bacterianas a través de un filtro de celulosa *Sartorius* con un diámetro de poro más bajo que el tamaño de las bacterias. El filtro cargado de bacterias (membrana bacteriana) fue fijado en la membrana de polímero del electrodo de oxígeno de Clark.

Las membranas bacterianas con características reproducibles fueron obtenidas al variar el volumen de la biomasa filtrada de acuerdo con su concentración, conservando constante el producto de estas dos variables. La punta del electrodo de oxígeno fue modificada con respecto a las disponibles comercialmente, ya que se sustituyó el frente esférico por uno plano y el pequeño cátodo por un multicátodo de oro distribuido uniformemente en la superficie delantera (figura 3). Estas modificaciones aseguran una membrana bacteriana uniforme y una facilidad para la difusión del oxígeno.

Figura 3. Multicátodo de oro en el electrodo de oxígeno

Capítulo 4:

RESULTADOS Y DISCUSIÓN

4 RESULTADOS Y DISCUSIÓN

4.1 Sensor basado en acetilcolinesterasa

4.1.1 Principio de medición

Las determinaciones se basaron en el proceso de hidrólisis de acetiltiocolina a tiocolina, la cual es catalizada por la acetilcolinesterasa inmovilizada ACh:

$$RSCOCH_3 + H_2O \xrightarrow{ACh} RSH + CH_3COOH$$

$R = (CH_3)_3N^+(CH_2)_2$

En presencia del inhibidor, ya sea clorofos o fluoruro, la concentración de tiocolina producida disminuye, esto es debido a la acción inhibitoria de la actividad enzimática del compuesto.

Dos principios fueron probados en los ensayos (Principio 1 y Principio 2) con el fin de recibir una respuesta sensible del sensor, en función de la concentración de clorofos. El Principio 1 consistió en el registro de la corriente de oxidación de la tiocolina a un potencial de +0.80V vs Ag/AgCl (Goodson, L., 1976; Stoytcheva, M. ,1995a; 1995b):

$$2RSH \rightarrow RS\text{-}SR + 2H^+ + 2e^-$$

El Principio 2 se basó en las reacciones siguientes (Neufeld, T., 2000):

$$2RSH + 2[Fe(CN)_6]^{3-} \rightarrow RS\text{-}SR + 2[Fe(CN)_6]^{4-}$$

$$[Fe(CN)_6]^{4-} \rightarrow [Fe(CN)_6]^{3-} + e^-$$

La tiocolina reaccionó con el cianuro de hierro (III), presente en la solución amortiguadora con una concentración de 1mM. El $[Fe(CN)_6]^{3-}$ se redujo a $[Fe(CN)_6]^{4-}$.

La señal analítica que es registrada pertenecía a la corriente de la oxidación del cianuro de hierro (II) a un potencial de +0.45/Ag (para las mediciones de clorofos) y +0.35/Ag (para F^-), AgCl. Para este valor de potencial las reacciones concurrentes (reducción de $[Fe(CN)_6]^{3-}$ o tiocolina y la oxidación de yoduro) no se llevan a cabo.

Por otro lado, la Figuras 4 y 5 ilustran la respuesta del biosensor amperométrico para acetiltiocolina y para el clorofos a concentración constante de acetiltiocolina, medida a un potencial de +0.80 V/Ag y a +0.35V/Ag, AgCl. La corriente correspondiente a una concentración de substrato fija [S] en ausencia de inhibidor (I_o) fue compensada usando una técnica electrónica (Fig. 4b, Fig. 5b). La corriente disminuye con el aumento de la concentración del inhibidor. Este mismo comportamiento fue observado cuando se usó fluoruro de sodio como inhibidor.

La aplicación del Principio 2 ofrece la ventaja de operar el electrodo a un potencial bajo. Sin embargo, los datos presentados desde la Figura 4 a la 7 permiten establecer la sensibilidad de las determinaciones demostrando que en este caso disminuyen significativamente (de 274.73μA/mM a 6.45μA/mM para acetiltiocolina y de 8.87μA/mM a 0.24μA/mM para el clorofos con una concentración de acetiltiocolina de 0.6 mM, a +0.80V y +0.45V respectivamente; para NaF de 16.85μA/mM a 6.95μA/mM con una concentración de acetiltiocolina de 1mM a +0.35V). Debido a lo anterior, los experimentos adicionales se llevaron a cabo con el primer acercamiento (Principio 1).

El clorofos y el NaF se midieron estableciendo la relación entre su concentración y la inhibición inducida de la enzima, expresado como una disminución de la corriente de oxidación de la tiocolina. El grado de inhibición ($D = \Delta I/I_o$, %) se definió como la relación de la disminución de la respuesta del biosensor debido a la adición del inhibidor (ΔI) y la respuesta inicial del biosensor (I_o) (NCIUB, 1982; Tran-Minh, C., 1985).

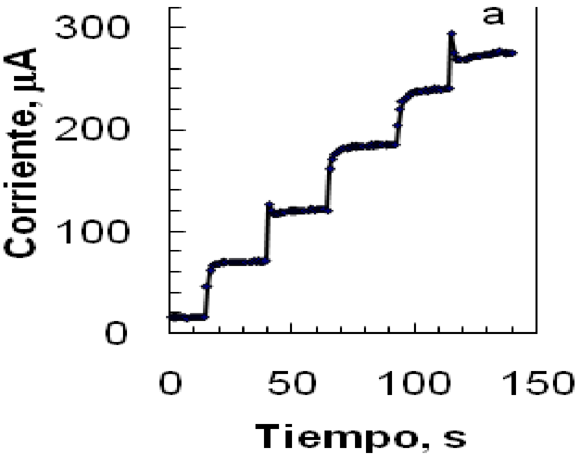


Figura 4. Respuesta del biosensor amperométrico con el incremento de la concentración de acetiltiocolina (0.2 mM; 0.4 mM; 0.6 mM; 0.8 mM; 1.0 mM) a un potencial de +0.80V/Ag, AgCl.

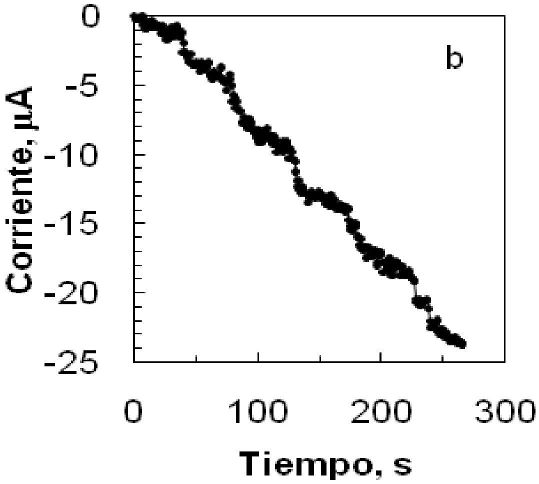


Figura 5. Respuesta del biosensor amperométrico con el incremento de la concentración de clorofos (0.2 mM; 0.4 mM; 0.6 mM; 0.8 mM; 1.0 mM) para una concentración constante de acetiltiocolina (0.6 mM) a un potencial de +0.80V/Ag, AgCl.

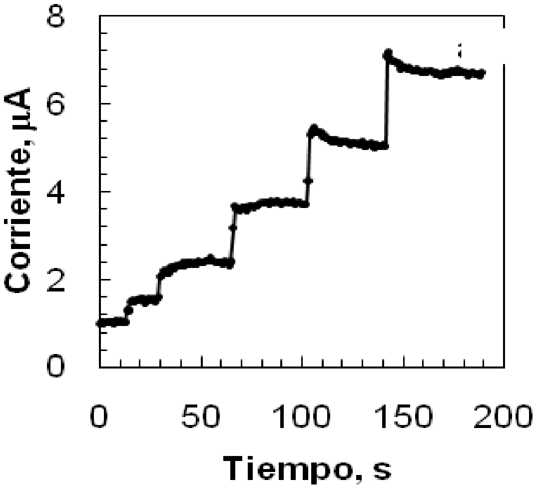


Figura 6. Respuesta del biosensor amperométrico con el incremento de la concentración de acetiltiocolina (0.2 mM; 0.4 mM; 0.6 mM; 0.8 mM; 1.0 mM) a un potencial de +0.45V /Ag, AgCl.

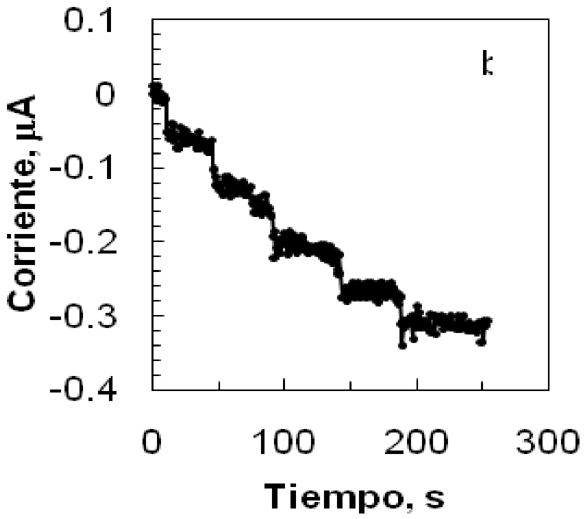


Figura 7. Respuesta del biosensor amperométrico con el incremento de la concentración de clorofos (0.2 mM; 0.4 mM; 0.6 mM; 0.8 mM; 1.0 mM) para una concentración constante de acetiltiocolina (0.6 mM) a un potencial de +0.45V /Ag, AgCl.

4.1.2 Aspectos cinéticos y analíticos de la determinación

Como se describió anteriormente, la determinación del clorofos y de NaF influye en la disminución de la actividad de la acetilcolinesterasa, la cual es expresada como la disminución de la corriente a una concentración de substrato constante [S].

Los datos experimentales presentados en figura 8 demuestran que el grado de inhibición de la acetilcolinesterasa inmovilizada se incrementa al aumentar la concentración del inhibidor y disminuye con la concentración del substrato. Este tipo de inhibición es considerada como competitiva.

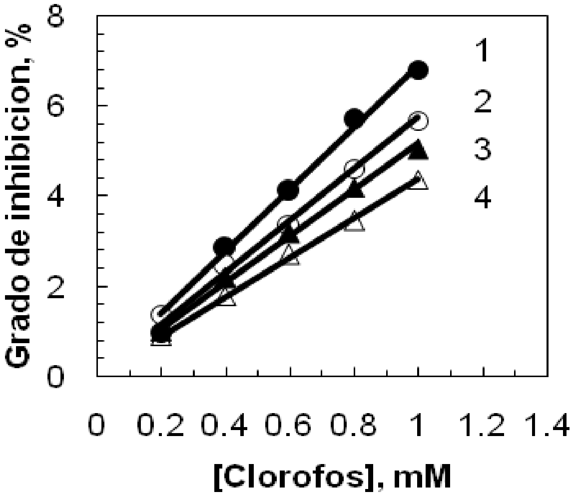


Figura 8. Grado de inhibición (D, %) de la acetilcolinesterasa inmovilizada en respuesta a la concentración de clorofos para concentraciones de substrato diferentes: 1) 0.2 mM; 2) 0.4 mM; 3) 0.6 mM; 4) 1.0 mM.

Se calcularon los parámetros cinéticos como la constante de Michaelis-Menten aparente K_M^{app} y la velocidad máxima de la reacción enzimática I_{max} ajustando los datos obtenidos en la región del control enzimático ([S] ≥ 0.8 mM) al modelo $I = I_{max}[S]/(K_M^{app} + [S])$, aplicando regresión no lineal.

Para este propósito, se usó un software especializado en cinética enzimática (GraphPad software, Inc.). Como se esperaba, se presenta una inhibición competitiva, ya que la velocidad máxima de la reacción enzimática no varía en presencia del inhibidor (Keleti, T.,1986). En cambio, como se observa en las tablas 3 y 4, el valor de K_M^{app} se incrementa con el aumento de la concentración del inhibidor (Keleti T., 1986).

Tabla 3. Parámetros cinéticos de la reacción enzimática, catalizada por la acetilcolinesterasa en ausencia y en presencia de NaF a diferente pH (26 ±0.5°C).

[NaF], mmol dm^{-3}	Kmapp, mmol dm^{-3}			
	pH 6	**pH 7**	**pH8**	**pH9**
0.0	0.37±0.01	1.04±0.01	0.91±0.01	1.53±0.02
0.2	0.39±0.01	1.07±0.01	0.94±0.01	1.59±0.02
0.4	0.41±0.01	1.09±0.01	0.98±0.01	1.64±0.02
0.6	0.43±0.01	1.12.±0.01	1.01±0.01	1.69±0.02
0.8	0.44±0.01	1.15±0.01	1.04±0.01	1.76±0.02
1.0	0.47±0.02	1.18±0.01	1.08±0.01	1.82±0.03
	K_I = 4.05mmoldm^{-3}	KI = 7.56mmoldm^{-3}	KI = 5.55mmoldm^{-3}	KI = 5.20mmoldm^{-3}
	I_{max} = 237.76μA	I_{max} = 423.59μA	I_{max} =401.15μA	I_{max} = 535.05μA

Tabla 4. Parámetros cinéticos de la reacción enzimática, catalizada por la acetilcolinesterasa inmovilizada en ausencia y presencia de fluoruro de sodio a diferentes temperaturas (pH 8).

[NaF], mmol dm^{-3}	Kmapp, mmol dm^{-3}			
	26± 0.5°C	**30± 0.5 °C**	**35± 0.5 °C**	**40± 0.5 °C**
0.0	0.91±0.01	1.21±0.01	0.92±0.01	0.88±0.01
0.2	0.94±0.01	1.26±0.01	0.97±0.01	0.90±0.01
0.4	0.98±0.01	1.31±0.01	1.00±0.01	0.95±0.01
0.6	1.01±0.01	1.35.±0.01	1.05±0.01	0.98±0.01
0.8	1.04±0.01	1.39±0.01	1.09±0.01	1.02±0.01
1.0	1.08±0.01	1.43±0.01	1.13±0.02	1.04±0.01
	K_I = 5.55mmoldm^{-3}	K_I = 5.47mmoldm^{-3}	K_I = 6.02mmoldm^{-3}	K_I = 5.29mmoldm^{-3}
	I_{max} =401.15μA	I_{max} =568.19μA	I_{max} = 572.25μA	I_{max} = 554.63μA

La constante de inhibición K_I se determinó según el método de Dixon (Dixon M., 1953). Esta presentación (Figuras 9a y 9b) confirmó el carácter competitivo de la inhibición de acetilcolinesterasa debido a los clorofos y al fluoruro de sodio.

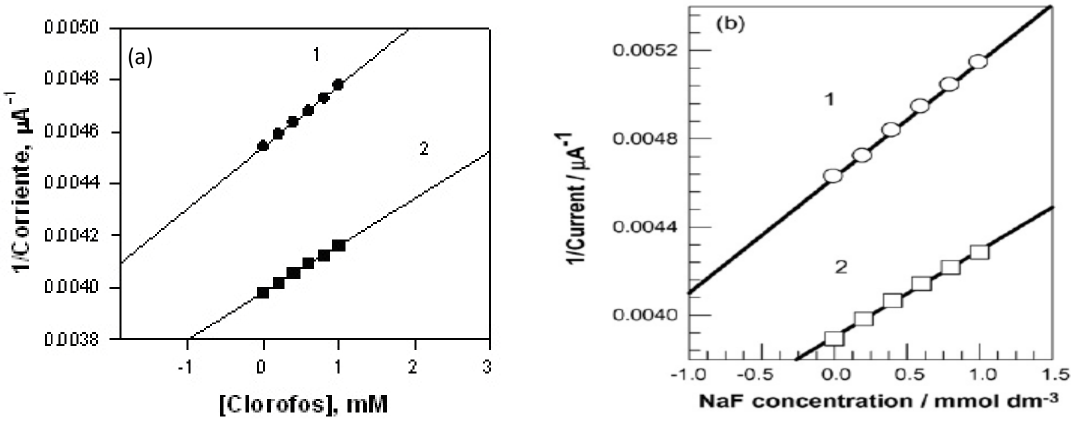


Figura 9. Gráfica de Dixon: a.) con clorofos, b.) con NaF 1) [S] = 0.8 mM; 2) [S] = 1.0 mM.

El proceso de inhibición para la enzima inmovilizada se puede describir con el siguiente esquema cinético:

$$E + S \rightleftharpoons ES \rightarrow P \qquad K_M^{app} = [E]\,[S]\,/\,[ES]$$

$$E + Inh \rightleftharpoons EInh \qquad K_I = [E]\,[Inh]\,/[\,EInh]$$

donde:

E es la acetilcolinesterasa inmovilizada, S es el substrato acetiltiocolina, P es el producto tiocolina, ES es el complejo enzima-substrato, EInh es el complejo enzima-inhibidor, K_M^{app} es la constante de Michaelis-Menten aparente y K_I es la constante de inhibición.

El comportamiento del clorofos $(CH_3O)_2POCH(OH)CCl_3$ como un inhibidor competitivo de la acetilcolinesterasa puede explicarse por su analogía estructural con el substrato de la enzima acetiltiocolina $(CH_3)_3N^+(CH_2)_2SCOCH_3$. Debido a que el substrato y el inhibidor compiten por el mismo lugar, el grado de la inhibición es reducido con el aumento de la concentración de substrato.

Los experimentos realizados demostraron que la disminución de la concentración del inhibidor con la dilución de la muestra analizada, manteniendo constante la concentración del substrato conduce a una recuperación de la actividad enzimática, es decir el proceso de la inhibición es reversible (Figuras 10a y 10b).

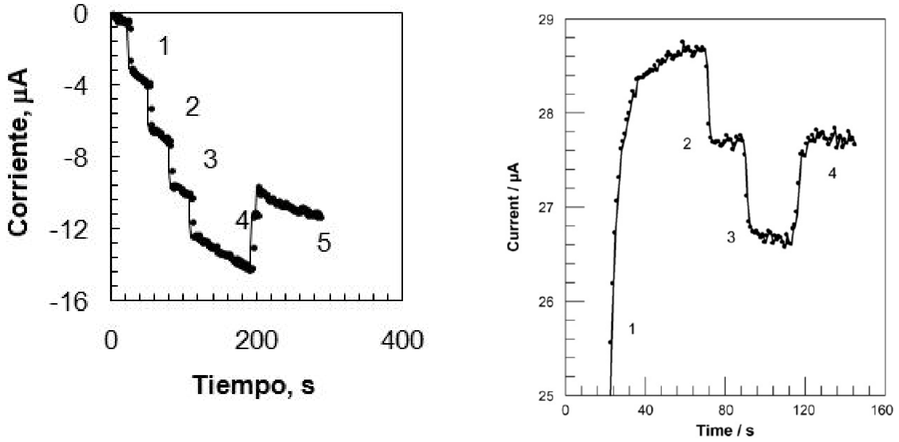


Figura 10. Reversibilidad de la respuesta del biosensor. a) concentración de substrato 0.2 mM; concentraciones de clorofos:1) 1.0 mM; 2) 2.0 mM; 3) 3.0 mM; 4) 4.0 mM; 5) 2.0 mM. b) (1) concentración de acetiltiocolina 0.1mM; concentración de fluoruro de sodio: (2) 0.4mM; (3) 0.8mM; (4) 0.4mM (doble dilución para la misma concentración de substrato).

La presentación de los datos experimentales en coordenadas de Lineweaver-Burk (Lineweaver H., 1934) (Figura 11) permite suponer que para concentraciones de substrato bajas ([S] < 0.8 mM) el proceso es controlado por la difusión. Esta suposición fue confirmada al investigar la dependencia del valor de corriente de estado estacionario con la raíz cuadrada de la velocidad de rotación de electrodo para concentraciones diferentes de substrato.

La relación observada puede describirse por la ecuación $I=0.65nAD^{2/3}\nu^{1/6}\omega^{1/2}[S]$, que es idéntica a la ecuación hidrodinámica de Levich para un electrodo de disco rotatorio (Shu F.,1976; Levich

V., 1959), donde I es la corriente del estado estacionario, n es el número de los electrones intercambiados, F es la constante de Faraday, A - el área geométrica del electrodo, D - el coeficiente de difusión, ν - la viscosidad cinemática, ω - la velocidad de rotación de electrodo y [S] - la concentración de substrato.

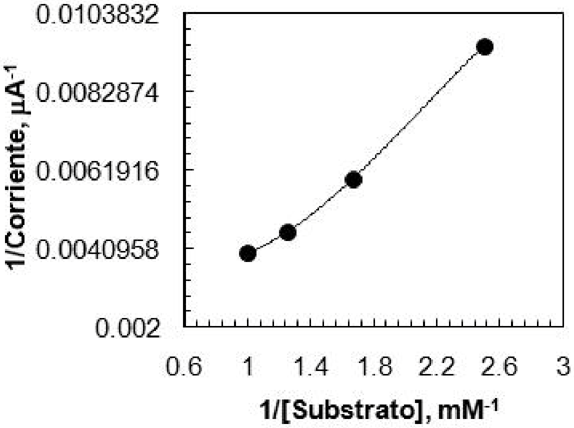


Figura 11. Grafica de Lineweaver-Burk para los datos experimentales

Los datos que se muestran en la Figura 8 presentan que la sensibilidad de la determinación del clorofos, debido al carácter competitivo de la inhibición, es mayor para concentraciones bajas de acetiltiocolina. Esto es igual a 6.89%/mM para [S]= 0.2 mM y 36.28 % más en comparación a la sensibilidad determinada para [S] = 1.0 mM. La inhibición puede ser superada por concentraciones de substrato altas. Es por esto que, en el último caso, sólo las altas concentraciones de clorofos pueden ser detectadas.

Como se mencionó arriba, el proceso estudiado es controlado por la transferencia de masa cuando las concentraciones de substrato son bajas. Este hecho impone la realización de las mediciones a velocidad de rotación del electrodo constante.

La reversibilidad del proceso inhibitorio permitió la recuperación de la actividad enzimática lavando la sonda con solución amortiguadora, es decir que el biosensor puede ser usado múltiplemente. La desviación estándar relativa (RSD) de la respuesta del biosensor para sucesivas determinaciones (N=5), se llevó a cabo en las mismas condiciones y se encontró que era 2.87% ([S]=1.0 mM). La simplicidad y el buen conocimiento de la técnica para la fabricación del biosensor aseguró una buena reproducibilidad de las determinaciones (RSD = 4.22 %, N=5, [S]=1.0 mM).

Se halló el tiempo de respuesta del biosensor, definido como el tiempo necesario para alcanzar el 90% del valor de la corriente en estado estacionario (Thévenot, 1999), el cual fue de 10 segundos.

4.2 Sensor basado en *Arthrobacter globiformis*

4.2.1 Cultivo Bacteriano

En la figura 12 se presenta la curva de crecimiento realizada a la bacteria *A. globiformis* que permite observar el comportamiento de la bacteria. Esta gráfica permitió construir una curva de calibración (OD vs. CFUx10^6) la cual es lineal en la gama de concentración investigada con intercepto en *y* de 0.0332 y una pendiente de 9x10^{-5} (r^2 = 0.9649). Esta gráfica se empleó más adelante para determinar la concentración de las bacterias midiendo solamente la densidad óptica de la muestra.

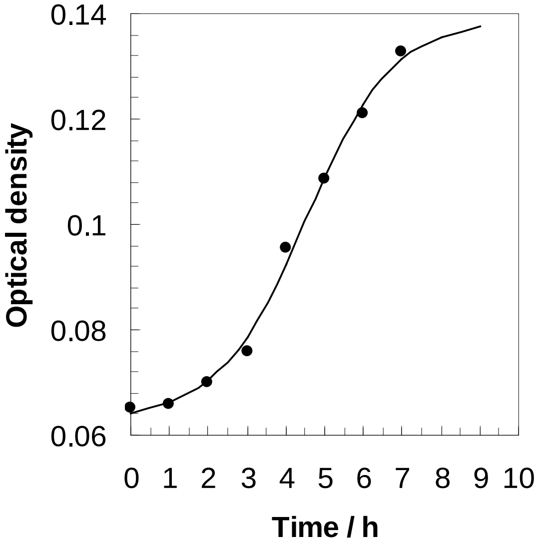


Figura 12. Curva de crecimiento de *A. globiformis*

4.2.2 Principio de medición

El principio de medición se basó en las siguientes reacciones:

$$\text{acetilcolina} + H_2O \xrightarrow{\text{ACh}} \text{colina} + CH_3COOH$$

$$\text{colina} + 2O_2 + H_2O \xrightarrow{\text{ChO}} \text{betaina} + 2H_2O_2$$

donde ACh es la acetilcolinesterasa y ChO es la colina-oxidasa bacteriana.

En la figura 13 se presenta un esquema para la oxidación de colina a betaína en las células de *A. globiformis* acoplado con el consumo de oxígeno.

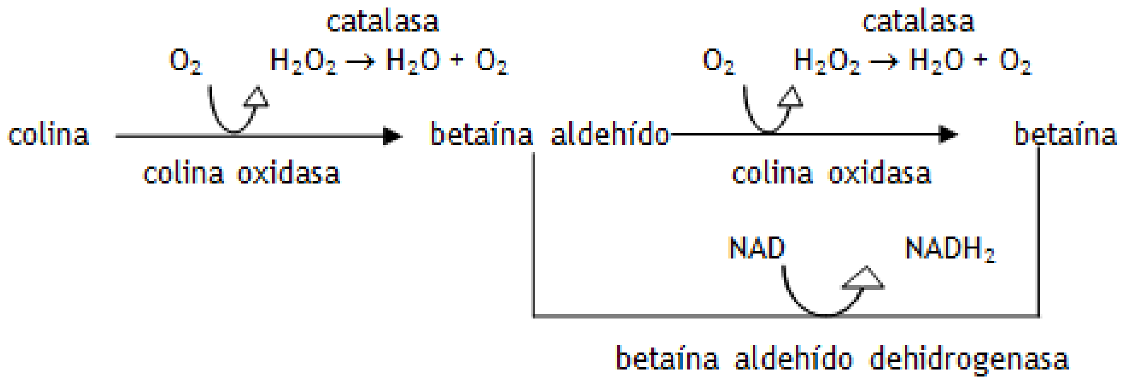


Figura 13. Posible camino oxidativo de colina a betaína en las células de *A. globiformis*, según Ikuta et al.

Para una concentración constante de acetilcolina y condiciones experimentales idénticas, la producción de colina depende de la actividad de la acetilcolinesterasa, afectada por los inhibidores OP. Al disminuir la cantidad de colina generada debido a la inhibición de la ACh, el consumo de oxígeno durante el proceso de oxidación a betaína en las células de la bacteria disminuye. Es por esto que, la corriente proporcional a la respiración de las bacterias fue registrada como respuesta del sensor y correlacionada a la concentración del pesticida OP.

4.2.3 Aspectos analíticos de la determinación

Como se mencionó arriba, de acuerdo a la ecuación presentada anteriormente, el consumo de oxígeno de las bacterias depende de la cantidad de colina. Teniendo en cuenta esto, se estudió la respuesta del biosensor a la colina y los resultados se presentan en un trabajo previo (M. Stoytcheva et al, 2006). Esto se resume en la tabla 5.

Tabla 5. Características analíticas del biosensor para la determinación de colina (I_s es la corriente de estado estacionario, μA; C es la concentración de colina, μmol dm^{-3}).

Sensibilidad	0.4 μA μmol^{-1}
Concentración lineal de la curva de calibración	I_s = 0.40C, r^2=0.9978
Límite de detección	8x10^{-8} mol dm^{-3}
Límite de cuantificación	2.6x10^{-7} mol dm^{-3}
Tiempo de respuesta en estado estacionario	200 s
Tiempo de vida de almacenamiento	t_{L10}=14 días; t_{L50}=49 días
Interferencias	Glucosa
Reproducibilidad	RSD<3%

La caracterización analítica del biosensor para la determinación de la colina incluyó la evaluación de los siguientes parámetros: sensibilidad, rango de concentración lineal de la curva de calibración, límite de detección, límite de cuantificación, tiempo de respuesta en estado estacionario, tiempo de vida de almacenamiento, selectividad y reproductibilidad, según lo definido por la IUPAC (D. R. Thévenot et al, 1999).

La figura 12a presenta la respuesta típica en estado estacionario del biosensor a la acetilcolina en el rango de concentración de 0.1 μmol dm^{-3} a 0.5 μmol dm^{-3}. El diagrama correspondiente de la calibración (figura 14b) fue lineal con una pendiente de 0.4338 μA dm^{3} μmol^{-1} y con un coeficiente de correlación 0.9966. El tiempo de respuesta del biosensor fue alrededor de 200 s, según lo ilustrado en figura 14a. Este valor no se diferencia del divulgado anteriormente para la detección de colina.

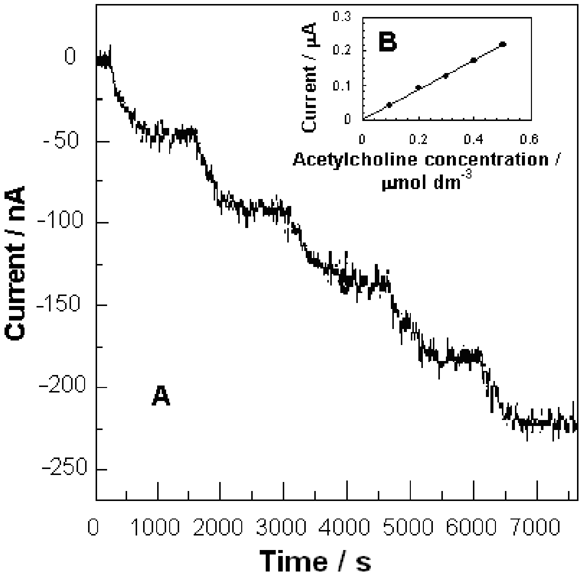


Figura 14. a) Corriente de respuesta en estado estacionario del biosensor al incrementar las concentraciones de acetilcolina con un incremento de 0.1 µmol dm-3 en el rango de 0.1 µmol dm-3 a 0.5 µmol dm-3 (0.425 µg cm-1 Ach, pH 6, 25oC, 300rpm); b) Curva de calibración de acetilcolina.

El tiempo de respuesta del biosensor, en función principalmente del proceso heterogéneo de oxidación de colina, no cambió. El biosensor, que combina *A. globiformis* inmovilizado y una sonda de oxígeno de tipo Clark muestra una estabilidad a largo plazo a temperatura ambiente. Se observó una pérdida de actividad del 10 % depués de 14 días de almacenamiento en agua desionizada y se registró un 50% de pérdida de actividad después de 49 días de almacenamiento en la mismas condiciones.

El dispositivo es reutilizable, la función de las enzimas ubicadas en las bacterias no se ve afectada por los inhibidores de OP. El biosensor tiene una excelente reproducibilidad (RSD <3%, N = 5), debido más que todo a la fiabilidad de la técnica de inmovilización de las bacterias, que garantizan la elaboración de las membranas bacterianas con características idénticas.

4.2.4 Determinación de Clorofos

Los datos anteriores que se obtuvieron al caracterizar la respuesta del biosensor con colina y acetilcolina se utilizaron para determinar la concentración de clorofos a unas condiciones que garantizaran una respuesta estable y reproducible del biosensor (concentración de acetilcolina: 20 μmol dm^{-3}, actividad inicial de la acetilcolinesterasa: $11.04Ucm^{-3}$, pH: 6, 25ºC y velocidad de agitación: 300 rpm). Una solución diluída de clorofos fue inyectada en el sustrato que contiene la muestra y se registró el cambio de la corriente en estado estacionario como respuesta del sensor.

Se obtuvo una curva de calibración construída a partir de una concentración de clorofos en el rango de 10^{-4} a 10^{-8} mol dm^{-3}, (corriente (μA) vs. -log [clorofos]). Esta curva fue lineal (r^2= 0,9987) con una pendiente de 0,0252 μA /p(mol dm^{-3}), el intercepto en y fue de 0,2534. El límite de detección para clorofos fue 10^{-9} mol dm^{-3}, esto indica que el sensor propuesto está en el mismo orden de magnitud o inferior, que los sensores basados en inhibición enzimática para la cuantificación de clorofos.

Algunos datos comparativos relacionados los sensores basados en inhibición enzimática para la cuantificación de clorofos se presentan en la Tabla 6.

Tabla 6. Límite de detección (LOD) de algunos sensores basados en inhibición enzimática para la cuantificación de clorofos

Electrodo de trabajo	Matriz enzimatica	LOD (M)	Referencias
Grafito	ACh/ Enlace covalente	1×10^{-9}	Stoytcheva, M., 1994
Electrodo de pH	ChO+HRP	2×10−13	Ghindilis, A. et al, 1996
Electrodo *“screen-printed”*	BuChE cross linked with GA	3.5×10−7	Gogol, E. et al, 2000
Pt	Ach/PVA	5×10−6	Turdean, G. et al, 2002
Carbon vítreo, Au	BuChE/polyethyleneimine/BSA-GA membrane	>1×10−7	Reybier, K. et al, 2002
Transistor de Efecto campo	BChE/BSA-GA membrane	1×10−6	Krifi, B. et al, 2002
GC	BChE/PAN/cross linked with GA	8×10−7	Ivanov, A.N., et al. 2003
Carbon vítreo	ACh/PAN/cross linked with GA	1×10−6	Ivanov, A.N., et al. 2003
Au	ACh + ChO/dendrimer	3×10−12	Snejdarkova, M., et al, 2004
Au	ACh + ChO/polyaniline	2×10−7	Snejdarkova, M., et al, 2004
Carbon vítreo	ACh/manganese (III) meso-tetraphenylporphyrin nanoparticles	5×10−10	Li, X. et al, 2007

La exactitud de la determinación se comprobó al realizar mediciones paralelas utilizando el sensor de acetilcolinesterasa descrito por Stoytcheva M, 1994. La comparación de los resultados demostró que el error relativo no superó el 3%.

El tiempo de respuesta del biosensor, el cual depende principalmente del proceso de oxidación heterogénea de la colina, no presentó cambios. El biosensor, que combina *A. globiformis* inmovilizada y la sonda de oxígeno de Clark, muestra una estabilidad a largo plazo a temperatura ambiente, como se señaló en la Tabla 6. Se observó una pérdida de actividad del 10% después de 14 días de almacenamiento en agua desionizada y se registró 50% de pérdida de actividad después de 49 días de almacenamiento en las mismas condiciones. El dispositivo fue reutilizable, las propiedades de las enzimas de las bacterias no se vieron afectadas por los inhibidores de la OP. El biosensor tenía una excelente reproducibilidad (RSD <3%, n = 5), debido más que todo a la técnica de inmovilización de las bacterias, que garantizó la elaboración de membranas bacterianas con características idénticas.

4.3 Sensor basado en *Leptospirillum ferrooxidans*

4.3.1 Cultivo bacteriano

El crecimiento de las bacterias debe concluirse entre el final de la fase exponencial y al principio de la fase de latencia para mantener la actividad de las bacterias, es por esto, que es necesario un control exacto del crecimiento del cultivo bacteriano.

Se aplicó el método potenciométrico combinado con las medidas espectrométricas. Como se observa en la figura 15 la inflexión registrada en un potencial cerca de 625 milivoltio en la curva del potencial redox (a), se refleja la variación del cociente de concentración de Fe^{3+}/Fe^{2+} la cual corresponde exactamente a la inflexión que aparece en la curva espectrométrica (b) que demuestra la caída de la concentración de Fe^{2+} respecto al tiempo, durante la fase exponencial de crecimiento de las bacterias.

Este hecho permite usar el método potenciométrico para la supervisión bacteriana en línea del crecimiento, substituyendo el tiempo y el reactivo que consumen las determinaciones espectrométricas de Fe^{2+}.

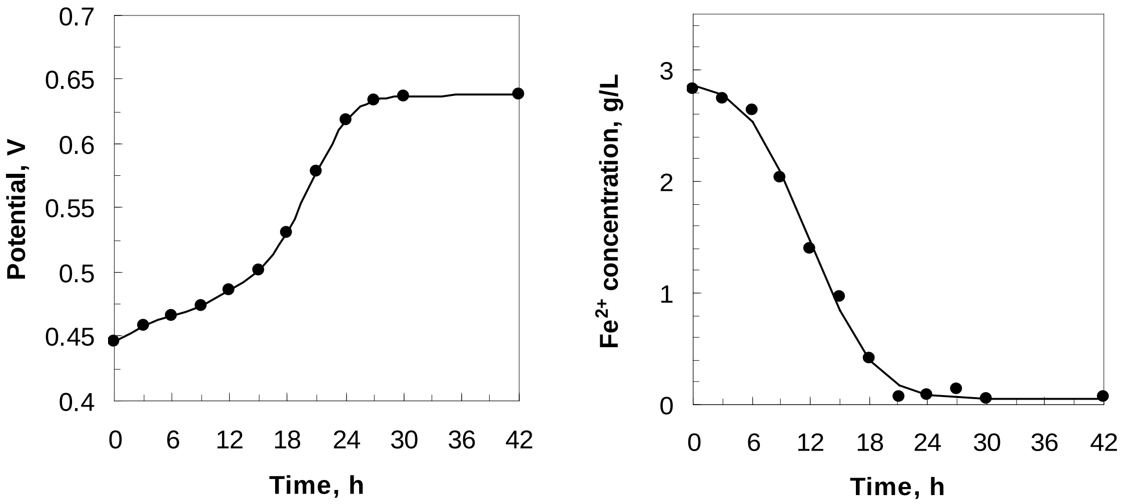


Figura 15. Monitoreo del crecimiento del cultivo bacteriano. a) Potenciométrico b) Espectrométrico

4.3.2 Caracterización del electrodo de oxígeno

4.3.2.1 Determinación del tiempo de respuesta del transductor

El tiempo de respuesta de la sonda de oxígeno (transductor) es un componente importante del tiempo de respuesta total de los biosensores. Este tiempo, se ve afectado por la velocidad de la reducción del oxígeno en el cátodo de la sonda de Clark, es decir, por el área del cátodo, la permeabilidad de la membrana de plástico permeable al oxígeno y el gradiente de concentraciones de oxígeno, manteniendo todas las demás condiciones constantes (espesor de la capa del electrolito entre la membrana de plástico y el cátodo de la sonda, composición del electrolito, potencial aplicado, material del electrodo, y temperatura). El mejoramiento del tiempo de respuesta se obtuvo al incrementar el consumo interno de oxígeno y aumentando la permeabilidad de la membrana al escoger un plástico con el material y el grosor adecuado.

El incremento del consumo del oxígeno interno se consiguió al aumentar el diámetro efectivo del cátodo de la sonda de Clark utilizando para ello un multicátodo, es decir, se distribuyó uniformemente en toda la superficie de acrílico del electrodo, discos de oro que se encontraban en contacto con el electrolito de la sonda (Zlatev et al. 2006a).

Se determinaron experimentalmente dos tiempos de respuesta de la sonda de oxígeno creando cambios positivos y negativos de la concentración de oxígeno al cambiar la velocidad de agitación (de 0 a 800 rpm) y con la adición de Na_2SO_3, respectivamente. La agitación provoca un aumento de la concentración de oxígeno en la interfaz que hay entre la solución y la membrana de la sonda de oxígeno haciendo que aumente también el intercambio de masa entre la solución y la interfaz de aire resultando un aumento de la concentración de oxígeno de equilibrio. Lo opuesto ocurre cuando se adiciona Na_2SO_3 el cual se oxida fácilmente por el oxígeno disuelto para obtener Na_2SO_4, esto hace que disminuya inmediatamente la concentración de oxígeno, lo anterior se presenta en la figura 16.

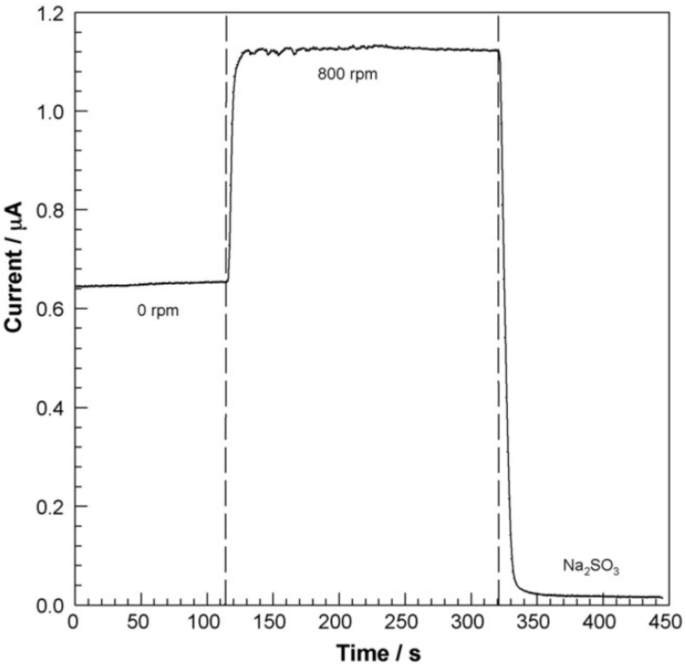


Figura 16. Respuesta de la corriente de la sonda de oxígeno al aumento y disminución de las concentraciones de oxígeno (25 ◦ C, -0,90 V).

Los resultados experimentales presentaron un tiempo de respuesta de 8.7 s es decir un 90% de la respuesta máxima a los cambios de concentración positiva, mientras que a los cambios negativos de la respuesta se encontró un tiempo de respuesta de 12.5 s, es decir, aproximadamente 1.5 veces más. Desafortunadamente, la respuesta del sensor bacteriano se basa en la respuesta del transductor al disminuir la concentración de oxígeno.

4.3.3 Parámetros de transporte a través de la membrana

Dado que la respuesta de los biosensores se debe a la reducción del oxígeno al cruzar la membrana de la sonda tipo Clark, los parámetros de transporte y el espesor de ésta, influencian fuertemente sobre el tiempo de respuesta del sensor y en la sensibilidad de las determinaciones; debido a esto, fueron evaluados y optimizados. De acuerdo con las siguientes ecuaciones (Tarnowski et al., 1995), la sensibilidad de la determinación de oxígeno es una función de la permeabilidad de la membrana y los coeficientes de difusión de oxígeno:

$$I_d = 4FA\ (P_m / Z_m)\ PO_2$$

$$P_m = D_m S_m$$

donde: I_d es la corriente límite de difusión de oxígeno, F es la constante de Faraday, A es el área de los cátodos, P_m es el coeficiente de permeabilidad de la membrana para el oxígeno, Z_m es el espesor de la membrana, P_{O2} es la presión parcial del oxígeno en la solución, D_m es el coeficiente de difusión de oxígeno a través de la membrana, y S_m es la solubilidad del oxígeno en la fase de la membrana. De esta manera, el coeficiente de permeabilidad de la membrana para el oxígeno, se evaluó registrando la corriente de respuesta en estado estacionario de la sonda en agua desionizada con agitación a temperatura constante. La velocidad de agitación elegida fue de 1000 rpm manteniendo el flujo de oxígeno casi constante con el aumento de la corriente. En estas condiciones, la capa de difusión se limita a la membrana y, por consiguiente, la corriente de estado estacionario sólo depende de las propiedades de transporte de oxígeno a través de la membrana (Hitchman, 1978).

El coeficiente de permeabilidad de oxígeno (P_m) resultó ser ($1.19 \pm 0.05) \times 10^{-12}$ molm^{-1} s^{-1} kPa^{-1} (N=12) calculado aplicando la ecuación anterior. (I_d=1.123×10^{-6} A; A=2.2×10^{-6}m^2; Z=15×10^{-6}m; P_{O2}=1.8×10^4 Pa). El coeficiente de difusión de oxígeno a través de la membrana

(D_m) se basó en la pendiente de la línea, descrita por la siguiente ecuación (Hitchman, 1978):

$$\ln \frac{1-(I_t/I_s)}{2} = -\frac{\pi^2 D_m t}{Z_m}$$

donde: I_t es la corriente de respuesta transitoria y I_s es la corriente de respuesta en estado estacionario de la sonda de oxígeno. La corriente de respuesta transitoria se obtuvo al cambiar la velocidad de agitación de la solución de 0 a 800 rpm (Figura 14), de esta forma se garantiza el máximo flujo de O_2 y así se puede mantener la concentración de oxígeno en la superficie externa de la membrana en los mismos niveles.

En la figura 17 se muestra la representación logarítmica para evaluar el coeficiente de difusión de oxígeno en la membrana (D_m), la cual es descrita por la ecuación:

$$y= -0.3074x - 1.3056 \ (r^2=0.9875)$$

Se encontró que $D_m=(4.6 \pm 0.2) \times 10^{-5}$ cm^2s^{-1} (N=7). Todos los valores obtenidos se relacionan con los 15μm de espesor de la membrana de polietileno. Este espesor de la membrana, combinado con la gran superficie del cátodo, se encontró que es óptima para garantizar un compromiso entre el tiempo de respuesta del transductor y la sensibilidad.

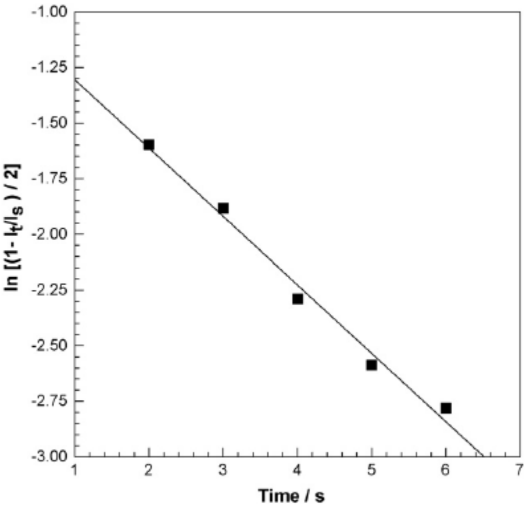


Figura 17. Gráfica logarítmica para evaluar el coeficiente de difusión de oxígeno (D_m) en la membrana de polímero de la sonda de oxígeno.

4.3.4 Determinación de Fe^{2+}.

Cada serie de determinaciones se realizó en el mismo día (es decir en la misma actividad bacteriana de la membrana) y no se hizo ninguna corrección. Debido a la dependencia de la temperatura de algunos parámetros, por ejemplo: la respuesta del electrodo de oxígeno, la concentración de oxígeno disuelta en equilibrio y la actividad enzimática, todas las medidas fueron realizadas en condiciones termostáticas (30°C), de esta manera no se afectan parámetros como la respuesta del transductor de oxígeno (alrededor del 2%/°C), el equilibrio de la concentración de oxígeno disuelto (aproximadamente 1,4% /°C), así como la actividad bacteriana la cual provocaría un cambio de la respuesta del sensor de 5%/°C. Al trabajar en estas condiciones termostáticas es posible evitar las fluctuaciones del equilibrio de la concentración de oxígeno disuelto, además, la velocidad de agitación constante mantiene invariable el gradiente de la concentración de oxígeno en la interfaz que existe entre la membrana bacteriana y la solución.

La respuesta típica del sensor en presencia de Fe^{2+} se presenta en la figura 18. La curva de calibración correspondiente (corriente del estado estacionario I_s/nA vs. concentración de Fe^{2+} C_{Fe}/μmol L^{-1}) es lineal en el rango de concentración hasta 80 μmol L^{-1}. La curva de calibración puede ser descrita por la ecuación: $I_s = 3.94C_{Fe}$, con $r^2 = 0.9935$, es decir que la sensibilidad del sensor es 3.94 nA L $μmol^{-1}$.

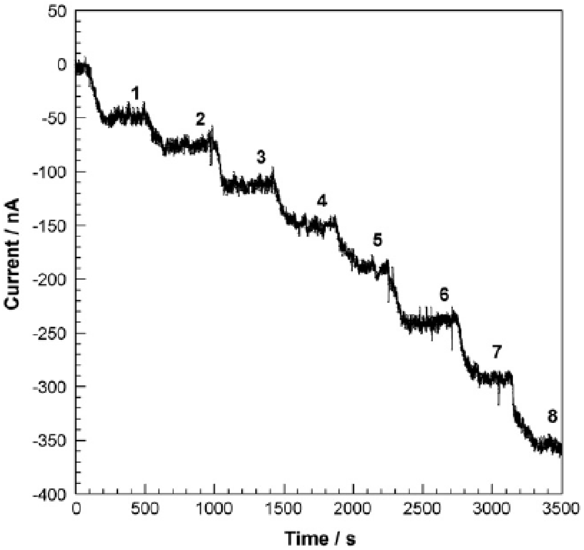


Figura 18. Respuesta amperométrica del sensor de *L. ferrooxidans* al incremento de concentración de Fe^{2+}: (1) 10μmol L^{-1}; (2) 20 μmol L^{-1}; (3) 30 μmol L^{-1}; (4) 40 μmol L^{-1}; (5) 50 μmol L^{-1}; (6) 60 μmol L^{-1}; (7) 70 μmol L^{-1}; (8) 80 μmol L^{-1} (agua desionizada a 30 °C, pH 1.8 y 100 rpm).

La respuesta del sensor aumenta con la disminución de la velocidad de agitación. Es por esto que se aplicó agitación moderada (100 rpm) durante los experimentos. El límite de detección, determinado experimentalmente en las condiciones mencionadas anteriormente, fue de 10^{-7} mol dm^{-3}. El límite de cuantificación se obtuvo según la ecuación: LOQ = 3.3 LOD, es por esto que el LOQ = 3.3×10^{-7} mol dm^{-3}.

La curva de calibración correspondiente (corriente en estado estacionario I_s/nA vs. la concentración de Fe^{2+} C/μmol dm^{-3}) es lineal y puede ser descrita por la ecuación: $I_s = -5C_{Fe} + 400$, con un $r^2 = 0.9978$. La sensibilidad fue de $S = -5$ nA μmol dm^{-3}. El tiempo de respuesta en estado estacionario es una función de la medida de la concentración de Fe^{2+}, de la membrana bacteriana y de la temperatura, considerando los parámetros anteriores, se encontró un tiempo de respuesta del biosensor de 200 s para una concentración de Fe^{2+} de 10 μmol dm^{-3}.

4.3.5 Determinación indirecta de $Cr_2O_7^{2-}$

Gracias a la sensibilidad del sensor bacteriano basado en *L. Ferrooxidans* para Fe^{2+}, puede ser utilizado para determinaciones indirectas de cualquier agente que reaccione con Fe^{2+} y que varíe estequiométricamente su concentración sin afectar a las bacterias. El $Cr_2O_7^{2-}$ satisface completamente a estas dos condiciones. El mecanismo se puede resumir con la ecuación siguiente:

$$Cr_2O_7^{2-} + 14H^+ + 6Fe^{2+} \rightarrow 2Cr^{3+} + 6Fe^{3+} + 7H_2O$$

Un mol de $Cr_2O_7^{2-}$ oxida 6 moles de Fe^{2+}, reduciendo así su concentración lo que produce el descenso del consumo de oxígeno por la bacteria, dando lugar a un cambio en la respuesta del biosensor proporcional a la concentración de $Cr_2O_7^{2-}$, como se muestra en la figura 19.

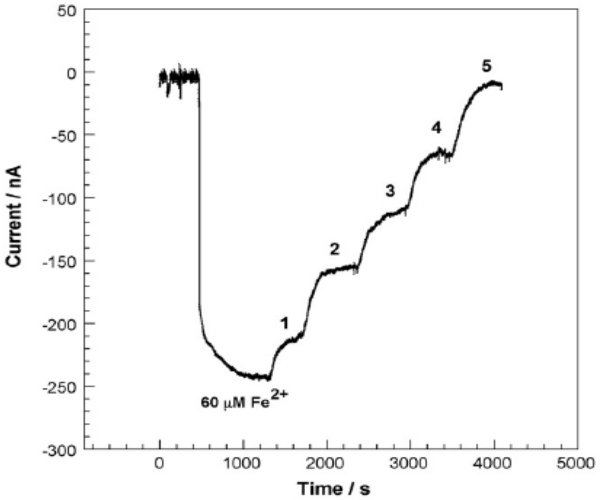


Figura 19. Respuesta amperométrica del sensor de *L. ferrooxidans* al incremento de $Cr_2O_7^{2}$: (1) 2μmol L^{-1}; (2) 4μmol L^{-1}; (3) 6μmol L^{-1}; (4) 8μmol L^{-1}; (5) 10μmol L^{-1}, en agua desionizada a 30 ◦C, pH 1.8, 100 rpm, y una concentración de Fe^{2+} de 60μmol L^{-1}.

Esta reacción consume Fe^{2+}, la cual es concurrente con la oxidación metabólica bacteriana de Fe^{2+} causando una disminución de la concentración inicial, ocasionando una disminución del consumo de oxígeno por parte de la bacteria, dando como resultado un aumento de la respuesta del biosensor.

La respuesta del sensor se registró durante sucesivas adiciones de alícuotas de $Cr_2O_7^{2-}$ a una solución que contenía una concentración constante de Fe^{2+} (60 μmol L^{-1}), a 30ºC, pH de 1.8 y una velocidad de agitación de 100 rpm. La curva de calibración correspondiente (corriente en estado estacionario I_s/nA vs. la concentración de $Cr_2O_7^{2-}$ C_{Cr}/μmol dm^{-3}) se describe por la ecuación lineal: $I_s=2.47C_{Cr}$, con $r^2=0.9817$. El rango lineal de la determinación de $Cr_2O_7^{2-}$ depende de la concentración inicial de Fe^{2+}. A partir de 1 mol de $Cr_2O_7^{2-}$ se oxidan 6 mol de Fe^{2+}, es decir, que el rango lineal del sensor para $Cr_2O_7^{2-}$ es 1/6 de la escala lineal de la determinación del Fe^{2+}.

CONCLUSIONES

5 CONCLUSIONES

1. Se desarrollaron nuevos sensores electroquímicos enzimáticos, bacterianos e híbridos para la cuantificación sencilla, rápida, sensible y fiable de contaminantes ambientales (pesticidas organofosforados, fluoruros y metales pesados).

2. Las características metrológicas y analíticas de los biosensores tales como límite de detección, sensibilidad, reproductibilidad, tiempo de vida y tiempo de respuesta al estado estacionario se evaluaron en diversas condiciones experimentales (pH, temperatura, concentraciones, etc.) y se optimizaron.

3. Se alcanzó un límite de detección del orden de nmol L^{-1} para la determinación del pesticida clorofos usando un biosensor hibrido estable a temperatura ambiente.

4. Se logró la cuantificación especifica del Fe^{2+}, aprovechando la restricción metabólica de la bacteria *Leptospirillum ferrooxidans*.

5. Se propusieron métodos electroquímicos basados en biosensores, convenientes para la determinación en campo y en proceso de fluoruros y dicromatos la cual es una alternativa viable a las técnicas convencionales.

6. Usando un método electroquímico apropiado, se obtuvo información que falta en la literatura científica sobre los parámetros cinéticos (K_m, K_I, I_{max}) de las reacciones catalizadas por enzimas inmovilizadas.

6. REFERENCIAS

6 REFERENCIAS

Alarcón-Herrera, M. T., Martín-Domínguez, I. R., Trejo-Vázquez, R., Rodríguez-Dosal, S. 2001. *Well water fluoride, dental fluorosis, and bone fractures in the Guadiana Valley of Mexico*. Fluoride, 34, 2, 139–149.

Bautista-Zúñiga, F. 1999. *Introducción al estudio de la contaminación del suelo por metales pesados*. Lib. UADY, 35-48.

Cámara de Diputados, Gaceta Parlamentaria, número 2704-V, martes 24 de febrero de 2009.

Carmona-Fonseca, Jaime. 2007. *Correlación y conversión entre los valores de actividad de la colinesterasa plasmática medida por tres técnicas diferentes*. Rev. Panam. Salud Pública, 22, 1, 35-40.

Christensen, H. N.; Cárdenas, J., Palmer G. A., Vega, J. M. 1980. *Cinética enzimática*. Ed. Reverté.

Eggins, B. 1996. *Biosensors, an introduction*. John Wiley & Sons Ltd.

EIA report, 2004. *Mexico: Environmental Issues* (http://www.eia.doe.gov/emeu/cabs/mexenv.html)

Ghindilis, A., Morzunova, H., Barmin, A., Kurochkin, I. 1996. Biosens. Bioelectron., 11, 837.

Gómez–Álvarez, A., Villalba–Atondo, A., Acosta–Ruiz, G., Castañeda–Olivares, M., y Kamp, D. 2004. *Metales pesados en el agua superficial del Río San Pedro durante 1997 y 1999*. Rev. Int. Contam. Ambient., 20, 5–12.

Gogol, E.V., Evtugyn, G.A., Marty, J.-L., Budnikov, H.C., Winter, V.G. 2000. Amperometric biosensors based on nafion coated screen-printed electrodes for the determination of cholinesterase inhibitors. Talanta, 53, 2, 379-389.

Ivanov, A.N., Evtugyn, G.A., Lukachova, L.V., Karyakina, E.E., Budnikov, H.C., Kiseleva, S.G., Orlov, A.V., Karyakin, A.A. 2003. New poly aniline-based potentiometric biosensor for pesticides detection. IEEE Sensors Journal, 3, 3, 333-340.

Koenig, A., Zaborosch, C., Muscat, A., Vorlop, K. D. y Spener, F. 1996. *Microbial sensors for naphthalene using Sphingomonas sp. B1 or Pseudomonas fluorescens WW4*. Appl. Microbiol. Biotechnol., 45, 844–850.

Krifi, B., Metche, M. (2002) MHA, 14, p. 66.

Lauwerys, Robert. 1994. *Toxicología industrial e intoxicaciones profesionales*. Ed. Masson. Barcelona, España. 493 – 502.

Li, X.-H., Xie, Z.-H., Min, H., Xian, Y.-Z., Jin, L.-T. 2007. Amperometric biosensor based on immobilization acetylcholinesterase on manganese porphyrin nanoparticles for detection of trichlorfon with flow-injection analysis system. *Electroanalysis*, 19, 24, 2551-2557.

Loyola-Rodriguez, J. P, Pozos-Guillen, A., Hernandez-Guerrero, J. C. y Hernandez-Sierra, J. F. 2000. *Fluorosis en dentición temporal en un área con hidrofluorosis endémica*. Salud pública Méx., 42, 3, 194-200.

Norma Oficial Mexicana NOM-041-SSA1-1993, *Bienes y Servicios. Agua purificada y envasada*. Especificaciones sanitarias. México, DF; Diario Oficial de la Federación, 24 de marzo de 1995.

Norma Oficial Mexicana NOM-127-SSA1-1994. *Salud ambiental, agua para uso y consumo humano. Límites permisibles de calidad y tratamientos a que debe someterse el agua para su potabilización*. México, DF: Diario Oficial de la Federación, 18 de enero de 1996.

Reviejo, A. J. y Pingarrón, J. M. 2000. *Biosensores electroquímicos. Una Herramienta útil para el análisis medioambiental, alimentario y clínico*. Anales de la Real Sociedad Española de Química, 96, 2, 5-15.

Pauw, T. 1994. *New River Pollution in Mexico*. TED Case Studies, June: 3, 2.

Reybier, K., Zairi, S., Jaffrezic-Renault, N., Fahys, B. 2002. The use of polyethyleneimine for fabrication of potentiometric cholinesterase biosensors Talanta, 56, 6, 1015-1020.

Sanchez-Pena, L C., Reyes, B. E., Lopez-Carrillo, L., Recio, R., Moran-Martinez, J., Cebrian, M. E. y Quintanilla-Vega, B. 2004. *Organophosphorous pesticide exposure alters sperm chromatin structure in Mexican agricultural workers*. Toxicol. Appl. Pharmacol. 196, 108-13.

Snejdarkova, M., Svobodova, L., Evtugyn, G., Budnikov, H., Karyakin, A., Nikolelis, D.P., Hianik, T. 2004. Acetylcholinesterase sensors based on gold electrodes modified with dendrimer and polyaniline: A comparative research. Analytica Chimica Acta, 514, 1, 79-88.

Stoytcheva, M. 1994. Acetylcholinesterase Based Amperometric Sensor Inhibitor Detection. Analytical Letters, 27, 15, 3065 – 3080

Stoytcheva, M., Zlatev, R., Valdez, B., Magnin, J.-P., Velkova, Z., *Electrochemical sensor based on Arthrobacter globiformis for cholinesterase activity determination*. Biosensors and Bioelectronics. 22, 1, 1-9.

Thevenot, D. R., Toth, K., Durst, R. A. y. Wilson, G. S. 2001, *Electrochemical biosensors: recommended definitions and classification*. Biosensors and Bioelectronics, 16, 1-2, 121-131.

Turdean, G., Popescu, I.C., Oniciu, L. 2002. Amperometric biosensors based on cholinesterases for the determination of organophosphorous pesticides [Biocapteurs ampérométriques à cholinestérases pour la détermination des pesticides organophosphorés]. Canadian Journal of Chemistry, 80, 3, 315-331

Waliszewski, S. y Pardio, S. 1992. *Plaguicidas en México*. Ciencia y Desarrollo, Vol. XVIII, 105, 139-144.

WHO (World Health Organization). 1984. *Fluorine and fluorides*, *Environmental Health Criteria* 36. Geneva.

WPCF (Water Pollution Control Federation). 1989. *Métodos normalizados para el análisis de aguas potables y residuales*. Ediciones Diaz-Santos. 17 ed. 4-95-116.

Wyatt, C., Fimbres, C., Romo, L., Méndez, R. O. y Grijalva, M. 1998. *Incidence of Heavy Metal Contamination in Water Supplies in Northern Mexico*. Env. Research., 76, 2, 114-119.

Zlatev, R., Magnin, J-P., Ozil, P., Stoytcheva, M.: *Bacterial sensors based on Acidithiobacillus ferrooxidans: Part I.* Fe^{2+} *and* $S_2O_3^{2-}$ *determination*. Biosensors and Bioelectronics, 21, 8, 1493-1500.

ANEXOS

ANEXO A

“Electrochemical study on the type of immobilized acetylcholinesterase inhibition by sodium fluoride”

M. Ovalle, M. Stoytcheva, R. Zlatev, B. Valdez, Z. Velkova

Revista: Electrochimica Acta, 53, 6344-6350, 2008.

ISSN: 0013-4686 IF 3.078.

Electrochimica Acta 53 (2008) 6344–6350

Contents lists available at ScienceDirect

Electrochimica Acta

journal homepage: www.elsevier.com/locate/electacta

Electrochemical study on the type of immobilized acetylcholinesterase inhibition by sodium fluoride

Marcela Ovalle[a], Margarita Stoytcheva[a,*], Roumen Zlatev[a], Benjamin Valdez[a], Zdravka Velkova[b]

[a] *Universidad Autónoma de Baja California, Instituto de Ingeniería, Blvd. B. Juárez y Calle de la Normal s/n, Col. Insurgentes Este, 21280 Mexicali, Baja California, Mexico*
[b] *University of Food Technologies, Department of Inorganic and Physical Chemistry, Bld. Maritza 26, 4002 Plovdiv, Bulgaria*

ARTICLE INFO

Article history:
Received 4 April 2008
Received in revised form 23 April 2008
Accepted 23 April 2008
Available online 30 April 2008

Keywords:
Acetylcholinesterase
Sodium fluoride inhibition
Kinetics
Electrochemical biosensor
Thiocholine oxidation

ABSTRACT

Homogeneous cholinesterases inhibition by sodium fluoride was studied by many authors, using conventional techniques. Controversial results however were reported on the inhibition kinetics and mechanism. In this work electrochemical methods were applied to identify the type of sodium fluoride inhibition of the immobilized acetylcholinesterase taking advantage of the capabilities of the electrochemical biosensors. The acetylcholinesterase inhibition was evaluated by current measurement, at constant potential, of the mediated by $K_3[Fe(CN)_6]$ and of the non-mediated oxidation of thiocholine iodide, produced by enzymatic acetylthiocholine iodide hydrolysis. Direct amperometric thiocholine detection was preferred for further studies, being more sensitive.

The biosensor transducer response to thiocholine iodide was investigated by cyclic and hydrodynamic voltammetry. The reversibility and pH dependence ($E_{1/2} = 1.08–0.06\,\mathrm{pH}$) of thiocholine oxidation were demonstrated.

The amperometric biosensor response to acetylthiocholine iodide obtained on a modified by acetylcholinesterase adsorption carbon electrode at +0.80 V/Ag, AgCl was characterised according to IUPAC recommendations. The enzyme reaction kinetic parameters: $I_{\max}$, K_M^{app}, and K_I were evaluated under kinetically controlled conditions, for various pH and temperature values. The reversibility of the inhibition was confirmed by dilution. Based on the kinetic analysis, the inhibition of the immobilized acetylcholinesterase by sodium fluoride was found to be of a competitive type.

1. Introduction

Sodium fluoride is known to cause cholinesterases inhibition [1–10]. The effect of fluoride concentration on the enzyme activity in vitro and in vivo has been studied by several authors [3,4,7,9,11] and related to the toxic levels of this compound. However, the mechanism and the kinetics of the process have not been fully elucidated. The detailed investigations of Krupka [8] demonstrate that the inhibition is reversible and depends on binding of one fluoride ion per active centre. In contrast, Westendorf attributes the inhibitory action of sodium fluoride to the presence of HF along with it [9]. The inhibition is found to be competitive and reversible. Heilbronn on the other hand observes, for different substrates, purely non-competitive or a mixed competitive and non-competitive inhibition [5]. Cimasoni reports that the cholinesterase inhibition by sodium fluoride is of uncompetitive type, with acetylthiocholine as a substrate [7]. Therefore, the existing controversy imposes further studies on this process. In addition, the accurate kinetic analysis and the identification of the type of inhibition could throw light on the protective effect of fluorides against the irreversible inhibition of the cholinesterases by organophosphorous compounds (insecticides or warfare agents) [6] and could be useful for the management of organophosphorous poisoning.

Taking into consideration that enzymes in the living cell constitute a part of a highly organised solid-state structure and that the behaviour of such immobilized enzymes differs from that in a homogeneous phase, an unconventional approach was applied in this work in order to investigate the inhibition of acetylcholinesterase by sodium fluoride. The enzyme activity, in the absence and in the presence of the inhibitor, was monitored using an immobilized acetylcholinesterase amperometric sensing device.

---

* Corresponding author at: Instituto de Ingeniería, Universidad Autónoma de Baja California, Blvd. B. Juárez y Calle de la Normal s/n, Col. Insurgentes Este, 21280 Mexicali, Baja California, Mexico. Tel.: +52 686 566 41 50; fax: +52 686 566 41 50.
E-mail address: margarita@iing.mxl.uabc.mx (M. Stoytcheva).

0013-4686/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.electacta.2008.04.062

As known, this type of enzyme sensors provides a fast response in concert with sensitive detection. The elimination of the inhibitor is performed without dialysis, gel filtration or other separation techniques. The reversibility of inhibition is easily confirmed through the recovery of the enzyme activity after the probe washing. Thus, electrochemical biosensors are very convenient for enzyme inhibition investigations [12].

The review of the literature demonstrated that various types of electrochemical biosensors have been created exploiting the process of inhibition of the immobilized cholinesterases by sodium fluoride [13–15]. However, the studies were axed only to fluoride quantification. The type of immobilized acetylcholinesterase inhibition by sodium fluoride was not been studied.

The goal of this work is to obtain new information on the type of immobilized acetylcholinesterase inhibition by sodium fluoride, taking advantage of the electrochemical biosensor-based analysis. This includes investigations on the electrode process, characterization of the biosensor response in the absence and in the presence of the inhibitor, identification of the type of inhibition and evaluation of the main kinetic parameters of the enzyme reaction in different conditions.

2. Experimental

2.1. Reagents

The chemicals, purchased from Sigma, Merck and Chemapol (Czechoslovakia) were of analytical reagent grade and were used without further purification. Acetylthiocholine iodide solutions were prepared daily and kept at 4 °C. Thiocholine iodide was obtained as a result of the spontaneous acetylthiocholine iodide hydrolysis at 30 °C during 24 h. The supporting electrolyte was a Britton–Robinson buffer (0.04 M H_3PO_4, 0.04 M CH_3COOH, 0.04 M H_3BO_3 and 0.2 M NaOH).

Membrane fraction of rat brain was used as a source of acetylcholinesterase. Rat brains were collected immediately after death into iced Tris buffer, pH 7.4, and homogenized to slurry. The resulting tissue was centrifuged at 3000 rpm at 4 °C for 15 min. The supernatant was removed, and centrifuged at 16,000 rpm for 15 min. The supernatant was removed once again and conserved frozen.

2.2. Instrumentation and procedures

The electrochemical studies were performed using an amperometric unit PRG-DEL Tacussel, coupled to a lab made data acquisition system, as well as a potentiostat Gamry PC4 FAS1.

The investigations were carried out in an electrolysis cell of conventional type (5 cm^3 of volume). Pt was used as auxiliary electrode and Ag, AgCl/KCl_{sat} as a reference. The working electrode was either an unmodified disk (6 mm of diameter) from spectrally pure graphite (Ringsdorf Werke, Germany), preliminary treated by polishing, degreasing with alcohol and ultrasonic cleaning or the same electrode modified with immobilized acetylcholinesterase. The immobilization was performed by adsorption, following the scheme: 30 μl of the supernatant, containing the enzyme were injected onto the electrode surface and kept for 30 min at room temperature; after that the electrode surface was cleaned for 5 min in the buffer solution, at an electrode rotation speed of 1000 rpm, in order to eliminate fairly strongly fixed enzyme.

pH and temperature of the solutions were controlled using respectively a pH-meter Orion 250 A and TU-16D thermostat.

3. Results and discussion

3.1. Measuring principle

The determinations were based on the process of acetylthiocholine hydrolysis to thiocholine, which was catalyzed by the immobilized acetylcholinesterase ACh:

$$RSCOCH_3 + H_2O \xrightarrow{ACH} RSH + CH_3COOH$$

where R is $(CH_3)_3N^+(CH_2)_2$.

In the presence of sodium fluoride, the concentration of the produced thiocholine decreased, because of the inhibition of the enzyme activity by this compound, at constant other conditions.

Two measuring principles were tested (Principle 1 and Principle 2) in order to obtain an analytical signal, function of the sodium fluoride concentration. Principle 1 consisted in the registration of the oxidation current of thiocholine at a potential of +0.80 V/Ag, AgCl [16–18] and its decrease in the presence of sodium fluoride:

$$2RSH \rightarrow RS\text{–}SR + 2H^+ + 2e^-$$

Principle 2 was based on the following reactions [19]:

$$2RSH + 2[Fe(CN)_6]^{3-} \rightarrow RS\text{–}SR + 2[Fe(CN)_6]^{4-}$$

$$[Fe(CN)_6]^{4-} \rightarrow [Fe(CN)_6]^{3-} + e^-$$

Thiocholine reacted with cyanoferrate(III), present in the buffer solution in concentration equal to 1 mmol dm^{-3}. $[Fe(CN)_6]^{3-}$ was reduced to $[Fe(CN)_6]^{4-}$. The registered analytical signal was the current of the oxidation of cyanoferrate(II) at a potential of +0.35 V/Ag, AgCl, in the absence and in the presence of sodium fluoride.

Figs. 1 and 2 illustrate the amperometric response of the biosensor to acetylthiocholine and to sodium fluoride at constant acetylthiocholine concentration measured at a potential of +0.80 V/Ag, AgCl and at +0.35 V/Ag, AgCl. The current, corresponding to a fixed substrate concentration in the absence of inhibitor (I_0) was compensated to zero using electronic technique. It decreased with the increase of sodium fluoride concentration.

A Principle 2 application offers the advantage to operate at low electrode potential, avoiding by this manner electrochemical interferences. However, data presented in Figs. 1 and 2 allowing to establish the sensitivity of the determinations from the constructed calibration plots (not shown) demonstrated that in the latter case it decreased significantly (from 212.36 μA dm^3 $mmol^{-1}$ to 91.76 nA dm^3 $mmol^{-1}$ for acetylthiocholine and from 16.85 μA dm^3 $mmol^{-1}$ to 6.95 nA dm^3 $mmol^{-1}$ for NaF for an acetylthiocholine concentration of 1.0 mmol dm^{-3}, at +0.80 and +0.35 V, respectively). In addition, a rapid decay of the signal was observed after the injection of acetylthiocholine in the presence of cyanoferrate(III), as shown in Fig. 1b. Because of these facts, further experiments were carried out according to the first approach (Principle 1). Sodium fluoride was quantified establishing the relationship between its concentration and the induced enzyme inhibition, expressed as a diminution of the thiocholine oxidation current. The degree of inhibition ($D = \Delta I/I_0$, %) was defined as the ratio between the decrease of the biosensor response due to the addition of inhibitor (ΔI) and the initial biosensor response (I_0) [12,20].

3.2. Characteristics of the biosensor response

The electrochemical oxidation of thiocholine, reaction that generates the biosensor response, could be described by the following

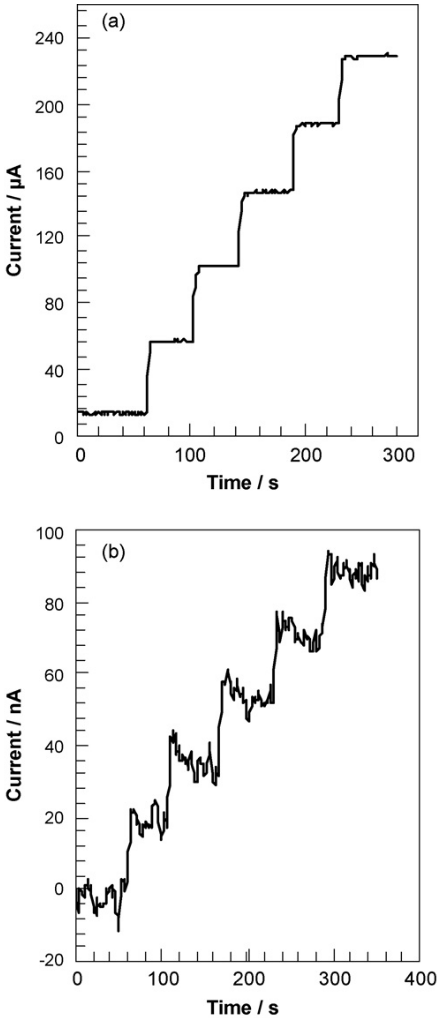


Fig. 1. Amperometric response of the biosensor to increasing acetylthiocholine concentrations (equal to 0.2; 0.4; 0.6; 0.8 and 1.0 mmol dm^{-3}): (a) applied potential +0.80 V/Ag, AgCl; 26 ± 0.5 °C, pH 8; (b) applied potential +0.35 V/Ag, AgCl; 26 ± 0.5 °C, pH 8.

equation:

$$\mathrm{RSH - e^- - H^+ \rightarrow RS^\bullet \rightarrow RS{-}SR}$$

The process involves the transfer of one electron from the thiol and a dimerisation of the intermediate to disulfide [13,21]. It is known that it occurs at a potential higher than +0.70 V/SCE when conventional electrodes are used [22–26]. The reaction is pH dependent, as well as the spontaneous [27] and the enzymatic hydrolysis of acetylthiocholine to thiocholine. However, the transducer response to thiocholine (including working potential and oxidation current) is not fully characterized at various experimental conditions [22,27]. In order to obtain information about the electrochemical oxidation of thiocholine iodide on a graphite electrode at different pH values, cyclic and hydrodynamic voltammetry methods were used. The registered typical cyclic voltammograms of the unmodified working electrode in the presence of 1.0 mmol dm^{-3} spontaneously hydrolyzed acetylthiocholine iodide are presented in Fig. 3.

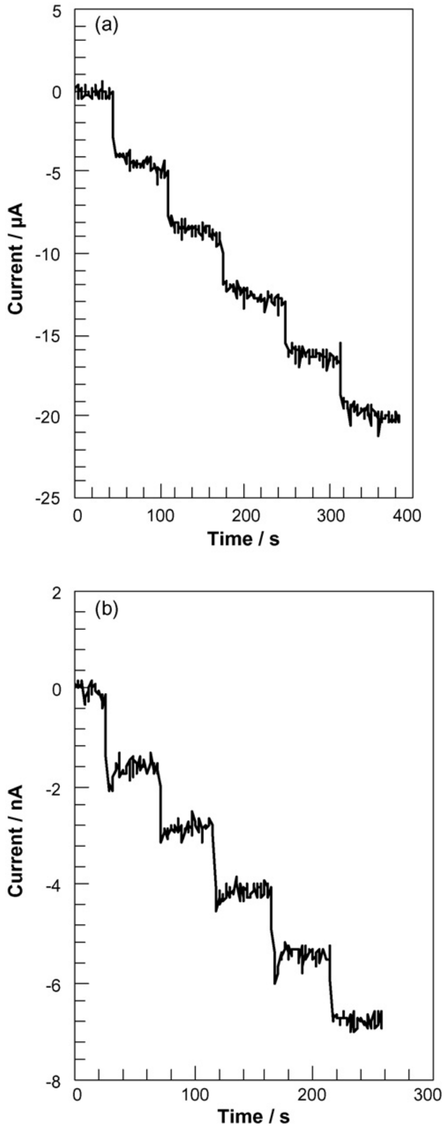


Fig. 2. Amperometric response of the biosensor to increasing sodium fluoride concentrations (equal to 0.2; 0.4; 0.6; 0.8; and 1.0 mmol dm^{-3}) and a constant acetylthiocholine concentration (1.0 mmol dm^{-3}): (a) applied potential +0.80 V/Ag, AgCl; 26 ± 0.5 °C, pH 8; (b) applied potential +0.35 V/Ag, AgCl; 26 ± 0.5 °C, pH 8.

The appearance of two pairs of well-defined redox peaks was observed in the pH range from 4 to 8. The first one (at lower potential) was attributed to the reversible process of iodide oxidation. In this case, the anodic and the cathodic peak potentials, as well as the anodic and the cathodic peak currents did not depend on pH. The second pair of redox peaks was considered as due to the reversible thiocholine oxidation. Their peak potentials shifted in the negative direction with pH increase. That shift led to the overlap of the two anodic peaks and of the two cathodic peaks at pH 9. A change of the peak current was also observed.

The relationship between the limiting current value and pH for the process of thiocholine oxidation was established exploiting the methods of the hydrodynamic voltammetry. The obtained polarization curves are presented in Fig. 4.

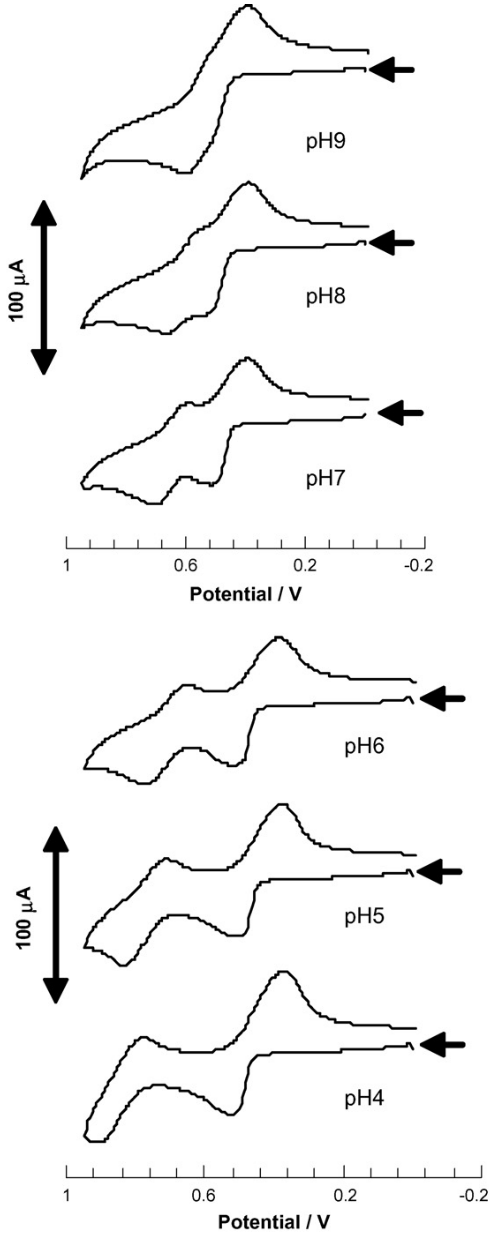


Fig. 3. Cyclic voltammograms of graphite electrode in Britton–Robinson buffer solutions (pH 4–9) in the presence of 1.0 mmol dm^{-3} spontaneously hydrolyzed acetylthiocholine iodide (scanning potential range 0.00 to +0.95 V/Ag, AgCl; scan rate 10 mV s^{-1}; 26 ± 0.5 °C).

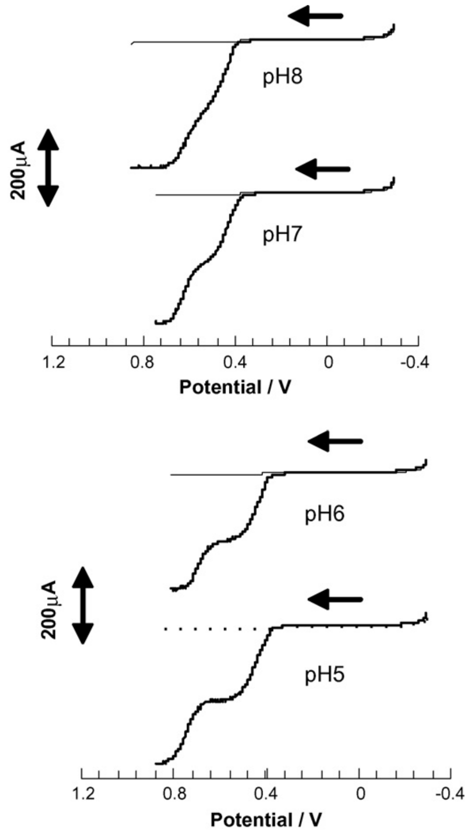


Fig. 4. Hydrodynamic voltammograms of graphite electrode in Britton–Robinson buffer solutions (pH 5–8) in the presence of 1.0 mmol dm^{-3} spontaneously hydrolyzed acetylthiocholine iodide (electrode rotation speed 1000 rpm; scan rate 2 mV s^{-1}; 26 ± 0.5 °C). The dotted curves correspond to the supporting electrolyte voltammograms.

It was found that between pH 5 and pH 6 the value of the anodic limiting current for thiocholine oxidation decreases significantly (from 163.33 to 117.65 μA). At pH 7 and 8, the corresponding limiting current values were 133.33 and 132.33 μA, i.e. independent of pH. The last allows suggesting that the kinetics of spontaneous hydrolysis of acetylthiocholine iodide leading to thiocholine production does not change in this pH range.

Between pH 5 and 8 the value of the half-wave potential ($E_{1/2}$) of thiocholine oxidation decreased linearly obeying the equation: $E_{1/2}$ (V) = +1.08 − 0.06 pH ($r^2 = 0.9939$). The limiting current was reached at a potential of +0.80 V/Ag, AgCl, suitable for the subsequent amperometric studies.

To note that in a previous work [28] performed in similar conditions, it was demonstrated, investigating the electrochemical behavior of KI, acetylthiocholine iodide, thiocholine iodide and thiocholine perchlorate by hydrodynamic voltammetry, that the first oxidation wave (at lower potential) could be associated with the process: $3I^- - 2e^- \rightleftharpoons I_3^-$ and the second one—with the thiocholine oxidation. The interpretation of the presented cyclic and hydrodynamic voltammograms was based on these results.

Further amperometric studies were performed at a potential of +0.80 V/Ag, AgCl in order to characterize the biosensor response to acetylthiocholine iodide in different conditions, according to the IUPAC recommendations [29]. It was found that the analytical signal, i.e. the steady-state current of oxidation of the enzymatically generated thiocholine, function of the enzyme activity for a constant substrate concentration, exhibited a maximum at pH 8 and a temperature of 38 °C. The plotted pH and temperature profiles (not shown) were typical for enzyme reactions. The activation energy of the process, catalyzed by the immobilized acetylcholinesterase was evaluated exploiting the Arrhenius equation in the form: $\ln I_{max} = B - U/RT$ [30], where I_{max} (μA) is the maximal velocity of the enzyme reaction, B is a constant, independent of the temperature, U (J mol^{-1}) is the energy of activation, R (J K^{-1} mol^{-1}) is the gas constant and T (K) is the temperature. I_{max} was calculated using experimental data, as commented below. The activation energy was found to be equal to 29.25 kJ mol^{-1}.

The amperometric response of the biosensor to acetylthicholine iodide in the concentration range up to 1.0 mmol dm^{-3} at pH 8 and 26 ± 0.5 °C is presented in Fig. 2a. The corresponding calibration plot: steady-state current I_0 (μA) versus substrate concentration C (mmol dm^{-3}) is linear, passes through the zero and it is described by the equation: $I_0 = 212.36 \times C$, with a correlation coefficient $r^2 = 0.9893$. The detection limit (LOD) of 6 μmol dm^{-3} was estimated on the basis of an S/N = 3. Consequently, the limit of quantification (LOQ) is 19.8 μmol dm^{-3} (LOQ = 3.3 LOD [29]).

The steady-state biosensor's response time was determined to be 10 s for a 0.10 mmol dm^{-3} thiocholine concentration (pH 8 and 26 ± 0.5 °C).

The R.S.D. of the biosensor response for successive determinations (N = 5), carried out in the same conditions was found to be 2.87% (C = 1.0 mmol dm^{-3}). The well-known and simple technique for biosensor fabrication ensured a good reproducibility of the determinations (R.S.D. = 4.22%, N = 5, C = 1.0 mmol dm^{-3}).

The shape of the curve, plotted in coordinates of Lineweaver–Burk [31] using experimental data allowed suppose, that for low substrate concentrations (C < 0.8 mmol dm^{-3}) the process is controlled by the diffusion, like shown and commented in a previous work [18]. This suggestion was confirmed investigating the dependence of the steady-state current value from the square root of the electrode rotation speed for different substrate concentrations. The observed relationship could be described by the equation $I_0 = 0.65\, nAD^{2/3}\nu^{-1/6}\omega^{1/2}C$, identical to the hydrodynamic equation of Levich for the rotating disk electrode [32], with I_0, steady-state current; n, number of exchanged electrons; F, Faraday constant; A, geometrical area of the electrode; D, diffusion coefficient; ν, cinematic viscosity; ω, electrode rotation speed; C, substrate concentration. This fact imposed to perform the measurements at a constant electrode rotation speed (1000 rpm).

3.3. Sodium fluoride inhibition of the immobilized acetylcholinesterase

As described above, the evaluation of the sodium fluoride inhibitory action involved the establishment of the decrease of the acetylcholinesterase activity, which was expressed as current (I) decrease at a constant substrate concentration C. The experimental data presented in Fig. 5 demonstrate that the degree of inhibition of the immobilized acetylcholinesterase increases with the increase of the inhibitor concentration and decreases with substrate concentration. This type of inhibition is considered as a competitive one.

The kinetic parameters apparent Michaelis–Menten constant K_M^{app} and maximal velocity of the enzyme reaction I_{max} were calculated fitting the model $I = I_{max}C/(K_M^{app} + C)$ to the obtained data in the enzyme controlled domain ($C \geq 0.8$ mmol dm^{-3}) and analyzing them applying nonlinear regression. For this purpose, specialized software was used (GraphPad Prism Version 5.00 for Windows, GraphPad Software, San Diego, California, USA, www.graphpad.com). As expected for a competitive inhibition, the

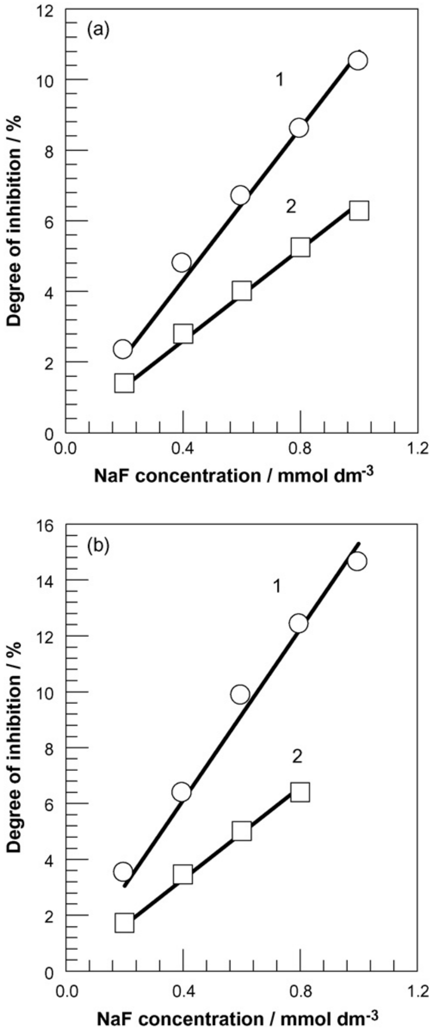


Fig. 5. Degree of inhibition of the immobilized acetylcholinesterase in response to sodium fluoride concentration. (a) pH 7, 26 ± 0.5 °C, substrate concentration: (1) 0.2 mmol dm^{-3}, $r^2 = 0.9911$; (2) 1.0 mmol dm^{-3}, $r^2 = 0.9928$; (b) pH 8; 26 ± 0.5 °C, substrate concentration: (1) 0.2 mmol dm^{-3}, $r^2 = 0.9853$; (2) 1.0 mmol dm^{-3}, $r^2 = 0.9938$.

Table 1
Kinetic parameters of the enzyme reaction, catalyzed by the immobilized acetylcholinesterase in the absence and in the presence of sodium fluoride at different pH (26 ± 0.5 °C)

[NaF] (mmol dm^{-3})	K_M^{app} (mmol dm^{-3})			
	pH 6	pH 7	pH 8	pH 9
0.0	0.37 ± 0.01	1.04 ± 0.01	0.91 ± 0.01	1.53 ± 0.02
0.2	0.39 ± 0.01	1.07 ± 0.01	0.94 ± 0.01	1.59 ± 0.02
0.4	0.41 ± 0.01	1.09 ± 0.01	0.98 ± 0.01	1.64 ± 0.02
0.6	0.43 ± 0.01	1.12. ± 0.01	1.01 ± 0.01	1.69 ± 0.02
0.8	0.44 ± 0.01	1.15 ± 0.01	1.04 ± 0.01	1.76 ± 0.02
1.0	0.47 ± 0.02	1.18 ± 0.01	1.08 ± 0.01	1.82 ± 0.03
	K_I = 4.05 mmol dm^{-3} I_{max} = 237.76 μA	K_I = 7.56 mmol dm^{-3} I_{max} = 423.59 μA	K_I = 5.55 mmol dm^{-3} I_{max} = 401.15 μA	K_I = 5.20 mmol dm^{-3} I_{max} = 535.05 μA

Table 2
Kinetic parameters of the enzyme reaction, catalyzed by the immobilized acetylcholinesterase in the absence and in the presence of sodium fluoride at different temperatures (pH 8)

[NaF] (mol dm^{-3})	K_M^{app} (mol dm^{-3})			
	26 ± 0.5 °C	30 ± 0.5 °C	35 ± 0.5 °C	40 ± 0.5 °C
0.0	0.91 ± 0.01	1.21 ± 0.01	0.92 ± 0.01	0.88 ± 0.01
0.2	0.94 ± 0.01	1.26 ± 0.01	0.97 ± 0.01	0.90 ± 0.01
0.4	0.98 ± 0.01	1.31 ± 0.01	1.00 ± 0.01	0.95 ± 0.01
0.6	1.01 ± 0.01	1.35. ± 0.01	1.05 ± 0.01	0.98 ± 0.01
0.8	1.04 ± 0.01	1.39 ± 0.01	1.09 ± 0.01	1.02 ± 0.01
1.0	1.08 ± 0.01	1.43 ± 0.01	1.13 ± 0.02	1.04 ± 0.01
	K_I = 5.55 mmol dm^{-3}	K_I = 5.47 mmol dm^{-3}	K_I = 6.02 mmol dm^{-3}	K_I = 5.29 mmol dm^{-3}
	I_{max} = 401.15 μA	I_{max} = 568.19 μA	I_{max} = 572.25 μA	I_{max} = 554.63 μA

maximal velocity of the enzyme reaction did not change in the presence of the inhibitor [33]. In contrast, the value of K_M^{app} increased with the increase of NaF concentration [33] (Tables 1 and 2).

The inhibition constant K_I was determined according to the Dixon method [34]. This presentation (Fig. 6) confirmed the competitive character of the acetylcholinesterase inhibition by sodium fluoride.

The values of the characteristic for the enzyme reactions kinetic parameters I_{max}, K_M^{app}, and K_I, evaluated for various pH and temperature values are reported in Tables 1 and 2.The experimental data in terms of amperometric response of the biosensor to acetylthiocholine in the presence and in the absence of sodium fluoride, obtained at different conditions (pH and temperature), were additionally treated using Eadie–Hofstee [35,36], Hanes–Woolf [37], and Lineweaver–Burk [31] transforms (not shown). These types of plots, as well as that of Dixon (Fig. 6) demonstrated that the character of the inhibition did not change in the studied pH and temperature ranges. Thus, the inhibition of the immobilized acetylcholinesterase by sodium fluoride could be described by the following kinetic scheme:

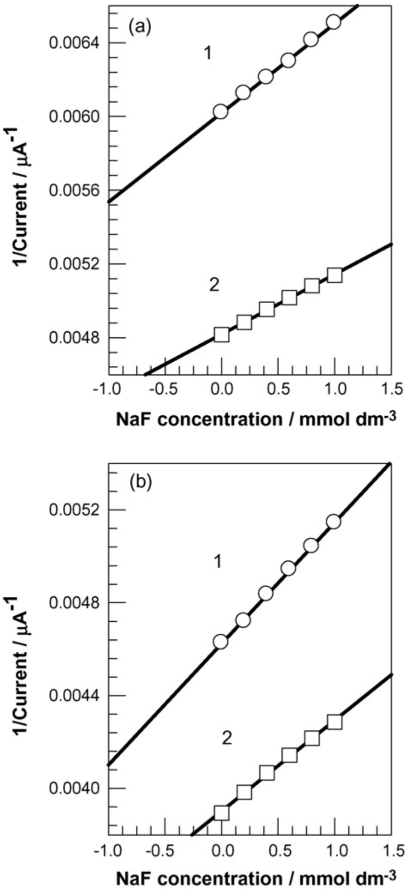


Fig. 6. Dixon plots of the experimental data. (a) pH 7, 26 ± 0.5 °C: (1) C = 0.8 mmol dm^{-3}, r^2 = 0.9985; (2) C = 1.0 mmol dm^{-3}, r^2 = 0.9988; (b) pH 8, 30 ± 0.5 °C: (1) C = 0.8 mmol dm^{-3}, r^2 = 0.9992; (2) C = 1.0 mmol dm^{-3}, r^2 = 0.9977.

$$
\begin{array}{lll}
E + S \rightleftharpoons ES \rightarrow E + P & & K_M{}^{app} = [E]\,[S]\,/\,[ES] \\
+ & & \\
\text{Inh} & K_I = [E]\,[\text{Inh}]\,/\,[\text{EInh}] & \\
\downarrow\uparrow & & \\
\text{EInh} & &
\end{array}
$$

where E is the immobilized acetylcholinesterase, S is the substrate acetylthiocholine, P is the product thiocholine, ES is the enzyme–substrate complex, EInh is the enzyme–inhibitor complex, K_M^{app} is the apparent Michaelis–Menten constant and K_I is the inhibition constant.However, it was observed (Fig. 5) that the degree of inhibition D (%) was pH dependent and varied as following: $D_{pH6} \approx D_{pH7} < D_{pH8} < D_{pH9}$. These results do not agree with those, reported by Westendorf [9]. They do not support the proposed model for acetylcholinesterase inhibition by sodium fluoride, involving HF [9]. Probably, as the investigations of Krupka [8] on the mechanism of the inhibition and the studies on the reactivation of phosphorylated cholinesterases by sodium fluoride [6] allow suggesting, fluoride is the main inhibitory specie and it is reversibly bound at the active centre.The reversibility of the process was confirmed by experiments involving dilution [12]. As shown in Fig. 7, first the amperometric response of the biosensor to acetylthiocholine was recorded in the absence of inhibitor. Then sodium fluoride was added with increasing concentrations. Finally, the inhibition was reversed by lowering the fluoride concentration keeping constant the concentration of the substrate. The registered steady-state current increased and reached the value corresponding to the lower inhibitor concentration.

The reversibility of the process of inhibition allowed recovering the enzyme activity by washing the probe with buffer solution, i.e. the multiple use of the biosensor, among others.

The reversible character of acetylcholinesterase inhibition by sodium fluoride in a homogeneous phase was demonstrated by several authors [5–9]. However, the chemical mechanism of the process still remains obscure. Krupka [8] suggests that fluoride binds directly to essential basic groups in the active center, and inhibits by keeping them in their protonated (inactive) forms. According to these investigations, the site of the inhibitor attachment in acetyl enzyme is different from that in the free enzyme and the enzyme–substrate complex, due to possible conformational changes, accompanying acetylation. Cimasoni [7] postulates

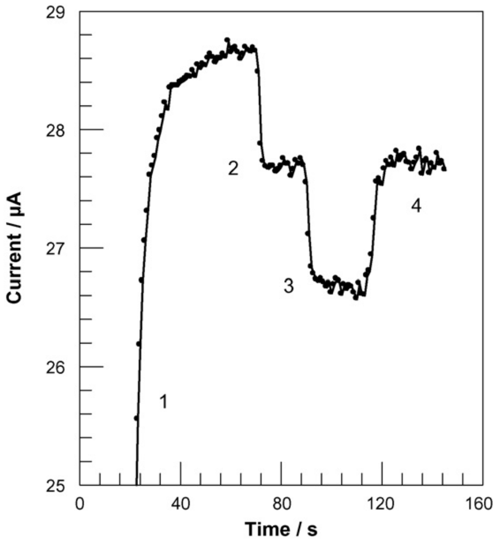


Fig. 7. Reversibility of the inhibition of the immobilized acetylcholinesterase by sodium fluoride: (1) 0.1 mmol dm^{-3} acetylthiocholine; (2) 0.4 mmol dm^{-3} NaF; (3) 0.8 mmol dm^{-3} NaF; (4) 0.4 mmol dm^{-3} sodium fluoride (2-fold dilution for the same substrate concentration); pH 8, 26 ± 0.5 °C.

that enzyme molecules are directly affected by the halide ions, but the binding site is probably outside of the active center. The hypothetical binding mechanism proposed by Westendorf [9] involves the formation of hydrogen bonds between HF molecules and histidine and serine residues in the acetylcholinesterase reactive center. Thus, the mechanism of acetylcholinesterase inhibition by sodium fluoride remains disconcerting and implies further studies.

4. Conclusion

The type of immobilized acetylcholinesterase inhibition by sodium fluoride was investigated, taking advantage of the simplicity, rapidity and sensitivity of the electrochemical biosensors. An efficient acetylcholinesterase immobilization procedure on carbon electrode surface was applied and an appropriate electrochemical approach was chosen in order to characterize the inhibition process.

Cyclic and hydrodynamic voltammetry were used to investigate the transducer response to thiocholine iodide. For the first time a quantitative expression of the pH dependence of the half-wave potential of thiocholine oxidation was derived.

New results demonstrating the reversible and competitive character of the inhibition of the acetylcholinesterase by sodium fluoride in a heterogeneous phase were reported. The main kinetic parameters of the enzyme reaction in the absence and in the presence of the inhibitor (I_{max}, K_M^{app}, and K_I) were estimated for different pH and temperature values.

References

[1] K.B. Augustinsson, Acta Physiol. Scand. 15 (1948) 1.
[2] B.N. Smallman, L.S. Wolfe, Enzymologia 17 (1954) 133.
[3] O. Dybing, L.V. Loe, Acta Pharmacol. Toxicol. (Copenh.) 12 (1956) 364.
[4] H. Harris, M. Whittaker, Ann. Hum. Genet. 27 (1963) 53.
[5] E. Heilbronn, Acta Chem. Scand. 19 (1965) 1333.
[6] E. Heilbronn, Biochem Pharmacol. 14 (1965) 1363.
[7] G. Cimasoni, Biochem. J. 99 (1966) 133.
[8] R.M. Krupka, Mol. Pharmacol. 2 (1966) 558.
[9] J. Westendorf, The kinetics of acetylcholinesterase inhibition and the influence of fluoride and fluoride complexes on the permeability of erythrocyte membranes, Dissertation, University of Hamburg, Hamburg, Germany, 1975, 66 pp., http://www.dartmouth/~masters/West7.doc.
[10] H.C. Froede, I.B. Wilson, Mol. Pharmacol. 27 (1985) 630.
[11] G. Wiesner, M. Gruber, K. Cornelius, A. Schneider, J. Drescher, J. Hobbhahn, Anesthesiology 95 (2001) 806.
[12] C. Tran-Minh, Ion-Selective Electrode Rev. 7 (1985) 41.
[13] G.A. Evtugyn, A.N. Ivanov, E.V. Gogol, J.L. Marty, H.C. Budnikov, Anal. Chim. Acta 385 (1999) 13.
[14] E.V. Gogol, G.A. Evtugyn, J.-L. Marty, H.C. Budnikov, V.G. Winter, Talanta 53 (2000) 379.
[15] R. Solná, S. Sapelnikova, P. Skládal, M. Winther-Nielsen, C. Carlsson, J. Emnéus, T. Ruzgas, Talanta 65 (2005) 349.
[16] M. Stoytcheva, Anal. Lett. 27 (1994) 3065.
[17] M. Stoytcheva, Electroanalysis 7 (1995) 560.
[18] M. Stoytcheva, Electroanalysis 7 (1995) 660.
[19] T. Neufeld, I. Eshkenazi, E. Cohen, J. Rishpon, Biosens. Bioelectron. 15 (2000) 323.
[20] Nomenclature Committee of the International Union of Biochemistry (NCIUB). Symbolism and terminology in enzyme kinetics, Eur. J. Biochem. 128 (1982) 281.
[21] G. Liu, S.L. Riechers, M.C. Mellen, Y. Lin, Electrochem. Commun. 7 (2005) 1163.
[22] D.Ž. Sužnjević, D.S. Veselinović, N.S. Vukelić, D.Ž. Pavlović, A.V. Nikolić, J. Serb. Chem. Soc. 50 (1985) 83.
[23] J.-L. Marty, N. Mionetto, R. Rouillon, Anal. Lett. 25 (1992) 1389.
[24] J.-L. Marty, N. Mionetto, T. Noguer, F. Ortega, C. Roux, Biosens. Bioelectron. 8 (1993) 273.
[25] D. Martorell, F. Cespedes, S. Alegret, E. Martinez-Faregas, Anal. Chim. Acta 290 (1994) 343.
[26] J.-L. Marty, N. Mionetto, S. Lacorte, D. Barceló, Anal. Chim. Acta 311 (1995) 265.
[27] P.C. Pandey, S. Upadhyay, H.C. Pathak, C.M.D. Pandey, I. Tiwari, Sens. Actuators B 62 (2000) 109.
[28] M. Stoytcheva, V. Sharkova, J.-P. Magnin, Electroanalysis 10 (1998) 994.
[29] D. Thévenot, K. Toth, R. Durst, G. Wilson, Pure Appl. Chem. 71 (1999) 2333.
[30] L.I. Antropov, Theoretical Electrochemistry, Vishaja Schola, Moscow, 1969.
[31] H. Lineweaver, D. Burk, J. Am. Chem. Soc. 56 (1934) 658.
[32] V.G. Levich, Fiziko-himicheskaja gidrodinamika, Physmathgys, Press, Moscow, 1959.
[33] T. Keleti, Basic enzyme kinetics, Akadémiai Kiadó Press, Budapest, 1986.
[34] M. Dixon, Biochem. J. 55 (1953) 170.
[35] G.S. Eadie, J. Biol. Chem. 146 (1942) 85.
[36] B.H.J. Hofstee, Nature 184 (1959) 1296.
[37] C.S. Hanes, Biochem. J. 26 (1932) 1406.

ANEXO B

“Hybrid electrochemical biosensor for organophosphorus pesticides quantification”

M. Stoytcheva, R. Zlatev, Z. Velkova, B. Valdez, M. Ovalle, L. Petkov

Revista: Electrochimica Acta, 54, 1721-1727, 2009
ISSN: 0013-4686, IF 3.078.

Electrochimica Acta 54 (2009) 1721–1727

Contents lists available at ScienceDirect

Electrochimica Acta

journal homepage: www.elsevier.com/locate/electacta

Hybrid electrochemical biosensor for organophosphorus pesticides quantification

Margarita Stoytcheva[a,*], Roumen Zlatev[a], Zdravka Velkova[b], Benjamin Valdez[a], Marcela Ovalle[a], Lubomir Petkov[c]

[a] *Universidad Autónoma de Baja California, Instituto de Ingeniería, Blvd. B. Juárez y Calle de la Normal s/n, Col. Insurgentes Este, 21280 Mexicali, Baja California, Mexico*
[b] *University of Food Technologies, Department of Inorganic and Physical Chemistry, Blvd. Maritza 26, 4002 Plovdiv, Bulgaria*
[c] *University of Chemical Technology and Metallurgy, Department of Technology of Inorganic and Electrochemical Production, Blvd. St. Kliment Ohridski 8, 1756 Sofia, Bulgaria*

ARTICLE INFO

Article history:
Received 19 August 2008
Received in revised form
23 September 2008
Accepted 28 September 2008
Available online 14 October 2008

Keywords:
Hybrid biosensor
Acetylcholinesterase
Arthrobacter globiformis
Organophosphorus pesticides

ABSTRACT

An electrochemical biosensor for organophosphorus (OP) pesticides trace level concentrations determination was developed and characterized. It integrates a hybrid biorecognition element consisting of immobilized *Arthrobacter globiformis* and free acetylcholinesterase (ACh) with a Clark type oxygen probe transducer. The bacteria convert the ACh-generated choline to betaine with oxygen consumption measured as a Clark probe current change. This change representing the sensor response correlates to the concentration of the OP pesticides inhibiting the Ach-catalyzed acetylcholine hydrolysis to choline. The conditions for maximal sensor response to choline were optimized according to the methodology of design of experiments. The analytical performances of the enzyme substrate determination in a wide concentration range (0.1–20 μmol dm^{-3} of acetylcholine) and different ACh activities were established. It was demonstrated that the biosensor ensures reproducible, accurate and reliable chlorophos quantification reaching a limit of detection (LOD) of 1 nmol dm^{-3} and a sensitivity of 0.0252 μA/p(mol dm^{-3}) under optimal experimental conditions. The biosensor response time is 200 s and the storage stability is t_{L50} = 49 days for the bacterial membrane at ambient temperature. The device is reusable, the bacterial membrane being not affected by OP. The biosensor was applied to chlorophos determination in contaminated milk.

1. Introduction

The worldwide increasing use of organophosphorus (OP) pesticides which are powerful neurotoxins and the resulting environmental and public concerns [1] created a demand for the development of reliable, fast, sensitive, simple and low-costing methods for their quantification, suitable for on-line and on-site measurements. The classical chromatographic, spectroscopic and immunoassay techniques for OP substances determination, although very accurate and sensitive are time-consuming and expensive, requiring trained personnel and sophisticated laboratory equipment [2–4]. In contrast, the electrochemical biosensors-based methods fulfill the above-mentioned requirements.

The electrochemical biosensors for OP pesticides analysis could be classed into two great groups according to the nature of the biological recognition element—enzymes or bacteria.

The function of the acylcholinesterases (acetylcholinesterase or butyrylcholinesterase) and phosphatases (acid or alkaline) electrochemical sensors is based on the ability of the PO compounds to inhibit these enzymes [5–15]. The determination is very sensitive, but indirect. Drawbacks of the method are also the lack of selectivity and the need, in some cases, of enzyme incubation and enzyme reactivation/regeneration.

Direct OP pesticides analysis could be achieved applying organophosphorus hydrolase (OPH) electrochemical sensors [13–20]. The enzyme OPH demonstrates substrate specificity toward paraoxon, parathion, coumaphos, diazinon, dursban, methyl parathion, etc., and toward some chemical warfare agents (sarin, soman, tabun, VX, etc.) [21–24]. The detection of parathion is also possible using parathion hydrolase (PH) [25]. The enzymatically catalyzed OP substrates hydrolysis involves pH changes and generates electroactive products. Thus, the detection could be

---

* Corresponding author. Tel.: +52 686 566 41 50; fax: +52 686 566 41 50.
E-mail address: margarita@iing.mxl.uabc.mx (M. Stoytcheva).

0013-4686/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.electacta.2008.09.063

performed in a single step, using potentiometric (pH sensitive) or amperometric transducers. OPH-based systems allow the selective determination of the family of the OP compounds, in contrast to the enzyme inhibition-based techniques, but the reported detection limit is higher [26]. An important drawback represents the complex, long-lasting, and expensive procedure for OPH or PH extraction and purification, performed in specialized microbiological laboratories (to note that these enzymes are not commercially available) [15].

Some reviews summarize the performances of the enzymes electrochemical sensors for OP pesticides determination and the principles of their operation [5–15]. The commune disadvantages of this group of biosensors are the instability of the response (due to enzyme leaking or deactivation), the observed interferences at high electrode potentials, the passivation of the electrode surface and the short life-time at ambient temperature.

The bacterial electrochemical sensors for OP pesticides quantification include, as biological recognition element, genetically engineered *Moraxella* sp., *Pseudomonas putida* or *Escherichia coli* with surface-expressed OPH [26–29]. The detection principle is identical to the described above, when employing the isolated and purified enzyme. Recently, microbial sensors based on Clark dissolved oxygen electrode modified with recombinant p-nitrophenol degrading/oxidizing bacteria endowed with OPH activity was reported [30,31]. The surface-displayed OPH catalyzes the hydrolysis of OP pesticides with nitrophenyl substituent to release products, metabolized by the bacteria while consuming oxygen. The oxygen consumption is measured and correlated to the OP concentration.

Lei et al. [32] report the construction of a hybrid biosensor for direct determination of OP pesticides using purified OPH for their initial hydrolysis and *Arthrobacter* sp. JS443 for the subsequent oxidation of the released p-nitrophenol to carbon dioxide through electroactive intermediates. The biocatalytic layer is prepared by bacteria and enzyme co-immobilization on a carbon paste electrode. The registered signal is the current of oxidation of the intermediates, function of the OP concentration.

The mentioned microbial and hybrid sensors for direct OP pesticides quantification display long-term stability, good reproducibility and accuracy, and relatively short response time. However, the reached limit of detection (LOD) is over the OP concentration in environmental samples and higher than that for acylcholinesterases inhibition-based sensors, immunoassays, and gas, liquid and thin layer chromatography [26].

In this paper we describe the development and the analytical characterization of a new hybrid electrochemical sensor for OP pesticides quantification. The biorecognition system includes immobilized choline-cultured cells of *Arthrobacter globiformis* and free acetylcholinesterase. Current proportional to bacteria respiration, indirectly depending on OP pesticides concentration, is registered as a sensor response, using a Clark type oxygen probe. The proposed approach combines the advantages of the bacterial sensors with those of the cholinesterases inhibition-based ones, namely: stable response and long life-time at ambient temperature, because of the conservation of the enzyme system of the bacteria in its natural environment; reproducible characteristics ensured controlling the bacterial charge and the bacterial activity; high sensitivity. In addition, it provides reliable, free of interferences measurement of the dissolved oxygen reduction current, the polymer membrane of the oxygen probe being permeable only for gases. The biosensor fabrication is simple and cost-effective, enzyme extraction and purification or genetic engineering being avoided.

The biosensor is suitable for general toxicity screening or for determining the concentration of isolated OP pollutants. In another format, described elsewhere [33], it was constructed by bacteria and enzyme co-immobilization.

2. Experimental

2.1. Reagents and apparatus

A. globiformis NBIMCC 2369 were obtained from the National Bank of Industrial Microorganisms and Cell Cultures (Bulgaria). Acetylcholinesterase (E.C.3.1.1.7, 1000 U mg^{-1} from bovine erythrocytes) was provided from BDH Chemicals Ltd., UK. The substrates acetylcholine chloride and choline chloride were purchased from Sigma. Fresh solutions were prepared daily. The OP compound 2,2,2-trichloro-1-dimethoxyphosphorylethanolchlorophos (chlorophos), used for the inhibition assays, was from Sigma. Chlorophos base stock solution with a concentration of 0.1 mol dm^{-3} was prepared directly by dissolving the product in deionized water. The solution is stable at room temperature. Stock solutions with lower concentrations were prepared by dilution of the base one. Concentrations close to the LOD were obtained by addition of microliter volumes of diluted stock solution directly into the measuring cell.

All the other reagents were of analytical grade. Phosphate buffer solutions (pH 6–8), glycylglycine buffer solutions (pH 9–11) and glycine buffer solutions (pH 9–11) were also employed.

Sartorius cellulose filters with 0.15 μm pore size served as support for bacterial membrane preparation.

The bacterial culture optical density (OD) measurements were performed using Gilford Stasar III spectrophotometer.

The Clark type probe used as a transducer was connected to a properly modified BAS 4LC amperometric detector coupled with a laboratory-made noise suppression unit and equipped with appropriated Windows software for data acquisition and data treatment.

The Clark type oxygen probe was integrated with a 10 cm^3 especially elaborated acrylic cell, which was used for all OP measurements.

The in situ OP quantification was carried out using a home-made hand held microprocessor controlled amperometric unit, coupled with 1 ml acrylic cell containing build in oxygen sensor.

All the measured concentrations were achieved by means of 10, 25 and 50 μl Hamilton precision syringes and microliter range automatic pipettes.

2.2. Bacterial culture

The bacterial strain *A. globiformis*, isolated and described by Conn and Dimmick [34], was cultivated aerobically in the medium proposed by Ikuta et al. [35,36], containing 10% choline chloride, 0.5% yeast extract, 0.5% KCl, 0.1% K_2HPO_4 and 0.05% $MgSO_4 \cdot 7H_2O$, at pH 7 and 30 °C, under stirring conditions. The bacterial growth was monitored measuring the OD of the cell suspension at 320 nm. A typical *A. globiformis* growth curve is presented in Fig. 1.

The optical density was related to the number of the viable cells established by parallel plate counts. The constructed calibration plot (OD versus CFU × 10^6) appeared to be linear in the investigated concentration range with a *y*-intercept of 0.0332 and a slope of 9×10^{-5} ($r^2 = 0.9649$). This plot was employed further for determining the bacteria concentration by measuring only the optical density of the sample.

Before reaching the beginning of the stationary growth phase (6 h of culture time), the bacterial suspension was filtered and centrifuged, in order to obtain a mass of concentrated cells. The latter were resuspended in deionized water.

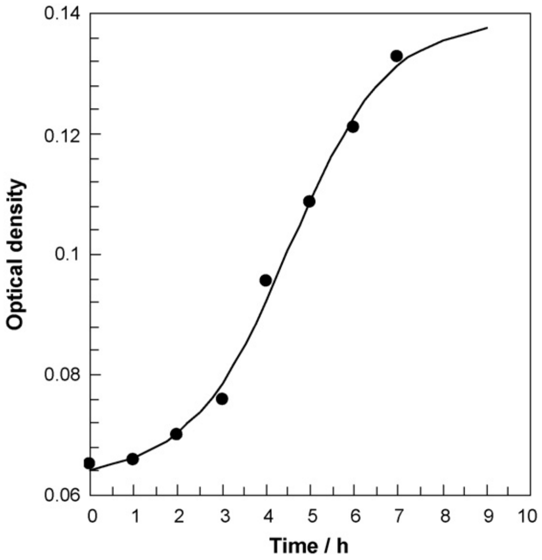


Fig. 1. *Arthrobacter globiformis* growth curve.

2.3. Biosensor design

The developed biosensor (Fig. 2) was constituted of an oxygen probe serving as a transducer and a bacterial layer, fixed on its polymer membrane, as described earlier [37–39]. The oxygen probe was modified in comparison to the commercially available ones by replacing the spherical front with a flat one and by substituting the small disk cathode by a gold multicathode uniformly dislocated on the whole flat-front surface (Fig. 2). This original construction ensured uniform thickness of the bacterial layer across the working surface of the probe, and facilitated oxygen diffusion.

The elaboration of the bacterial layer was achieved by filtration of the resuspended bacterial cells through a Sartorius cellulose filter with a pore diameter lower than the bacteria size. The bacteria loaded filter (bacterial membrane) was fixed on the polymer membrane of the oxygen probe. Bacterial membranes with reproducible characteristics were obtained varying the volume of the filtered biomass in accordance with its concentration, keeping constant the product of these two variables.

3. Results and discussion

3.1. Measuring principle

The measuring principle was based on the following reactions:

$$\text{acetylcholine} + H_2O \xrightarrow{\text{ACh}} \text{choline} + CH_3COOH \quad (1)$$

$$\text{choline} + 2O_2 + H_2O \xrightarrow{\text{ChO}} \text{betaine} + 2H_2O_2 \quad (2)$$

where ACh is the acetylcholinesterase and ChO is the bacterial choline oxidase.

The possible scheme [35] for the oxidation of choline to betaine in the cells of *A. globiformis*, coupled with oxygen consumption is presented in Fig. 3.

For a constant acetylcholine concentration and identical experimental conditions, choline production depends of the acetylcholinesterase activity, affected by OP inhibitors. With the diminution of the amount of the generated choline due to ACh inhibition, the oxygen consumption during the process of its oxidation to betaine in the cells of *A. globiformis* decreases. Thus, current proportional to bacteria respiration was regis-

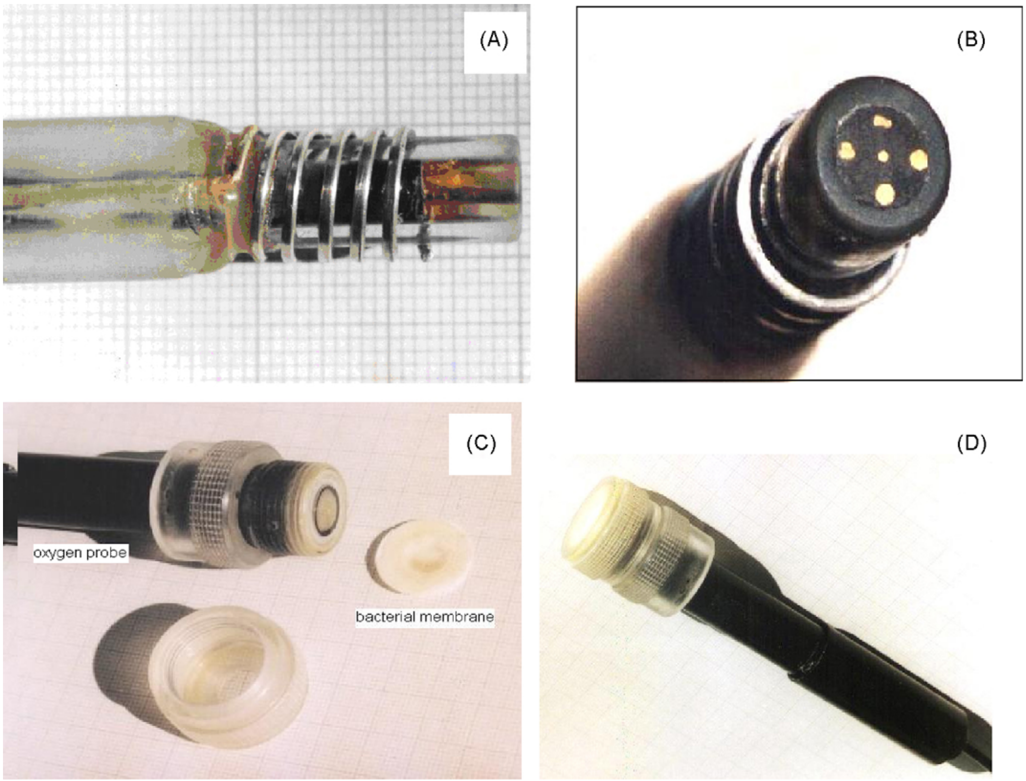


Fig. 2. Biosensor design: (A) the Ag anode of the oxygen probe; (B) the multicathode of the oxygen probe; (C) the elements of the biosensor; and (D) general view of the biosensor.

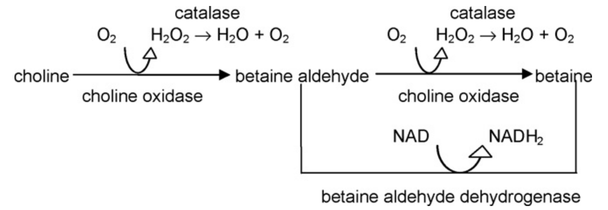


Fig. 3. Possible oxidative pathway of choline to betaine in the cells of *A. globiformis*, according to Ikuta et al. [35].

Table 1
Analytical characteristics of the biosensor for choline determination (I_s is the recorded steady-state current, μA; *C* is the choline concentration, μmol dm^{-3}).

Sensitivity	0.4 μA μmol^{-1}
Linear concentration range of the calibration curve	$I_s = 0.40C$, $r^2 = 0.9978$
Limit of detection	8×10^{-8} mol dm^{-3}
Limit of quantitation	2.6×10^{-7} mol dm^{-3}
Steady-state response time	200 s
Storage life-time at 25 °C	t_{L10} = 14 days; t_{L50} = 49 days
Interferences	Glucose
Reproducibility	R.S.D. < 3%

tered as a sensor response and correlated to the OP pesticide concentration.

3.2. Analytical characteristics of the biosensor

As noted above, bacteria oxygen consumption depends on the quantity of choline, in conformity to Eq. (2). Taking this into consideration, the response of the biosensor to choline was investigated and the results were presented in a previous work [39]. They are summarized in Table 1.

The analytical characterization of the biosensor for choline determination included the evaluation of the following parameters: sensitivity, linear concentration range of the calibration curve, limit of detection, limit of quantitation, steady-state response time, storage life-time, selectivity and reproducibility, as defined by IUPAC [40]. The analyses were performed at stirring rate of 300 rpm, temperature of 25 °C and pH 6. The investigations on the pH dependence of the biosensor response to choline in the concentration range up to 12 μmol dm^{-3} provided experimental data, useful for mathematical modeling and optimizing the determination according to the methodology of design of experiments (DOE). The inputs to the model were the choline concentration (*C*) and pH, and the output—the sensor response (steady-state current I_s). For describing the dependences between $I_s = f(\text{pH})$ and $I_s = f(C)$, a model based on second degree polynomial was chosen, taken into consideration the previously obtained experimental data:

$$z = a + bx + cy + dy^2 \tag{3}$$

with *z*, steady-state current (μA); *x*, choline concentration (μmol dm^{-3}); *y*, pH of the solution.

The coefficients *a*, *b*, *c* and *d* of the postulated polynomial model were determined by means of D-optimum composition plan [41], the independent factors varying as follow: 6 < pH < 11 and 1 < *C* < 12. The experimental matrix of the DOE, applied for the modeling and optimization of the response of the biosensor to choline is presented in Table 2.

The mathematical analysis of the results led to a suitable response model according to the following equation:

$$I_s = 1.636 + 0.407C + 0.421\text{pH} - 0.026\text{pH}^2 \tag{4}$$

The values of statistics r^2 and r^2_{adjusted} were respectively 0.9931 and 0.9923. Such values, greater than 0.85 mean that the descriptive power of the model is satisfactory. In Fig. 4 the model is presented in graphic form.

The maximization of the model allowed predicting the optimal set of parameters for choline determination, as shown in Table 3.

Table 3
Optimal set of parameters for choline determination.

No.	I_s (μA)	*C* (μmol dm^{-3})	pH
1	4.88	12	8.0
2	4.90	12	8.5
3	4.89	12	9.0

Table 2
Experimental matrix of the DOE, applied for the modeling and optimization of the response of the biosensor to choline with: *C*, choline concentration and I_s, steady-state current (stirring rate 300 rpm; 25 °C).

No.	pH	*C* (μmol dm^{-3})	I_s (μA)	No.	pH	*C* (μmol dm^{-3})	I_s (μA)
1	6.0	1	0.50	25	8.5	2	1.00
2	6.0	2	0.80	26	8.5	4	1.50
3	6.0	3	1.30	27	8.5	6	2.40
4	6.0	4	1.75	28	8.5	8	3.40
5	6.0	5	2.20	29	8.5	10	4.25
6	6.0	6	2.40	30	9.0	12	4.90
7	6.0	7	2.80	31	9.0	2	0.99
8	6.0	8	3.20	32	9.0	4	1.49
9	6.0	9	3.60	33	9.0	6	2.39
10	6.0	10	4.05	34	9.0	8	3.39
11	6.0	11	4.35	35	9.0	10	4.24
12	6.0	12	4.70	36	9.0	12	4.89
13	7.0	2	0.94	37	10.0	2	0.90
14	7.0	4	1.44	38	10.0	4	1.40
15	7.0	6	2.34	39	10.0	6	2.30
16	7.0	8	3.34	40	10.0	8	3.30
17	7.0	10	4.19	41	10.0	10	4.15
18	7.0	12	4.84	42	10.0	12	4.80
19	8.0	2	0.98	43	11.0	2	0.75
20	8.0	4	1.48	44	11.0	4	1.25
21	8.0	6	2.38	45	11.0	6	2.15
22	8.0	8	3.38	46	11.0	8	3.15
23	8.0	10	4.23	47	11.0	10	4.00
24	8.0	12	4.88	48	11.0	12	4.65

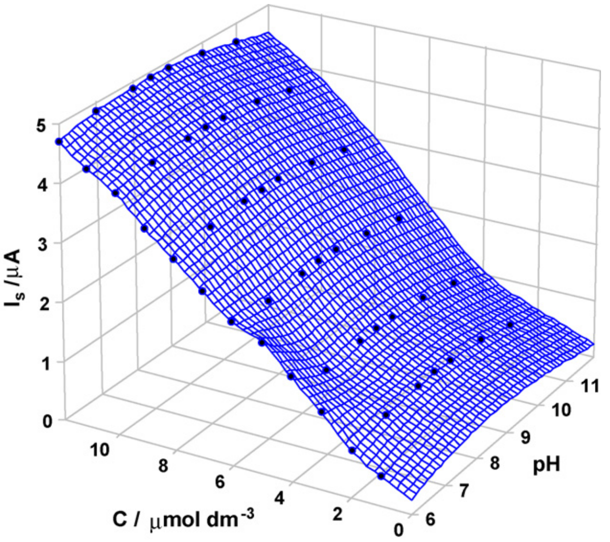


Fig. 4. Biosensor response in function of choline concentration and of pH (300 rpm, 25 °C).

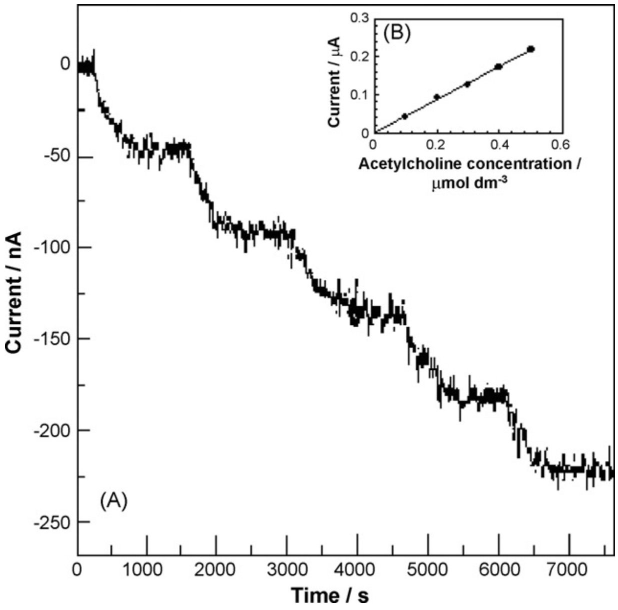


Fig. 5. (A) Steady-state current response of the biosensor to increasing acetylcholine concentrations with an increment of 0.1 μmol dm^{-3} in the range of 0.1–0.5 μmol dm^{-3} (0.425 μg cm^{-1} Ach, pH 6, 25 °C, 300 rpm) and (B) calibration curve for acetylcholine.

Therefore, the maximum predicted response should be obtained in the pH range 8–9 and maximal choline concentration (12 μmol dm^{-3}) in the fixed experimental conditions (300 rpm, 25 °C).

Although the pH optimum for choline determination was found to be in the range 8–9, further analyses performed in order to characterize the sensor response to acetylcholine were carried out at pH 6. As demonstrated by Lautié et al. [42], the acetylcholine undergoes an uncontrolled spontaneous non-enzymatic hydrolysis, which rate is pH dependent. This interfering reaction could be suppressed by increasing the acidity of the solution. Thus, the amperometric response of the biosensor to acetylcholine was evaluated in the following conditions: pH 6, temperature of 25 °C and stirring rate of 300 rpm, for different acetylcholine concentrations (0.1–20 μmol dm^{-3}) and amount of the free enzyme (0.2–0.85 μg cm^{-1}). Fig. 5A presents the typical steady-state current response of the biosensor to acetylcholine in the concentration range of 0.1–0.5 μmol dm^{-3} and acetylcholinesterase content of 0.425 μg cm^{-1}. The corresponding calibration plot (Fig. 5B) was linear with a slope of 0.4338 μA dm^3 μmol^{-1} and a correlation coefficient 0.9966. The response time of the biosensor was found to be about 200 s, as illustrated in Fig. 5A. This value do not differ from the reported above for choline detection. Evidently, choline oxidation inside the bacterial cells is the rate-limiting step, taking into consideration the high turnover number of the acetylcholinesterase [43–46] and the absence of diffusion constraints for the homogeneous enzyme reaction. Acetylcholinesterase is thought to be one of the fastest enzymes, which is a prerequisite for its role in nervous function.

The obtained data characterizing the response of the biosensor system to choline and to acetylcholine were used to perform chlorophos determination in conditions ensuring stable and reproducible biosensor response (acetylcholine concentration of 20 μmol dm^{-3}, initial acetylcholinesterase activity of 11.04 U cm^{-3}, pH 6, 25 °C and stirring rate of 300 rpm). Microliter volume of chlorophos diluted stock solution was injected into the substrate containing sample and the output steady-state current change was registered as sensor response without preliminary enzyme incubation in the presence of chlorophos.

The constructed calibration plot (current (μA) versus −log[chlorophos]) was linear ($r^2 = 0.9987$) with a slope of 0.0252 μA/p(mol dm^{-3}) and y-intercept of 0.2534 in the investigated concentration range (10^{-4} to 10^{-8} mol dm^{-3} of chlorophos). The detection limit for chlorophos (defined as signal to noise ratio of 3) was 10^{-9} mol dm^{-3}, i.e. of the same order of magnitude or lower, in most cases, than that for enzyme inhibition-based sensors for chlorophos quantification [47–54].

Some comparative data related to enzyme inhibition-based sensors for chlorophos quantification are presented in Table 4.

The accuracy of the determination was checked carrying out parallel measurements using the acetylcholinesterase sensor described in a previous work [47]. The comparison of the result demonstrated that the relative error did not exceed 3%. However, as mentioned above, the enzyme sensor-based determinations suffer from interferences at the applied high positive potential and instability of the analytical response due to enzyme activity loss.

The response time of the biosensor, depending mainly of the heterogeneous process of choline oxidation, did not change. The biosensor device, combining immobilized *A. globiformis* and a Clark type oxygen probe displayed a long-term stability at ambient temperature, as noted in Table 1. 10% loss of activity was observed after 14 days of storage in deionized water and 50% loss of activity was recorded after 49 days of storage in same conditions. The device was reusable, the bacteria enzyme machinery not being affected by OP inhibitors.

The biosensor had excellent reproducibility (R.S.D. < 3%, $n = 5$), due before all to the reliable bacteria immobilization technique, ensuring the elaboration of bacterial membranes with identical characteristics.

The developed hybrid biosensor was applied for chlorophos determination in contaminated milk according to the procedure described earlier [55], in the above-mentioned conditions: acetylcholine concentration 2×10^{-5} mol dm^{-3}, initial acetylcholinesterase activity of 11.04 U cm^{-3}, pH 6, 25 °C and stirring rate of 300 rpm. Milk samples were taken from lactating cows dermally treated with chlorophos for pest control. Milk, artificially contaminated with chlorophos with known concentration was used for calibration curve construction.

Table 4
Limit of detection (LOD) of some enzyme inhibition-based sensors for chlorophos quantification (C, graphite; SPE, screen-printed electrode; GC, glassy carbon; FET, field effect transistor; ACh, acetylcholinesterase; ChO, choline oxidase; HRP, horse radish peroxidase; BuChE, butyrylcholinesterase; PVA, polyvinyl alcohol; BSA, bovine serum albumine).

Working electrode	Enzyme matrix	LOD (M)	References
C	ACh/covalent bond	1×10^{-9}	[47]
pH electrode	ChO + HRP	2×10^{-13}	[48]
SPE	BuChE cross linked with GA	3.5×10^{-7}	[49]
Pt	Ach/PVA	5×10^{-6}	[10]
GC, Au	BuChE/polyethyleneimine/BSA–GA membrane	$>1 \times 10^{-7}$	[50]
FET	BChE/BSA–GA membrane	1×10^{-6}	[51]
GC	BChE/PAN/cross linked with GA	8×10^{-7}	[52]
GC	ACh/PAN/cross linked with GA	1×10^{-6}	[52]
Au	ACh + ChO/dendrimer	3×10^{-12}	[53]
Au	ACh + ChO/polyaniline	2×10^{-7}	[53]
GC	ACh/manganese (III) meso-tetraphenylporphyrin nanoparticles	5×10^{-10}	[54]

Milk samples preparation was carried out as follows, according to the Boehringer-Mannheim protocol [56]: 20 cm^3 of milk was transferred into a 100 cm^3 volumetric flask; 10 cm^3 Carrez I solution (3.6 g $K_4[Fe(CN)_6]\cdot 3H_2O$/100 cm^3), 10 cm^3 Carrez II solution (7.2 g $ZnSO_4\cdot 7H_2O$/100 cm^3) and 20 cm^3 NaOH (0.1 mol dm^{-3}) were added, shaking vigorously after each addition, in order to perform protein precipitation; the solution was diluted with deionized water up to 100 cm^3, mixed and filtered. The filtrate was used for the assays. To avoid the influence of glucose interference the samples were preliminary treated with glucose oxidase used as catalyst for glucose oxidation into gluconic acid, no affecting the bacterial membrane response to choline [39].

Chlorophos concentration was established employing the previously constructed calibration plot. 86 and 47 μg kg^{-1} of chlorophos were measured 8 and 24 h after the animal treatment. The relative error of the analysis, established carrying out parallel measurements with the developed earlier [47] acetylcholinesterase-based amperometric sensor did not exceed 4.63%.

To note that currently chlorophos concentration in milk is evaluated by thin layer chromatography, displaying a LOD of 5 μg kg^{-1} ($\sim 2 \times 10^{-8}$ mol kg^{-1}), i.e. higher that reached with the new biosensor technique.

4. Conclusion

Taking advantage of the ability of *A. globiformis* to convert choline to betaine with oxygen consumption coupled with the inhibition effect of the OP pesticides on the ACh-catalyzed hydrolysis of acetylcholine to choline, a new hybrid electrochemical biosensor for OP quantification was developed, applying a reproducible bacteria immobilization technique and original transducer design. It was optimized and characterized in different experimental conditions.

The reported inexpensive electrochemical biosensor combines the construction simplicity, the response stability and the long life-time at ambient temperature of the bacterial sensors with the sensitivity of the inhibition-based acylcholinesterases ones. The detection limit for chlorophos, as an example, was found to be of the order of nmol dm^{-3}, similar to that reached with enzyme inhibition-based sensors for chlorophos quantification.

The biosensor was applied for chlorophos determination in contaminated milk.

Acknowledgement

This work was supported by the grant from Secretaria de Educación Superior, Programa de Mejoramiento del Profesorado (PROMEP/103-5/08/0181), México.

References

[1] Centers for Disease Control and Prevention, Third National Report on Human Exposure to Environmental Chemicals, CDC, Atlanta, GA, 2005.
[2] NIOSH Manual of Analytical Methods, 4th edition, in: P.C. Schlecht, P.F. O'Connor (Eds.), 1994.
[3] R. Jeannot, T. Dagnac, in: L. Nollet (Ed.), Chromatographic Analysis of the Environmental, 3rd edition, CRC Press, Boca Raton/London, NY, 2006, Chapter 23.
[4] J.M. Van Emon, Immunoassay and Other Bioanalytical Techniques, CRC Press, Boca Raton/London, NY, 2006.
[5] C. Tran-Minh, Ion-Select. Electr Rev. 7 (1985) 41.
[6] F. Mazzei, F. Botré, C. Botré, Anal. Chim. Acta 336 (1996) 67.
[7] W.R. Everett, G.A. Rechnitz, Anal. Lett. 32 (1999) 1.
[8] T. Noguer, B. Leca, G. Jeanty, J.-L. Marty, Field Anal. Chem. Technol. 3 (1999) 171.
[9] N. Jaffrezic-Renault, Sensors 1 (2001) 60.
[10] G. Turdean, I.C. Popescu, L. Oniciu, Can. J. Chem. 80 (2002) 315.
[11] S. Solé, A. Merkoçi, S. Alegret, Crit. Rev. Anal. Chem. 33 (2003) 89.
[12] S. Solé, A. Merkoçi, S. Alegret, Crit. Rev. Anal. Chem. 33 (2003) 127.
[13] S. Rodriguez-Mozaz, M.-P. Marco, M.J. Lopez de Alda, D. Barceló, Pure Appl. Chem. 76 (2004) 723.
[14] J. Anzai, Yakugaku Zasshi 126 (2006) 1301.
[15] B. Prieto-Simón, M. Campàs, S. Andreescu, J.-L. Marty, Sensors 6 (2006) 1161.
[16] A. Mulchandani, W. Chen, P. Mulchandani, J. Wang, K.R. Rogers, Biosens. Bioelectron. 16 (2001) 225.
[17] P. Mulchandani, W. Chen, A. Mulchandani, Environ. Sci. Technol. 35 (2001) 2562.
[18] J. Wang, R. Krause, K. Block, M. Musameh, A. Mulchandani, M.J. Schöning, Biosens. Bioelectron. 18 (2003) 255.
[19] S.H. Chough, A. Mulchandani, P. Mulchandani, W. Chen, J. Wang, K. Rogers, Electroanalysis 14 (2002) 273.
[20] L. Chenghong, M. Michelle, K. Valenta, S. Prasad, E.J. Ackerman, J. Environ. Qual. 36 (2007) 233.
[21] D.M. Munnecke, J. Agric. Food Chem. 28 (1980) 105.
[22] D.P. Dumas, J.R. Wild, F.M. Raushel, Biotech. Appl. Biochem. 11 (1989) 235.
[23] D.P. Dumas, S.R. Caldwell, J.R. Wild, F.M. Raushel, J. Biol. Chem. 33 (1989) 19659.
[24] D.P. Dumas, H.D. Durst, W.G. Landis, F.M. Raushel, J.R. Wild, Arch. Biochem. Biophys. 227 (1990) 155.
[25] V. Sacks, I. Eshkenazi, T. Neufeld, C. Dosoretz, J. Rishpon, Anal. Chem. 72 (2000) 2055.
[26] P. Mulchandani, W. Chen, A. Mulchandani, Anal. Chim. Acta 568 (2006) 217.
[27] A. Mulchandani, P. Mulchandani, I. Kaneva, W. Chen, Anal. Chem. 70 (1998) 4140.
[28] P. Mulchandani, W. Chen, A. Mulchandani, J. Wang, L. Chen, Biosens. Bioelectron. 16 (2001) 433.
[29] R. Richins, I. Kaneva, A. Mulchandani, W. Chen, Nat. Biotechnol. 15 (1997) 984.
[30] Y. Lei, P. Mulchandani, W. Chen, A. Mulchandani, J. Agric. Food Chem. 9 (2005) 524.
[31] Y. Lei, P. Mulchandani, W. Chen, A. Mulchandani, Sensors 6 (2006) 466.
[32] Y. Lei, P. Mulchandani, W. Chen, J. Wang, A. Mulchandani, Biotechnol. Bioeng. 30 (2004) 706.
[33] M. Stoytcheva, R. Zlatev, M. Ovalle, B. Valdez, Z. Velkova, 214th ECS Meeting, Honolulu, October 12–17, 2008, (http://www.electrochem.org/meetings/scheduler/abstracts/214/2830/pdf).
[34] H.J. Conn, I. Dimmick, J. Bacteriol. 54 (1947) 291.
[35] S. Ikuta, K. Matuura, S. Imamura, H. Misaki, Y. Horiuti, J. Biochem. 82 (1977) 157.
[36] S. Ikuta, S. Imamura, H. Misaki, Y. Horiuti, J. Biochem. 82 (1977) 1741.
[37] R. Zlatev, J.-P. Magnin, P. Ozil, M. Stoytcheva, Biosens. Bioelectron. 21 (2006) 1493.
[38] R. Zlatev, J.-P. Magnin, P. Ozil, M. Stoytcheva, Biosens. Bioelectron. 21 (2006) 1501.
[39] M. Stoytcheva, R. Zlatev, B. Valdez, J.-P. Magnin, Z. Velkova, Biosens. Bioelectron. 22 (2006) 1.
[40] D.R. Thévenot, K. Toth, R.A. Durst, G.S. Wilson, Pure Appl. Chem. 71 (1999) 2333.

[41] V.V. Fedorov, Theory of Optimal Experiments, Academic Press, New York, 1972.
[42] A. Lautié, D. Aslanian, J. Merlin, A. Dupax, M. Balkanski, J. Raman Spectrosc. 7 (1978) 337.
[43] D. Nachmansohn, Chemical and Molecular Basis of Nerve Activity, Academic Press, Inc., New York, 1959.
[44] M.A. Rothenberg, D. Nachmansohn, J. Biol. Chem. 168 (1947) 223.
[45] O.H. Michel, S. Krop, J. Biol. Chem. 190 (1951) 119.
[46] I.B. Wilson, M.A. Harrison, J. Biol. Chem. 236 (1961) 2292.
[47] M. Stoytcheva, Anal. Lett. 27 (1994) 3065.
[48] A. Ghindilis, H. Morzunova, A. Barmin, I. Kurochkin, Biosens. Bioelectron. 11 (1996) 837.
[49] E. Gogol, G. Evtugyn, J.-L. Marty, H. Budnikov, V. Winter, Talanta 53 (2000) 379.
[50] K. Reybier, S. Zairi, N. Jaffrezic-Renault, B. Fahys, Talanta 56 (2002) 1015.
[51] B. Krifi, M. Metche, MHA 14 (2002) 66.
[52] A. Ivanov, G. Evtugyn, L. Lukachova, E. Karyakina, H. Budnikov, S. Kiseleva, A. Orlov, G. Karpacheva, A. Karyakin, IEEE Sens. J. 3 (2003) 333.
[53] M. Snejdarkova, L. Svobodova, G. Evtuyn, H. Budnikov, A. Karyakin, D.P. Nikolelis, T. Hianik, Anal. Chim. Acta 514 (2004) 79.
[54] X.-H. Li, Z.-H. Xie, H. Min, Y.-Z. Xian, L.-T. Jin, Electroanalysis 19 (2007) 2551.
[55] M. Stoytcheva, Z. Velkova, R. Zlatev, M. Ovalle, B. Valdez, Scientific Works of the Plovdiv University of Food Technologies 55 (2008) 285.
[56] Boehringer-Mannheim, Methods of Biochemical Analysis and Food Analysis using Test-Combinations, Boehringer Mannheim GmbH Biochemica, 1989.

ANEXO C

"*Leptospirillum ferrooxidans* based Fe^{2+} sensor"

M. Stoytcheva, R. Zlatev, J.-P. Magnin, M. Ovalle, B. Valdez

Revista: Biosensors and Bioelectronics, 25, 482-487, 2009.
ISSN 0956-5663 IF 5.143.

Biosensors and Bioelectronics 25 (2009) 482–487

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Leptospirillum ferrooxidans based Fe^{2+} sensor

Margarita Stoytcheva[a,*], Roumen Zlatev[a], Jean-Pierre Magnin[b], Marcela Ovalle[a], Benjamin Valdez[a]

[a] *Universidad Autónoma de Baja California, Instituto de Ingeniería, Blvd. B. Juárez S/N, 21280 Mexicali, Baja California, Mexico*
[b] *Laboratoire d'Electrochimie et de Physico-chimie des Matériaux et Interfaces (LEPMI), UMR 5631, CNRS/UJF/INPG, BP75, 38402 St. Martin d'Hères, France*

ARTICLE INFO

Article history:
Received 20 May 2009
Received in revised form 9 August 2009
Accepted 12 August 2009
Available online 22 August 2009

Keywords:
Bacterial sensor
Leptospirillum ferrooxidans
Ferrous ions

ABSTRACT

A novel electrochemical biosensor integrating the strictly autotrophic bacterial strain *Leptospirillum ferrooxidans* as a recognition element and a Clark type oxygen probe as a transducer was designed, metrologically and analytically characterized and applied for the specific Fe^{2+} determination. The bacterial Fe^{2+} oxidation involves O_2 consumption, thus the quantification was performed registering the decrease of the oxygen reduction current. The limit of detection was found to be $2.4\,\mu mol\,L^{-1}$ and the sensitivity of the determinations—$3.94\,nA\,L\,\mu mol^{-1}$. The response time of the biosensor is 18 s for Fe^{2+} concentrations of 10^{-5} to $10^{-4}\,mol\,L^{-1}$. The biosensor was applied as well for the indirect determination of Fe^{2+} oxidizing species such as $Cr_2O_7{}^{2-}$, reaching a sensitivity of $2.47\,nA\,L\,\mu mol^{-1}$.

The transducer characteristics were evaluated and optimized to obtain short response time and high sensitivity.

The analytical performances of the biosensor subject of the present work were found to be similar to that of the *At. ferrooxidans* based one developed by the authors earlier, avoiding however the sulfur compounds interference, because of the substrate specificity of the applied bacterial strain.

1. Introduction

Leptospirillum ferrooxidans is known as an acidophilic chemolithotrophic iron-oxidizing bacterium (Balashova et al., 1974; Harrison, 1984; Harrison and Norris, 1985; Hippe, 2000; Johnson, 2001; Markosyan, 1972; Norris, 1983). Its metabolic activity, similar to that of the most extensively characterized iron-oxidizing acidophil *Acidithiobacillus ferrooxidans* (Colmer and Hinkle, 1947; Harrison, 1984; Kelly and Wood, 2000; Leduc and Ferroni, 1994; Temple and Colmer, 1951; Trudinger, 1971), involves ferrous iron oxidation using molecular oxygen as electron acceptor:

$$4Fe^{2+} + O_2 + 4H^+ = 4Fe^{3+} + 2H_2O \qquad (1)$$

Hence, this process could be exploited for Fe^{2+} determination measuring the bacterial oxygen consumption. However, while Fe^{2+} sensors based on *At. ferrooxidans* have been already designed, characterized and described (Mandl and Macholan, 1990; Zlatev et al., 2006a,b), a *L. ferrooxidans* based Fe^{2+} sensor has not been reported until now.

Unlike *At. Ferrooxidans*, growing in both ferrous iron and sulfur media, *L. ferrooxidans* is a strictly autotroph and uses Fe^{2+} as a sole source of energy. The substrate specificity of *L. ferrooxidans* promotes the expectation that the Fe^{2+} sensor based on this bacterium will provide no sensitivity to sulfur compounds, thus overcoming the main drawback of the *At. ferrooxidans* based Fe^{2+} sensors (Mandl and Macholan, 1990; Zlatev et al., 2006a). In addition, *L. ferrooxidans* displays greater affinity to ferrous iron, combined with about 10 times higher tolerance to ferric iron inhibition, compared to *At. ferrooxidans* (Norris et al., 1988).

The goal of the present work is the development and the analytical and metrological characterization of a *L. ferrooxidans* based electrochemical sensor for direct Fe^{2+} determination. Additionally, its application for indirect Fe^{2+} mediated $Cr_2O_7{}^{2-}$ determination is presented, as well as the characteristics of the Clark type oxygen probe used as a sensor transducer (Zlatev et al., 2006a), which were evaluated and optimized to obtain a maximal sensor response.

2. Experimental

2.1. Instrumentation

A special construction Clark type oxygen probe with a flat front end and uniformly distributed gold microcathodes equipped with a bacterial membrane holder (Zlatev et al., 2006a) connected to an appropriately modified Model LC-4B Amperometric Detector (BAS, USA) was applied for the sensor response registration. An especially developed noise suppression unit (subject of another publication) was connected between the current to voltage converter and the ADC, allowing about 50-fold suppres-

---

* Corresponding author. Tel.: +52 6865664150; fax: +52 6865664150.
E-mail address: margarita@iing.mxl.uabc.mx (M. Stoytcheva).

0956-5663/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.bios.2009.08.019

sion of the noise without any signal distortion and response time degradation.

Model Unimax 1010 DT turntable (Heidolf, Germany) and Model 64R temperature controller (Brookfield, USA) were used for the bacterial culture development. Model 350 pH/ion analyzer (Corning, USA) connected to a PC was applied for pH measurements and redox potential monitoring. The Fe^{2+} photometric determinations were carried out applying Stasar III photometer (Gilford, USA).

2.2. Reagents

All the reagents were of analytical grade and were purchased from Merck and Sigma. Deionized water produced by MilliQ system of Milipore was used for the experiments and bacterial culture media preparation.

2.3. Bacterial strain and culture media

The bacterial strain *L. ferrooxidans*, DSM 2705^T, isolated and described by Markosyan (1972) was cultured in DSM medium 882 with the following composition: (i) solution A: $(NH_4)_2SO_4$—132 mg, $MgCl_2{\cdot}6H_2O$—53 mg, KH_2PO_4—27 mg, $CaCl_2{\cdot}2H_2O$—147 mg, H_2O—950 mL; (ii) solution B: $FeSO_4{\cdot}7H_2O$—20 g, H_2SO_4 0.25N—50 mL; (iii) trace element solution: $MnCl_2{\cdot}2H_2O$—62 mg, $ZnCl_2$—68 mg, $CoCl_2{\cdot}6H_2O$—64 mg, H_3BO_3—31 mg, Na_2MoO_4—10 mg, $CuCl_2{\cdot}2H_2O$—67 mg, H_2O—1000 mL. The medium, with final pH adjusted to 1.8, was prepared by mixing solution A and solution B and adding 1 mL of trace element solution. Prior to mix, the solutions were autoclaved separately at 112 °C for 30 min.

2.4. Bacterial cultures development

L. ferrooxidans bacterial cultures were developed in 0.5-L flasks mounted on a turntable at 100 rpm, ensuring thus a moderate bacterial culture oxygenation. The turntable was placed in air thermostated reactor maintaining 30 °C. Photometric determinations of the Fe^{2+} concentration applying the ortho-phenantroline method (Charlot, 1961) were carried out periodically and the redox potential was continuously monitored in parallel. The bacteria development was stopped at the end of the exponential—the beginning of the stationary growth phase (90% of the ferrous ions oxidized), and followed by culture centrifugation. The concentrated bacterial mass was washed, resuspended in deionized water, obtaining protein concentrations between 5 and 10 $\mu g\,L^{-1}$, and kept at 4 °C. It was used for bacterial membrane preparation.

2.5. Fe^{2+} determination and monitoring

2.5.1. Photometric method

Samples of 10 μL were taken from the bacterial culture periodically for Fe^{2+} determination by the ortho-phenanthroline spectrophotometric method. The formed extremely stable over the time red–orange complex absorbs light in the range of 380–580 nm (Charlot, 1961) with a maximal absorption at 505 nm in the pH interval from 2 to 9. The Fe^{2+} concentration was evaluated using a preliminary built calibration curve: light absorption vs. Fe^{2+} concentration.

2.5.2. Potentiometric method (Pesic et al., 1989)

Unlike the photometric method, the potentiometric one allowed continuous monitoring of the Fe^{2+} concentration during bacterial culture development. Very precise control of the bacterial culture development can be achieved, since the redox potential E depends on the Fe^{3+}/Fe^{2+} concentration ratio, according to the Peters–Nernst equation:

$$E = E^{\circ} + \left(\frac{RT}{F}\right)\ln\left(\frac{[Fe^{3+}]}{[Fe^{2+}]}\right) \tag{2}$$

where E° is the standard redox potential, R is the gas constant, T is the temperature, F is the Faraday constant, and $[Fe^{3+}]$ and $[Fe^{2+}]$ are the ionic concentrations at equilibrium.

A Pt wire sensing electrode and Ag, AgCl/3 M KCl reference electrode was applied connected to the PC controlled Corning pH/ion analyzer for Fe^{2+} concentration monitoring.

2.6. Determination of the protein concentration

The Bradford method (Bradford, 1976) was applied initially for the resuspended bacterial mass protein concentration determination. Since this method is complicated and time-consuming, it was replaced by the simpler and more rapid turbidimetric method (Layne, 1957) based on the preliminarily built calibration plot: light absorption–protein concentration determined by the Bradford method.

It was found experimentally that the maximal light absorption by the *L. ferrooxidans* concentrated bacterial mass occurs at 275 nm, in the ultraviolet light range. The absorption value however remained sufficiently high and usable even for wavelengths up to 400 nm. Thus, a standard curve was built at 340 nm using a simple visible photometer, by dilution of an initial concentrated bacterial mass whose protein concentration was previously determined by the Bradford method. The curve appeared to be linear in the concentration range under study up to 25 $mg\,L^{-1}$ of protein.

2.7. Sensor construction and bacterial membrane preparation

The biosensor, including a Clark type oxygen probe as a transducer and a bacterial membrane as a sensing element was fabricated like described in a previous work (Zlatev et al., 2006a).

The bacterial membrane was prepared by filtration of a defined volume of the resuspended bacterial mass through a 0.15 μm pore size Sartorius cellulose filter. After the filtration, the cellulose filter charged with bacteria was attached onto the plastic membrane of the Clark oxygen probe forming a sandwich type bacterial membrane. The bacterial mass was confined between the Clark probe plastic membrane and the cellulose filter, permeable for the oxygen and the analyte.

The biosensor construction was schematically presented and discussed by the authors earlier (Zlatev et al., 2006a; Stoytcheva et al., 2009).

Even kept at 4 °C, the bacteria mortality caused a diminution of the resuspended bacterial mass activity with time, resulting in decrease of the sensor sensitivity and LOD. Although the deviations could be corrected by sensor calibration, the proposed method for bacteria membrane preparation allowed avoiding the great dispersion of the initial sensor characteristics. For this purpose the filtered volume of the resuspended bacterial mass was determined as a function of the bacterial activity. A two steps procedure was applied for the bacterial membranes preparation: (i) determination of the response R_0 to 50 $\mu mol\,L^{-1}$ Fe^{2+} of the sensor with bacterial membrane prepared by filtering of 1 mL or known volume (V_0) of the resuspended bacterial mass; (ii) calculation of the volume V_1 to be filtered to achieve the desired sensor response R_1 applying the equation:

$$V_1 = \frac{(R_1/R_0)}{V_0}. \tag{3}$$

2.8. Measuring procedure

Bacterial oxygen consumption, proportional to Fe^{2+} concentration was measured registering, as a sensor response, the current of O_2 reduction. The Fe^{2+} concentration was evaluated by the current diminution resulting from the sample insertion, using a preliminary built calibration plot, taking into account the sample dilution. The measurements were performed in a cell, containing 28 mL of diluted H_2SO_4 aqueous solution (pH 1.8), under stirring.

Since very small current changes caused by the sample injection were measured in the presence of high background current due to the bulk oxygen concentration, a current compensation was applied. It allowed the potentiostat *I*–*E* converter sensitivity augmentation, in order to achieve sufficiently high amplitude of the measured response. Unfortunately, the current compensation does not eliminate the noise which is amplified too with sensitivity increase, thus causing signal-to-noise ratio (*S*/*N*) and hence—LOD and precision degradation. The application however of an especially developed noise suppression unit (subject of another publication) allowing increasing about 50 times the *S*/*N* ratio helped avoiding this problem.

3. Results and discussion

3.1. Bacterial culture growth monitoring

The development of active bacteria requires their growth to be terminated near the end of the exponential phase. The end point determination can be achieved by precise monitoring of the Fe^{2+} concentration applying the on-line potentiometric method after preliminary calibration done by results obtained by the photometric method application.

The redox potential vs. time curve (Fig. 1A) strictly corresponds to the Fe^{2+} concentration ratio vs. time (Fig. 1B) curve allowing the precise on-line monitoring of the bacterial growth instead of the time and reagent consuming off-line photometric method usually applied.

It was found (Fig. 1A and B) that 90% of the ferrous ions oxidation (determined by the photometric method) corresponds to redox potential value of 625 mV which is the end point of the bacterial culture development, followed by centrifugation and resuspension, as described above.

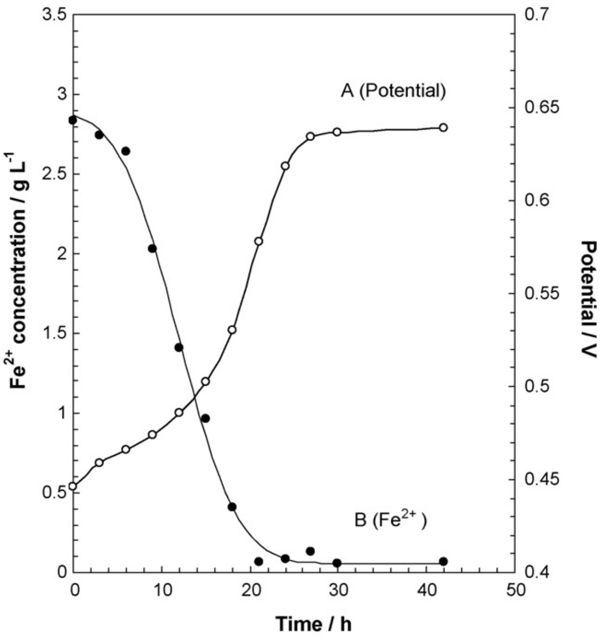


Fig. 1. Bacterial culture growth monitoring: on-line potentiometric (A); off-line photometric (B).

3.2. Oxygen probe characterization

3.2.1. Transducer response time determination

The oxygen probe (transducer) response time is an important component of the total biosensor response time. It is affected by the rate of the oxygen reduction on the Clark probe cathode, i.e. by the cathode area, by the permeability of the plastic oxygen permeable membrane, and by the oxygen gradient change mode, keeping all the other conditions constants (thickness of the electrolyte layer between the plastic membrane and the probe cathode, electrolyte composition, applied potential, electrode material, and temperature).

Response time refinement was performed by increasing the internal oxygen consumption and by membrane permeability improvement, choosing adequate plastic material and thickness. The increase of the internal oxygen consumption was achieved by increasing the effective diameter of the Clark probe cathode using for this purpose a multicathode, i.e. uniformly distributed on the entire acrylic support surface gold discs in contact with the Clark probe electrolyte (Zlatev et al., 2006a). The characteristics of this original Clark type oxygen probe were not published until now.

Two response times of the oxygen probe were determined experimentally creating positive and negative steps of the oxygen concentration by stirring rate change (from 0 to 800 rpm) and by Na_2SO_3 addition respectively. The stirring provokes an increase of the oxygen concentration at the solution/oxygen probe membrane interface intensifying also the mass exchange at the solution/air interface resulting in bulk equilibrium oxygen concentration increase. The addition of Na_2SO_3 which is easily oxidized by the dissolved oxygen yielding Na_2SO_4 immediately decreases its concentration. The two types of the oxygen probe current responses: positive and negative, are shown in Fig. 2.

The experimental results showed 8.7 s response time to 90% of the maximal response to positive concentration changes, while to

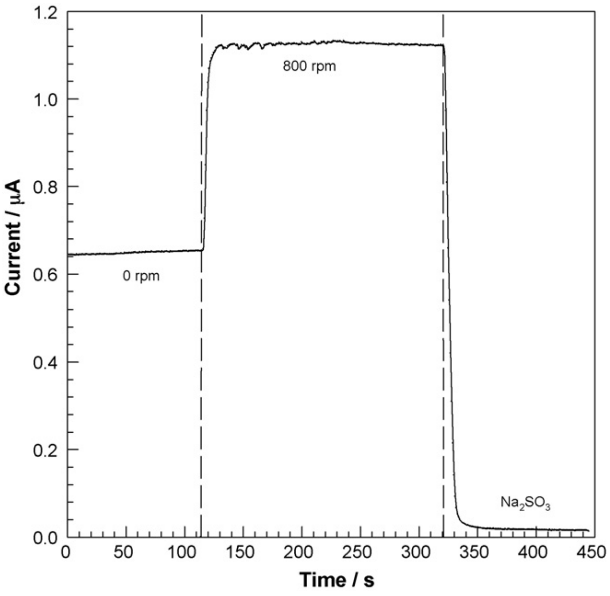


Fig. 2. Oxygen probe transducer current transient response to increasing and to decreasing oxygen concentrations (25 °C, −0.90 V).

the negative changes the response was found to be 12.5 s, i.e. about 1.5 times longer. Unfortunately, the bacterial sensor response is based on the transducer response to decreasing oxygen concentrations.

3.2.2. Membrane transport parameters

Since the biosensor response represents the reduction current of the oxygen, crossing the Clark type probe membrane, the transport parameters and the thickness of the latter strongly influenced the response time of the sensor and the sensitivity of the determinations. Thus, they were evaluated and optimized.

According to Eqs. (4) and (5) (Tarnowski et al., 1995), the sensitivity of the oxygen determination is a function of the membrane permeability and oxygen diffusion coefficients:

$$I_d = 4FA(P_m/Z_m)P_{O_2} \tag{4}$$

$$P_m = D_m S_m \tag{5}$$

where: I_d is the oxygen mass transport limited current, F is the Faraday constant, A is the area of the cathodes, P_m is the membrane oxygen permeability coefficient, Z_m is the membrane thickness, P_{O_2} is the partial pressure of the oxygen in the solution, D_m is the membrane oxygen diffusion coefficient, and S_m is the oxygen solubility in the membrane phase.

Thus, the membrane oxygen permeability coefficient was evaluated registering the steady-state current response of the probe in air-equilibrated deionized water under stirring at constant temperature. The chosen stirring rate of 1000 rpm maintained an oxygen flux, remaining almost constant with current rate increase. In these conditions, the diffusion layer is confined to the membrane and consequently, the steady-state current depends only on the oxygen transport properties of the membrane (Hitchman, 1978).

The oxygen permeability coefficient (P_m) was found to be $(1.19 \pm 0.05) \times 10^{-12}$ mol m^{-1} s^{-1} kPa^{-1} ($N = 12$) calculated applying Eq. (4) ($I_d = 1.123 \times 10^{-6}$ A; $A = 2.2 \times 10^{-6}$ m^2; $Z_m = 15 \times 10^{-6}$ m; $P_{O_2} = 1.8 \times 10^4$ Pa).

The membrane oxygen diffusion coefficient (D_m) was derived from the slope of the line, described by the following equation (Hitchman, 1978):

$$\ln \frac{1 - (I_t/I_s)}{2} = -\frac{\pi^2 D_m t}{Z_m} \tag{6}$$

where: I_t is the transient current response and I_s is the steady-state current response of the oxygen probe.

The transient current response was obtained by step changing of the solution stirring rate from 0 to 800 rpm (Fig. 2), sufficient to ensure maximal O_2 flux and able to maintain the oxygen concentration at the outer surface of the membrane at bulk levels. The logarithmic plot built to evaluate the oxygen diffusion coefficient in the membrane D_m and described by the equation $y = -0.3074x - 1.3056$ ($r^2 = 0.9875$) is presented in Fig. 3.

It was found that $D_m = (4.6 \pm 0.2) \times 10^{-5}$ cm^2 s^{-1} ($N = 7$).

All the values obtained are related to 15 μm thick polyethylene membrane. This membrane thickness, combined with a large cathode surface, was found to be the optimal to ensure a compromise between the transducer response time and sensitivity.

3.3. Analytical and metrological characterization of the L. ferrooxidans based Fe^{2+} sensor

Every series of measurements were carried out during the same day and no corrections were made for bacterial activity diminution, preliminary determined to be about 1% per day, applying the following procedure: the sensor response for a same value of Fe^{2+} concentration (50 μmol L^{-1}) was measured at identical experimental conditions (30 °C, pH 1.8, 100 rpm) within a time interval of 12 h and the response change was evaluated. A constant temperature was maintained, since parameters affecting the sensor response amplitude, such as transducer response to oxygen (about 2%/°C), equilibrium dissolved oxygen concentration (about 1.4%/°C), as well as the bacterial activity, are temperature dependent, thus provoking a 5%/°C sensor response change. Working at thermostatic conditions allowed avoiding fluctuations of the equilibrium dissolved oxygen concentration, while the constant stirring rate kept unchanged the oxygen concentration gradient at the bacterial membrane/solution interface.

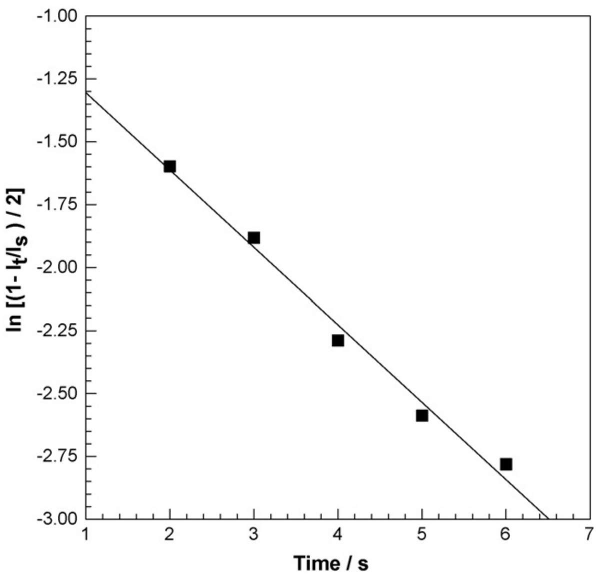


Fig. 3. Logarithmic plot built to evaluate the oxygen diffusion coefficient (D_m) in the polymer membrane of the oxygen probe.

The typical sensor response to Fe^{2+} is presented in Fig. 4. The corresponding calibration plot (steady-state current I_s/nA vs. Fe^{2+} concentration C_{Fe}/μmol L^{-1}) is linear in the investigated concen-

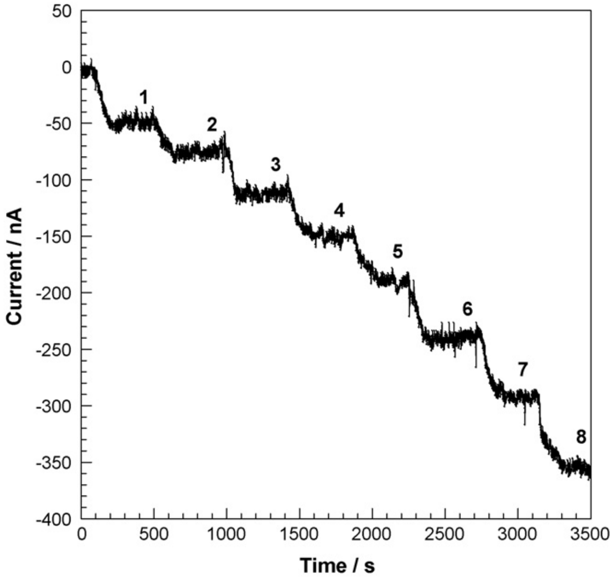


Fig. 4. Amperometric response of the *L. ferrooxidans* sensor for increasing Fe^{2+} concentrations: (1) 10 μmol L^{-1}; (2) 20 μmol L^{-1}; (3) 30 μmol L^{-1}; (4) 40 μmol L^{-1}; (5) 50 μmol L^{-1}; (6) 60 μmol L^{-1}; (7) 70 μmol L^{-1}; (8) 80 μmol L^{-1} (air-equilibrated deionized water at 30 °C, pH 1.8 and 100 rpm).

tration range up to 80 μmol L^{-1}, representing a part of the total dynamic linear range which was found to be 2.1 mmol L^{-1}, at the mentioned above experimental conditions. The calibration plot can be described by the equation: $I_s = 3.94C_{Fe}$, with $r^2 = 0.9935$, i.e. the sensor sensitivity is 3.94 nA L μmol^{-1}.

The limit of detection, experimentally determined in the same conditions was found to be 2.4 μmol L^{-1} when the signal-to-noise ratio equals to 3. According to the UPAC definition determining the limit of quantitation as LOQ = 3.3 LOD (Thévenot et al., 1999), the *L. ferrooxidans* based Fe^{2+} bacterial sensor LOQ was found to be 7.92 μmol L^{-1}.

The steady-state sensor response time was determined to be about 18 s for Fe^{2+} concentrations of 10^{-5} to 10^{-4} mol L^{-1}.

The *L. ferrooxidans* based Fe^{2+} sensor when not in use was stored in diluted aqueous solution of H_2SO_4 with pH 1.8, at ambient temperature. The sensor response to 50 μmol L^{-1} Fe^{2+} was measured daily at 30 °C, pH 1.8 and 100 rpm. After 5 days about 10% lost of activities was observed, while for 45 days the response was only 50% of the initial. Thus, the storage lifetime t_{L10} and t_{L50} were determined to be 5 and 45 days respectively, at ambient temperature. The long storage lifetime of the bacterial sensors at ambient temperature is one of their advantages compared to the enzymatic ones which loose activity due to the enzyme denaturation.

It was found that the sensor lifetime equals the free (non-immobilized) bacteria lifetime kept at the same conditions (H_2SO_4 aqueous solution, pH 1.8, ambient temperature), demonstrating that the nature of the membrane material does not affect this parameter.

The addition to the storage media of Fe^{2+}, involved in the bacterial metabolic activity, increased the sensor lifetime. However, this procedure was not applied to avoid the “cleaning” of the bacterial membrane before every application, by sensor immersion for a long time in running deionized water while the internal Fe^{2+} concentration decreases to zero.

The reproducibility of the bacterial sensors was determined by the ability of preparation of bacterial membranes with same bacterial activity, as presented in a previous authors work (Zlatev, 2002). Applying the procedure described above, combined with preliminary bacterial activity determination, a reproducibility of the response within 3% R.S.D. for 50 μmol L^{-1} Fe^{2+} was achieved at 30 °C and 100 rpm agitation speed ($I_s = 196 \pm 2$ nA, $N = 10$, $P = 95\%$).

Unlike *At. ferrooxidans*, it is known that *L. ferrooxidans* grows in Fe^{2+} containing media only and that is why it was not expected sulfur compounds influence on the sensor response. Nevertheless, because the *At. ferrooxidans* Fe^{2+} sensor reported by the authors (Zlatev et al., 2006a) is sensitive to thiosulfate, its influence on the *L. ferrooxidans* based Fe^{2+} sensor response was tested. The sensor response to 50 μmol L^{-1} Fe^{2+} and increasing up to 0.6 mmol L^{-1} thiosulfate concentrations made by successive microliter volumes additions of concentrated thiosulfate solution was registered and no effect was observed out of the negligible response fluctuations within the experimental error.

Because of the metabolic restriction of *L. ferrooxidans*, the sensor response to Fe^{2+} could be affected by bacteria metabolism inhibitors only. The Fe^{2+} oxidizing agents such as $Cr_2O_7^{2-}$ are not assumed to be interfering agents because they change the free Fe^{2+} concentration itself but not interfere with the ferrous iron determination.

As demonstrated by Sugio et al. (1987, 1992, 1994), the iron-oxidizing activity of *L. ferrooxidans* could be blocked in the presence of bisulfite ions, because of the iron oxidase inhibition. The bisulfite ions interference on the ferrous iron quantification was investigated by monitoring the sensor response amplitude to 10 μm L^{-1} Fe^{2+} and increasing HSO_3^- concentrations. Small volumes of Na_2SO_3 were added to the Fe^{2+} containing H_2SO_4 solution (pH 1.8) obtaining thus concentrations of: 0.1, 0.2, 0.5 and 1 μmol L^{-1}. In acidic media the sulfite ion is converted to bisulfite one according to the equation: $SO_3^{2-} + H^+ \rightleftharpoons HSO_3^-$. The Na_2SO_3 additions provoked respectively 27%, 52%, 81% and 92% diminution of the steady-state sensor response, measured 30 min after every addition. As known, SO_3^{2-} undergoes a rapid oxidation by the dissolved oxygen forming SO_4^{2-} and the rate constant increases with pH (Shi et al., 2006). This effect however was not taken into account since the experiments were performed at very low pH value (1.8).

As demonstrated (Table 1), the sensitivity of the Fe^{2+} quantification is almost the same, when using *L. ferrooxidans* and *At. ferrooxidans* based sensors. However, the *L. ferrooxidans* based sensor displays a shorter response time and ensures free of sulfur compounds interference determinations, because of the specific mechanism and kinetics of the Fe^{2+} oxidation (Sand et al., 1992).

Thus, the proposed Fe^{2+} sensor can be applied for continuous on-line monitoring of ferrous iron in acidic sulfate media without sample pretreatment. In contrast, the conventional photometric methods require preliminary sample treatment: Fe^{2+} extraction, pH adjustment, reagents addition, etc., which makes the on-line determination complicated or even impossible.

3.4. *Application of the L. ferrooxidans based sensor for indirect $Cr_2O_7^{2-}$ determination*

Since the bacterial sensor based on *L. ferrooxidans* is sensitive to Fe^{2+}, it can be used for indirect determinations of any agent reacting with Fe^{2+}, thus changing stoechiometrically its concentration without affecting the bacteria metabolism. $Cr_2O_7^{2-}$ completely fulfill these two conditions. The process which mechanism is investigated in details (Espenson and King, 1963; Laitinen and Harris, 1979) is described by the following equation:

$$Cr_2O_7^{2-} + 14H^+ + 6Fe^{2+} \rightarrow 2Cr^{3+} + 6Fe^{3+} + 7H_2O \qquad (7)$$

One mol of $Cr_2O_7^{2-}$ oxidizes 6 mol of Fe^{2+}, thus lowering its concentration causing decrease of the bacterial oxygen consumption, resulting in biosensor response change proportional to the $Cr_2O_7^{2-}$ concentration, as shown in Fig. 5.

The sensor response was registered during successive additions of $Cr_2O_7^{2-}$ aliquots to a solution containing a constant Fe^{2+} concentration (60 μmol L^{-1}), at 30 °C, pH 1.8, and 100 rpm stirring rate. The corresponding calibration curve (steady-state current I_s/nA vs. $Cr_2O_7^{2-}$ concentration $C_{Cr}/\mu mol\ L^{-1}$) was described by the linear equation: $I_s = 2.47C_{Cr}$, with $r^2 = 0.9817$.

The linear range of $Cr_2O_7^{2-}$ determination depends on the initial Fe^{2+} concentration. Since 1 mol of $Cr_2O_7^{2-}$ oxidizes 6 mol of Fe^{2+}, the linear range of the sensor toward $Cr_2O_7^{2-}$ is 1/6 of the linear range of the Fe^{2+} determination.

The proposed *L. ferrooxidans* sensor is not sensitive to $Cr_2O_7^{2-}$. It only senses the changes of the mediator Fe^{2+} concentration resulting from the reaction (7). Thus, the $Cr_2O_7^{2-}$ determination was

Table 1
Main characteristics of the *L. ferrooxidans* and *At. ferrooxidans* (Zlatev, 2002) based sensors for Fe^{2+} quantification.

Bacteria	Sensitivity (nA L μmol^{-1})	LOD (μmol L^{-1})	Response time (s)	t_{L50} days	R.S.D. (%)	Interferences
L. ferrooxidans	3.94	2.4	18	45	<3	No
At. ferrooxidans	4.09	0.9	45	–	<3	Thiosulphate

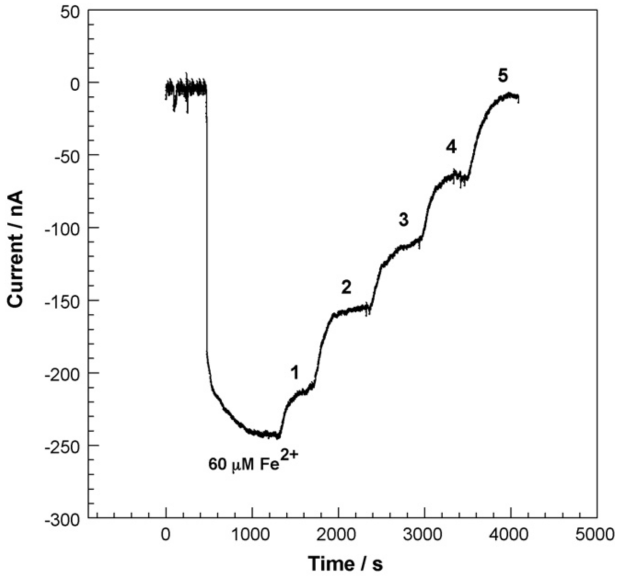


Fig. 5. Amperometric response of the *L. ferrooxidans* sensor for increasing $Cr_2O_7{}^{2-}$: (1) 2 μmol L^{-1}; (2) 4 μmol L^{-1}; (3) 6 μmol L^{-1}; (4) 8 μmol L^{-1}; (5) 10 μmol L^{-1}, in air-equilibrated deionized water at 30 °C, pH 1.8, 100 rpm, and Fe^{2+} concentration of 60 μmol L^{-1}.

influenced by the factors affecting the Fe^{2+} determination described above.

4. Conclusion

Taking advantage of the metabolic restriction of the bacterium *L. ferrooxidans*, a novel electrochemical biosensor was developed, providing specific Fe^{2+} quantification not interfered by sulfur compounds presence, thus overcoming the main drawback of the *At. ferrooxidans* based Fe^{2+} sensors.

The biosensor metrological and analytical characteristics, such as LOD, sensitivity, reproducibility, lifetime t_{L50}, and steady-state response time were evaluated and reported.

The Clark type oxygen probe parameters were optimized to obtain transducer short response time and high sensitivity.

The sensor was applied as well as for the indirect $Cr_2O_7{}^{2-}$ determination, using the Fe^{2+} as a mediator.

Acknowledgement

This work was supported by the Secretaria de Educación Superior, Programa de Mejoramiento del Profesorado (PROMEP/103-5/08/0181), México.

References

Balashova, V.V., Vedenina, I.Ya., Markosyan, G.E., Zavarzin, G.A., 1974. Mikrobiologiya 43, 491–494 (in Russian).
Bradford, M.M., 1976. Anal. Biochem. 72, 248–254.
Charlot, G., 1961. Les méthodes de la chimie analytique. Masson, Paris.
Colmer, A.R., Hinkle, M.E., 1947. Science 106, 253–256.
Espenson, J.H., King, E.L., 1963. J. Am. Chem. Soc. 85, 3328–3333.
Harrison Jr., A.P., 1984. Annu. Rev. Microbiol. 38, 265–269.
Harrison Jr., A.P., Norris, P.R., 1985. FEMS Microbiol. Lett. 30, 99–102.
Hippe, H., 2000. Int. J. Syst. Evol. Microbiol. 50, 501–503.
Hitchman, M.L., 1978. Measurement of Dissolved Oxygen. Wiley, NY.
Johnson, D.B., 2001. In: Garrity, G. (Ed.), Bergey's Manual of Systematic Bacteriology, vol. I, second ed. Springer-Verlag, Berlin, pp. 453–457.
Kelly, D.P., Wood, A.P., 2000. Int. J. Syst. Evol. Microbiol. 50, 489–500.
Laitinen, H.A., Harris, W.E., 1979. Chemical Analysis, second ed. McGraw-Hill, New York/London.
Layne, E., 1957. Methods Enzymol. 3, 447–454.
Leduc, L.G., Ferroni, G.D., 1994. FEMS Microbiol. Rev. 14, 103–119.
Mandl, M., Macholan, L., 1990. Folia Microbiol. 35, 363–367.
Markosyan, G.E., 1972. Biol. Zh. Armenia 25, 26–29 (in Russian).
Norris, P.R., 1983. In: Rossi, G., Torma, A.E. (Eds.), Recent Progress in Biohydrometallurgy. Associazione Mineraria Sarda, Iglesias, Italy, pp. 83–96.
Norris, P.R., Barr, D.W., Hinson, D., 1988. In: Norris, P.R., Kelly, D.P. (Eds.), Biohydrometallurgy. Science and Technology Letters, Kew, Surrey, UK, pp. 43–59.
Pesic, B., Oliver, D.J., Wichlacz, P., 1989. Biotechnol. Bioeng. 33, 428–439.
Sand, W., Rohde, K., Sobotke, B., Zenneck, C., 1992. Appl. Environ. Microbiol. 58, 85–92.
Shi, Y., Zhan, X., Lü, T., Li, L., Cao, C., Shu, X., Li, C., Li, L.-L., 2006. Huaxue Xuebao 64, 496–500 (in Japan).
Stoytcheva, M., Zlatev, R., Velkova, Z., Valdez, B., Ovalle, M., Petkov, L., 2009. Electrochim. Acta 54, 1721–1727.
Sugio, T., Noguchi, M., Tano, T., 1987. Agric. Biol. Chem. 51, 1431–1433.
Sugio, T., White, K., Shute, E., Choate, D., Blake, R., 1992. Appl. Environ. Microbiol. 58, 431–433.
Sugio, T., Uemura, S., Makino, I., Iwahori, K., Tano, T., Blake, R., 1994. Appl. Environ. Microbiol. 60, 722–725.
Tarnowski, D.J., Bekos, E.J., Korzeniewski, C., 1995. Anal. Chem. 67, 1546–1552.
Temple, K.L., Colmer, A.R., 1951. J. Bacteriol. 62, 605–611.
Thévenot, D.R., Toth, K., Durst, R.A., Wilson, G.S., 1999. Pure Appl. Chem. 71, 2333–2348.
Trudinger, P.A., 1971. Miner. Sci. Eng. 3, 13–25.
Zlatev, R., 2002. Etudes sur les possibilités d'application de la bactérie *Acidithiobacillus ferrooxidans* dans les méthodes électrochimiques d'analyse – biocapteurs et voltammétrie. Thesis work. LEPMI, INPG, France.
Zlatev, R., Magnin, J.-P., Ozil, P., Stoytcheva, M., 2006a. Biosens. Bioelectron. 21, 1493–1500.
Zlatev, R., Magnin, J.-P., Ozil, P., Stoytcheva, M., 2006b. Biosens. Bioelectron. 21, 1501–1506.

ANEXO D

“Electrochemical study of rat brain acetylcholinesterase inhibition by chlorofos: kinetic aspects and analytical applications”

M. Ovalle, M. Stoytcheva, R. Zlatev, B. Valdez

Revista: Electrochimica Acta, 55, 516-520, 2009.
ISSN: 0013-4686 IF 3.078

Electrochimica Acta 55 (2009) 516–520

Contents lists available at ScienceDirect

Electrochimica Acta

journal homepage: www.elsevier.com/locate/electacta

Electrochemical study of rat brain acetylcholinesterase inhibition by chlorofos: Kinetic aspects and analytical applications

Marcela Ovalle, Margarita Stoytcheva*, Roumen Zlatev, Benjamin Valdez

Universidad Autónoma de Baja California, Instituto de Ingeniería, Blvd. B. Juárez y Calle de la Normal s/n, Col. Insurgentes Este, 21280 Mexicali, Baja California, México

ARTICLE INFO

Article history:
Received 10 June 2009
Received in revised form 24 August 2009
Accepted 4 September 2009
Available online 9 September 2009

Keywords:
Chlorofos
Acetylcholinesterase
Biosensor
Kinetics
Quantification

ABSTRACT

An electrochemical sensor was applied for investigating the immobilized rat brain acetylcholinesterase inhibition by chlorofos. Two alternative routes were explored as response-generating reactions: (i) direct electrochemical oxidation of thiocholine produced upon acetylthiocholine enzymatic hydrolysis and (ii) reduction of the produced thiocholine with hexacyanoferrate (III), followed by hexacyanoferrate (II) electrochemical detection. The advantages of the direct way are simplicity and higher sensitivity compared to the indirect one, which however avoids the interferences because of the lower potential applied.

Enzyme inhibition was identified as competitive, the K_M^{app} increasing from 1.31 to 1.43 mmol L^{-1} with chlorofos concentration in the range 0.2–1.0 mmol L^{-1} and the maximal rate of the enzyme reaction remaining constant ($I_{max} = 579.30 \pm 5.71$ μA) in the presence of chlorofos. The inhibition constant was calculated using the Dixon method ($K_I = 10.07$ mmol L^{-1}).

The suppression of the acetylcholinesterase activity by the inhibitor, expressed as current decrease at a constant substrate concentration, was exploited for chlorofos quantification optimized by the design of experiments methodology. Optimal response was obtained for an acetylthiocholine concentration of 0.2 mmol L^{-1}, at 26 °C and pH 7.

1. Introduction

Chlorofos (2,2,2-trichloro-1-dimethoxyphosphoryl-ethanol) is an extensively used, broad-spectrum, moderately toxic organophosphorus insecticide. It is applied in the agricultural and veterinary practices for protection of field and fruit crops, and for parasites control in domestic animals [1]. Under the generic name of metrifonate, it is used for the treatments of schistosomiasis caused by Schistosoma haematobium in humans [2]. Over-exposure and accidental or intentional ingestion of chlorofos may cause serious poisoning. Its high acute toxicity, rapid absorption by the organism and fast degradation in the environment [1] call for the development of simple, express, sensitive, reliable and low-costing methods for its "*in situ*" determination.

The commonly used analytical methods for chlorofos quantification include various chromatographic techniques, such as gas-chromatography [3], gas-chromatography with mass spectrometry detection [4,5] thin-layer chromatography [6–9], and high performance liquid chromatography [10–12]. However, although selective and sensitive, these methods are not applicable for in field determinations: they are time-consuming, require trained personnel and sophisticated laboratory equipment.

The electrochemical biosensors, developed during the last decades, offer a new approach to organophosphorus pesticides quantification. Taking advantage of the ability of the organophosphorus compounds to inhibit the acylcholinesterases, they allow their determination evaluating the enzyme activity suppression, applying electrochemical techniques. For instance, several acylcholinesterases-based electrochemical sensors have been suggested for chlorofos determination [13–32]. Because of the inexpensive instrumentation, the simple operation procedure and the high sensitivity [33], the electrochemical biosensors were becoming the devices of choice for organophosphorus pesticides "*in situ*" analysis.

As known, enzyme fixation on the electrode surface modifies its three-dimensional structure and causes the appearance of steric, partition, micro-environmental and diffusion constraints [34], thus affecting the enzyme activity and the enzyme catalytic rate. Consequently it is reflected on the analytical and metrological characteristics of the determination (sensitivity, selectivity, linear dynamic range, and sensor response time).

Only few of the kinetics of the inhibition of the immobilized acylcholinesterases used as recognition element in electrochemical biosensors for chlorofos quantification has been investigated until now [35]. Thus, in this work, applying a simple, inexpensive, and enzyme activity preserving immobilization technique (adsorption of membrane fraction of rat brain), an acetylcholinesterase based amperometric sensor was developed and applied for

---

* Corresponding author. Tel.: +52 686 566 41 50; fax: +52 686 566 41 50.
E-mail address: margarita@iing.mxl.uabc.mx (M. Stoytcheva).

0013-4686/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.electacta.2009.09.008

elucidating the kinetics of acetylcholinesterase inhibition by chlorofos. The analysis of the obtained data allowed identifying the type of the immobilized enzyme inhibition and to evaluate the kinetic parameters of the heterogeneous enzyme reaction. Finally, the analytical aspects of the inhibition were discussed and condition optimization of the chlorofos determination was performed by mathematical modeling.

2. Experimental

2.1. Reagents

Acetylthiocholine iodide (Chemapol, Czechoslovakia) and 2,2,2-trichloro-1-dimethoxyphosphoryl-ethanol (chlorofos) (Sigma) were used as such without any purification pretreatment. Acetylthiocholine iodide solutions were prepared daily and kept at 4 °C. The supporting electrolyte was a Britton–Robinson buffer, pH 7 (0.04 mol L^{-1} H_3PO_4, 0.04 mol L^{-1} CH_3COOH, 0.04 mol L^{-1} H_3BO_3, and 0.2 mol L^{-1} NaOH). All the reagents were analytical reagent grade.

Membrane fraction of rat brain was used as a source of acetylcholinesterase. Rat brains were collected immediately after death into iced Tris buffer, pH 7.4, and homogenized to slurry. The resulting tissue was centrifuged at 3000 rpm at 4 °C for 15 min. The supernatant was removed, and centrifuged at 16000 rpm for 15 min. The supernatant was removed once again and conserved frozen.

2.2. Instrumentation and procedures

The electrochemical studies were performed using an amperometric unit PRG-DEL Tacussel, coupled to a lab-made data acquisition system.

The investigations were carried out in an electrolysis cell of conventional type (5 cm^3 of volume), at a temperature of 26 °C, pH 7 (Britton–Robinson buffer), with a rotation speed of the enzyme working electrode of 1000 rpm. Pt was used as auxiliary electrode and Ag, AgCl/KCl (sat.) as a reference.

pH and temperature of the solution were controlled using respectively a pH-meter Orion 250 A and TU-16D thermostat.

2.3. Preparation of the acetylcholinesterase based sensor

The enzyme working electrode was a rotating disk with a diameter of 6 mm, fabricated from spectrally pure graphite (Ringsdorf Werke, Germany). The electrode surface was preliminary treated by polishing, degreasing with alcohol and ultrasonic cleaning. The acetylcholinesterase immobilization was performed by adsorption, following the scheme: 30 μl of the supernatant, containing the enzyme were injected onto the electrode surface and kept for 30 min at room temperature; after that the electrode surface was cleaned for 5 min in the buffer solution, at an electrode rotation speed of 1000 rpm, in order to eliminate fairly strongly fixed enzyme.

3. Results and discussion

3.1. Measuring principles comparison

The determinations were based on the process of acetylthiocholine hydrolysis to thiocholine, which was catalyzed by the immobilized acetylcholinesterase ACh (Eq. (1)):

$$(CH_3)_3N^+(CH_2)_2SCOCH_3 + H_2O \xrightarrow{\text{A Ch}} (CH_3)_3N^+(CH_2)_2SH + CH_3COOH \qquad (1)$$

In the presence of chlorofos, the concentration of the produced thiocholine decreased, because of the inhibition of the acetylcholinesterase activity by this compound, through phosphorylation of the enzyme's serin group, according to the reaction (Eq. (2)):

$$\mathrm{EnzSer-OH} + \underset{\text{cholorofos}}{\mathrm{CCl_3CH_2O-PO-(OCH_3)_2}}$$
$$\xrightarrow{\text{inhibition}} \mathrm{EnzSer-O-PO-(OCH_3)_2 + CCl_3CH_2OH} \qquad (2)$$

Two alternative routes were explored as response-generating electrochemical reactions:

1. Electrochemical oxidation of thiocholine at +0.80 V/Ag, AgCl (Eq. (3)) produced upon enzymatic hydrolysis of the substrate acetylthiocholine (Eq. (1)) [36–38] (route 1):

$$2(CH_3)_3N^+(CH_2)_2SH \rightarrow (CH_3)_3N^+(CH_2)_2S\text{-}S(CH_2)_2N^+(CH_3)_3 + 2H^+ + 2e^- \qquad (3)$$

2. Indirect way [53,54] comprising the following stages: (i) acetylthiocholine hydrolysis (Eq. (1)); (ii) a redox interaction between the produced thiocholine and 1 mmol L^{-1} solution of hexacyanoferrate (III) (Eq. (4)); (iii) electrochemical oxidation of the formed hexacyanoferrate (II) at a potential of +0.45 V/Ag, AgCl (Eq. (5)) (route 2):

$$2(CH_3)_3N^+(CH_2)_2SH + 2[Fe(CN)_6]^{3-}$$
$$\rightarrow (CH_3)_3N^+(CH_2)_2S\text{-}S(CH_2)_2N^+(CH_3)_3 + 2[Fe(CN)_6]^{4-} \qquad (4)$$

$$[Fe(CN)_6]^{4-} \rightarrow [Fe(CN)_6]^{3-} + e^- \qquad (5)$$

The process of direct thiocholine oxidation occurring at +0.80 V/Ag, AgCl at conventional metal and graphite transducers [39–43] involves the transfer of one electron from the thiol and a dimerization of the intermediate to disulfide [44,45]. The high potential value however causes the appearance of a high background current, as well as electroactive compounds interferences.

Several types of electrodes overcoming these drawbacks were reported, such as the ones chemically modified with phtalocyanines [46–48], Prussian blue [49], tetracyanoquinodimetane [50,51] and ferrocene [52]. A carbon nanotube modified glassy carbon electrode [40], providing a sensitive electrochemical detection of enzymatically generated thiocholine at low potential was described, too.

In this work, instead of electrode modification, a simpler approach (route 2) for potential lowering was applied in some of the experiments as an alternative to the direct thiocholine oxidation (route 1).

The typical amperometric response of the biosensor to acetylthiocholine and to chlorofos at constant acetylthiocholine concentration registered at a potential of +0.80 V and at +0.45 V respectively, is presented in Fig. 1(A and B, inset) and Fig. 2 (A and B, inset). The current corresponding to a fixed substrate concentration was compensated, before inhibitor injection, using electronic technique. Thus, the potentiostat *I–E* converter sensitivity was augmented, in order to achieve sufficiently high amplitude of the measured response, in the presence of high background current, in conjunction with an especially developed noise suppression unit (subject of another publication) allowing increasing about 50 times the S/N ratio.

Chlorofos was quantified establishing the relationship between its concentration and the induced enzyme inhibition. The degree of inhibition ($D = \Delta I/I_o, \%$) was defined as the ratio between the decrease of the biosensor response due to the addition of inhibitor

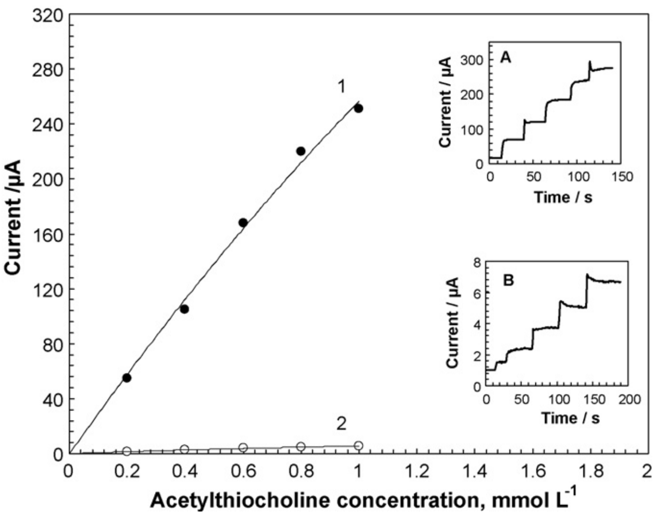


Fig. 1. Calibration plot for acetylthiocholine quantification. Applied potential: (1) +0.80 V/Ag, AgCl; (2) +0.45 V/Ag, AgCl. pH 7, 26 ± 0.5 °C, 1000 rpm. (A, inset) Amperometric response of the biosensor to increasing acetylthiocholine concentrations (equal to 0.2, 0.4, 0.6, 0.8 and 1.0 mmol L^{-1}) at a potential of +0.80 V/Ag, AgCl; (B, inset) Amperometric response of the biosensor to increasing acetylthiocholine concentrations (equal to 0.2, 0.4, 0.6, 0.8 and 1.0 mmol L^{-1}) at a potential of +0.45 V/Ag, AgCl.

(ΔI) and the initial biosensor response (I_o) [55,56]. The measurements were performed according to the following three-step procedure: (i) recording of the amperometric biosensor response to acetylthiocholine; (ii) acetylthiocholine oxidation current compensation and noise suppression; (iii) chlorofos injection and current decrease recording.

The calibration plots, presented in Figs. 1 and 2 respectively, demonstrate that although the second measurement principle offers the advantage to operate at low electrode potential thus avoiding the interferences, the first one based on the direct thiocholine oxidation ensures better analytical performances of the biosensor in terms of sensitivity. For example, the sensitivity of the determinations, carried out at a potential of +0.80 V and at +0.45 V respectively decreased from 274.73 µA L $mmol^{-1}$ to 6.45 µA L $mmol^{-1}$ for acetylthiocholine and from 8.87 µA L $mmol^{-1}$ to 0.24 µA L $mmol^{-1}$ for chlorofos for an acetylthiocholine concentration of 0.6 mmol L^{-1}. Because of the fact that the biosensor response is controlled by the rates of a number of conjugated processes [33], the sensitivity of the biosensors determinations depends, among other, on the kinetics of the involved reactions. Thus, the kinetic aspects of rat brain acetylcholinesterase inhibition by chlorofos were investigated. The direct electrochemical approach was preferred for further studies because of its greater sensitivity.

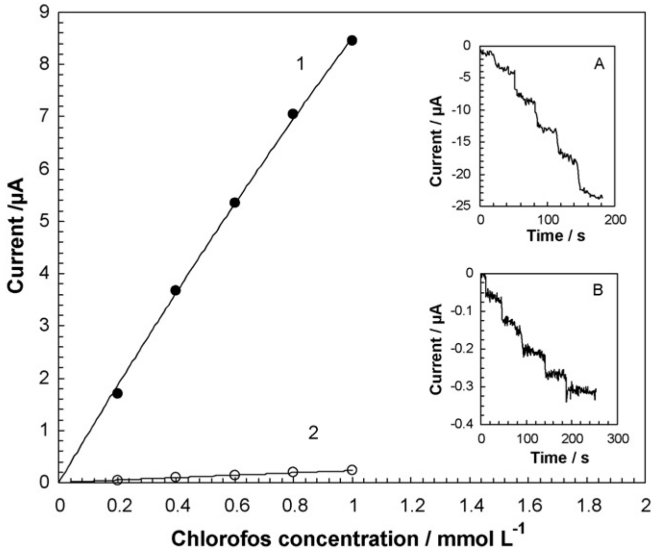


Fig. 2. Calibration plot for chlorofos quantification. Applied potential: (1) +0.80 V/Ag, AgCl; (2) +0.45 V/Ag, AgCl. pH 7, 26 ± 0.5 °C, 1000 rpm. (A, inset) Amperometric response of the biosensor to increasing chlorofos concentrations (equal to 0.2, 0.4, 0.6, 0.8 and 1.0 mmol L^{-1}) and a constant acetylthiocholine concentration (0.6 mmol L^{-1}) at a potential of +0.80 V/Ag, AgCl. pH 7, 26 ± 0.5 °C, 1000 rpm. (B, inset) Amperometric response of the biosensor to increasing chlorofos concentrations (equal to 0.2, 0.4, 0.6, 0.8 and 1.0 mmol L^{-1}) and a constant acetylthiocholine concentration (0.6 mmol L^{-1}) at a potential of +0.45 V/Ag, AgCl. pH 7, 26 ± 0.5 °C, 1000 rpm.

3.2. *Kinetic aspects of the acetylcholinesterase inhibition by chlorofos*

A reliable mathematical model predicting the kinetic parameters of the immobilized enzymes was proposed by Shu and Wilson [57], taking advantage of the well-defined diffusion behavior of the rotating disk electrode. It was demonstrated that the steady-state current under mass transport limiting conditions at low substrate concentrations could be expressed by the equation (Eq. (6)):

$$I_s = 0.65zFAD^{2/3}\nu^{-1/6}\omega^{1/2}[S], \qquad (6)$$

identical to the Levich equation for the steady-state current of the conventional rotating disk electrode (with: I_s, steady-state current; z, number of exchanged electrons; F, Faraday constant; A, geometrical area of the electrode; D, diffusion coefficient; ν, kinematic viscosity of the solution; ω, angular electrode rotation rate[1]; $[S]$, substrate concentration).

Under catalysis controlled conditions, the transfer function of the electrode could be presented in the form (Eq. (7)):

$$\frac{I_{\max}}{I_s} = \frac{K_M^{app}}{[S]} + 1, \qquad (7)$$

with $I_{\max}$, maximal steady-state current and K_M^{app}, apparent Michaelis–Menten constant.

As demonstrated in our previous works [38,54], presenting the experimental data in coordinates of Lineweaver-Burk [58], at low substrate concentrations ($[S] < 0.8$ mmol L^{-1}) the acetylthiocholine hydrolysis to thiocholine catalyzed by the immobilized acetylcholinesterase is controlled by the diffusion. Thus, the kinetic parameters apparent Michaelis–Menten constant K_M^{app} and maximal velocity of the enzyme reaction $I_{\max}$ were calculated fitting the model $I_s = I_{\max}[S]/(K_M^{app} + [S])$ (Eq. (7)) to the obtained data in the enzyme controlled domain ($[S] \geq 0.8$ mmol L^{-1}) and analyzed applying nonlinear regression. For this purpose, specialized for enzyme kinetics software was used (GraphPad Prism Version 5.00 for Windows, GraphPad Software, San Diego, California, USA, http://www.graphpad.com). It was found that the maximal velocity of the enzyme reaction did not change in the presence of the inhibitor. In contrast, the value of K_M^{app} increased with the increase of the chlorofos concentration. This type of inhibition is considered as a competitive one, taking into account the criteria, establishing and analyzing the kinetics of the inhibition of the immobilized enzymes i.e., the competitive inhibitors increase K_M^{app} and leave $I_{\max}$ unaffected [56]. This suggestion was confirmed, determining the inhibition constant K_I according to the Dixon method [59], as shown in Fig. 3.

---

[1] ω (rad s^{-1}) = $2\pi n/60$ with n – the rotation rate expressed in rpm.

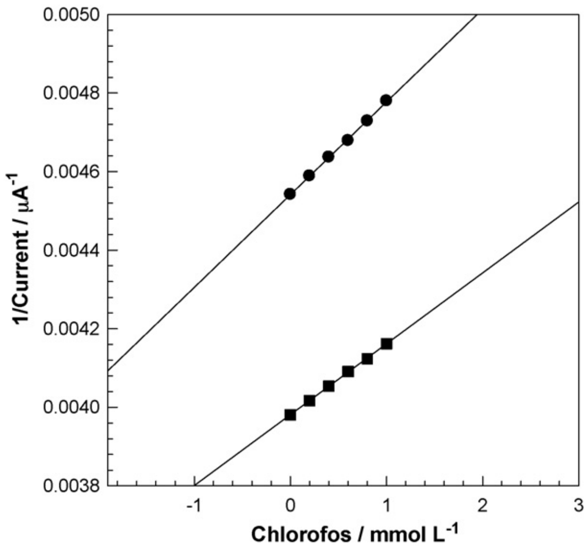


Fig. 3. Dixon plots of the experimental data (pH 7, 26 ± 0.5 °C, 1000 rpm): (1) $[S]=0.8\,\text{mmol}\,\text{L}^{-1}$, $r^2=0.9993$; 2) $[S]=1.0\,\text{mmol}\,\text{L}^{-1}$, $r^2=0.9998$.

The values of the characteristic for the enzyme reactions kinetic parameters I_{max}, $K_{\text{M}}^{\text{app}}$, and K_{I} are shown in Table 1.

Thus, the inhibition of the immobilized acetylcholinesterase by chlorofos could be described by the following kinetic scheme:

$$\text{E}+\text{S}\rightleftharpoons\text{ES}\rightarrow\text{E}+\text{P},\quad K_{\text{M}}^{\text{app}}=\frac{[\text{E}][\text{S}]}{[\text{ES}]}$$

$$\text{E}+\text{Inh}\rightleftarrows\text{EInh},\qquad K_{\text{I}}=\frac{[\text{E}][\text{I}n\text{h}]}{[\text{EI}n\text{h}]}$$

where E is the immobilized acetylcholinesterase, S is the substrate acetylthiocholine, P is the product thiocholine, ES is the enzyme–substrate complex, EInh is the enzyme–inhibitor complex, $K_{\text{M}}^{\text{app}}$ is the apparent Michaelis–Menten constant and K_{I} is the inhibition constant.

The behavior of chlorofos $(\text{CH}_3\text{O})_2\text{POCH(OH)CCl}_3$ as a competitive inhibitor of the acetylcholinesterase could be explained by its structural analogy with the enzyme substrate acetylthiocholine $(\text{CH}_3)_3\text{N}^+(\text{CH}_2)_2\text{SCOCH}_3$. Because the substrate and the inhibitor compete for the same site, the degree of inhibition is reduced with the increase of the substrate concentration, as demonstrated below.

The performed experiments showed that the decrease of the inhibitor concentration by dilution of the analyzed probe, keeping constant the substrate concentration leads to a recovery of the enzyme activity, i.e. the process of inhibition is reversible (Fig. 4).

Table 1
Kinetic parameters of the enzyme reaction, catalyzed by the immobilized acetylcholinesterase in the absence and in the presence of chlorofos.

Chlorofos concentration $(\text{mmol}\,\text{L}^{-1})$	$K_{\text{M}}^{\text{app}}$ $(\text{mmol}\,\text{L}^{-1})$
0.0	1.2763 ± 0.0119
0.2	1.3073 ± 0.0122
0.4	1.3377 ± 0.0149
0.6	1.3327 ± 0.0149
0.8	1.3987 ± 0.0186
1.0	1.4337 ± 0.0222
$I_{\text{max}}=579.30\pm5.71$ (µA)	
$K_{\text{I}}=10.07$ $(\text{mmol}\,\text{L}^{-1})$	

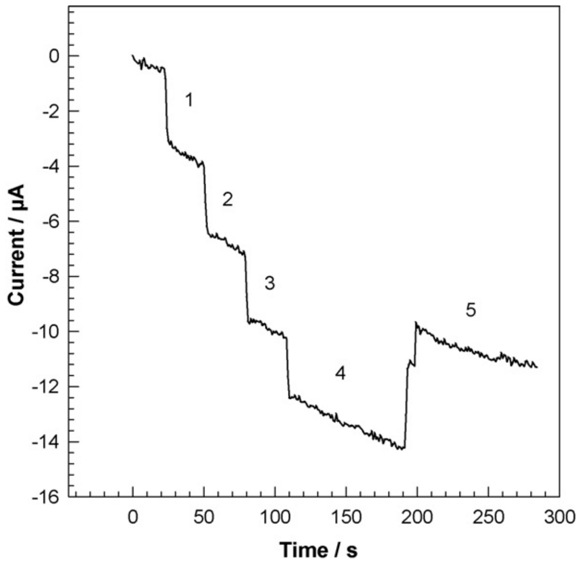


Fig. 4. Reversibility of the inhibition of the immobilized acetylcholinesterase by chlorofos: substrate concentration $0.2\,\text{mmol}\,\text{L}^{-1}$; chlorofos concentrations: (1) $1.0\,\text{mmol}\,\text{L}^{-1}$; (2) $2.0\,\text{mmol}\,\text{L}^{-1}$; (3) $3.0\,\text{mmol}\,\text{L}^{-1}$; (4) $4.0\,\text{mmol}\,\text{L}^{-1}$; (5) $2.0\,\text{mmol}\,\text{L}^{-1}$ (2-fold dilution for the same substrate concentration). pH 7, 26 ± 0.5 °C, 1000 rpm.

3.3. Analytical aspects of the acetylcholinesterase inhibition by chlorofos

As mentioned above, chlorofos determination involves the establishment of the decrease of the acetylcholinesterase activity, expressed as current decrease at a constant substrate concentration. The obtained data were used for the mathematical modeling and the optimizing of the quantification, according to the methodology of design of experiments (DOE). The input parameters of the model were the chlorofos concentration (C) and the acetylthiocholine concentration (S), and the output – the degree of inhibition of the immobilized enzyme (D). The following polynomial model was chosen to describe the dependences between $D=f(C)$ and $D=f(S)$ (Eq. (8)):

$$z=a+bx+c/y+dx^2+e/y^2+fx/y \tag{8}$$

with z, degree of inhibition (%); x, chlorofos concentration $(\text{mmol}\,\text{L}^{-1})$; y, acetylthiocholine concentration $(\text{mmol}\,\text{L}^{-1})$.

The coefficients a, b, c, d, e and f were determined by means of D-optimum composition plan [60]. The independent factors were varied as follows: $0.2<C<1.0$ and $0.2<S<1.0$.

The mathematical analysis of the results led to a suitable model, described by the equation (Eq. (9)):

$$D=-0.552+4.607C+0.448/S-0.785C^2-0.088/S^2+0.722C/S \tag{9}$$

The values of the statistics r^2 and r^2_{adjusted}, respectively equal to 0.9966 and 0.9951 demonstrate that the descriptive power of the model is satisfactory. Fig. 5 exhibits the model in graphic form.

The presented in Fig. 5 data seem to be adequate, demonstrating that the degree of inhibition of the immobilized acetylcholinesterase increases with the increase of the inhibitor concentration and decreases with substrate concentration. The maximization of the model allowed predicting that the optimal acetylthiocholine concentration for chlorofos determination is $0.2\,\text{mmol}\,\text{L}^{-1}$. The sensitivity of the chlorofos determination was found to be $6.89\%\,\text{L}\,\text{mmol}^{-1}$ for $S=0.2\,\text{mmol}\,\text{L}^{-1}$, i.e. with 36.28% more important in comparison to the sensitivity, determined for

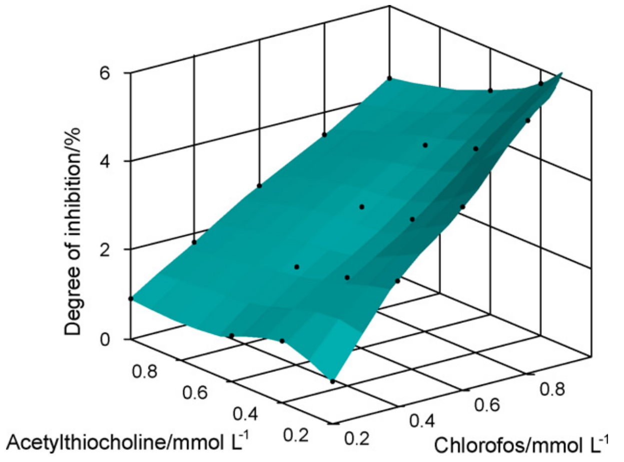


Fig. 5. Degree of inhibition of the immobilized acetylcholinesterase in response to different chlorofos and substrate concentrations. pH 7, 26 ± 0.5 °C, 1000 rpm.

$S = 1.0\ \mathrm{mmol\ L^{-1}}$. Such analytical performances could be expected, taking into account the competitive character of the inhibition [56]. The inhibition could be overcome by high substrate concentrations. That is why, in the latter case, only high chlorofos concentrations could be detected.

The reversibility of the process of inhibition allowed recovering of the enzyme activity by washing the probe with buffer solution, i.e. the multiple use of the biosensor. The R.S.D. of the biosensor response for successive chlorofos determinations ($N = 5$), carried out in the same conditions was found to be 2.87% ($S = 1.0\ \mathrm{mmol\ L^{-1}}$). The well known and simple technique for biosensor fabrication ensured a good reproducibility of the determinations (R.S.D. = 4.22%, $N = 5$, $S = 1.0\ \mathrm{mmol\ L^{-1}}$).

4. Conclusion

The immobilized acetylcholinesterase inhibition by chlorofos was investigated choosing an appropriate electrochemical approach. The main kinetic parameters of the enzyme reaction (K_M^{app}, I_{max}, and K_I) were evaluated to establish the type of the enzyme inhibition. The kinetic analysis demonstrated the competitive character of the process, which fits very well with the increase of the sensitivity of the chlorofos quantification with substrate concentration decrease. The methodology of design of experiments was applied for analysis condition optimization.

Acknowledgements

Marcela Ovalle expresses her thanks to CONACyT, México for the grant received (CVU 228597/Becario 208762) to complete her Ph.D. thesis work (Programa de Maestría y Doctorado en Ciencias e Ingeniería, Instituto de Ingeniería, UABC).

References

[1] G. Matolessy, M. Nadasy, V. Andrisca, Pesticide Chemistry, Studies in Environmental Science, vol. 32, Elsevier, Amsterdam, 1988.
[2] A. Ross, P. Bartley, A. Sleigh, G. Olds, Y. Li, G. Williams, D. McManus, New England J. Med. 346 (2002) 1212.
[3] G. Zweig, J. Sherma, Analytical Methods for Pesticides and Plant Growth Regulators, vol. 6, Academic Press, New York, London, 1972, p. 387.
[4] H. Stan, B. Abraham, J. Jung, M. Kellert, K. Steinland, Z. Fresenius, Anal. Chem. 287 (1977) 271.
[5] H. Stan, Z. Lebensm. Unters. Forsch 164 (1977) 153–159.
[6] J. Guth, Pflanzenschutzberichte 35 (1967) 129.
[7] M. Getz, H. Wheeler, J. Assoc. Off. Anal. Chem. 51 (1968) 1101.
[8] O. Antoine, G. Mees, J. Chromatogr. 58 (1971) 247.
[9] A. Ambru, E. Hargitai, G. Karoly, A. Fulop, J. Lantos, J. Assoc. Off. Anal. Chem. 64 (1981) 743.
[10] G. Szalontai, J. Chromatogr. 124 (1976) 9.
[11] T. Daldrup, F. Susanto, P. Michalke, Z. Fresenius, Anal. Chem. 308 (1981) 413.
[12] T. Daldrup, P. Michalke, W. Boehme, Chromatogr. Newsl. 10 (1982) 1.
[13] J. Diehl-Faxon, A. Ghindilis, P. Atanasov, E. Wilkins, Sens. Actuators B 35–36 (1996) 448.
[14] A. Ghindilis, H. Morzunova, A. Barmin, I. Kurochkin, Biosens. Bioelectr. 11 (1996) 873.
[15] R. Gyurçsányi, Z. Vágföldi, K. Tóth, G. Nagy, Electroanalysis 11 (1999) 712.
[16] N. Jaffrezic-Renault, A. Senillou, C. Martelet, K. Wan, J.-M. Chovelon, Sens. Actuators B 59 (1999) 154.
[17] A. Ivanov, G. Evtugyn, R. Gyurçsányi, K. Tóth, H. Budnikov, Anal. Chim. Acta 404 (2000) 55.
[18] E. Gogol, G. Evtugyn, J.-L. Marty, H. Budnikov, V. Winter, Talanta 53 (2000) 379.
[19] N. Jaffrezic-Renault, Sensors 1 (2001) 60.
[20] V. Arkhypova, S. Dzyadevych, A. Soldatkin, A. El'skaya, N. Jaffrezic-Renault, H. Jaffrezic, C. Martelet, Talanta 55 (2001) 919.
[21] K. Reybier, S. Zairi, N. Jaffrezic-Renault, B. Fahys, Talanta 56 (2002) 1015.
[22] A. Ivanov, L. Lukachova, G. Evtugyn, E. Karyakina, S. Kiseleva, H. Budnikov, A. Orlov, G. Karpacheva, A. Karyakin, Bioelectrochemistry 55 (2002) 75.
[23] S. Solé, A. Merkoçi, S. Alegret, Crit. Rev. Anal. Chem. 33 (2003) 89.
[24] A. Ivanov, G. Evtugyn, L. Lukachova, E. Karyakina, H. Budnikov, S. Kiseleva, A. Orlov, G. Karpacheva, A. Karyakin, IEEE Sens. J. 3 (2003) 333.
[25] M. Snejdarkova, L. Svobodova, G. Evtugyn, H. Budnikov, A. Karyakin, D. Nikolelis, T. Hianik, Anal. Chim. Acta 514 (2004) 79.
[26] S. Dzyadevych, A. Soldatkin, V. Arkhypova, A. El'skaya, J.-M. Chovelon, C. Georgiou, C. Martelet, N. Jaffrezic-Renault, Sens. Actuators B 105 (2005) 81.
[27] M. Shi, J. Xu, S. Zhang, B. Liu, J. Kong, Talanta 68 (2006) 1089.
[28] A. Amine, H. Mohammadi, I. Bourais, G. Palleschi, Biosens. Bioelectr. 21 (2006) 1405.
[29] Jun-Ichi Anzai, Yakugaku Zasshi 126 (2006) 1301.
[30] Z. Talebpour, A. Ghassempour, M. Zendehzaban, H. Bijanzadeh, M. Mirjalili, Anal. Chim. Acta 576 (2006) 290.
[31] S. Andreescu, J.-L. Marty, Biomol. Engin. 23 (2006) 1.
[32] Y. Wei, Y. Li, Y. Qu, F. Xiao, G. Shi, L. Jin, Anal. Chim. Acta 643 (2009) 13.
[33] G. Evtugyn, H. Budnikov, E. Nikolskaya, Talanta 46 (1998) 465.
[34] L. Goldstein, in: K. Mosbach (Ed.), Methods in Enzymology, Acad. Press, NY, 1976, p. 44.
[35] G. Turdean, I. Popescu, L. Oniciu, D. Thevenot, J. Enz. Inh. Med. Chem. 17 (2002) 107.
[36] L. Goodson, W. Jacobs, in: K. Mosbach (Ed.), Immobilized Enzymes 44 (1976) 647.
[37] M. Stoytcheva, Electroanalysis 7 (1995) 560.
[38] M. Stoytcheva, Electroanalysis 7 (1995) 660.
[39] D.Ž. Sužnjević, D.S. Veselinović, N.S. Vukelić, D.Ž. Pavlović, A.V. Nikolić, J. Serb. Chem. Soc. 50 (1985) 83.
[40] J.-L. Marty, N. Mionetto, R. Rouillon, Anal. Lett. 25 (1992) 1389.
[41] J.-L. Marty, N. Mionetto, T. Noguer, F. Ortega, C. Roux, Biosens. Bioelectron. 8 (1993) 273.
[42] D. Martorell, F. Cespedes, S. Alegret, E. Martinez-Faregas, Anal. Chim. Acta 290 (1994) 343.
[43] J.-L. Marty, N. Mionetto, S. Lacorte, D. Barceló, Anal. Chim. Acta 311 (1995) 265.
[44] G. Evtugyn, A. Ivanov, E. Gogol, J.-L. Marty, H. Budnikov, Anal. Chim. Acta 385 (1999) 13.
[45] G. Liu, S. Riechers, M. Mellen, Y. Lin, Electrochem. Commun. 7 (2005) 1163.
[46] P. Skladal, Anal. Chim. Acta 252 (1991) 11.
[47] J. Hart, I. Hartley, Analyst 119 (1994) 259.
[48] M. Harlbert, R. Baldwin, Anal. Chem. 57 (1985) 591.
[49] F. Ricci, F. Arduini, A. Amine, D. Moscone, G. Palleschi, J. Electroanal. Chem. 563 (2004) 229.
[50] D. Martorell, F. Cespedes, E. Fabreagas, S. Alegret, Anal. Chim. Acta 337 (1997) 305.
[51] J. Julis, E. D'costa, Biosens. Bioelectron. 6 (1991) 109.
[52] G. Evtugyn, Yu. Budnikov, E. Galyametdinov, Zh. Suntsov, Anal. Khim. 51 (1996) 424.
[53] T. Neufeld, I. Eshkenazi, E. Cohen, J. Rishpon, Biosens. Bioelectron. 15 (2000) 323.
[54] M. Ovalle, M. Stoytcheva, R. Zlatev, B. Valdez, Z. Velkova, Electrochim. Acta 53 (2008) 6344.
[55] Nomenclature committee of the International Union of Biochemistry (NCIUB), Eur. J. Biochem. 128 (1982) 281.
[56] C. Tran-Minh, Ion-Selective Electrode Rev. 7 (1985) 41.
[57] F. Shu, G. Wilson, Anal. Chem. 48 (1976) 1679.
[58] H. Lineweaver, D. Burk, J. Am. Chem. Soc. 56 (1934) 658.
[59] M. Dixon, Biochem. J. 55 (1953) 170.
[60] V. Fedorov, Theory of Optimal Experiments, Academic Press, New York, 1972.

ANEXO E

Otras Producciones

Los estudios realizados en la presente Tesis Doctoral han dado lugar a la siguiente producción (revistas indexadas, en extenso, ponencias, citas y premios):

ARTICULOS CIENTÍFICOS INDEXADOS

1. M. Stoytcheva, R. Zlatev, Z. Velkova, B. Valdez, and M. Ovalle
Electrochemical Study on the Kinetic Behavior of the Immobilized Acetylcholinesterase
ECS Transactions, 20, 1, 175-184, 2009, ISSN: 1938-6737 online, ISSN: 1938-5862 print

2. M. Ovalle, M. Stoytcheva, R. Zlatev, B. Valdez
Electrochemical study of rat brain acetylcholinesterase inhibition by chlorofos: kinetic aspects and analytical applications
Electrochimica Acta, 55, 516-520, 2009. ISSN: 0013-4686 IF 3.078

3. M. Stoytcheva, R. Zlatev, J.-P. Magnin, M. Ovalle, B. Valdez
Leptospirillum ferrooxidans based Fe2+ sensor
Biosensors and Bioelectronics, 25, 482-487, 2009. ISSN 0956-5663 IF 5.143

4. M. Stoytcheva, R. Zlatev, Z. Velkova, B. Valdez, M. Ovalle, L. Petkov
Hybrid electrochemical biosensor for organophosphorus pesticides quantification
Electrochimica Acta, 54, 1721-1727, 2009 ISSN: 0013-4686, IF 3.078

5. M. Stoytcheva, R. Zlatev, B. Valdez, M. Ovalle
Características analíticas de los biosensores electroquímicos
Portugaliae Electrochimica Acta. 27, 3, 353-362, 2009. ISSN 1647-1571

6. M. Ovalle, M. Stoytcheva, R. Zlatev, B. Valdez, Z. Velkova

Electrochemical study on the type of immobilised acetylcholinesterase inhibition by sodium fluoride.
Electrochimica Acta, 53, 6344-6350, 2008 ISSN: 0013-4686, IF 3.078

7. M. Stoytcheva, R. Zlatev, Z. Velkova, B. Valdez, M. Ovalle
Electrochemical evaluation of the properties of the immobilized enzyme glucose oxidase.
ECS Transactions, 15, 1, 429-438, 2008. ISSN: 1938-6737 online, ISSN: 1938-5862 print

8. M. Stoytcheva, Z. Velkova, R. Zlatev, M. Ovalle, B. Valdez
Bioelectrochemical method of organophosphorous pesticides determination in food samples
Scientific Works of the University of Food Technologies, Plovdiv, 2008, 55, 2, 285-290. ISSN 0477-0250

ARTICULOS EN EXTENSO

1. M. Stoytcheva, R. Zlatev, B. Valdez, M. Carrillo, M. Ovalle.
Methods for biological sensing elements immobilization in electrochemical biosensors.
VI Simposio Internacional: Investigación Química en la Frontera, Noviembre 15-17, 2006. Tijuana. Baja California, Mexico, pp. 247-248

2. M. Ovalle, M. Stoytcheva, R. Zlatev
Cuantificación bioelectroquímica de pesticidas organofosforados
Coloquio de Postgrado, Maestría y Doctorado en Ciencias e Ingeniería, UABC, 2007, pp. 16-24, ISBN 978-970-735-098-4

3. M. Stoytcheva, R. Zlatev, B. Valdez, M. Ovalle
Características analíticas de los biosensores electroquímicos
XVIII Congreso de la Sociedad Iberoamericana de Electroquímica, Medellín, Colombia, 10-14 de marzo de 2008, pp. 1-6.

4. M. Stoytcheva, R. Zlatev, Z. Velkova, B. Valdez, M. Ovalle
Electrochemical evaluation of the properties of the immobilized enzyme glucose oxidase.
XIII Congreso de la Sociedad Mexicana de Electroquímica (SMEQ), Ensenada, Baja California, México, 1-6 de junio de 2008

5. M. Stoytcheva, R. Zlatev, Z. Velkova, M. Ovalle, B. Valdez
Electrochemical study on the kinetic behavior of the immobilized acetylcholinesterase
XIV Congreso de la Sociedad Mexicana de Electroquímica (SMEQ), Puerto Vallarta, Jalisco, México, 31de mayo-5 de junio de 2009. ISBN 978-970-764-739-8

PONENCIAS EN CONGRESOS

1. M. Stoytcheva, R. Zlatev, B. Valdez, M. Carrillo, M. Ovalle.
Methods for biological sensing elements immobilization in electrochemical biosensors.
VI Simposio Internacional: Investigación Química en la Frontera, Noviembre 15-17, 2006. Tijuana. Baja California, Mexico, pp. 247-248

2. M. Ovalle, M. Stoytcheva, R. Zlatev
Cuantificación bioelectroquímica de pesticidas organofosforados
Coloquio de Postgrado, Maestría y Doctorado en Ciencias e Ingeniería, UABC, 2007, pp. 16-24, ISBN 978-970-735-098-4

3. M. Ovalle, M. Stoytcheva, R. Zlatev, B. Valdez
Compartimiento electroquímico de la tiocolina
XIII Simposio en Ciencia de Materiales, UNAM, Centro de Ciencias de la Materia Condensada, Ensenada, Baja California, México, 12-15 de febrero 2008

4. M. Stoytcheva, R. Zlatev, M. Ovalle, B. Valdez
Desarrollo y aplicación de biosensores electroquímicos ambientales
XIII Simposio en Ciencia de Materiales, UNAM, Centro de Ciencias de la Materia Condensada, Ensenada, Baja California, México, 12-15 de febrero 2008

5. M. Stoytcheva, R. Zlatev, B. Valdez, M. Ovalle
Características analíticas de los biosensores electroquímicos
XVIII Congreso de la Sociedad Iberoamericana de Electroquímica, Medellín, Colombia, 10-14 de marzo de 2008, pp. 1-6.

6. M. Stoytcheva, R. Zlatev, Z. Velkova, B. Valdez, M. Ovalle
Electrochemical evaluation of the properties of the immobilized enzyme glucose oxidase.
XIII Congreso de la Sociedad Mexicana de Electroquímica (SMEQ), Ensenada, Baja California, México, 1-6 de junio de 2008

7. M. Stoytcheva, R. Zlatev, M. Ovalle, B. Valdez, Z. Velkova
Organophosphorous electrochemical sensor base on combined bacterial-enzyme membrane
214th ECS Meeting, Honolulu, October 12-17, 2008. ISSN 1091-8213

8. R. Zlatev, M. Stoytcheva, B. Valdez, J. A. Valera, M. Ovalle
High resolution voltammetric techniques: application for direct simultaneous species determination.
214th ECS Meeting, Honolulu, October 12-17, 2008. ISSN 1091-8213

9. M. Stoytcheva, Z. Velkova, R. Zlatev, M. Ovalle, B. Valdez
Bioelectrochemical method of organophosphorous pesticides determination in food samples
Scientific Conference with International Participation: Food Science, Engineering and Technologies 2008. Plovdiv, Bulgaria, October 24-25, 2008.

10. M. Stoytcheva, R. Zlatev, B. Valdez, S. Bratkova, M. Ovalle, M. Schorr
Desarrollo de un sensor bacteriano para el monitoreo de contaminantes ambientales organofosforados
I Congreso Latinoamericano de Biotecnología Ambiental, Xalapa, Veracruz, México, Octubre 5-10, 2008.

11. M. Stoytcheva, R. Zlatev, B. Valdez, M. Ovalle, Z. Velkova, S. Cosnier
Electrochemical biosensors systems for organophosphorus aerosols quantification
215th ECS Meeting, San Francisco, USA, May 24-29, 2009. ISSN 1091-8213

12. M. Stoytcheva, R. Zlatev, Z. Velkova, M. Ovalle, B. Valdez
Electrochemical study on the kinetic behavior of the immobilized acetylcholinesterase
XIV Congreso de la Sociedad Mexicana de Electroquímica (SMEQ), Puerto Vallarta, Jalisco, México, 31de mayo-5 de junio de 2009. ISBN 978-970-764-739-8

13. M. Ovalle, M. Stoytcheva
Characterization and application of electrochemical environmental biosensors
Central Regional Meeting of the American Chemical Society (CERMACS), USA, Cleveland, Ohio, May 20-23, 2009

14. M. Stoytcheva, R. Zlatev, M. Ovalle, B. Valdez
Limit of detection improvement of bacterial biosensors for Fe2+ and Cr6+ determination.
216th ECS Meeting, Vienna, Austria, October 4-9, 2009.

PREMIOS Y DISTINCIONES

Primer lugar en el concurso de carteles obtenido con el trabajo: “Methods for biological sensing elements immobilization in electrochemical biosensors”, M. Stoytcheva, R. Zlatev, B. Valdez, M. Carrillo, M. Ovalle, presentado en el marco de las actividades del VI Simposio Internacional: Investigación Química en la Frontera, Noviembre 15-17, 2006. Tijuana. Baja California, México.

CITAS A ARTÍCULOS EN REVISTAS

Hybrid electrochemical biosensor for organophosphorus pesticides quantification”: Margarita Stoytcheva, Roumen Zlatev, Zdravka Velkova, Benjamín Valdez, **Marcela Ovalle**, Lubomir Petkov. Electrochimica Acta, Volume 54, Issue 6, 15 February 2009, Pages 1721–1727. ISSN: 0013-4686, IF 3.078

1. Jingxia Qiu, Jin Chen, Qianqian Ma, Yuqing Miao, Development of square wave voltammetry method for the assessment of organophosphorus compound impact on the cholinesterase of pheretima with 2,6-dichloroindophenol as a redox indicator, Chemosphere, Volume 77, Issue 1, September 2009, Pages 129-132, ISSN 0045-6535.

2. Xiangling Ren, Fangqiong Tang, Rui Liao, Lin Zhang, Using gold nanorods to enhance the current response of a choline biosensor, Electrochimica Acta, Volume 54, Issue 28, 1 December 2009, Pages 7248-7253, ISSN 0013-4686.

“*Electrochemical study on the type of immobilized acetylcholinesterase inhibition by sodium fluoride*”. **M. Ovalle,** M. Stoytcheva, R. Zlatev, B. Valdez, Z. Velkova. Electrochimica Acta, Volume 53, Issue 22, 20 September 2008, Pages 6344-6350. ISSN: 0013-4686, IF 3.078.

1. F. Arduini, A. Amine, D. Moscone, G. Palleschi. Reversible Enzyme Inhibition-Based Biosensors: Applications And Analytical Improvement Through Diagnostic Inhibition; Analytical Letters 42 (9): 1258-1293 2009

2. Kai Sun, Jingxia Qiu, Keming Fang, Wangyao Zhang, Yuqing Miao, Square wave voltammetry assay of organophosphorus inhibition on cholinesterase in two phases of isooctane/water, Electrochemistry Communications, Volume 11, Issue 5, May 2009, Pages 1022-1025, ISSN 1388-2481

3. Jingxia Qiu, Jin Chen, Qianqian Ma, Yuqing Miao, Development of square wave voltammetry method for the assessment of organophosphorus compound impact on the cholinesterase of pheretima with 2,6-dichloroindophenol as a redox indicator, Chemosphere, Volume 77, Issue 1, September 2009, Pages 129-132, ISSN 0045-6535.