Universitat Autònoma de Barcelona

BIOSISTEMES ANALÍTICS INTEGRATS PER A APLICACIONS INDUSTRIALS I MEDIAMBIENTALS

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Tesi Doctoral

Programa de Doctorat en Química

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Grup de Sensors i Biosensors

Memòria presentada per aspirar al Grau de Doctor per Xavier Llopis i Gonzàlez

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Resum

En el marc d'aquesta tesi s'han desenvolupat tres biosistemes analítics integrats que mostren: 1) la millora substancial que suposa realitzar un procés d'integració, encara que aquest sigui parcial, en el rendiment analític de les metodologies proposades; 2) com la miniaturització, en diferents graus, permet augmentar de forma significativa la robustesa, la sensibilitat o la selectivitat dels sistemes proposats; i 3) noves solucions a la problemàtica comuna dels biosensors i biosistemes analítics consistent en la pèrdua d'activitat de l'element biològic.

El primer treball descriu la construcció d'un biosensor amperomètric per a glucosa basat en un biocompòsit de grafit i resina epoxi. L'enzim glucosa oxidasa (GOD) i la sal orgànica conductora tetratiafulvalé-tetracianoquinodimetà (TTF·TCNQ) s'incorporen al compòsit per formar un biosensor renovable. S'avaluen diferents proporcions grafit/TTF·TCNQ/GOD per tal d'optimitzar el sensor pel que fa a les seves prestacions analítiques i posteriorment integrar-lo en un sistema d'anàlisi per injecció en flux (FIA), amb una cel·la dissenyada especialment a tal efecte. Els millor resultats en la determinació de glucosa en begudes refrescants s'obtenen amb una composició de 5% de GOD, 76% de polímer, 9.5% de grafit i 9.5% de TTF·TCNQ.

El segon treball descriu el disseny, construcció i avaluació d'un analitzador miniaturitzat basat en la tecnologia de ceràmiques verdes (Low temperature co-fired ceramics, LTCC) per a la

determinació de pesticides, que integra una etapa de pretractament mitjançant dos mescladors tridimensionals i un sistema de detecció amperomètrica per mesurar el producte de una reacció d'inhibició enzimàtica. El sistema de detecció s'integra de forma monolítica a la plataforma de fluídica, i consisteix en dos làmines de platí com a elèctrodes de treball i auxiliar, i d'un elèctrode de referència serigrafiat. El sistema és caracteritzat i avaluat satisfactòriament per la determinació de carbofuran a nivell nanomolar.

Per últim, es descriu un mètode ultrasensible per determinar toxicitat deguda a pesticides en un lab-on-a-chip de vidre mitjançant la inhibició de l'enzim acetilcolinesterasa immobilitzat sobre partícules magnètiques. S'optimitzen la introducció de forma reproduïble d'una quantitat controlada de partícules modificades amb l'enzim al canal del xip i la seva captura en una zona determinada mitjançant l'ús d'un camp magnètic extern. Aquest procediment permet de forma fàcil i altament reproduïble la renovació del material biosensor després de cada determinació. S'avaluen o optimitzen el potencial de treball per a la detecció selectiva de tiocolina en un elèctrode platí, la reproductibilitat i sensibilitat en la detecció de tiocolina, el voltatge del flux electroosmòtic i el temps de la reacció d'inhibició. S'aconsegueix la determinació del pesticida carbofuran (un dels pesticides carbamats més tòxics) a nivell nanomolar.

Abstract

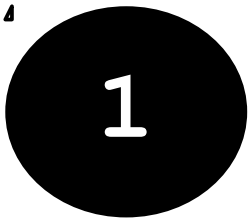
Three integrated analytical biosystems have been developed within the scope of this thesis, showing: 1) the substantial improvement in the analytical performance of the proposed methodologies thanks to the Integration process; 2) how miniaturisation provides significant increases of the robustness, sensitivity and selectivity of the proposed systems; and 3) new solutions to the loss of activity of the biological element of biosensors and analytical biosystems.

The first work describes how an amperometric glucose biosensor based on graphite and non-conducting epoxy resin biocomposite was constructed. Glucose oxidase (GOD) and the tetrathiafulvalene-tetracyanoquinodimethane (TTF•TCNQ) conducting organic salt were incorporated into the bulk of the composite to form a renewable biosensor. Several graphite-TTF•TCNQ ratios (w/w) were studied in order to select the best biosensor to be integrated in a FIA system for the automated detection of glucose. The optimal amount of GOD in the composite was studied as well. The selection was based on the analytical response of the electrodes. Best results were obtained by an electrode whose composition was 5% GOD, 76% polymer, 9.5% graphite and 9.5% TTF•TCNQ. An especially designed flow amperometric cell was constructed so that the biosensor could be integrated into a FIA system and glucose in beverage samples could be determined.

In the second work the design, construction and evaluation of a miniaturized analyser for pesticides determination that integrates a pre-treatment stage, based on two three-dimensional mixers or reactors, and an amperometric detection system to measure the product of an enzymatic inhibition reaction are presented. The detection system was monolithically integrated in the microfluidic platform and it consisted of a screen-printed reference electrode and two platinum sheets, acting as auxiliary and working electrodes, which were embedded within the ceramic structure. The miniaturized system was characterized and successfully evaluated by determining carbofuran at nanomolar level.

Finally, in the third work an ultrasensitive method to determine toxicity due to pesticides in a glass lab-on-a-chip by means of enzymatic inhibition of acetylcholinesterase immobilised on magnetic beads is described. The reproducible insertion of a controlled amount of enzyme-coupled magnetic beads inside the chip channel and their immobilisation in a capture region with the aid of a magnetic field has been optimised. This procedure enables the easy renewal of the biosensing material after each determination in a highly reproducible manner. Several operational parameters such as the working potential for the selective detection of thiocholine (TCh) on a platinum disc electrode, the TCh detection reproducibility and sensitivity, the electroosmotic flow driving voltage and the inhibition time were also evaluated or optimised. The detection of carbofuran (one of the most toxic carbamate pesticides) has been achieved down to the nanomolar level.

TAULA DE CONTINGUTS



INTRODUCCIÓ GENERAL

1.1 Sistemes Analítics Integrats

En el context de la instrumentació analítica, un sistema analític es defineix com un conjunt de dispositius que formen un tot, i que permeten obtenir informació química seguint una seqüència de diferents etapes ben establerta. Aquestes etapes no són altres que les habituals de qualsevol procés analític: el mostreig, el transport i processament de la mostra, la separació, la reacció, la transducció, i l'adquisició i el processament del senyal. La majoria dels sistemes analítics es dissenyen seguint totalment o en part aquestes operacions, establint una seqüència ben definida per a la òptima resolució del problema analític objecte d'estudi.

La simplificació, automatització i miniaturització de la instrumentació i de les etapes del procés analític són des de fa dues dècades les tendències predominants tant a nivell acadèmic i recerca com industrial, ja que permeten la obtenció de metodologies i dispositius més eficients, robustos, barats, sensibles, acurats i versàtils. De forma genèrica, una manera efectiva d'aconseguir aquestes millores és mitjançant la **integració** (acció o efecte d'unir part per aconseguir un tot), que en el context de la tecnologia només té sentit si el tot resultant presenta unes funcions i unes característiques que milloren d'alguna manera significativa les prestacions de les parts constituents per separat.

Un **sistema analític integrat** (IAS, Integrated Analytical System)[1], idealment, estaria constituït per un reduït número d'elements capaços de realitzar les operacions unitàries bàsiques que requereix el problema analític. L'objectiu del procés d'integració és el de conferir connectivitat a aquestes etapes, de manera que es minimitzi tant la mida del dispositiu final com la intervenció humana. Com a resultat, com ja s'ha esmentat, s'aconsegueixen tant la reducció de costos (consum de reactius, necessitat de personal especialitzat, temps d'anàlisi), com l'increment de la robustesa i fiabilitat del mètode.

Cada IAS porta associades una sèrie de característiques que el defineixen i donen una mesura del seu potencial:

- El número d'unitats que constitueixen tot el sistema.
- El tipus d'integració (relació, interconnexió, comunicació, confinament espacial).
- Les noves funcions generades.
- L'objectiu del sistema complert.

Gràcies a la integració es poden implementar nous procediments i instruments analítics obrint millors perspectives en la simplificació, miniaturització, automatització, comunicació, informació, qualitat, rapidesa, portabilitat i cost de les anàlisis químiques. Algunes de les formes més habituals d'integració es troben resumides a la Taula 1.1-1.

Taula 1.1-1 Diferents formes i nivells d'integració[1]

Sistema	Elements integrats	Relació entre elements integrats	Exemples
Reactius immobilitzats	Reactius i suports	Continuïtat	Tires analítiques, reactors, columnes cromatogràfiques
Sensors químics	Receptors i transductors	Continuïtat	Quimiosensors i biosensors

Sistemes d'anàlisi en flux	Operacions analítiques, sensors i actuadors	Canals de fluídica	Cromatografia líquida o gasosa, analitzadors de flux en continu, analitzadors automatitzats, sistemes totals d'anàlisi (TAS)
Sistemes de laboratori	Mòduls analítics, estacions de treball i instruments	Interfícies de comunicació	Sistemes de gestió de la informació dels laboratoris (LIMS), estacions analítiques robotitzades
Sistemes analítics distribuïts	Instruments analítics distribuïts	Interfícies de comunicació	Instruments analítics en xarxa
Microsistemes analítics	Operacions analítiques i dispositius confinats en un espai micromètric	Plataformes i microcanals de flux monolítics	Microsistemes totals d'anàlisi (uTAS), laboratori en un xip (LoC)
Nanosistemes	Dispositius atòmics i moleculars confinats en un espai nanomètric	Forces o enllaços atòmics o moleculars	Nanosensors, nanosondes

En el marc d'aquesta tesi, s'han desenvolupat tres sistemes analítics integrats que tenen en comú els següents objectius:

1. Demostrar la millora substancial que suposa realitzar un procés d'integració, encara que aquest sigui parcial, en el rendiment analític de les metodologies proposades.
2. Demostrar com la miniaturització, en diferents graus, permet augmentar de forma significativa la robustesa, la sensibilitat o la selectivitat dels sistemes proposats.
3. Aportar solucions a la problemàtica comuna dels biosensors i biosistemes analítics consistent en la pèrdua d'activitat de l'element biològic. Es proposen i demostren tres sistemes diferents de recuperar aquesta activitat.

La present introducció pretén fer una breu descripció de l'estat de l'art en cada un dels processos d'integració que s'han utilitzat: biosensors de glucosa, tècnica FIA convencional i modular (basada en ceràmiques verdes, LTCC), i ús combinat de partícules magnètiques com a suport per a la immobilització de reactius i lab-on-a-chip.

1.2 Sensors i Biosensors

Els **sensors** es poden definir com uns dispositius analítics que permeten fer mesures de paràmetres físic, químics o biològics, i es caracteritzen per ser simples (poden ser emprats per personal no especialitzat), portàtils (i per tant robustos, de dimensions reduïdes i amb capacitat de ser utilitzats fora del laboratori), i són capaços de generar informació de tipus discreta (sensors d'un sol ús etc.) o en temps real, permetent l'obtenció d'informació en continu i, eventualment, poden ser implementats en sistemes de flux (industrials), al cos humà (implantats o de tipus "wearable"), per al control de la seguretat dels aliments, etc.

Una particularitat intrínseca dels sensors químics és el fet que integren en una petita porció d'espai la major part de les etapes dels procediments analítics tradicionals, reduint-les a dues: el reconeixement i la transducció. La primera etapa correspon a la codificació de la quantitat d'analit present en la mostra en un senyal primari (de tipus òptic, elèctric, màssic o tèrmic), originat per la interacció selectiva d'un element de reconeixement molecular amb l'analit. En la segona etapa es produeix la conversió del senyal primari, mitjançant un transductor que el transforma en un senyal secundari de tipus elèctric fàcilment mesurable i interpretable.

Un **biosensor** pot ser definit breument com un sensor químic que conté un element biològic o biomimètic com a element de reconeixement. Les dues propietats més importants dels biosensors són l'especificitat i la sensibilitat enfront l'analit. L'especificitat és una conseqüència de la capacitat del component biològic per reaccionar només amb l'analit d'interès. La sensibilitat depèn de l'eficàcia de la detecció d'aquesta reacció específica per

part del transductor. Els materials biològics utilitzats en els desenvolupament de biosensors es poden dividir en quatre grups principals:

- Enzims: són proteïnes que catalitzen una determinada reacció, transformant de manera selectiva uns substrats determinats en productes/metabòlits fàcils de detectar.
- Anticossos: són proteïnes del tipus globulines, que són generades pel sistema immunològic dels organismes animals en resposta a una substància estranya, anomenada antigen. En la majoria dels casos un anticòs reconeix un únic antigen.
- Àcids nucleics: l'ADN està format per dues cadenes simples que tenen la particularitat de ser complementàries.
- Aptàmers: es consideren anticossos sintètics i representen sondes de DNA hibridades (i seleccionades mitjançant un procés anomenat 'selex') en part creant unes estructures capaces de detectar proteïnes i altres analits.
- Receptors químics: són proteïnes cel·lulars que s'uneixen específicament a determinats compostos químics, ocasionant-se un canvi conformacional en l'estructura de la proteïna.

En el desenvolupament i construcció dels biosensors el component biològic ha de ser immobilitzat en íntim contacte amb el transductor. L'elecció d'una tècnica particular d'immobilització depèn de diverses variables: de la naturalesa del bioelement, dels mediadors i estabilitzadors químics o bioquímics, de la superfície transductora, de les condicions d'emmagatzematge del sensor i del medi on s'aplicarà el biosensor. La condició indispensable que ha de complir tota tècnica d'immobilització és que l'activitat del material biològic no es vegi afectada significativament i, a més, és convenient que la cinètica involucrada en l'accessibilitat del centre actiu per part de l'analit es vegi afavorida.

Les tècniques generals d'immobilització es poden classificar en cinc grups:

- Retenció mitjançant una membrana inerta.
- Adsorció física.

- Retenció o copolimerització en una matriu polimèrica.
- Entrecreuament mitjançant agents bifuncionals.
- Enllaç covalent amb el transductor.

1.2.1 RECUPERACIÓ DE LA BIOACTIVITAT

Un dels inconvenients dels biosensors és el fet que l'activitat del bioelement sovint disminueix en utilitzacions successives del dispositiu, situació condicionada principalment per la seva interacció amb l'analit, que pot ser de caràcter irreversible, o bé perquè el contacte continuat amb la matriu de la mostra redueix la quantitat efectiva de material biològic actiu.

La regeneració de les superfícies dels biosensors és una de les principals novetats que, a part de recuperar la bioactivitat, permet evitar els inconvenients deguts a l'existència de senyals de memòria de les mesures prèvies, els efectes de la desnaturalització o pèrdua de l'element biològic i la passivació de la superfície del transductor. En general es poden descriure dues estratègies per recuperar la bioactivitat del dispositiu: mètodes que es basen en una regeneració química (fer que el material torni a ser actiu) i mètodes que consisteixen en renovar al material per un de fresc, que no hagi tingut contacte amb la mostra.

1.2.1.1 Regeneració mitjançant reactius químics

Consisteix en recuperar la bioactivitat mitjançant variacions de pH o mitjançant la utilització de reactius específics que aconsegueixen desplaçar l'analit del centre actiu del component biològic. Sempre s'intenta conservar la capacitat de reconeixement del material immobilitzat i les característiques sensores del transductor, però sovint això no és possible degut a les condicions extremes a les quals es sotmet el biosensor.

1.2.1.2 Regeneració mitjançant biosensors d'un sol ús

Consisteix en aprofitar la possibilitat d'utilitzar biosensors construïts mitjançant tècniques de producció massiva i configuració planar (thin film, thick film). Aquestes tècniques,

especialment la de capes gruixudes o thick film, permeten la construcció de sensors de forma massiva amb una infraestructura mínima i amb una molt bona reproductibilitat entre els diferents sensors d'un mateix lot de fabricació.

1.2.1.3 Regeneració mitjançant membranes bescanviables

El material biològic s'immobilitza en una membrana sintètica fàcilment manipulable i adaptable a la superfície del transductor. Després de cada anàlisi aquesta membrana es substitueix per una de nova.

1.2.1.4 Regeneració mitjançant l'ús de biocompòsits

Aquesta tècnica és el resultat de grans esforços duts a terme en el camp dels biosensors amb l'objectiu d'adaptar el disseny clàssic, construït de forma manual, a configuracions compatibles amb la fabricació industrial.[2] L'estratègia més habitual es basa en elèctrodes modificats en què una fase conductora es troba dispersa en una matriu polimèrica. Aquests dispositius combinen les propietats elèctriques del grafit, nanotubs de carboni, grafè (o altres materials conductors)[2–4] amb la facilitat de processament dels plàstics, obtenint-se dispositius a baix preu.

Aquesta tècnica de construcció presenta un seguit d'avantatges: flexibilitat pel que fa a la composició de la matriu sensora, possibilitant la integració de material biològic, cofactors, mediadors, additius, etc.; augment de la sensibilitat degut al contacte íntim que s'estableix entre el material biològic i el material conductor; i versatilitat pel que fa a les formes i dimensions en el moment de la seva construcció, facilitant la seva aplicació. Però l'avantatge més important pel que fa a la recuperació de la bioactivitat és la possibilitat que ofereixen aquests dispositius d'obtenir superfícies fresques reproduïbles per a cada mostra mitjançant un simple poliment de la superfície. Si tots els components que formen el biocompòsit estan distribuïts de forma uniforme en tot el volum de la pasta sensora, cada nova superfície proporciona resultats reproduïbles. A més a més, presenten una elevada estabilitat.

El desenvolupament d'elèctrodes basats en materials compòsits ha aportat importants avenços en el camp dels biosensors. Aquests compòsits conductors rígids combinen les propietats elèctriques del material conductor (grafit, nanotubs de carboni, graf)[4,5] amb la facilitat de manipulació dels plàstics, amb excel·lents propietats físiques, mecàniques, electroquímiques i econòmiques.[2,6] A més, permeten la incorporació d'un gran nombre de materials biològics[3], de manera que el material transductor serveix a la vegada de reservori de l'element de reconeixement. Així, s'ha desenvolupant sensors enzimàtics,[7–10] immunologics[11,12] i genètics.[13,14]

1.2.2 BIOSENSORS AMPEROMÈTRICS ENZIMÀTICS

Indubtablement, la detecció electroquímica és el més emprat de tots els mètodes de transducció del senyal primari generat per la interacció de l'element de reconeixement amb l'analit. Destaquen els sensors amperomètrics i els sensors potenciomètrics. Les dues tècniques es diferencien en que, en el cas dels sensors amperomètrics, cal aplicar un potencial extern que provoqui la reacció electroquímica utilitzada per detectar i quantificar (mesurant el corrent elèctric produït) l'analit, mentre que en els potenciomètrics es provoca un equilibri químic local en la superfície sensora, mesurant el potencial generat entre l'elèctrode i la solució.

Els sensors amperomètrics es basen en la mesura de la intensitat resultant de la oxidació o reducció d'una espècie electroactiva en la superfície de l'elèctrode sotmesa a un potencial constant. L'elecció adequada d'aquest potencial aporta certa selectivitat electroquímica, ja que només permet l'oxidació/reducció de les espècies químiques que reaccionen a un potencial inferior a l'aplicat.

La intensitat de corrent és una mesura directa de la velocitat de la reacció electroquímica descrita per la llei de Faraday. Aquesta velocitat de reacció depèn de la velocitat de transferència dels electrons entre la superfície de l'elèctrode i les espècies electroquímiques; de les característiques de l'elèctrode i del transport de massa de l'analit a la superfície, que al mateix temps depèn de la concentració de l'analit a la solució (C_A); de l'àrea de l'elèctrode;

i de les condicions de difusió, migració i convecció. Si la migració es redueix mitjançant la presència d'un electròlit inert i la convecció es manté constant amb una agitació controlada, es crea una zona propera a l'elèctrode anomenada capa de difusió, en què el transport de matèria és degut únicament a la difusió i, aleshores, la intensitat de corrent compleix l'expressió:

$$I = \frac{nFADC_A}{\delta}$$

on n és el número d'electrons que participen en el procés, A és l'àrea de l'elèctrode de treball, F és la constant de Faraday, D és el coeficient de difusió de les espècies electroactives de concentració C_A a la mostra i δ és el gruix de la capa de difusió. Cal assumir que la concentració de l'analit en la superfície de l'elèctrode és un valor molt proper a zero, situació que s'aconsegueix aplicant un sobrepotencial respecte el potencial redox per maximitzar la velocitat de transferència de carrega heterogènia. Sota aquestes condicions, l'equació anterior es pot simplificar a:

$$I = KC_A$$

Les mesures del senyal amperomètric es realitzen utilitzant sistemes de tres elèctrodes: el de treball, sobre el qual es produeix la reacció electroquímica indicadora; l'auxiliar, que actua de contraelèctrode i tanca el circuit; i el de referència que, connectat a un potenciostat, manté i assegura que el potencial aplicat sigui el desitjat i constant.

Els avantatges de la tècnica amperomètrica es resumeixen en els següents:

- Hi ha una relació directa entre la magnitud física observada (intensitat de corrent) i la concentració de l'espècie d'interès.
- Les reaccions es produeixen en la superfície de l'elèctrode i, per tant, són independents del volum.
- És una tècnica analítica sensible, simple i alhora robusta i econòmica.
- S'eviten interferències de tipus físic (terbolesa, coloració, viscositat).
- Els materials utilitzats són simples i accessibles.

La mesura d'intensitats és, a efectes pràctics, una mesura de la velocitat de consum d'una determinada espècie. Quan s'utilitza un enzim com element de reconeixement molecular, la catàlisi enzimàtica és la que controla el procés de resposta del biosensor. La velocitat de consum d'un determinat substrat per part del corresponent enzim ve expressada per l'equació de Michaelis-Menten:

$$V = \frac{V_{màx} C_S}{K_m + C_S}$$

on $V_{màx}$ és la velocitat màxima de la reacció, K_m és la constant de Michaelis-Menten i CS és la concentració de substrat.

Si substituïm els valors de V i $V_{màx}$ d'aquesta equació pels valors de I i $I_{màx}$, s'obté una equació equivalent:

$$I = \frac{I_{màx} C_S}{K_m^{ap} + C_S}$$

En aquest cas I és el valor de la intensitat faradaica que circula per l'elèctrode indicador, $I_{màx}$ és el valor màxim de la intensitat que pot assolir el sistema i K_m^{ap} és la constant aparent de Michaelis-Menten.

1.2.3 BIOSENSORS AMPEROMÈTRICS PER A GLUCOSA

La idea d'un elèctrode enzimàtic per a glucosa fou proposada per Clark i Lyons el 1962.[15] El primer dispositiu consistia en una fina capa de l'enzim glucosa oxidasa (GOD) atrapada sobre un elèctrode d'oxigen mitjançant una membrana de diàlisi semipermeable. Aquest elèctrode mesurava el consum d'oxigen per la reacció catalitzada per l'enzim:

$$glucosa + O_2 \xrightarrow{GOD} \text{àcid glucònic} + H_2O_2$$

Al 1973 Guilbault i Lubrano[16] descriuen un elèctrode enzimàtic per determinar glucosa en sang basat en la monitorització amperomètrica del peròxid d'hidrogen generat. Des

d'aleshores s'ha descrit un gran nombre d'elèctrodes amperomètrics enzimàtics, que difereixen en el disseny o material de l'elèctrode, la composició de la membrana o l'estratègia d'immobilització.[17–20]

1.2.3.1 Biosensors per a glucosa de primera generació

La primera generació de sensors per a glucosa es basa en l'ús de l'oxigen dissolt de forma natural en la mostra com a cosubstrat de la reacció enzimàtica i en la generació i detecció de peròxid d'hidrogen. El mecanisme de catàlisi inclou la reducció del grup flavina (FAD) en l'enzim per reacció amb la glucosa, i la seva posterior oxidació a $FADH_2$ mitjançant l'oxigen molecular dissolt en el medi.

La mesura amperomètrica del peròxid d'hidrogen és molt senzilla, mitjançant un elèctrode (generalment de platí) a un potencial anòdic de +0.6 V (vs. Ag/AgCl). A aquest potencial, però, moltes altres espècies presents en la mostra també són electroactives (àcid ascòrbic, àcid úric, certs medicaments), i per tant potencials interferents. A finals dels anys 80 bona part de l'activitat acadèmica en aquest àmbit anava encaminada a minimitzar els efectes d'aquests interferents. A la literatura es poden trobar incomptables aproximacions al problema, que es poden englobar en tres grans grups: a) ús de recobriments permselectius per minimitzar l'accés dels interferents a la superfície de l'elèctrode; b) reducció del sobrepotencial necessari a l'elèctrode per detectar de forma sensible el peròxid d'hidrogen; c) combinació de les dues anteriors.

Per exemple, s'han utilitzat diferents polímers i multicapes amb diferents propietats de transport basades en la càrrega, la mida o la polaritat per bloquejar el pas de compostos electroactius, i a la vegada confinar la GOD en contacte íntim amb el transductor. Films electropolimeritzats com la poli(fenilendiamina) el polifenol i el polipirrol es poden dipositar amb gran precisió sobre superfícies extremadament petites i amb geometries complexes.[21–23] Altres materials utilitzats són els films d'acetat de cel·lulosa,[24] els ionòmers Nafion o Kodak AQ,[25] i capes lipídiques hidrofòbiques.[26]

Pel que fa a la reducció del sobrepotencial per a la detecció amperomètrica del peròxid d'hidrogen, els materials més utilitzats en la construcció dels transductors han estat els basats en complexes metall-hexacianoferrat, com el Blau de Prússia.[27–31] De forma semblant, el carboni metal·litzat, per exemple amb rodi o ruteni,[32–34] permet la detecció del peròxid d'hidrogen a potencials al voltant dels 0.0 V, on la majoria d'interferents no són detectats. Si a més es combinen les dues aproximacions, com per exemple dispersant partícules de rodi en una pel·lícula de Nafion,[35] s'obtenen millores addicionals. Posteriorment es va estendre l´ús de nanotubs de carboni,[4,36] que presentaven una millora significativa en el comportament electroquímic del peròxid d'hidrogen sobre l'elèctrode, possiblement degut a la presència d'impureses metàl·liques.

Un segon inconvenient dels sensors per a glucosa de primera generació és la seva dependència de la concentració d'oxigen dissolt en la mostra, ja que habitualment aquesta és 1 ordre de magnitud inferior a la de la glucosa. Per resoldre-ho s'han proposat diferents alternatives encaminades a facilitar l'accés de l'oxigen a l'elèctrode en comparació a la glucosa, com ara pel·lícules limitadores del transport de massa[37–39] o elèctrodes basats en pasta de carboni enriquida amb oxigen.[40,41]

1.2.3.2 Biosensors per a glucosa de segona generació

En els sensors per a glucosa de segona generació l'oxigen és substituït per una altra espècie electroactiva capaç de transferir electrons des del centre redox de l'enzim fins a la superfície del transductor. Idealment, aquests mediadors han de ser capaços de reaccionar ràpidament amb l'enzim reduït, la seva cinètica heterogènia ha de ser reversible, el sobrepotencial de regeneració de la seva forma oxidada ha de ser baix i independent del pH, les dues formes (oxidada i reduïda) han de ser estables i la forma reduïda no ha de reaccionar amb l'oxigen.[42] Malgrat tots aquest requeriments teòrics, la majoria de mediadors usats tradicionalment (ferrocè i els seus derivats, tetratiafulvalé, alguns pigments orgànics, ferricianur) són solubles en com a mínim un dels seus estats redox. Això fa necessari l'ús de sistemes de retenció com ara membranes, polimeritzacions o electrodeposicions.

Com a resultat de la utilització d'aquest mediadors, la mesura esdevé pràcticament independent de la pressió parcial d'oxigen i es pot dur a terme a potencials prou baixos com per no provocar reaccions interferents. Actualment la majoria de dispositius comercials per al control de glucosa en sang utilitzen algun tipus de mediador.[20]

Una altra manera d'afavorir la transferència electrònica entre el centre redox de l'enzim i el transductor consisteix en connectar-los directament creant una mena de cable conductor. Per exemple, amb un esquelet polimèric hidròfil de poli(vinilpiridina) o de poli(vinilimidazol) amb complexos d'osmi entrellaçats.[43,44] El polímer penetra i s'enllaça amb l'enzim reduint significativament la distància entre els seus centres redox i el de l'enzim (FAD). Així es facilita enormement el pas d'electrons entre el grup FAD i l'elèctrode, oferint corrents elevades i per tant major sensibilitat. De forma semblant, l'ús de nanomaterials com nanotubs de carboni o AuNPs permeten afavorir la transferència electrònica entre el centre redox de l'enzim i el transductor.[45]

1.2.3.3 Biosensors per a glucosa de tercera generació

Les sals orgàniques conductores com el tetratiafulvalé-tetracianoquinodimetà (TTF·TCNQ) permeten la transferència directa d'electrons amb diversos enzims, particularment flavoproteïnes, a través del seu grup prostètic. El NADH també pot ser oxidat sobre sals orgàniques, de manera que aquestes també es poden utilitzar en combinació amb deshidrogenases. De tota manera el mecanisme de transferència electrònica no és del tot clar, però s'han proposat explicacions com la mediació homogènia per part de petites quantitats de TTF·TCNQ dissolt,[46] catàlisi heterogènia[47] i transferència directa d'electrons, on la sal actua directament com a transductor en el que són els biosensors per glucosa de tercera generació.[48]

El TTF·TCNQ s'ha incorporat com a part de membranes biosensores de diferents maneres. Es pot trobar empaquetat amb l'enzim immobilitzat a la seva superfície,[49,50] adsorbit[51] o fet créixer sobre la superfície de films.[52–54] Totes aquestes tècniques són o difícils de realitzar per personal no qualificat o requereixen massa temps per a poder ser considerades per a la

producció en massa de biosensors. La seva incorporació a una matriu de grafit, resina epoxi i GOD mitjançant una simple barreja i posterior enduriment permet obtenir de forma ràpida i senzilla un biocompòsit conductor rígid amb excel·lents propietats analítiques i funcionals.

El primer treball d'aquesta tesi consisteix en la construcció i avaluació d'un biosensor per a glucosa de tercera generació basat en un biocompòsit que integra l'enzim GOD i la sal orgànica TTF·TCNQ. Posteriorment aquest biosensor s'integra en un sistema FIA mitjançant una cel·la dissenyada a tal efecte que permet treure i posar el sensor de forma fàcil i ràpida per procedir a la regeneració de l'activitat enzimàtica mitjançant poliment mecànic.

1.3 Sistemes d'Anàlisi en Flux

1.3.1 SISTEMES CONVENCIONALS: FIA, SIA

L'anàlisi per injecció en flux (FIA) va ser descrit originàriament per Ruzicka i Hansen el 1975,[55] i fins ara el seu desenvolupament com a sistema d'anàlisi on-line de multitud de components químics i bioquímics ha estat molt ràpid.

Els avantatges de la tècnica són:

- Gran nombre d'aplicacions potencials degut a la gran varietat de reaccions o detectors que permet utilitzar o incorporar.
- Preparació automatitzada de la mostra, per exemple per difusió o dilució.
- Calibratge continu del sensor sense necessitat d'interrompre el procés de monitorització en curs.
- Detecció simultània de diferents analits utilitzant reactors o sensors diferents en sèrie o en paral·lel.

FIA es basa en el control i reproductibilitat de la dispersió de la mostra introduïda en un flux no segmentat de solució portadora.[56] El disseny bàsic d'un sistema FIA s'inicia amb una etapa de presa de mostra, que pot incloure un pretractament com ara la dilució. En un segon bloc

es realitzen operacions com la separació o el reconeixement de l'analit d'interès per tal d'eliminar interferències o per produir espècies que podran ser mesurades en etapes posteriors. Finalment se situa l'etapa de detecció, la magnitud de la qual es basa en la concentració de les espècies d'interès.

A efectes pràctics s'utilitza una bomba peristàltica com element impulsor dels fluids, una vàlvula d'injecció per introduir un determinat volum de mostra en la solució portadora i un sistema de tubs per on circulen els líquids. La detecció té lloc en el detector en forma de pic transitori, l'alçada i l'àrea del qual són proporcionals a la concentració d'analit a la mostra. Aquests dos paràmetres s'utilitzen per a la quantificació de l'analit mitjançant un procés de comparació amb mostres de concentració coneguda (interpolació en una recta de calibratge). Segons la complicació del mètode d'anàlisi es poden incorporar diferents etapes de processament, com ara la dilució o concentració, l'extracció, la modificació de la matriu, l'eliminació d'interferències, etc.

Una variant de la tècnica FIA és l'anàlisi per injecció seqüencial (SIA). Introduïda per Ruzicka i Marshall el 1990,[57] el seu funcionament es basa en el principi d'operació en discontinu, el que permet programar el flux per tal d'optimitzar les etapes individuals del protocol analític. Per exemple, permet un gran estalvi de reactius i solucions portadores. El sistema bàsic consta d'una bomba bidireccional, un serpentí, una vàlvula multiposició i un detector.

1.3.2 SISTEMES CONVENCIONALS INTEGRATS: LTCC

Els muntatges experimentals dels sistemes de flux convencionals (FIA, SIA) es basen en la combinació de diferents dispositius discrets, cada un amb una funcionalitat determinada. Així, es fan necessaris una sèrie de tubs de diferents diàmetres per on circularà la solució portadora, els reactius i la mostra, un sistema d'impulsió (bomba peristàltica, xeringues), columnes de separació, distribuïdors i concentradors de flux, membranes, cel·les de detecció, etc. A la literatura és poden trobar infinites configuracions amb elements disponibles comercialment o bé construïts a mida per l'aplicació objecte d'estudi.

Malgrat la gran versatilitat que ofereix la tècnica FIA, presenta una sèrie d'inconvenients com per exemple lentitud en el processament de la mostra, limitada selectivitat, consum molt elevat de reactius i reduïda portabilitat. Per tal de donar solució a aquestes problemàtiques a principis de la dècada dels 90 sorgeix el concepte de microsistemes totals d'anàlisi o μ-TAS o Lab-on-a-chip (LOC),[58] que es basa en l'escalat de totes les operacions unitàries i el seu confinament espacial. Es poden trobar a la literatura diferents revisions de l'evolució i el progrés d'aquests sistemes.[59,60] Aquesta aproximació permet la fabricació massiva de dispositius de baix cost, i permet explotar nous fenòmens només viables a microescala, com els processos de difusió en flux laminar. És necessari puntualitzar que en moltes ocasions es parla de microsistemes quan en realitat es tracta de sistemes miniaturitzats o bé híbrids. Ríos i Zougagh[61] proposen tres nivells de miniaturització:

1. Sistemes analítics "mini", que inclouen dispositius específics de mida reduïda, com ara minireactors. Sovint a la literatura es troben descrites tècniques que substitueixen de forma no acurada el prefix "mini" per "micro".
2. Microsistemes analítics, que integren estructures analítiques de mida de l'ordre de μm, i que s'han fabricat mitjançant tècniques de microfabricació.
3. Nanosistemes analítics, que tenen alguna estructura de mida de l'ordre dels nanòmetres.

Entre els diversos materials utilitzats amb la finalitat de miniaturitzar procediments analítics,[59,62] en els darrers 10 anys les ceràmiques verdes (Low Temperature co-fired ceramics, LTCC) han esdevingut una bona alternativa degut a diversos avantatges.[63] Des del punt de vista tècnic, permeten el prototipat ràpid i senzill d'estructures tridimensionals complexes. A més, la metodologia multicapa que utilitza facilita la integració de materials de diferent naturalesa en el cos ceràmic. Entre aquest materials es troben per exemple les tintes conductores usades en el serigrafia, fet que permet integrar sistemes de detecció electroquímica (sensors) i el circuit elèctric per al control de l'analitzador miniaturitzat.

Un altre avantatge remarcable és el perfecte segellat entre capes i amb els elements integrats que s'aconsegueix durant el procés de fabricació (sinterització), fet que evita l'ús de gomes

d'enganxar, taps o procediments complicats.[64–66] Originàriament el vidre i el silici eren els materials més utilitzats per a fabricar dispositius miniaturitzats, però tenen limitacions com la dificultat per aconseguir estructures tridimensionals i la impossibilitat d'integrar-hi certs elements necessaris per al procediment analític.[67] Per aquest motiu es van desenvolupar metodologies basades en l'ús de materials polimèrics, que permeten la construcció d'estructures més complexes.[68,69] La tecnologia LTCC combina els avantatges de les dues tecnologies anteriors, ja que permet la construcció ràpida i barata de estructures tridimensionals, i permet la integració de diferents elements sensors i actuadors sense problemes de fugues de líquid. A més a més la infraestructura requerida és molt simple i barata ja que no es necessari l'ús de sales blanques.

El procés de fabricació consta de les següents etapes:[70]

1. Disseny del dispositiu mitjançant un programa CAD.
2. Mecanitzat de les capes constituents del dispositiu (Làser, CNC, serigrafia).
3. Serigrafiat de les pistes conductores.
4. Integració de diferents component a les ceràmiques (sensors, membranes, etc).
5. Laminació de les capes aplicant pressió.
6. Sinteritzat de les capes aplicant temperatura.

En els darrers anys el nostre grup han dissenyat i construït dispositius que incorporen sensors potenciomètrics[65,66,71–73] i òptics,[74–80] diferents tipus de mescladors,[74] reactors,[75,76,78,79] i control tèrmic,[81] o bé que integren de forma modular l'electrònica de control.[82,83]

La segona part d'aquesta tesi descriu el disseny, construcció i avaluació d'un dispositiu basat en LTCC que integra per primera vegada un sensor amperomètric, i que permet la detecció de pesticides mitjançant inhibició enzimàtica.

1.4 Sistemes de Microfluídica

1.4.1 Lab-on-a-chip i partícules magnètiques

El camp dels microsistemes totals d'anàlisi o Lab-on-a-chip (LOC) ha assolit un elevat grau de maduresa en els darrers anys. Moltes són les disciplines científiques que han contribuït al seu desenvolupament, ja sigui des del punt de vista de funcionament o solució tecnològica com des de la seva aplicació. A la literatura es poden trobar excel·lents reculls que descriuen l'estat de l'art des del punt de vista generalista,[59,60,84,85] de la tecnologia o operació integrada,[86–90] o de l'aplicació.[91–95]

La utilització de partícules com a suport físic per al material biològic en sistemes bioanalítics és una alternativa atractiva per resoldre la problemàtica de la renovació de la activitat d'aquest material, especialment en sistemes de flux automatitzats.[96]

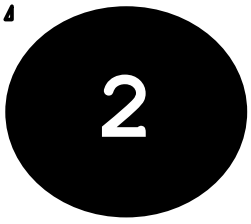
Les partícules magnètiques típicament consisteixen en un nucli magnètic generalment de Fe_3O_4, i d'una coberta externa no magnètica que confereix estabilitat i biocompatibilitat. Per a la seva aplicació en bioassajos aquesta coberta es pot funcionalitzar per tal de permetre l'enllaç covalent o l'absorció de biomolècules. Una particularitat molt important és la seva gran relació superfície/volum, que incrementa l'àrea disponible per a funcionalitzacions, augmentant l'eficiència de la captura de l'analit. Això és d'especial rellevància a l'hora d'immobilitzar-hi enzims i que a la vegada mantinguin la seva activitat.[97] La segona propietat més important és la capacitat que tenen de ser magnetitzades sota la influència d'un camp magnètic extern. De preferència són les partícules superparamagnètiques, que es desmagnetitzen en deixar d'aplicar el camp magnètic i per tant eviten la formació d'aglomeracions. Es troben disponibles en diferents diàmetres (de pocs nanòmetres a desenes de micròmetres), distribucions de mida de partícula i funcionalitzacions.[98]

Les partícules magnètiques han estat utilitzades abastament en el nostre grup com a estratègia de separació o concentració en sistemes bioanalítics[99–102] i en sistemes FIA.[96,103]

Pel que fa a la seva utilització en sistemes de microfluídica, el principal avantatge és permeten dur a terme assajos amb volums molt petits i molt localitzats. La seva manipulació més bàsica consisteix en la seva retenció en una zona concreta del xip mitjançant un camp magnètic extern i, un cop utilitzades, eliminar el camp i deixar-les sortir impulsades pel fluid.[88,98] Més recentment s'han desenvolupat microsistemes totals d'anàlisi en què l'ús de les partícules magnètiques permet realitzar de forma automatitzada diverses operacions sobre la mostra, com la extracció, separació i detecció.[104–109]

S'han descrit diferents dispositius de microfluídica per a la detecció de pesticides organofosforats, però els límits de detecció que presenten no són satisfactoris. Per exemple, l'electroforesi en xip amb detecció per reducció amperomètrica directa del pesticida ofereix límits de detecció en l'ordre dels ppms.[110,111] Amb dispositius Lab-on-chip amb una precolumna per la hidròlisi enzimàtica del pesticida amb posterior detecció per mesura de la conductivitat també s'aconsegueixen límits de detecció en aquest ordre.[112]

La tercera part d'aquest treball mostra com la utilització de quantitats controlades de partícules magnètiques com a suport físic de l'enzim colinesterasa permet assolir sensibilitats molt elevades en la determinació de pesticides combinant assajos d'inhibició enzimàtica i determinació amperomètrica. A més, l'ús de partícules magnètiques permet la regeneració de l'activitat enzimàtica de forma simple i efectiva.



2 OBJECTIUS

De manera general, l'objectiu d'aquesta tesi és el disseny i construcció de dispositius bioanalítics per als sectors industrial i mediambiental utilitzant diferents estratègies d'integració de les diferents etapes del procés analític, per tal d'aportar millores substancials en la sensibilitat i especificitat, que la seva usabilitat sigui senzilla i que siguin de baix cost.

En concret, es desenvoluparan tres sistemes analítics integrats que tenen en comú els següents objectius:

1. Demostrar la millora substancial que suposa realitzar un procés d'integració, encara que aquest sigui parcial, en el rendiment analític de les metodologies proposades.
2. Demostrar com la miniaturització, en diferents graus, permet augmentar de forma significativa la robustesa, la sensibilitat o la selectivitat dels sistemes proposats.
3. Aportar solucions a la problemàtica comuna dels biosensors i biosistemes analítics consistent en la pèrdua d'activitat de l'element biològic. Es proposen i demostren tres sistemes diferents de recuperar aquesta activitat.

Així, es dissenyaran, construiran i avaluaran biosensors amperomètrics de tercera generació per a glucosa, basats en compòsits epoxi-grafit-TTF•TCNQ-GOD. S'integrarà en la seva matriu els elements necessaris per determinar selectivament glucosa (glucosa oxidasa, GOD) i per reduir significativament l'efecte de les possibles interferències presents a la mostra mitjançant la dràstica reducció del potencial de treball aplicat sobre l'elèctrode (efecte de la

sal conductora TTF•TCNQ). A més a més, aquests biosensors s'integraran en un sistema d'anàlisi per injecció en flux (FIA) mitjançant una cel•la dissenyada especialment. D'aquesta manera, serà possible el seguiment on-line i a temps real de la concentració de glucosa en processos industrials.

D'altra banda, es dissenyaran, construiran i avaluaran dos sistemes d'anàlisi miniaturitzats per a la determinació de pesticides en aigües superficials mitjançant la tècnica de la inhibició enzimàtica selectiva. En el primer s'utilitzarà la tecnologia LTCC (Low Temperature co-fired ceramics), que permet la fabricació d'estructures tridimensionals de forma ràpida i sense la necessitat d'emprar sales d'ambient controlat i que, a més, permet la integració de diferents elements com per exemple elèctrodes de platí per deteccions amperomètriques.

El segon dispositiu es basarà en un microxip de fluídica comercial, en l'interior del qual s'introduirà una determinada quantitat de partícules magnètiques modificades amb enzim a mode de bioreactor. Aquest sistema permetrà la renovació controlada del material biològic mitjançant la simple acció d'un camp magnètic proporcionat per un imant permanent mòbil.

3 RESULTATS I DISCUSSIÓ

3.1 FIA WITH BIOSENSORS

3.1.1 INTRODUCTION

An amperometric glucose biosensor based on graphite and non-conducting epoxy resin biocomposite was constructed. Glucose oxidase (GOD) and the tetrathiafulvalene-tetracyanoquinodimethane (TTF•TCNQ) conducting organic salt were incorporated into the bulk of the composite to form a renewable biosensor. Several graphite-TTF•TCNQ ratios (w/w) were studied in order to select the best biosensor to be integrated in a FIA system for the automated detection of glucose. The optimal amount of GOD in the composite was studied as well. The selection was based on the analytical response of the electrodes. Best results were obtained by an electrode whose composition was 5% GOD, 76% polymer, 9.5% graphite and 9.5% TTF•TCNQ. An especially designed flow amperometric cell was constructed so that the biosensor could be integrated into a FIA system and glucose in beverage samples could be determined.

3.1.2 MATERIALS AND METHODS

3.1.2.1 Reagents

Graphite powder with a particle size of 50 µm (BDH Laboratory Supplies), epoxy resin Epo-Tek H77 (Epoxy Technology), TTF and TCNQ (Fluka), and GOD type VII 185 U mg^{-1} from *Aspergillus niger* (Sigma) were used to prepare the biocomposite paste.

TTF·TCNQ organic salt was prepared by mixing identical volumes of equimolar solutions (0.1 M) of each compound in hot anhydrous acetonitrile. The reaction was let to complete overnight with stirring and the black crystals formed were rinsed with cold acetonitrile and diethyl ether, and then dried under vacuum.

Glucose stock solutions were prepared with D-(+)-glucose monohydrate (Fluka) in phosphate buffer, and were left at 4 ºC overnight to allow the equilibration of the anomers. A commercially available Glucose Assay Kit (Sigma) was used for comparison purposes.

Aqueous solutions of 0.1 M phosphate and 0.1 M KCl, buffered at pH 7.5 were used to perform both batch and FIA experiments. All other reagents used were of analytical grade.

3.1.2.2 Instrumentation

Current intensities were measured with an LC-4C amperometric controller (BAS Bioanalytical Systems Inc.) connected to a Labograph 517 graphic recorder (Metrohm). Cyclic voltammetries were performed with an Autolab PGSTAT20 (Eco Chemie). The reference electrode was a double junction Ag/AgCl electrode (Orion 900200) with 0.1 M phosphate and 0.1 M KCl as external reference solution. In batch experiments, a platinum electrode (Crison 52-67 1) was used as counter electrode. pH measurements were performed with a glass electrode (Crison 52-03) connected to a potentiometer (Crison MicropH 2002).

The flow system, shown in Figure 3.1-1, consisted in a Miniplus 3 peristaltic pump (Gilson), a six-way injection valve (Omnifit) and a homemade methacrylate flow cell which integrated both working and counter electrodes. The flow cell volume was 140 μl. This flow cell was formed by a stain-less steel counter electrode (block A) that was screwed in methacrylate block B, where the biosensor (C) was allocated. Water tightness was achieved by two O-rings. The biosensor was fixed with a second methacrylate block (D), also screwed in block B. A magnetic bar was inserted in the cavity to stir the solution and minimize sample dispersion.

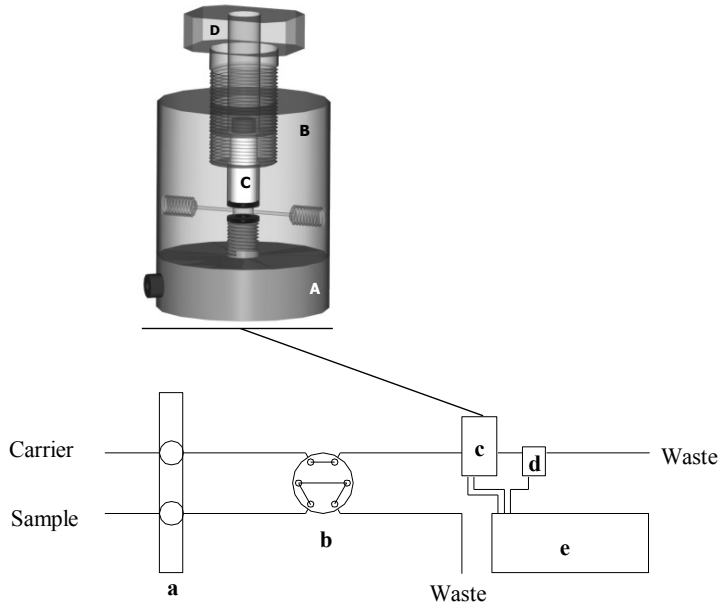


Figure 3.1-1 Flow injection system diagram and flow-through cell detail. The cell (c) has a stain-less steel part (A) that serves as counter electrode. The methacrylate cell body (B) is attached to this lower block by screwing forming an inner compartment where the biosensor (C) is placed and fixed by means of a methacrylate sealing screw (D). The space between the counter electrode and the biosensor defines the cell volume. A standard double junction Ag/AgCl reference electrode (d) is positioned next to the flow-through cell, in the downstream flow. A peristaltic pump (a), a 6-way injection valve (b) and the amperometric detection unit (e) complete the flow system.

3.1.2.3 Construction of the graphite-epoxy-TTF·TCNQ-GOD amperometric biosensors

The biosensor material was easily prepared by hand-mixing epoxy resin, graphite powder, TTF·TCNQ and GOD until a homogeneous paste was obtained. The electrodes constructed had the composition shown in Table 3.1-1. The paste was placed into a cylindrical PVC body (6 mm inner diameter, 2 cm length), which has an electrical contact, to a depth of 3 mm. Finally, the composite material was cured at 40 ºC (higher temperatures, recommended for curing the epoxy-resin, could damage the enzyme) for one week. Once cured, biocomposite excess was eliminated with abrasive paper and finally the surface was smoothed with alumina paper (polishing strips 301044-001, Moyco Precision Abrasives Inc). When not in use, electrodes were stored at 4 ºC.

Table 3.1-1 Composition (% weight) of the biocomposite paste used for the biosensors construction

Biosensor	Epoxy resin	Graphite	TTF·TCNQ	GOD
A	80.0	14.0	4.0	2.0
B	80.0	9.0	9.0	2.0
C	80.0	4.0	14.0	2.0
D	80.0	-	18.0	2.0
E	77.4	9.7	9.7	3.2
F	76.0	9.5	9.5	5.0
G	75.0	9.4	9.4	6.2

3.1.3 RESULTS AND DISCUSSION

3.1.3.1 Electrochemical behaviour of TTF·TCNQ-based graphite-epoxy electrodes

The sensitivity of the glucose biosensor will depend on its polarization potential, and this is limited by the potential in which the organic salt suffers decomposition and leaching occurs. The cyclic voltammetry of the TTF·TCNQ based graphite-epoxy electrodes in a typical PBS buffer (phosphate 0.1 M and KCl 0.1 M), conditions used for glucose measuring, was performed to study the organic salt decomposition inside the polymeric matrix. Positive and negative scans (Figure 3.1-2) were applied to observe the TTF·TCNQ behaviour. As shown in Figure 3.1-2a, the TTF·TCNQ starts being oxidized at 0.30 V vs. Ag/AgCl. The oxidised species obtained during the oxidation until the final potential (0.6V) will be reduced on reversal giving a well-defined peak at -0.01V. Current intensities observed may be attributed to the following reactions:[113]

E > 0.30 V: $TTF{\cdot}TCNQ + 2X^- \rightarrow TTFX_2 + TCNQ + 2e^-$

E = -0.01 V: $TCNQ + M^+ + e^- \rightarrow MTCNQ$

E = 0.22 V: $MTCNQ \rightarrow TCNQ + M^+ + e^-$

where M^+ and X^- represents cations and anions present in the working solution. The nature of these spices determines the solubility of the corresponding salts.

On the other hand, the negative scan (Figure 3.1-2b) shows that the reduction of the organic salt starts at –0.2 V, giving insoluble TTF and TCNQ. If sodium or potassium ions are present in the electrolyte, an insoluble TCNQ salt is deposited, which is also electro active (oxidation peaks between 0 and 0.3 V shown in Figure 3.1-2b).

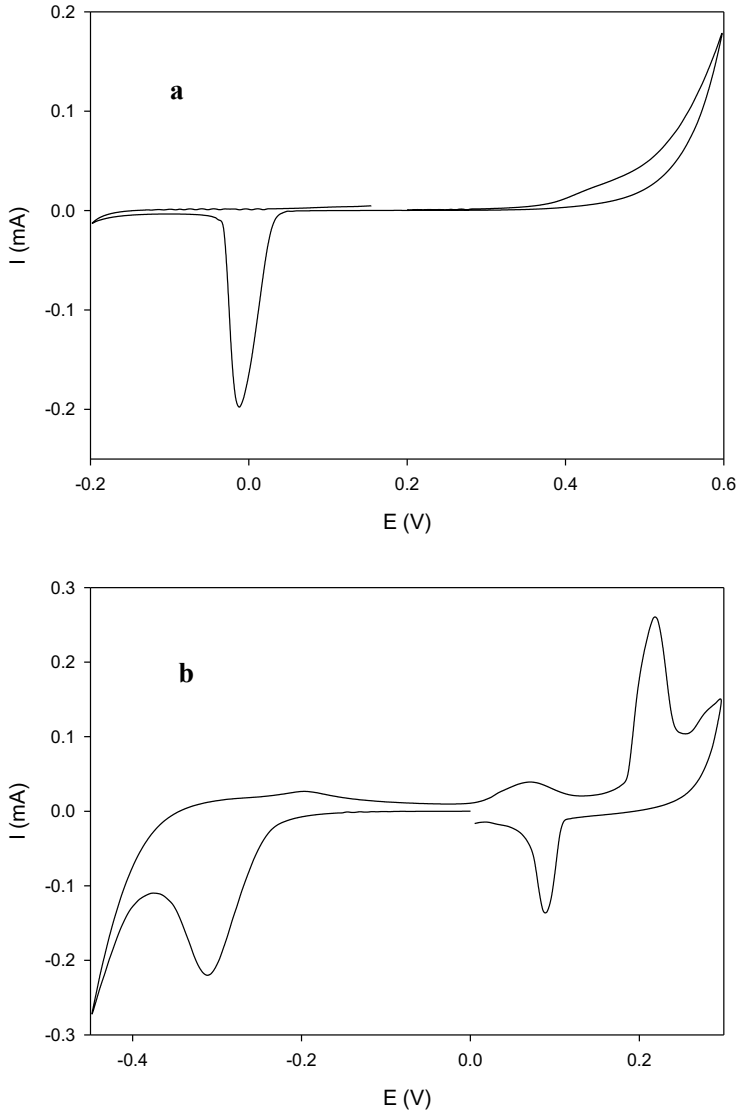


Figure 3.1-2 Cyclic voltammograms of the TTF-TCNQ biosensor in PBS pH 7.5 (phosphate 0.1 M and KCl 0.1 M): a) Initial positive scan with oxidation of the electrode surface and resultant peaks on reversal. Start potential: 0.2 V, forward final potential: 0.6 V, backward final potential: -0.2 V; b) Initial negative scan with reduction of the electrode surface. Start potential: 0 V, forward final potential: -0.45 V, backward final potential: 0.3 V. Scan rate: 0.005 V/s.

Results obtained in both scans show that composite electrodes containing TTF·TCNQ can be polarized at any potential between –0.1 and 0.3 V vs. Ag/AgCl without risk of decomposition of the organic salt. The working potential was finally established by using cyclic voltammetry studies (results not shown) of the biosensor in the presence of increasing concentrations of glucose. Maximum sensitivity was achieved at 0.3 V vs. Ag/AgCl, but lower applied potentials could be selected in order to minimize interferences.

3.1.3.2 Effect of oxygen upon biosensors response

Oxygen may induce a diminution on the response of the biosensor towards glucose because a competition between itself and the organic salt to carry out the oxidation of the $FADH_2$ group of GOD can be established. The responses of the prepared glucose biosensors, in the presence or absence of oxygen, were compared to clarify the possible competitive reaction mechanisms (Figure 3.1-3). When oxygen was completely removed from the working solution (before and during the experiment, not allowing diffusion from the atmosphere), all the active individual enzyme molecules were oxidised back onto the TTF·TCNQ surface due to the applied potential (inset mechanism A in Figure 3.1-3), and the observed sensitivity to glucose concentration changes was high. On the other hand, when some oxygen was present in the solution (i.e. not removing it or removing it only before the experiment and, thus, allowing some diffusion from the atmosphere), mechanism B (Figure 3.1-3 inset) took place simultaneously with mechanism A. Taking into account that 1.15 V[114] should be necessary to oxidise hydrogen peroxide and that the actual applied potential was 0.15 V, hydrogen peroxide could not be detected by the biosensor. For that reason, the number of active sites performing mechanism A was lower than in the absence of oxygen, resulting in a decreased sensitivity. When oxygen was completely consumed, all the enzyme molecules performed mechanism A, and an increase of the sensitivity was observed.

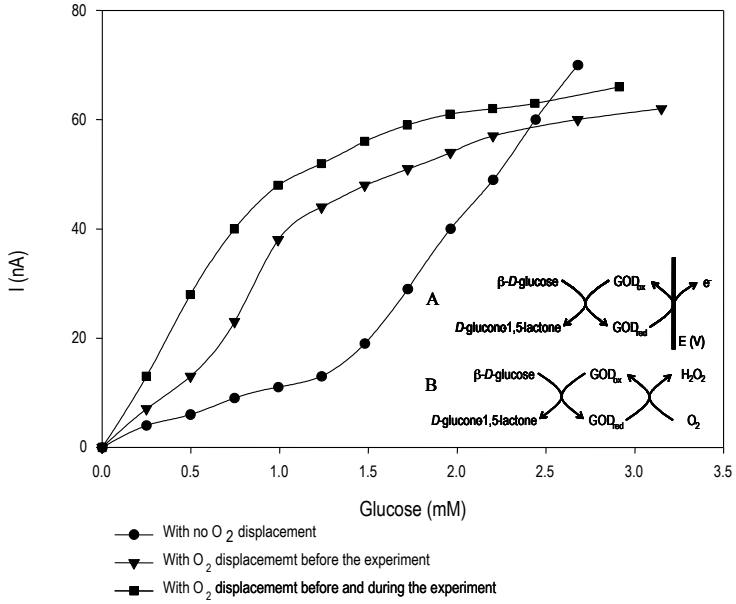


Figure 3.1-3 Effect of the oxygen in the response of the TTF·TCNQ biosensor. The two possible mechanisms for the oxidation of the enzyme are indicated. A: direct regeneration of reduced GOD onto TTF·TCNQ. B: regeneration of reduced GOD via natural cofactor and hydrogen peroxide production. Applied potential: 0.15 V. PBS pH 7.5 (phosphate 0.1 M and KCl 0.1 M). Oxygen was removed using a nitrogen flow.

The above results demonstrate that, in order to obtain reproducible glucose signals, the biosensor must operate under absence of oxygen, responding through TTF·TCNQ mechanism only and avoiding a non-reproducible 'bi-mechanism' response due to the oxygen effect.

3.1.3.3 Biocomposite composition

Epoxy resin Epo-Tek H77 has very interesting properties concerning chemical stability but, compared to other commercially available epoxy resins, the amount of solid material that can be incorporated in its matrix is limited to 20 % in weight. Whenever this amount is overtaken, the plastic phase loses continuity and the material does not cure properly.

To improve the conductivity of the biocomposite, and thus the sensitivity, graphite powder was added into the paste because TTF·TCNQ is not a pure conductor and the electron flow could be limited if no other conductive material was added. On the other hand, adding graphite powder could reduce the effective TTF·TCNQ surface in which the prosthetic group of the enzyme could be oxidised, decreasing the biosensor sensitivity. It was necessary, then, the optimisation of the graphite/TTF·TCNQ ratio. For that reason, biosensors with various graphite/TTF·TCNQ ratios (A, B, C and D from Table 3.1-1) were studied, and the corresponding responses toward glucose additions are shown in Figure 3.1-4.

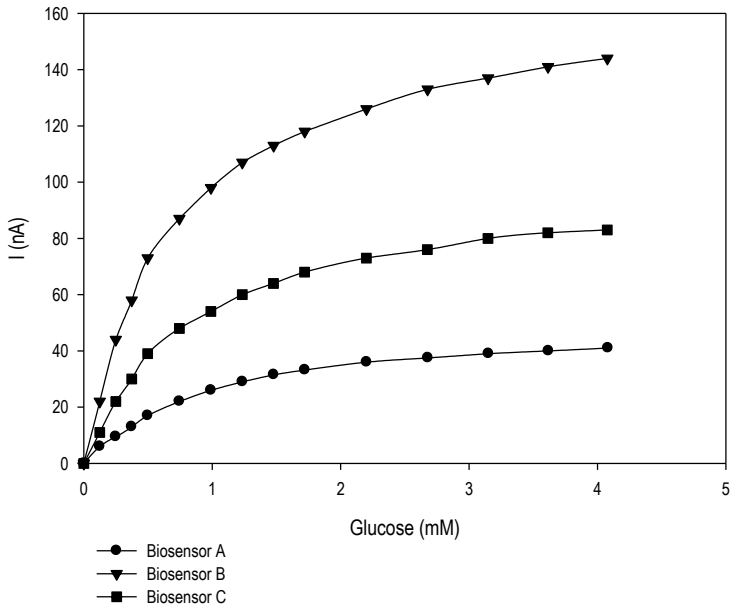


Figure 3.1-4 Effect of the graphite/TTF-TCNQ ratio (w/w) on the biosensor response. Biosensor A: 14% graphite, 4% TTF·TCNQ. Biosensor B: 9% graphite, 9% TTF·TCNQ. Biosensor C: 4% graphite, 14% TTF·TCNQ. For all biosensors: 2% GOD. Applied potential: 0.15 V. PBS pH 7.5 (phosphate 0.1 M and KCl 0.1 M).

Both from the point of view of sensitivity and linear range of response best results were obtained from biosensor B, with biocomposite containing a 1:1 graphite/TTF·TCNQ ratio. When graphite was the predominant material (biosensor A), the signal was limited by the available TTF·TCNQ surface. On the other hand, when the organic salt was the predominant

material (biosensor C, 4% graphite), the biocomposite was not conductive enough. The curve for biosensor D (0% graphite) could not be constructed because the measured current was not stable, indicating that some amount of graphite is necessary to facilitate the flow of electrons through the biocomposite.

Regarding the quantity of GOD, it was expected from Michaelis-Menten mechanism that the higher the active enzyme concentration, the higher sensitivity and the higher linear range of response should be obtained. Biosensors with various GOD contents were prepared and their responses to glucose additions were measured. As expected, increasing quantities of GOD in the biocomposite formulation resulted in better sensitivities. Best responses were obtained from biosensors reaching 5% GOD (see Figure 3.1-5). Biosensors "F" and "G" presented the same response, indicating that there is an optimal amount of enzyme an excess of which is not active or it is not retained in the matrix.

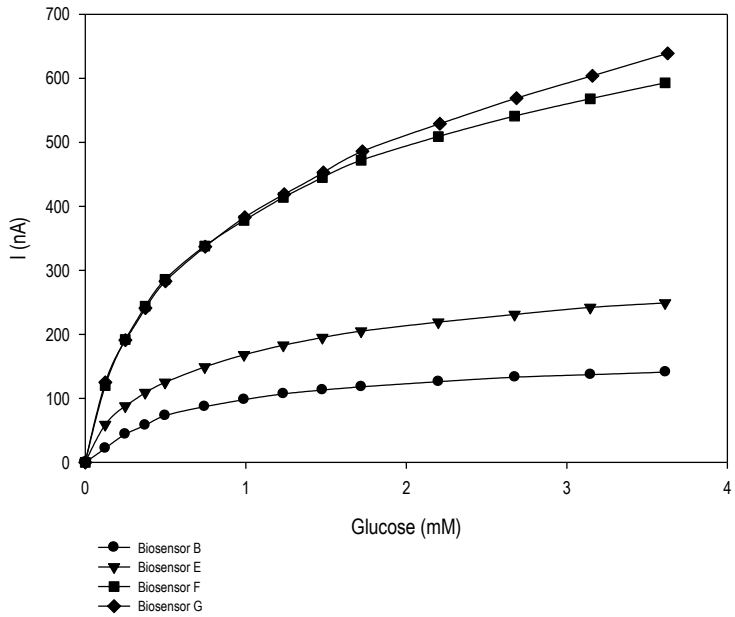


Figure 3.1-5 Effect of the quantity of GOD in the sensing material on the biosensor response. Biosensor B: 2% GOD. Biosensor E: 3.2% GOD. Biosensor F: 5% GOD. Biosensor G: 6.2% GOD. Applied potential: 0.15 V. PBS pH 7.5 (phosphate 0.1 M and KCl 0.1 M).

In conclusion, the biosensor that showed better analytical characteristics had the following composition: 76% resin, 9.5% graphite, 9.5% TTF·TCNQ and 5% GOD, and it was selected to perform the flow injection analysis system. The apparent Michaelis constant, K_m, calculated using the Lineweaver-Burk plot method[115] was 0.38 mM.

3.1.3.4 Biosensor response in batch measurements

Cyclic voltammetries (in the stability range of potentials of the organic salt, from –0.1 to 0.3 V) at different glucose concentrations over biosensor F were performed, in order to determine the optimum applied potential in terms of sensitivity. As expected the maximum sensitivity for glucose determination was achieved at 0.3 V, so this potential value was selected as the working potential of the biosensors. Nevertheless, lower polarisation potentials could be selected, if necessary, in order to minimize interferences depending on the sample matrix.

It was also necessary to verify that the recorded signal during glucose additions was exclusively due to the electrons generated by the glucose oxidation reaction. In other words, it was necessary to check that no other reaction was being performed at the biosensor surface (directly either over graphite or TTF·TCNQ). For that reason, electrodes with various compositions of graphite, epoxy and TTF·TCNQ, but without GOD, were constructed and their responses to glucose were checked. None of them responded to glucose concentration variations. This indicates that glucose cannot be oxidized directly by the electrode surface without the presence of the GOD. Electrodes containing GOD, but without TTF·TCNQ, were also constructed, and no response towards glucose was observed. This experiment confirmed that the signal obtained for the biosensors was due to the oxidation of the reduced enzyme onto the organic salt surface.

Reproducibility was the last characterisation parameter of the biosensors being evaluated. Because of the complex composition of the sensing material, it is difficult to ensure that its analytical properties are not affected after several uses. Enzyme immobilisation, for example, is not 100% effective, and some leaching, on introducing the biosensor under stirring

conditions (in batch or FIA systems), can occur. On the other hand, applying a constant potential over the electrode surface can result in the polymerisation of electro active species in the solution forming a film over the surface (fouling). When this occurs, the electrode is passivated and sensitivity decreases. This fact was checked performing three consecutive evaluations of the biosensor response. Figure 3.1-6 shows the resulting calibration curves. It can be seen that if biosensor is used without any surface treatment (apart from rinsing with water) the sensitivity decreases around 60%. To recover the optimal response a simply polishing procedure (as described in the experimental section) was carried out. In this way, fresh GOD replaces the used one and the response is recovered nearly 100%.

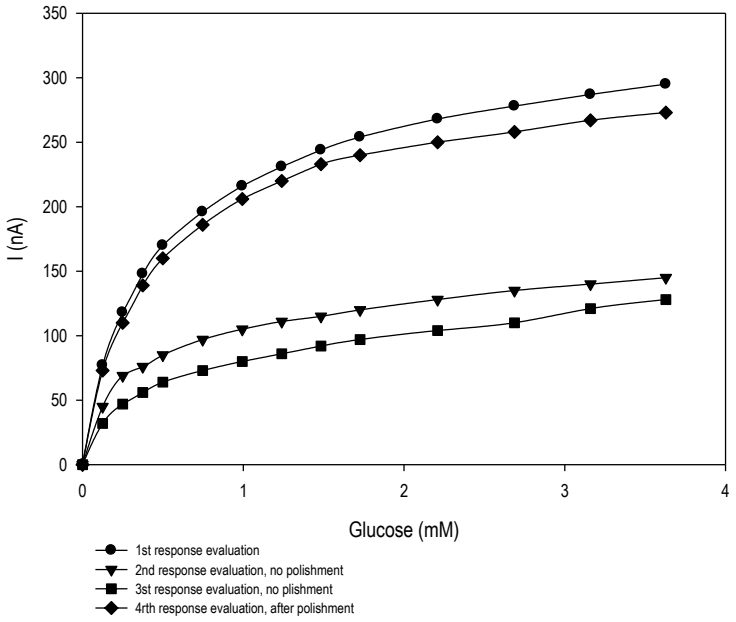


Figure 3.1-6 Consecutive drop of biosensor response without surface renewal, including recovering after polishing. Applied potential: 0.15 V. PBS pH 7.5 (phosphate 0.1 M and KCl 0.1 M).

3.1.3.5 Biosensor integration into a FIA system and glucose determination

An amperometric flow cell for the integration of surface renewable biocomposite electrodes had been previously designed and constructed in our group.[7] Nevertheless, the previous designed cell presented two main drawbacks: the irreproducibility of the cell volume due to the compressibility of the silicone sheet between the two bodies of the cell, and the mechanical instability of the silver-loaded epoxy counter electrode. The new flow cell (see Figure 3.1-1) avoids the drawbacks of the old cell showing an improved reproducibility during the operational cycles along with an enhanced mechanical stability of the stainless steel electrode, which also improved the signal-to-noise ratio.

The optimum flow rate and sample injection volume were found to be 1 ml min^{-1} and 350 μl respectively. The reproducibility of the designed cell was checked by 15 consecutive injections of a 1 mM glucose solution (see Figure 3.1-7). A relative standard deviation of 1.9 % indicates a good reproducibility of the signals obtained by this flow-through cell.

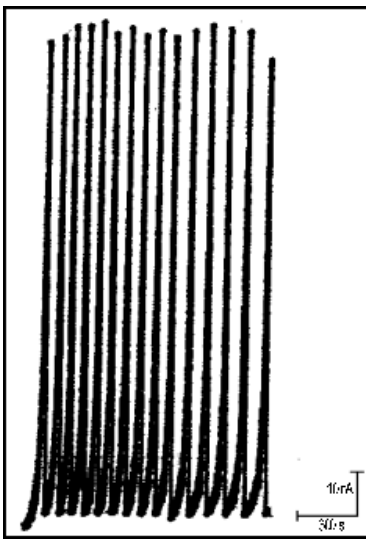


Figure 3.1-7 Record of 15 successive injections of 1 mM glucose. Flow rate: 1mL min^{-1}. Injected volume: 350 μL. Applied potential: 0.3 V.

Three measurements were performed for each standard used in the construction of the calibration curve, which is shown in Figure 3.1-8. A sensitivity of 322 ± 19 nA mg^{-1} ml, an ordinate of −1 ± 5 nA and a linear regression coefficient (*r*) of 0.9994 were obtained. The graphical register of the obtained signals, being A to G the standards and I to IV the samples, are also shown in Figure 3.1-8 as insets. These real samples were commercial cola beverages, being III and IV "diet" type. Samples were diluted in PBS at pH 7.5 (phosphate 0.1 M and KCl 0.1 M) to fit the linear range of response of the analytical system and to adjust the pH. As stated previously, samples and standards were degassed with N_2, eliminating any possibility of interference from O_2.

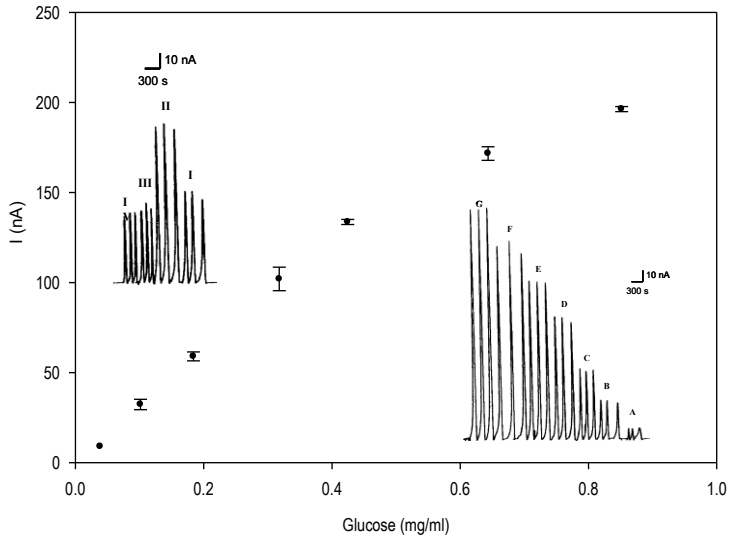


Figure 3.1-8 Calibration curve obtained for standards (ranging from 0.05 to 0.9 mg mL^{-1} glucose) and the corresponding records (Inset: A-G standards, I-IV samples). Flow rate: 1mL min^{-1}. Injected volume: 350 µL. Applied potential: 0.15 V.

The same samples were also analysed by the commercially available *Glucose Assay Kit* for comparison purposes. The results obtained showed no significant differences between results from both methods (see Table 3.1-2).

Table 3.1-2 Results for glucose concentration in cola beverage samples obtained by two methods (mg mL^{-1}).

Sample	Method	
	Biosensor-FIA	Colorimetric
I	17.96 ± 0.70	18.75 ± 0.23
II	61.815 ± 1.48	62.39 ± 1.26
III	0.60 ± 0.03	0.70 ± 0.01
IV	0.94 ± 0.05	1.05 ± 0.01

3.2 LTCC Biosystem

3.2.1 INTRODUCTION

In this work, the fabrication of a LTCC device integrating some of the stages needed to perform the analytical process such as sample/reagent mixing, incubation steps and detection system is presented. To demonstrate the operational capacity of this technology, a microanalytical system for pesticide determination in water streams based on its inhibition effect on acetylcholiesterase enzymatic activity has been designed and evaluated. It is well known that pesticides derived from carbamates and organo-phosphorus compounds are potent acetylcholiesterase inhibitors. This inhibition effect can be followed amperometrically using acetylthiocholine (ATCh) as substrate because thiocholine, the product of its hydrolysis, is an electroactive substance that can be oxidised at the surface of a working electrode polarised between 450 and 600 mV. Many approaches have been proposed to carry out this determination, such us the immobilisation of the enzyme in different materials to produce bioreactors[116–118] or the development of biosensors incorporating the enzyme and its use both in batch and flow systems.[96,119,120] To simplify the development of the microfluidic system, the enzymatic inhibition was carried out using the enzyme in solution. In this case the steps needed previous to the detection are the incubation of the inhibitor and the

enzyme in a first stage and, afterwards, the incubation of the inhibited enzyme with the substrate.

Some work has already been done miniaturizing devices aimed at the detection of pesticides or other toxic compounds. Some have scaled down one of the pre-treatment steps needed,[121,122] another has automated this process[123] or integrated a separation step and the detection system.[110] One of the most interesting requirements of a miniaturized analyser is its portability, so that *in-situ* and real time analysis of samples can be performed.

The microflow system consists of two mixing reactors that will act efficiently as efficient incubation stages for the two main reactions because of their multiple 90º angles designed to enhance chaotic mixing.[124] The detection system used combines screen-printed conductors and the integration of two platinum sheets acting as counter and working electrodes, proving the versatility of this technology. The reference electrode consists of a silver-based screen-printed conductor integrated inside the LTCC structure. After the oxidation of the silver in chloride, the reference electrode potential will be kept constant by means of a flowing solution of chloride ions.[65]

As starting point, the integrated amperometric system was characterized for sensitivity and repetitivity without enzymatic reaction. After that, the potentiality of both the reactors and the detection system to carry out the determination of a pesticide, carbofuran, by enzymatic inhibition will be tested.

3.2.2 EXPERIMENTAL SECTION

3.2.2.1 LTCC device construction

Dupont 951 LTCC tapes (thickness of 254 µm) were used. A LPKF ProtoMat® C100/HF (LPKF, Germany) milling machine was used to mill the designed layers. The general fabrication procedure was described in detail elsewhere.[65] Figure 3.2-1 shows all the layers involved in the fabrication procedure that, once overlapped, turned out to be the desired device. Layer

D is the bottom one, and A is the top one. A total amount of 10 layers were used: 2xA, 2xB, 4xC and 2xD. After sintering, the height of the device was ~2.2 mm. The four holes in each corner of the layers were used to align them during the lamination process.[65]

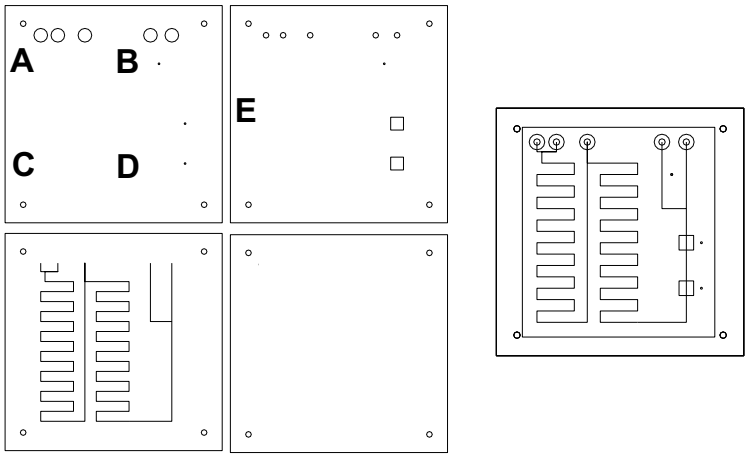


Figure 3.2-1 Layers involved in the miniaturised analyser. A is the top one (where the fluidic connectors are attached), B is the layer that supports the platinum sheets, C are the layers where the channels are mechanised and D the bottom one (the base). E represents all the layers once overlapped.

Figure 3.2-2A shows a picture of the final device and Figure 3.2-2B shows the inner three-dimensional structure. Once the device was burnt-out, the inner channels dimensions were 1.7 mm width and 430 µm height. Each reactor had a total volume of 164 µL.

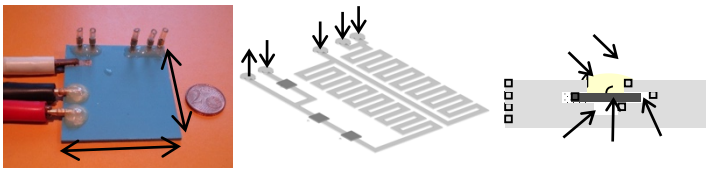


Figure 3.2-2 A. Device picture once burn-out. B: inner three-dimensional structure. C: integration of the platinum sheets in the LTCC device. *a*: waste. *b*: 0.1M Cl^- solution inlet. *c*: acetylthiocholine solution in buffer A inlet. *d*: enzyme solution in buffer B inlet. *e*: water/sample inlet. *f*: conductor path (connected to the inner reference electrode). *g*: platinum counter-electrode. *h*: platinum working electrode. d_1: 5 cm. d_2: 5 cm. R1: reactor 1. R2: reactor 2.

The two platinum sheets (Aldrich, foil thickness 0.5 mm) of 5 x 5 mm each were used as counter and working electrode. Their integration in the LTCC device is showed in Figure 3.2-2C. In order to compensate the shrinkage caused by the sintering process (12.3% in the x-y axis and 15% in the z axis),[125] the cavities designed to embed the platinum sheets were slightly bigger. In order to ensure a perfect sealing between the ceramic tapes and the metal sheets, sinterable glass (Dupont 9615) was deposited. After the sintering step, commercial connectors were attached to the conductive paths (see Figure 3.2-2A) and finally insulated with epoxy glue.

The reference electrode (indicated as f in Figure 3.2-2) consisted of an internal screen-printed Ag-based path (Dupont 6142) with its anodised surface in direct contact with the flow channel. The anodisation of the Ag path to cover it with an AgCl layer was achieved by applying a voltage of 0.8 V for 20 minutes in presence of a 0.2 M Cl^- solution. In order to achieve a stable reference potential, a 0.1 M Cl^- solution flowed constantly through the channel in contact with this path (indicated as b in Figure 3.2-2B). The electric contact between the anodised path and the device surface was achieved by means of a hole filled with dense Ag paste (Dupont 6141). A solderable Ag-based path (Dupont 6146) was printed onto the device surface to provide contact between the miniaturised device and the external electronic set-up.

3.2.2.2 Reagents

Carbofuran (analytical standard) was purchased from Riedel-de Haën. Pesticide standard solutions were prepared daily in double-distilled water by dilution from a 2.3×10^{-4} M stock in ethanol. Acetylthiocholine chloride (Sigma) standard solutions were prepared in 0.1 M phosphate and 0.1 M potassium chloride aqueous solutions buffered at pH 7 (buffer A). Acetylcholinesterase type VI-S from Electric Eel (Sigma) was dissolved in 0.2 M phosphate and 0.2 M potassium chloride aqueous solutions buffered at pH 7.4 (buffer B). Sodium dihydrogenphosphate, disodium hydrogenphosphate and potassium chloride were obtained from Fluka. 0.1 M potassium chloride was used to assure a constant concentration of chloride ions at the reference electrode.

A 0.1 M thiocholine stock solution of was prepared by adding 25 units of acetylcholinesterase to 100 mL of a 0.1 M acetylthiocholine chloride solution in buffer A and allowing the hydrolysis to take place for 3 hours. Thiocholine standard solutions were prepared by dilution of this stock in buffer A.

Safety Considerations. OP pesticides are highly toxic and should be handled in an extractor hood. Skin and eye contact and accidental inhalation or ingestion should be avoided.

3.2.2.3 Flow injection manifold

The continuous flow system set-up consisted of a peristaltic pump (Minipuls 3, Gilson), a 6-port distribution valve (Hamilton MVP), silicon tubing with different inner diameters (ISMATEC) and 0.8 mm inner diameter Teflon tubing (SCHARLAB, S.L.).

Current intensities were measured with an LC-4C amperometric controller (Bioanalytical Systems Inc.) connected to a Duo 18 data recording system (World Precision Instruments Ltd.).

3.2.3 RESULTS AND DISCUSSION

The first experiments ran to characterise the miniaturised system in terms of sensitivity and repetitivity were carried out with thiocholine obtained as previously described. During the performance of these experiments, a common drawback was observed. It is well known that the oxidation of thiocholine at the electrode surface produces a passivation (fouling) of this surface due to the electropolymerisation of thiocholine.[126] The consequence of this passivation is a decrease in the sensitivity of the detection system, because the effective electrode surface is reduced. This was observed qualitatively after ten consecutive injections of thiocholine (Figure 3.2-3). It can be seen that the peak height decreases dramatically until a certain value and, at the same time, the baseline increases.

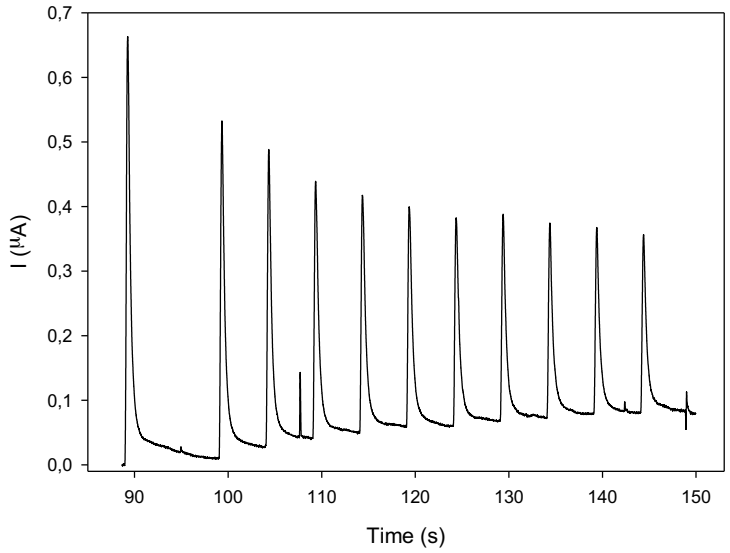


Figure 3.2-3 Consecutive injections of 0.1 M thiocholine to show the decrease of sensitivity due to the fouling effect (channel *e*: buffer A; channel *d*: buffer A/thiocholine; channel *c*: blocked). Flow rate: 1 mL·min^{-1}. Volume injected: 100µL. Applied potential: 500 mV vs. Ag/AgCl.

The undesirable consequence of this passivation process is the lack of reproducibility of the system. In order to solve this problem an initial cleaning step of the electrode surface previous to each inhibition test was evaluated. This pre-treatment consisted in a switch of the polarity of the potential applied to the working electrode, producing the solubilisation of the polymerised substances on the electrode surface. Unfortunately, it was difficult to optimise the time necessary to achieve the desired results in a reproducible manner, and this cleaning procedure was discarded. As alternative, and further to the confirmation that the decrease of sensitivity due to the passivation occurred only to a limit, a controlled fouling pre-treatment step was tested. In this case, and previous to each inhibition test, a continuous stream (0.5 mL·min^{-1}) of a highly concentrated thiocholine solution was allowed to flow through the electrode surface polarised at 500 mV vs. Ag/AgCl. The measured intensity increased up to a maximum until the extent of the passivation was high enough to keep on reducing the sensitivity. Afterwards, the intensity decreased to a constant value after

approximately 30 minutes. The current recorded at this stage was the maximum current that the electrode could measure under these controlled fouling conditions.

After this pre-treatment step the reproducibility of the detection system was evaluated. Ten consecutive injections (25 µL) of a 0.5 mM thiocholine standard at a flow rate of 0.75 mL·min^{-1} were performed. The resulting RSD% was 2.1%, showing a good repetitivity and the adequacy of the pre-treatment step.

Hydrodynamic voltammetries for buffer A and for 2 mM thiocholine solution were performed to determine the optimum potential to be applied to the working electrode for the amperometric detection of thiocholine. Firstly, the background current provided by buffer A flowing at a constant rate of 0.5 mL·min at increasing applied potentials (from 0 to 700 mV and steps of 100 mV) was recorded. In a second run, the same procedure was repeated using a 2 mM thiocholine solution instead of buffer A. The maximum analyte/background current ratio was obtained at 500 mV vs. Ag/AgCl., so this was selected as the potential to be applied.

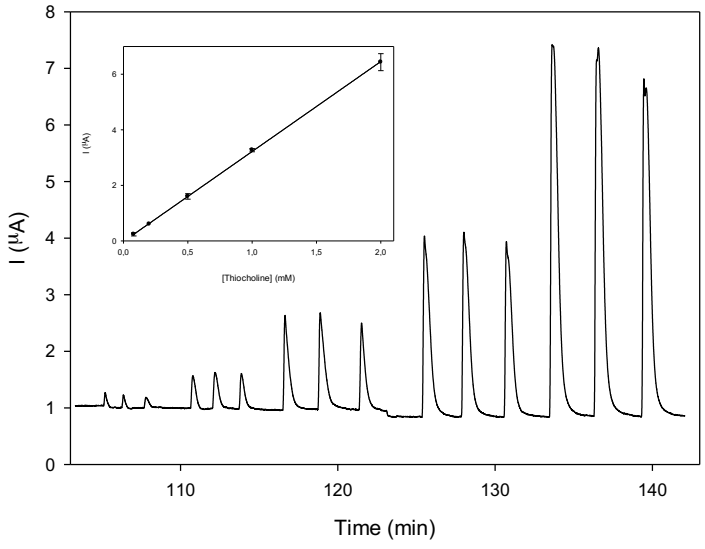


Figure 3.2-4 Registry plot and calibration curve (inset) for thiocholine standard solutions (0.08, 0.2, 0.5, 1 and 2 mM) (channel *e*: buffer A; channel *d*: buffer A/thiocholine; channel *c*: blocked). Flow rate: 1mL·min^{-1}. Volume injected: 25µL. Applied potential: 500 mV vs. Ag/AgCl.

A further optimisation of the system performance towards the detection of thiocholine was achieved with the evaluation of the sensitivity of the electrodes. In this case, five thiocholine standard solutions were injected by triplicate through channel ***d*** by means of a 6-way injection valve into a stream of buffer A. The signals measured at the working electrode, polarised at 500 mV vs. Ag/AgCl, are shown in Figure 3.2-4. These values were used to construct a calibration curve (Figure 3.2-4 in-set) that presents a presented slope of 3.239 $\mu A \cdot mM^{-1}$ and r^2 of 0.9994, showing a sensitivity good enough to detect the expected production of thiocholine with the enzymatic system.

A previous optimisation of the enzymatic system parameters, such us the enzyme and substrate concentrations, was achieved by means of the construction of the pseudo Michaelis-Menten curves (Figure 3.2-5). Each curve was constructed recording the current intensity after the injection of 25 µL of four different standard solutions of acetylcholinesterase into a flow of ATCh at a given concentration. This was repeated for five concentrations of substrate, namely 2.5, 5, 10, 20 and 40 mM.

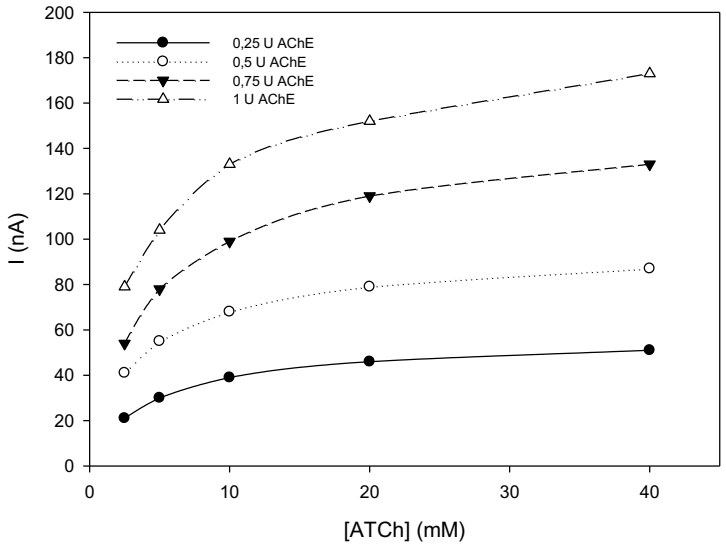


Figure 3.2-5 Michaelis-Menten curves. Flow rate: 0.5 $mL \cdot min^{-1}$. Volume injected: 25 µL. Applied potential: 500 mV vs. Ag/AgCl.

From Figure 3.2-5 it can be concluded that substrate concentrations lower than 10 mM should not be used because the dependency of the rate of conversion with the substrate concentration could lead to irreproducible results. Although the initial concentration of enzyme tested was 0.5U, to improve detection limits lower concentrations were used in subsequent experiments.

Once the detection system had been tested and the enzymatic system had been optimised, the following procedure (as described in Figure 3.2-2) was used to perform the inhibition test. With water and ATCh dissolved in buffer A running through channels ***e*** and ***c***, respectively, the enzyme dissolved in buffer B was injected (25µL) into the microfluidic system through channel ***d*** by means of the 6-way injection valve. Under these conditions no inhibition should take place, so the signal obtained was considered as the maximum signal that could be reached. After that, water in channel ***e*** was replaced by a carbofuran standard solution and the procedure was repeated in triplicate. This time, due to the inhibition of the enzyme, the signal obtained was lower than the blank signal, with a carbofuran concentration-dependent decrease. After the blank measurement, each standard took 20 minutes to be analysed by triplicate, which means a frequency of 3 analysis·h^{-1}. The degree of inhibition was calculated as:

$$\%I = \frac{S_{blank} - S_{inhibition}}{S_{blank}} \times 100$$

Four carbofuran standard solutions were tested (68, 113, 226 and 453 nM), and the resulting calibration plot is shown in Figure 3.2-6. The method showed a good correlation between the percentage of inhibition and the concentration of carbofuran (r^2 0.9983), with a sensitivity of 0.128 %I·nM^{-1}. The LOD, defined as the concentration that produces 5% of inhibition, was 36 nM carbofuran. This value can be considered as excellent compared to those values found in the literature taking into account that the inhibition takes place in continuous mode, without any pre-incubation of the enzyme and the inhibitor. This shows the capability of the two passive mixers to act as reactors without the need to work with stopped-flow conditions,

which would decrease the amperometric system response and would lower the analysis frequency. Moreover, in spite of the common ceramic wall roughness (~5 μm), which could favour enzyme adsorption, no contamination effects due to enzyme residues along the mixers trajectory have been observed.

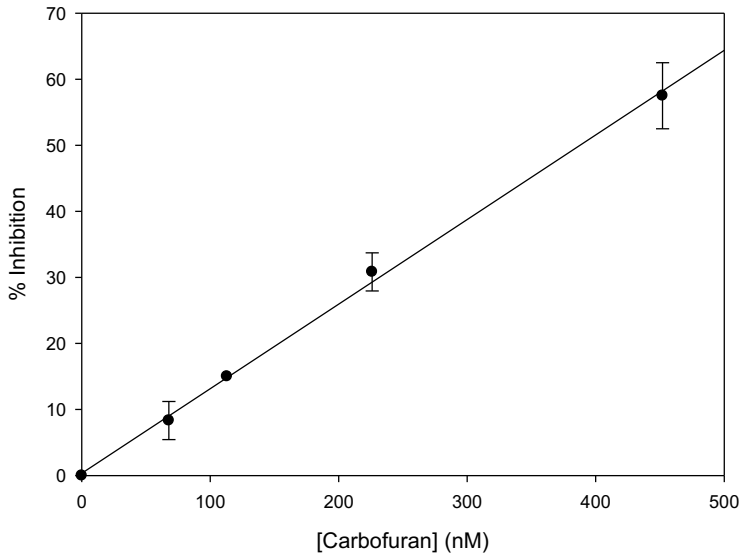


Figure 3.2-6 Calibration curve for carbofuran standard solutions (68, 113, 226 and 453 nM). Flow rate: 0.1 mL·min^{-1}; Volume of enzyme injected: 25μL of 0.5 U·mL^{-1} stock (0.0125 U). [ATCh] = 10 mM. Applied potential: 500 mV vs. Ag/AgCl.

3.3 Magnetic Biosystem in a Lab-on-a-chip

3.3.1 INTRODUCTION

The European Council Directive 98/83/CE, on the quality of water intended for human consumption, sets individual maximum values for toxic substances. Thus, the maximum concentration of any individual pesticide is fixed at 0.1 ppb, and the total amount must not exceed 0.5 ppb. Carbamates and organophosphates represent the most widely used

pesticides. Although they are not as persistent as the banned organochlorate compounds, they are extremely toxic due to their molecular structure, which is very similar to that of acetylcholine (ACh), so they can irreversibly bond to the active site of the enzyme acetylcholinesterase (AChE) and inhibit its activity, which is essential in the transmission of the nerve impulse.[127] The pronounced toxicity of these compounds makes necessary their detection in a sensitive, rapid and efficient way. The methods generally used are based on chromatographic techniques involving time-consuming extraction and purification procedures that are not compatibles with the strict requests for in-field monitoring of contamination episodes. In the screening field a number of batch- and flow-based systems have been developed using enzymes in solution,[128] biosensors and bioreactors containing immobilised enzymes,[119,120,129,130] tissues[131] and micro-organisms[132–134] or antibodies.[99,135] Among these biological detection systems, those based in the inhibition of the activity of cholinesterases offer excellent sensitivity and specificity.

Despite the large number of advantages offered by biosensors and bioreactors based on enzyme inhibition, it is important to consider that enzymatic activity is lost after a measuring cycle. This phenomenon affects the lifetime of the biosensing device that contains a certain amount of immobilised enzymatic material. The use of special re-activators, such as 2-PAM (pralidoxime chloride) in the case of cholinesterases,[136] has been proposed to lengthen the life of the biosensor. Nevertheless these additional procedures besides complicating the measurement protocol,[137] may increase the analysis time or induce other experimental errors. The automation of the analytical procedure by coupling the biosensor with a flow system to facilitate the regeneration steps represents another alternative.[138]

An interesting approach to overcome the regeneration problem is the use of bio-labelled magnetic beads (MBs)[96,139,140] that have shown attractive performance even for DNA[141] and protein[142] sensing. MBs can be introduced into a microfluidic channel and immobilised in a capture region with the aid of a magnetic field, constructing a bioreactor before the detection system or directly onto the surface of an electrochemical transducer. Once the inhibition has taken place, MBs can be easily removed and substituted by a fresh new set.

The use of magnetic beads as a versatile platform for an easy *in-situ* replacement of the inhibited enzyme inside a glass lab-on-a-chip[143] coupled to an amperometric detection system to detect thiocholine (TCh) is presented. Thiocholine is the electrochemically active product of the AChE-catalysed hydrolysis when acetylthiocholine (ACTh) is used as substrate (Figure 3.3-1). This approach allows sensitive detection of carbofuran (one of the most toxic carbamate pesticides) down to the nanomolar (sub-ppb) level.

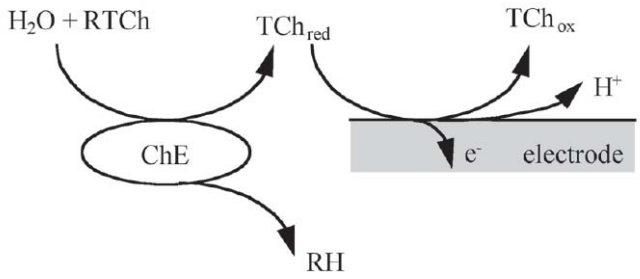


Figure 3.3-1 Amperometric determination of cholinesterase activity. ATCh is used instead of ACh, because once it has been hydrolysed by the enzyme the resulting product, TCh, can be oxidised in a polarised working electrode, and the resulting current can be related to the enzyme activity.

3.3.2 EXPERIMENTAL SECTION

3.3.2.1 Reagents

Sodium dihydrogenphosphate, disodium hydrogenphosphate and potassium chloride were obtained from Fluka. ATCh (Sigma) standard solutions were prepared in 10 mM phosphate and 10 mM potassium chloride aqueous solution buffered at pH 7.4 (running buffer). Carbofuran (analytical standard) was purchased from Riedel-de Haën. Pesticide standard solutions were prepared daily in double-distilled water by dilution from a 250.89 ppm stock in ethanol. AChE type VI-S from Electric Eel was purchased from Sigma. Epoxy group surface-activated magnetic beads of 2.8 μm diameter (surface area 24.63 μm^2), Dynabeads M-270 Epoxy, were purchased from Dynal BioTech. The specific surface area of these beads is 2-5 m^2/g, and the active chemical functionality is between 0.1 and 0.2 mmol/g. Once inserted and immobilised in the capture region, and assuming close packing of spherical beads, the

inter-bead volume was around 3.33 nL (calculated from the diameter of beads, 2-8 μm, and the amount of beads inserted, around $8.25x10^5$).

A 0.1 M TCh stock solution was prepared by adding 25 units of AChE to 100 mL of a 0.1 M ATCh solution and allowing the hydrolysis to take place for 3 hours. TCh standard solutions were prepared by dilution of this stock solution in running buffer.

All reagents were of analytical grade and all experiments were performed at room temperature.

3.3.2.2 Safety considerations

Carbofuran is highly toxic and should be handled in an extractor hood. Skin and eye contact as well as accidental inhalation or ingestion should be avoided.

3.3.2.3 Apparatus

The lab-on-a-chip device used in this study consisted of a glass chip fabricated by means of wet chemical etching and thermal bonding techniques, and it was obtained from Micralyne (Model MC-BF4-001, Edmonton, Canada). The 88 mm x 16 mm chip consisted of a four-way injection cross, with a 74 mm main channel and side arms of 5 mm long each. The original waste reservoir was cut off so the working electrode could be placed just opposite to the channel outlet, thus facilitating the end-channel amperometric detection (as described below). The channels were 50 μm wide and 20 μm deep. The microchannels of the glass chip were treated before use by rinsing with 0.1 M NaOH during 10 minutes and deionised water for another 10 minutes.

A Plexiglas holder was fabricated for holding the chip and housing the detector and reservoirs as described elsewhere.[144] An NdFeB permanent magnet (cylinder-shaped, 6 mm height and 6 mm diameter) was used for handling and positioning the magnetic beads. The detection system consisted of a platinum wire counter, an Ag/AgCl wire reference electrode and a

platinum disc working electrode (CH Instruments, Austin, TX, USA) with diameter of 2 mm. Reference and counter electrodes were inserted through holes drilled in the Plexiglas holder. The working electrode, housed in a plastic screw, was placed opposite to the channel outlet at distance of 50 μm, measured under microscope. The surface of the working electrode was renewed and smoothed with alumina paper (polishing strips 301044-001, Moyco Precision Abrasives Inc., Montgomeryville, PA, USA) between measurements. Amperometric detection was performed with an electrochemical analyser 621 (CH Instruments, Austin, TX, USA) connected to a personal computer. A laboratory-made high-voltage power supply with an adjustable voltage range between 0 and +5000 V was used as driving force for the electroosmotic flow. Substrate and sample injections were performed after stabilisation of the baseline. In Figure 3.3-2 a schematic (not in scale) drawing (A) and a picture (B) of the lab-on-a-chip used are shown.

3.3.2.4 Magnetic beads coating procedure

The immobilisation of the enzyme onto the MB surface was achieved following the coating procedure proposed by Dynal Biotech. Dry beads were washed and equilibrated three times with 0.1 M sodium phosphate buffer (PBS) at pH 7.4 for 10 minutes and resuspended in dimethyl formamide (DMF) to a final concentration of $2 \cdot 10^9$ MB·mL^{-1}, and then stored in this organic solvent at 4º C when not in use. 90 μL from this stock solution ($1.8 \cdot 10^8$ MB) were washed four times with PBS and re-suspended in a vial with 60 μL of this buffer. Then 60 μL of 3 M ammonium sulphate and 60 μL of enzyme solution (1500 μg·mL^{-1}) were added to the vial and the mixture was incubated for 24 hours at 37ºC with slow tilt rotation to allow the covalent bonding of the enzyme with the epoxy groups of the beads. After four washing steps with PBS, the MBs were re-suspended to a concentration of $1 \cdot 10^9$ MB·mL^{-1} with running buffer and were ready to use.

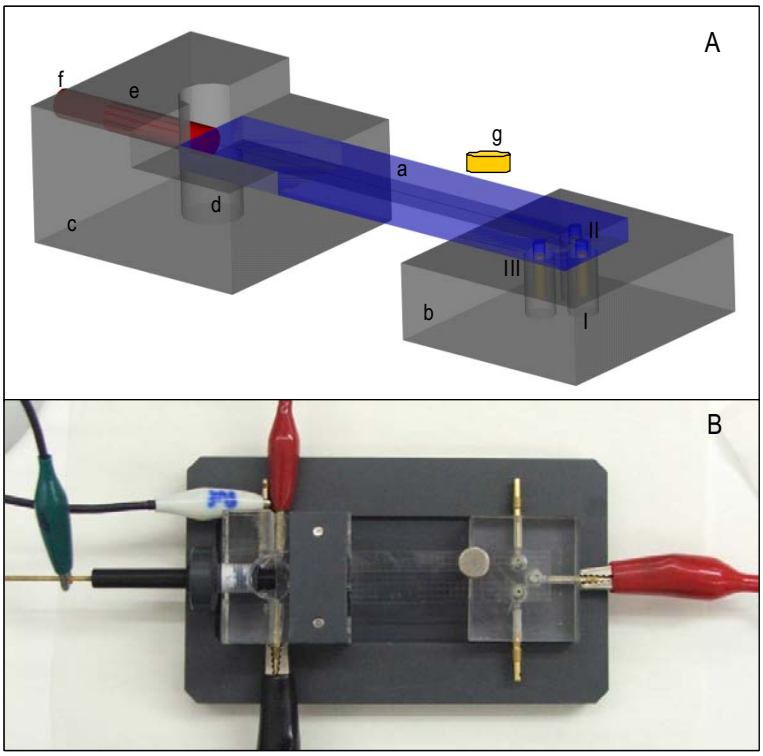


Figure 3.3-2 A) Schematic drawing of the lab-on-a-chip system with electrochemical detection for enzymatic inhibition determination. (a) Glass microchip, (b) Plexiglas body for reservoirs, (c) Plexiglas body for detection system, (d) detection reservoir, (e) hole for working electrode placement, (f) working electrode, (g) magnet, (I) buffer reservoir, (II) ATCh reservoir, (III) pesticide reservoir. B) Top view of the experimental setup, showing the connections of the detection system and high voltage electrodes.

3.3.3 RESULTS AND DISCUSSION

3.3.3.1 Hydrodynamic voltammograms

Hydrodynamic voltammograms (HDVs) were recorded in order to determine the optimum working potential to be applied to the platinum electrode in the determination of TCh. The HDV curve (current vs. applied potential) was obtained point-wise by making 100 mV changes in the applied potential from 0 to +0.9 V and measuring the produced current corresponding to TCh oxidation while injecting a fixed volume of 5mM ATCh solution over the enzyme-

labelled magnetic beads immobilised in the main channel. Results, depicted in Figure 3.3-3, showed a rising tendency of the produced current from +0.2 V to higher potentials. The background current also showed a similar trend in terms of current but with an increase starting at +0.7 V. The potential value of +0.6 V, which offered the most favourable signal-to-noise ratio was selected as working potential for future measurements.

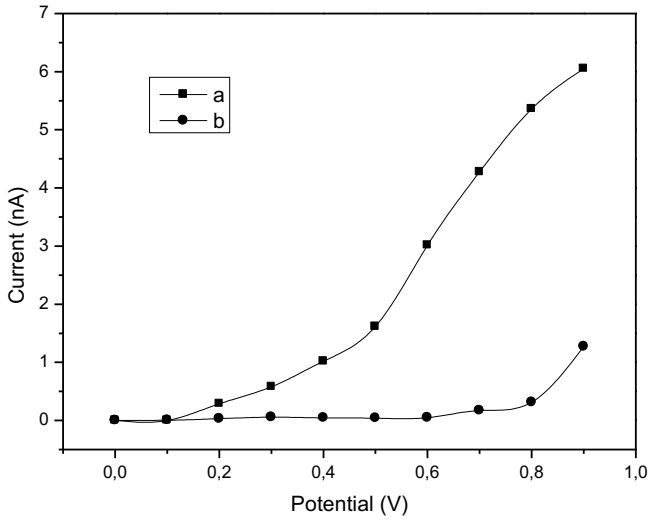


Figure 3.3-3 Hydrodynamic voltammogram for TCh (a) and the corresponding background current obtained using running buffer (b). Conditions: ATCh concentration, 5 mM; amount of magnetic beads, approx. $8.25x10^5$; electroosmotic flow voltage, 1.5 kV; injection voltage, 1.5 kV; injection time, 10 s; running buffer, 10 mM phosphate and 10 mM KCl at pH 7.4.

3.3.3.2 Response stability towards TCh

In order to prove the reproducibility of the electrode performance towards the detection of TCh a series of repetitive injections of a 5 mM ACTh solution through a fixed amount of enzyme-labelled magnetic beads was carried out. Results for the average current response and peak time were 5.16 nA (relative standard deviation, RSD=2.13%, n=6) and 69.22 s (RDS=0.84%, n=6) respectively. Apparently, the detection of TCh does not produce fouling of

the electrode surface and the substrate injection procedure is highly reproducible. Figure 3.3-4 depicts the current peaks obtained in this characterisation.

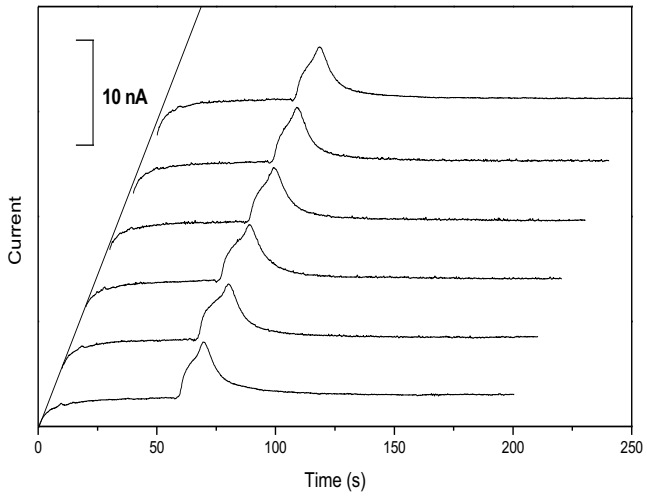


Figure 3.3-4 Precision of repetitive injections of ATCh through the enzyme-labelled magnetic beads. Conditions: working potential, +0.6V; ATCh concentration, 5 mM; amount of magnetic beads, approx. $8.25x10^5$; electroosmotic flow voltage, 1.5 kV; injection voltage, 1.5 kV; injection time, 5 s; running buffer, 10 mM phosphate and 10 mM KCl at pH 7.4.

3.3.3.3 Calibration curve for TCh

Apart from the reproducibility of the detection performance it was also important to determine the capability of the detection system to provide proportional response to different concentrations of TCh. The previous evaluation of the capability for TCh detection is crucial due to the fact that the degree of inhibition would be calculated as the relative decrease of substrate conversion caused by the reduction of the enzyme activity. This evaluation was achieved by performing a calibration curve (Figure 3.3-5) after the injection by triplicate of four ATCh standard solutions, ranging from 5 to 20 mM. The calibration

measurements were carried out replacing consecutively the substrate standard solution from reservoir II after each injection. Sensitivity (0.18 nA mM^{-1})

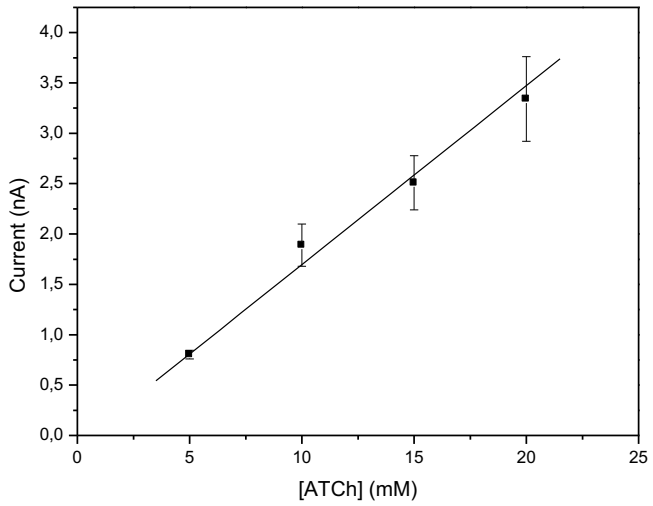


Figure 3.3-5 Calibration curve for ATCh standard solutions (5, 10, 15 and 20 mM). Conditions: working potential, +0.6V; amount of magnetic beads, approx. $8.25x10^5$; electroosmotic flow voltage, 1.5 kV; injection voltage, 1.5 kV; injection time, 5 s; running buffer, 10 mM phosphate and 10 mM KCl at pH 7.4.

and correlation coefficient (0.9946) prove the linear dependence of the current response towards the substrate concentration in the range from 5 to 20 mM, and the adequacy of the detection system to perform the inhibition tests.

3.3.3.4 Electroosmotic flow driving voltage

The field strength applied to produce the electro-osmotic flow was also optimised. As expected, the elution time decreased almost 10 times when the applied voltage between the outlet and inlet reservoirs was increased from +500 V (t=190.6 s) to +4000 V (t=22.9 s). Figure 3.3-6 shows this effect, together (as in-set) with the peak heights obtained for each applied voltage. The maximum current signal was obtained at 1500 V so this was selected as the

optimum flow driving voltage for further applications. At higher flow driving voltages the flow rate was so high that the magnetic field of the permanent magnet was not strong enough to retain the magnetic beads and some of them escaped from the main plug. At 4000 V magnetic beads were completely disaggregated and short and broad double peaks were obtained.

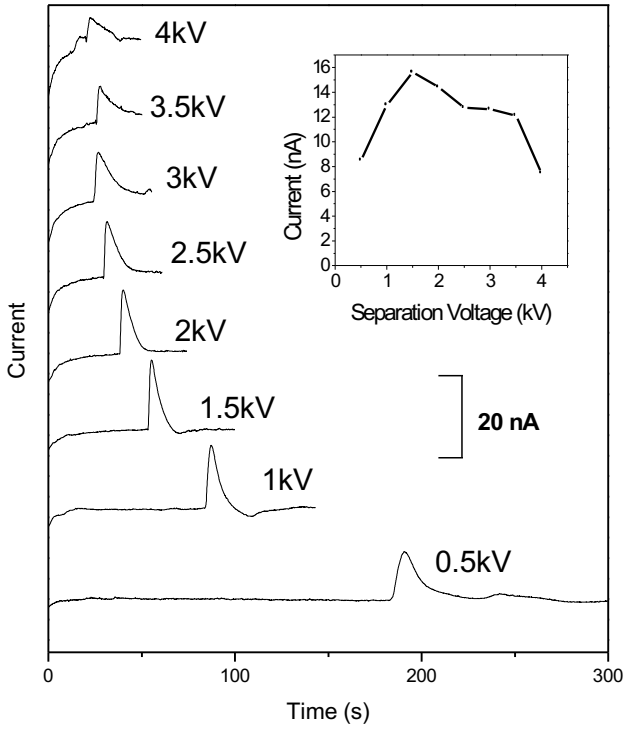


Figure 3.3-6 Optimisation of the electroosmotic flow driving voltage. Conditions: working potential, +0.6V; amount of beads, aprox. $8.25x10^5$; ATCh concentration, 10mM; injection voltage, 1.5 kV; injection time, 5 s; running buffer, 10 mM phosphate and 10 mM KCl at pH 7.4.

3.3.3.5 Acetylcholinesterase inhibition

Figure 3.3-7 schematically shows the steps (1 to 6) involved in the determination of pesticides. Initially (Step 1) a defined amount of MB-AChE was introduced and positioned

into the main channel of the chip dragging them from reservoir I with the aid of a permanent magnet. In order to ensure the maximum reproducibility between consecutive inhibition measurements, all the magnetic beads introduced in the reservoir must be flushed through the channel.

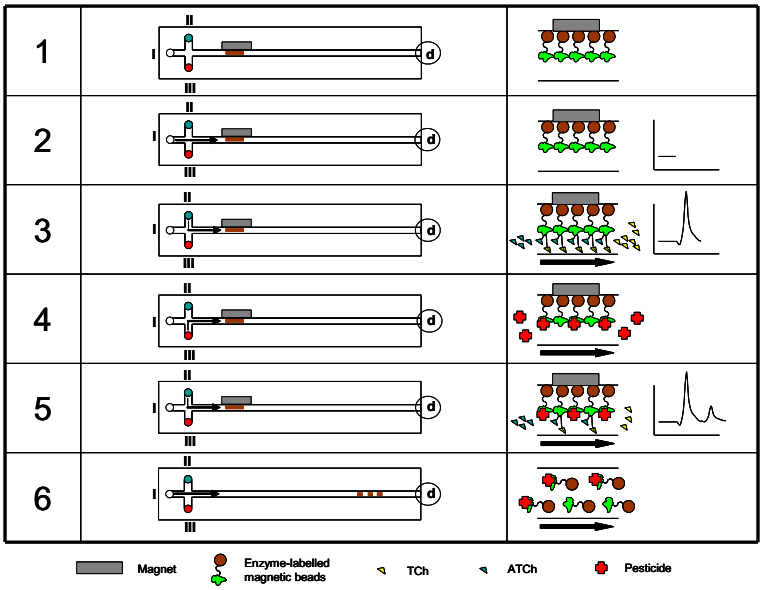


Figure 3.3-7 Schematic drawing of the steps involved in the determination of pesticide by enzymatic inhibition using enzyme-modified magnetic beads on chip: 1) Introduction and retention of the magnetic beads, 2) Injection of running buffer and obtaining of the background signal, 3) Injection of substrate (ATCh) and obtaining of the blank signal, 4) Injection of the inhibitor (carbofuran) and enzyme inhibition, 5) Injection of substrate and obtaining of the inhibition signal, 6) Removal of the magnetic field and flush out of the beads. Shown are the reservoirs for injection of MBs-AChE suspension and running buffer (I), ATCh solution (II), inhibitor solution (III) and electrochemical measurements (d).

In case some beads aggregated and blocked the entrance of the channel, they could be easily disaggregated by rapid movements of the magnet. Once the magnetic plug had been correctly positioned inside the channel, a high voltage was applied between reservoir I and the detection reservoir (d) to produce the electro-osmotic flow of the buffer solution and to achieve the stabilisation of the background current (Step 2). Next, the application of the high voltage was switched from reservoir I to reservoir II for a fixed time (ranging from 5 to 30 s) to introduce a volume of ATCh solution and push it through the MB-AChE plug (Step 3). When

the substrate ATCh passed through the magnetic bioreactor it was hydrolysed producing TCh (Figure 3.3-1). TCh is an electro-active substance that can be detected on the electrode surface polarised at +0.6 V (optimum value), leading to the blank signal. Inhibition (Step 4) occurred when a certain amount of inhibitor solution (carbofuran) was introduced in the main channel from reservoir III. Then the injection of ATCh was repeated (Step 6) and the inhibition peak obtained. Finally, the permanent magnet was removed (Step 6) in order to flush out the magnetic beads and prepare the chip for further measurements.

The degree of AChE inhibition was calculated as the relative decrease of the amperometric signal:

$$\%I = \frac{S_{blank} - S_{inhibition}}{S_{blank}} \times 100$$

where S_{blank} is the current obtained after the first injection of ATCh (Step 3) and $S_{inhibition}$ is the current obtained after inhibition of the enzyme (Step 5).

The AChE inhibition depends on the pesticide concentration as well as on the time during which the AChE is exposed (or incubated) to the pesticide. The former factor, which corresponds to the injection time of the pesticide (step 4), can be significant at low pesticide concentrations. Thus, AChE inhibition was evaluated by measuring the %I for various pesticide concentrations at two different injection times (60 and 90 seconds). The obtained %I for increasing concentrations of carbofuran ranging from 0.1 to 20 ppb were used to construct the AChE inhibition calibration curves for each of the inhibition times assayed (Figure 3.3-8). Results showed a good linear correlation between the percentage of inhibition and the concentration of carbofuran, with a sensitivity of 2.58 %I ppb^{-1} (r=0.9989) for 60 seconds of injection and 9.28 %I ppb^{-1} (r=0.9996) for 90 seconds. The limits of detection, calculated as the concentration of carbofuran that would produce a 5% of inhibition, were 0.96 ppb and 0.34 ppb respectively.

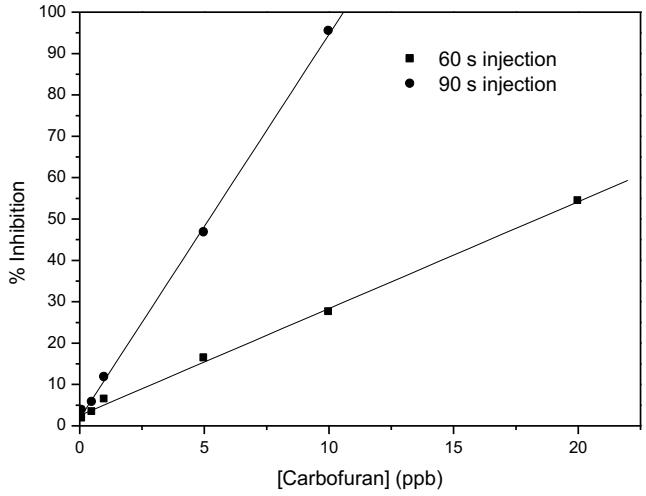


Figure 3.3-8 Calibration curves for carbofuran standards (0.1, 0.5, 1, 5, 10 and 20 ppb) by on-chip enzymatic inhibition for 60 and 90 seconds injections. Conditions: working potential, +0.6V; amount of beads, aprox. $8.25x10^5$; ATCh concentration, 10mM; injection voltage, 1.5 kV; substrate injection time, 5 s; running buffer, 10 mM phosphate and 10 mM KCl at pH 7.4.

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5 Conclusions

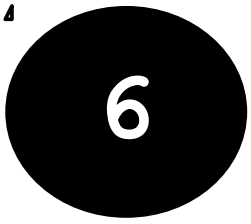
En el primer treball s'ha dissenyat, construït i optimitzat un nou biosensor basat en un biocompòsit de grafit, resina epoxi no conductora, GOD i TTF·TCNQ, i s'ha integrat en un sistema FIA per a la determinació de glucosa. S'ha demostrat l'efectivitat de la sal orgànica conductora TTF·TCNQ com a mediador un cop integrada en el compòsit. El mecanisme de transferència electrònica pot ser degut a la mediació homogènia de petites quantitats de TTF·TCNQ dissoltes que actuen de mediadors. El biosensor és estable a potencial compresos entre -0.1 i +0.3 V (vs. Ag/AgCl), on es comporta com un elèctrode de material inert capaç de de dur a terme reaccions electroquímiques sense oxidar-se o reduir-se ell mateix. Això permet la seva utilització a potencials relativament baixos, mostrant la màxima sensibilitat a +0.3V vs Ag/AgCl. En aquestes condicions s'eliminen totes aquelles interferències provocades per espècies químiques que s'oxiden a potencials superiors.

El biosensor proposat té els avantatges inherents als biosensors basats en biocompòsits. És fàcil de fabricar, és de baix cost i mitjançant un simple poliment mecànic s'obté una superfície biosensora totalment regenerada.

En la segona part de la tesi es mostra la construcció i avaluació mitjançant un test de inhibició enzimàtica per a pesticides d'un microsistema basat en LTCC amb detecció amperomètrica. Aquesta detecció es basa en dos làmines de platí que actuen com a elèctrodes de treball i auxiliar, i en un elèctrode de referència serigrafiat de Ag/AgCl. La sensibilitat del sistema en la detecció de tiocolina va ser de 3.239 $\mu A \cdot mM^{-1}$. S'ha demostrat l'efectivitat dels dos

mescladors passius integrats en la inhibició de l'acetilcolinesterasa i en la hidròlisi de l'acetiltiocolina. Pel que fa a la determinació de pesticides, els paràmetres analítics són una sensibilitat de 0.128 %I·nM^{-1} amb un límit de detecció de 36 nM. Essent un sistema pensat per mesures in situ, aquests resultats són excel·lents. El mateix dispositiu podria ser utilitzat en altres aplicacions que requereixin una o dues etapes de mescla i incubació i detecció amperomètrica d'elevada sensibilitat.

Amb el tercer treball s'han aconseguit límits de detecció extremadament baixos en la determinació del pesticida carbofuran amb la utilització d'un Lab-on-a-chip per inhibició enzimàtica i detecció amperomètrica gràcies a la introducció controlada de petites quantitats de partícules magnètiques amb acetilcolinesterasa immobilitzada. La seva combinació amb la elevada sensibilitat en xip de la detecció amperomètrica confereix al sistema una resposta sensible i estable a la presència de pesticides. Aquest mètode pot trobar aplicacions no només en el camp mediambiental sinó també en seguretat i anàlisis forènsiques, ja que els pesticides poden ser emprats amb finalitats terroristes com a agents nerviosos.



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Integration of a glucose biosensor based on an epoxy-graphite-TTF·TCNQ-GOD biocomposite into a FIA system

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Abstract

An amperometric glucose biosensor based on graphite and non-conducting epoxy resin biocomposite was constructed. Glucose oxidase (GOD) and the tetrathiafulvalene–tetracyanoquinodimethane (TTF·TCNQ) conducting organic salt were incorporated into the bulk of the composite to form a renewable biosensor. Several graphite-TTF·TCNQ ratios (w/w) were studied in order to select the best biosensor to be integrated in a FIA system for the automated detection of glucose. The optimal amount of GOD in the composite was studied as well. The selection was based on the analytical response of the electrodes. Best results were obtained by an electrode whose composition was 5% GOD, 76% polymer, 9.5% graphite and 9.5% TTF·TCNQ. An especially designed flow amperometric cell was constructed so that the biosensor could be integrated into a FIA system and glucose in beverage samples could be determined.

Keywords: Glucose; Biosensor; Graphite-epoxy biocomposite; TTF·TCNQ; FIA; Amperometric detection

1. Introduction

The search for novel materials to design electrochemical biosensors is a problem of great concern. The development of composite-based electrodes may lead to important advances in biosensor devices. These rigid conducting composites combine the electrical properties of graphite with the ease of processing of plastics, showing excellent electrochemical, physical, mechanical and economical properties [1,2]. It should also be taken into account that a great number of biological materials can be incorporated by blending them with the polymer and the graphite to form a biocomposite material [3]. In this way, the sensing material acts as a reservoir of the biological components, and the polishing of the surface allows its renewal. Rigid conducting graphite-polymer biocomposites have been extensively used in our laboratories for electrochemical sensing. Thus, enzymatic [4–7], immunologic [8,9] and genetic [10,11] sensors have been developed in the last decade.

Enzymatic amperometric glucose biosensors have been widely studied in the last four decades [12], and many approaches referring to their design, materials, membrane composition or enzyme immobilisation strategies have been described [13–15]. The first devices developed (first-generation of glucose biosensors) were based on the use of the oxygen as natural co-substrate and on the detection of the produced hydrogen peroxide. The main problem of this simple approach was that the amperometric measurement of hydrogen peroxide required the application of a potential in which other species could be oxidized (i.e. ascorbic and uric acids) limiting the selectivity of the method. Another drawback was the errors produced by the fluctuations in the oxygen tension, known as the "oxygen deficit". In the second-generation of glucose biosensors, the oxygen is replaced by another electro active species able to transfer electrons from the redox centre of the enzyme to the surface of the electrode. Ideally, these mediators should be able to react rapidly with

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the reduced enzyme, its heterogeneous kinetics should be reversible, they should have a low and pH independent overpotential for the regeneration of its oxidized form, both oxidized and reduced forms should be stable and finally, the reduced form should not react with oxygen [16]. Despite all these theoretical requirements, most of the mediators traditionally used (ferrocene and its derivatives, tetrathiafulvalene, some organic dyes, ferricyanide) are soluble in both or at least one of their redox states. This fact requires them to be used in solution or entrapped onto the electrode surface by means of membranes. Other elaborated strategies to keep mediators in contact or as close as possible to the immobilised enzyme in the biosensor are polymerisation and electrodeposition.

Organic conducting salts, such as tetrathiafulvalene–tetracyanoquinodimethane (TTF·TCNQ) allow direct electron-transfer with a number of enzymes, particularly flavoproteins (glucose oxidase) through their prosthetic group. NADH can also be oxidized onto the organic salt surface and thus, dehydrogenases can be used together with the organic salt as well. However, the electron-transfer mechanism is not clearly understood. Different approaches have been proposed: homogeneous mediation due to small amounts of TTF·TCNQ dissolved [17], heterogeneous catalysis [18] and direct electron transfer, which is the so-called Third-Generation of glucose biosensors [19].

TTF·TCNQ has been incorporated as part of biosensing membranes in different ways. Thus, it can be found packed with the enzyme immobilised on its surface [20,21], adsorbed [22] or grown at the surface of electroconductive films [23–25]. All these techniques are either difficult to perform by non-qualified personnel or time-consuming, and therefore, non-suitable for electrode mass production. In this work TTF·TCNQ powder is hand-mixed together with graphite, glucose oxidase and epoxy resin to easily construct a glucose biosensor based on a rigid conducting biocomposite. This novel sensing material offers attractive features to be used in batch or FIA systems for glucose determinations.

2. Materials and methods

2.1. Reagents

Graphite powder with a particle size of 50 μm (BDH Laboratory Supplies), epoxy resin Epo-Tek H77 (Epoxy Technology), TTF and TCNQ (Fluka), and GOD type VII 185 U mg^{-1} from *Aspergillus niger* (Sigma) were used to prepare the biocomposite paste.

TTF·TCNQ organic salt was prepared by mixing identical volumes of equimolar solutions (0.1 M) of each compound in hot anhydrous acetonitrile. The reaction was let to complete overnight with stirring and the black crystals formed were rinsed with cold acetonitrile and diethyl ether, and then dried under vacuum.

Glucose stock solutions were prepared with D-(+)-glucose monohydrate (Fluka) in phosphate buffer, and were left at 4 °C overnight to allow the equilibration of the anomers. A commercially available Glucose Assay Kit (Sigma) was used for comparison purposes.

Aqueous solutions of 0.1 M phosphate and 0.1 M KCl, buffered at pH 7.5 were used to perform both batch and FIA experiments. All other reagents used were of analytical grade.

2.2. Instrumentation

Current intensities were measured with an LC-4C amperometric controller (BAS Bioanalytical Systems Inc.) connected to a Labograph 517 graphic recorder (Metrohm). Cyclic voltammetries were performed with an Autolab PGSTAT20 (Eco Chemie). The reference electrode was a double junction Ag/AgCl electrode (Orion 900200) with 0.1 M phosphate and 0.1 M KCl as external reference solution. In batch experiments, a platinum electrode (Crison 52-671) was used as counter electrode. pH measurements were performed with a glass electrode (Crison 52-03) connected to a potentiometer (Crison MicropH 2002).

The flow system, shown in Fig. 1, consisted in a Miniplus 3 peristaltic pump (Gilson), a six-way injection valve (Omnifit) and a homemade methacrylate flow cell which integrated both working and counter electrodes. The flow cell volume was 140 μL. This flow cell was formed by a stainless steel counter electrode (block A) that was screwed in methacrylate block B, where the biosensor (C) was allocated.

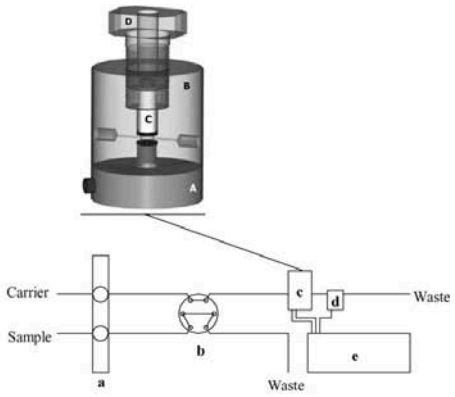

Fig. 1. Flow injection system diagram and flow-through cell detail. The cell (c) has a stain-less steel part (A) that serves as counter electrode. The methacrylate cell body (B) is attached to this lower block by screwing forming an inner compartment where the biosensor (C) is placed and fixed by means of a methacrylate sealing screw (D). The space between the counter electrode and the biosensor defines the cell volume. A standard double junction Ag/AgCl reference electrode (d) is positioned next to the flow-through cell, in the downstream flow. A peristaltic pump (a), a six-way injection valve (b) and the amperometric detection unit (e) complete the flow system.

Table 1
Composition (% weight) of the biocomposite paste used for the biosensors construction

Biosensor	Epoxy resin	Graphite	TTF·TCNQ	GOD
A	80.0	14.0	4.0	2.0
B	80.0	9.0	9.0	2.0
C	80.0	4.0	14.0	2.0
D	80.0	–	18.0	2.0
E	77.4	9.7	9.7	3.2
F	76.0	9.5	9.5	5.0
G	75.0	9.4	9.4	6.2

Water tightness was achieved by two O-rings. The biosensor was fixed with a second methacrylate block (D), also screwed in block B. A magnetic bar was inserted in the cavity to stir the solution and minimize sample dispersion.

2.3. Construction of the graphite-epoxy-TTF·TCNQ-GOD amperometric biosensors

The biosensor material was easily prepared by hand-mixing epoxy resin, graphite powder, TTF·TCNQ and GOD until a homogeneous paste was obtained. The electrodes constructed had the composition shown in Table 1. The paste was placed into a cylindrical PVC body (6 mm inner diameter, 2 cm length), which has an electrical contact, to a depth of 3 mm. Finally, the composite material was cured at 40 °C (higher temperatures, recommended for curing the epoxy-resin, could damage the enzyme) for one week. Once cured, biocomposite excess was eliminated with abrasive paper and finally the surface was smoothed with alumina paper (polishing strips 301044-001, Moyco Precision Abrasives Inc.). When not in use, electrodes were stored at 4 °C.

3. Results and discussion

3.1. Electrochemical behaviour of TTF·TCNQ-based graphite-epoxy electrodes

The sensitivity of the glucose biosensor will depend on its polarization potential, and this is limited by the potential in which the organic salt suffers decomposition and leaching occurs. The cyclic voltammetry of the TTF·TCNQ-based graphite-epoxy electrodes in a typical PBS buffer (phosphate 0.1 M and KCl 0.1 M) conditions used for glucose measuring, was performed to study the organic salt decomposition inside the polymeric matrix. Positive and negative scans (Fig. 2) were applied to observe the TTF·TCNQ behaviour. As shown in Fig. 2a, the TTF·TCNQ starts being oxidized at 0.30 V vs. Ag/AgCl. The oxidized species obtained during the oxidation until the final potential (0.6 V) will be reduced on reversal giving a well-defined peak at −0.01 V. Current intensities

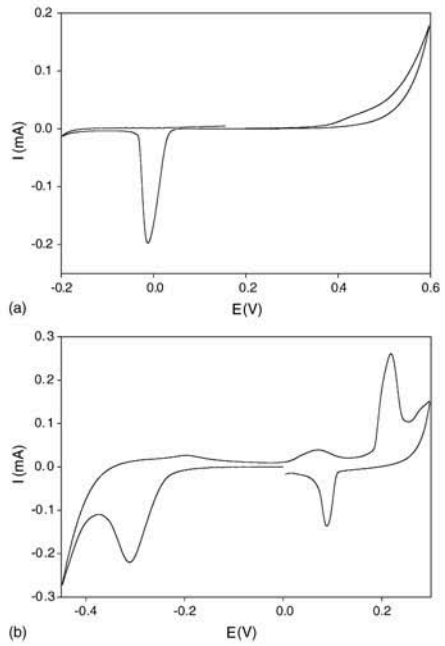

Fig. 2. Cyclic voltammograms of the TTF·TCNQ biosensor in PBS pH 7.5 (phosphate 0.1 M and KCl 0.1 M): (a) initial positive scan with oxidation of the electrode surface and resultant peaks on reversal. Start potential: 0.2 V, forward final potential: 0.6 V and backward final potential: −0.2 V; (b) initial negative scan with reduction of the electrode surface. Start potential: 0 V, forward final potential: −0.45 V and backward final potential: 0.3 V. Scan rate: 0.005 V/s.

observed may be attributed to the following reactions [26]:

$$E > 0.30\,\text{V}: \quad \text{TTF}\cdot\text{TCNQ} + 2\text{X}^- \rightarrow \text{TTFX}_2 + \text{TCNQ} + 2\text{e}^-$$

$$E = -0.01\,\text{V}: \quad \text{TCNQ} + \text{M}^+ + \text{e}^- \rightarrow \text{MTCNQ}$$

$$E = 0.22\,\text{V}: \quad \text{MTCNQ} \rightarrow \text{TCNQ} + \text{M}^+ + \text{e}^-$$

where M^+ and X^- represents cations and anions present in the working solution. The nature of these spices determines the solubility of the corresponding salts.

On the other hand, the negative scan (Fig. 2b) shows that the reduction of the organic salt starts at −0.2 V, giving insoluble TTF and TCNQ. If sodium or potassium ions are present in the electrolyte, an insoluble TCNQ salt is deposited, which is also electro active (oxidation peaks between 0 and 0.3 V shown in Fig. 2b).

Results obtained in both scans show that composite electrodes containing TTF·TCNQ can be polarized at any

potential between −0.1 and 0.3 V vs. Ag/AgCl without risk of decomposition of the organic salt. The working potential was finally established by using cyclic voltammetry studies (results not shown) of the biosensor in the presence of increasing concentrations of glucose. Maximum sensitivity was achieved at 0.3 V vs. Ag/AgCl, but lower applied potentials could be selected in order to minimize interferences.

3.2. Effect of oxygen upon biosensors response

Oxygen may induce a diminution on the response of the biosensor towards glucose because a competition between itself and the organic salt to carry out the oxidation of the $FADH_2$ group of GOD can be established. The responses of the prepared glucose biosensors, in the presence or absence of oxygen, were compared to clarify the possible competitive reaction mechanisms (Fig. 3).

When oxygen was completely removed from the working solution (before and during the experiment, not allowing diffusion from the atmosphere) all the active individual enzyme molecules were oxidized back onto the TTF·TCNQ surface due to the applied potential (inset mechanism A in Fig. 3) and the observed sensitivity to glucose concentration changes was high. On the other hand, when some oxygen was present in the solution (i.e. not removing it or removing it only before the experiment and thus, allowing some diffusion from the atmosphere). Mechanism B (Fig. 3 inset) took place simultaneously with mechanism A. Taking into account that 1.15 V [27] should be necessary to oxidize hydrogen peroxide and that the actual applied potential was 0.15 V, hydrogen peroxide could not be detected by the biosensor. For that reason, the number of active sites performing mechanism A was lower than in the absence of oxygen, resulting in a decreased sensitivity. When oxygen was completely consumed, all the enzyme molecules performed mechanism A, and an increase of the sensitivity was observed.

The above results demonstrate that, in order to obtain reproducible glucose signals, the biosensor must operate under absence of oxygen, responding through TTF·TCNQ mechanism only and avoiding a non-reproducible 'bi-mechanism' response due to the oxygen effect.

3.3. Biocomposite composition

Epoxy resin Epo-Tek H77 has very interesting properties concerning chemical stability but, compared to other commercially available epoxy resins, the amount of solid material that can be incorporated in its matrix is limited to 20% in weight. Whenever this amount is overtaken, the plastic phase loses continuity and the material does not cure properly.

To improve the conductivity of the biocomposite, and thus, the sensitivity, graphite powder was added into the paste because TTF·TCNQ is not a pure conductor and the electron flow could be limited if no other conductive material was added. On the other hand, adding graphite powder could reduce the effective TTF·TCNQ surface in which the prosthetic group of the enzyme could be oxidized, decreasing the biosensor sensitivity. It was necessary, then, the optimisation of the graphite/TTF·TCNQ ratio. For that reason, biosensors with various graphite/TTF·TCNQ ratios (A–D from Table 1) were studied, and the corresponding responses toward glucose additions are shown in Fig. 4. Both from the point of view of sensitivity and linear range of response best results were

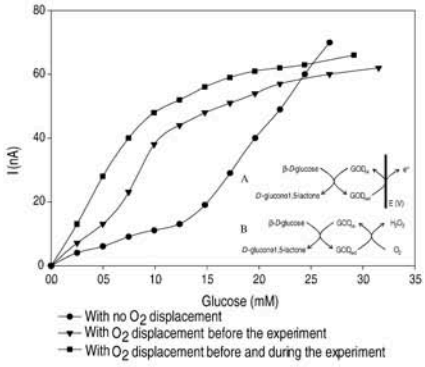

Fig. 3. Effect of the oxygen in the response of the TTF·TCNQ biosensor. The two possible mechanisms for the oxidation of the enzyme are indicated. (A) Direct regeneration of reduced GOD onto TTF·TCNQ and (B) regeneration of reduced GOD via natural cofactor and hydrogen peroxide production. Applied potential: 0.15 V and PBS pH 7.5 (phosphate 0.1 M and KCl 0.1 M). Oxygen was removed using a nitrogen flow.

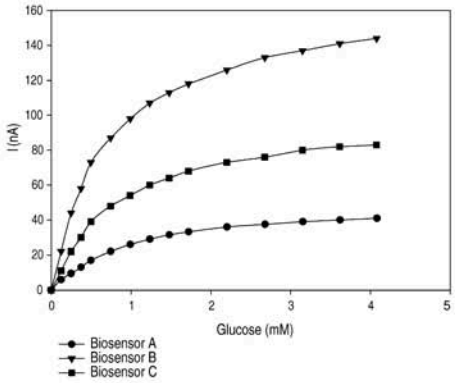

Fig. 4. Effect of the graphite/TTF·TCNQ ratio (w/w) on the biosensor response. Biosensor A: 14% graphite, 4% TTF·TCNQ; biosensor B: 9% graphite, 9% TTF·TCNQ; biosensor C: 4% graphite, 14% TTF·TCNQ. For all biosensors: 2% GOD, applied potential: 0.15 V and PBS pH 7.5 (phosphate 0.1 M and KCl 0.1 M).

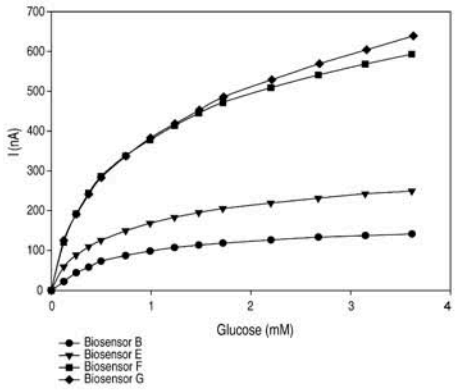

Fig. 5. Effect of the quantity of GOD in the sensing material on the biosensor response. Biosensor B: 2% GOD, biosensor E: 3.2% GOD, biosensor F: 5% GOD and biosensor G: 6.2% GOD. Applied potential: 0.15 V and PBS pH 7.5 (phosphate 0.1 M and KCl 0.1 M).

obtained from biosensor B, with biocomposite containing a 1:1 graphite/TTF·TCNQ ratio. When graphite was the predominant material (biosensor A) the signal was limited by the available TTF·TCNQ surface. On the other hand, when the organic salt was the predominant material (biosensor C, 4% graphite) the biocomposite was not conductive enough. The curve for biosensor D (0% graphite) could not be constructed because the measured current was not stable, indicating that some amount of graphite is necessary to facilitate the flow of electrons through the biocomposite.

Regarding the quantity of GOD, it was expected from Michaelis–Menten mechanism that the higher the active enzyme concentration, the higher sensitivity and the higher linear range of response should be obtained. Biosensors with various GOD contents were prepared and their responses to glucose additions were measured. As expected, increasing quantities of GOD in the biocomposite formulation resulted in better sensitivities. Best responses were obtained from biosensors reaching 5% GOD (see Fig. 5). Biosensors "F" and "G" presented the same response, indicating that there is an optimal amount of enzyme an excess of which is not active or it is not retained in the matrix.

In conclusion, the biosensor that showed better analytical characteristics had the following composition: 76% resin, 9.5% graphite, 9.5% TTF·TCNQ and 5% GOD, and it was selected to perform the flow injection analysis system. The apparent Michaelis constant (K_m) calculated using the Lineweaver–Burk plot method [28] was 0.38 mM.

3.4. Biosensor response in batch measurements

Cyclic voltammetries (in the stability range of potentials of the organic salt, from −0.1 to 0.3 V) at different glucose concentrations over biosensor F were performed, in order to determine the optimum applied potential in terms of sensitivity. As expected the maximum sensitivity for glucose determination was achieved at 0.3 V, so this potential value was selected as the working potential of the biosensors. Nevertheless, lower polarisation potentials could be selected, if necessary, in order to minimize interferences depending on the sample matrix.

It was also necessary to verify that the recorded signal during glucose additions was exclusively due to the electrons generated by the glucose oxidation reaction. In other words, it was necessary to check that no other reaction was being performed at the biosensor surface (directly either over graphite or TTF·TCNQ). For that reason, electrodes with various compositions of graphite, epoxy and TTF·TCNQ, but without GOD, were constructed and their responses to glucose were checked. None of them responded to glucose concentration variations. This indicates that glucose cannot be oxidized directly by the electrode surface without the presence of the GOD. Electrodes containing GOD, but without TTF·TCNQ, were also constructed, and no response towards glucose was observed. This experiment confirmed that the signal obtained for the biosensors was due to the oxidation of the reduced enzyme onto the organic salt surface.

Reproducibility was the last characterisation parameter of the biosensors being evaluated. Because of the complex composition of the sensing material, it is difficult to ensure that its analytical properties are not affected after several uses. Enzyme immobilisation, for example, is not 100% effective, and some leaching on introducing the biosensor under stirring conditions (in batch or FIA systems) can occur. On the other hand, applying a constant potential over the electrode surface can result in the polymerisation of electro active species in the solution forming a film over the surface (fouling). When this occurs, the electrode is passivated and sensitivity decreases. This fact was checked performing three consecutive evaluations of the biosensor response. Fig. 6 shows the resulting calibration curves. It can be seen that if biosensor is used without any surface treatment (apart from rinsing with water) the sensitivity decreases around 60%. To recover the optimal response a simply polishing procedure (as described in the Section 2) was carried out. In this way, fresh GOD replaces the used one and the response is recovered nearly 100%.

3.5. Biosensor integration into a FIA system and glucose determination

An amperometric flow cell for the integration of surface renewable biocomposite electrodes had been previously designed and constructed in our group [4]. Nevertheless, the previous designed cell presented two main drawbacks: the irreproducibility of the cell volume due to the compressibility of the silicone sheet between the two bodies of the cell, and the mechanical instability of the silver-loaded epoxy counter electrode. The new flow cell (see Fig. 1)

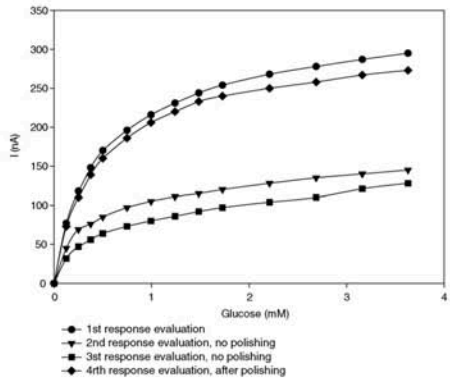

Fig. 6. Consecutive drop of biosensor response without surface renewal, including recovering after polishing. Applied potential: 0.15 V and PBS pH 7.5 (phosphate 0.1 M and KCl 0.1 M).

avoids the drawbacks of the old cell showing an improved reproducibility during the operational cycles along with an enhanced mechanical stability of the stain-less steel electrode, which also improved the signal-to-noise ratio.

The optimum flow rate and sample injection volume were found to be 1 mL min^{-1} and 350 μL, respectively.

The reproducibility of the designed cell was checked by 15 consecutive injections of a 1 mM glucose solution (see Fig. 7). A relative standard deviation of 1.9% indicates a good reproducibility of the signals obtained by this flow trough cell.

Three measurements were performed for each standard used in the construction of the calibration curve, which is shown in Fig. 8. A sensitivity of 322 ± 19 nA mg^{-1} mL, an

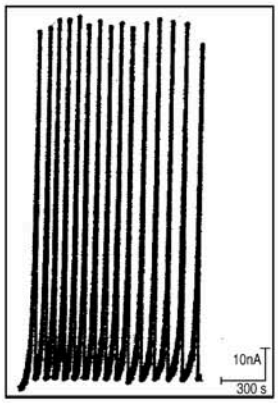

Fig. 7. Record of 15 successive injections of 1 mM glucose. Flow rate: 1 mL min^{-1}, injected volume: 350 μL and applied potential: 0.3 V.

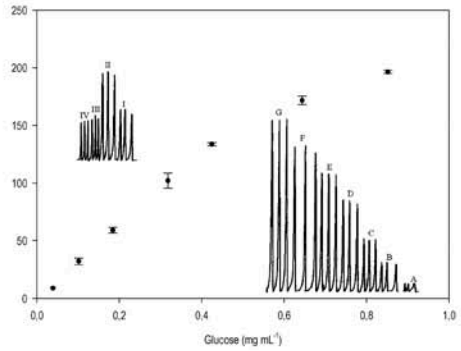

Fig. 8. Calibration curve obtained for standards (ranging from 0.05 to 0.9 mg mL^{-1} glucose) and the corresponding records (Inset: A–G standards, I–IV samples). Flow rate: 1 mL min^{-1}, injected volume: 350 μL and applied potential: 0.15 V.

Table 2
Results for glucose concentration in cola beverage samples obtained by two methods (mg mL^{-1}).

Sample	Method	
	Biosensor-FIA	Colorimetric
I	17.96 ± 0.70	18.75 ± 0.23
II	61.815 ± 1.48	62.39 ± 1.26
III	0.60 ± 0.03	0.70 ± 0.01
IV	0.94 ± 0.05	1.05 ± 0.01

ordinate of -1 ± 5 nA and a linear regression coefficient (r) of 0.9994 were obtained. The graphical register of the obtained signals, being A–G the standards and I–IV the samples, are also shown in Fig. 8 as insets. These real samples were commercial cola beverages, being III and IV "diet" type. Samples were diluted in PBS at pH 7.5 (phosphate 0.1 M and KCl 0.1 M) to fit the linear range of response of the analytical system and to adjust the pH. As stated previously, samples and standards were degassed with N_2, eliminating any possibility of interference from O_2.

The same samples were also analysed by the commercially available Glucose Assay Kit for comparison purposes. The results obtained showed no significant differences between results from both methods (see Table 2).

4. Conclusions

A new biosensor based on a biocomposite made of graphite, a non-conducting epoxy resin, GOD and TTF·TCNQ is prepared and integrated in FIA systems for glucose determination. TTF·TCNQ has shown to be an effective mediator when integrated in the biocomposite matrix. The electron transfer mechanism, not clarified yet, may involve homogeneous mediation by a small amount of dissolved

TTF-TCNQ. The biosensor has a stability potential range from −0.1 to +0.3 V (vs. Ag/AgCl) where it behaves as an inert electrode material, capable of carrying out electrochemical reactions without suffering oxidation or reduction. This fact allows the biosensor to be used at relatively low applied potential showing the maximum sensitivity at +0.3 V. In these conditions, chemical interferences, arising from species that can be oxidized at upper working potentials, are eliminated.

The proposed biosensor bears the inherent advantages of biocomposite-based biosensors. It is easy to be prepared, has a low cost and ensures a new biosensor surface after a simple mechanical polishing. Its application in FIA of glucose shows a good reproducibility and accuracy similar with those obtained by standard optical method.

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Pesticide Determination by Enzymatic Inhibition and Amperometric Detection in a Low-Temperature Cofired Ceramics Microsystem

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Among the several fabrication techniques used to construct microflow systems, the low-temperature cofired ceramics (LTCC) technology, taking advantage of its multilayer approach, is one of the most versatile ones. It permits the integration of several unitary operations of an analytical process in a modular or monolithic way. Moreover, due to its perfect compatibility with screen-printing techniques, it also permits the integration of electronic components used to control the whole system setup. In this work the design, construction, and evaluation of a miniaturized analyzer for pesticide determination that integrates a pretreatment stage, based on two mixers or reactors, and an amperometric detection system to measure the product of an enzymatic inhibition reaction are presented. The detection system was monolithically integrated in the microfluidic platform, and it consisted of a screen-printed reference electrode and two platinum sheets, acting as auxiliary and working electrodes, which were embedded within the ceramic structure. The miniaturized system was characterized and successfully evaluated by determining carbofuran at the nanomolar level.

Among the several materials used in analytical chemistry with miniaturization purposes[1] low-temperature cofired ceramics (LTCC) are becoming a good alternative due to some interesting advantages. From a technical point of view, they permit a fast and very simple prototyping of devices with complex three-dimensional structures. Moreover, the multilayer methodology used facilitates the integration of materials of different nature in the ceramic body. Among these materials, the integration of screen-printing conductors is one of the promising ones because they may be part of the circuitry needed to control the miniaturized analyzer or of the detection system. Another remarkable advantage is the perfect sealing achieved during the fabrication process (in the sintering stage), which avoids the use of glues, gaskets, or complicated procedures.[2–4] In spite of their multiple advantages, the use of these ceramics as microfabrication materials for chemical analysis has not been totally exploited. In the early years, silicon and glass have been the most widely used materials for this purpose; however, the use of these materials is mainly limited to bidimensional devices and the integration of some of the elements needed to carry out a whole analytical process provides an additional challenge.[5] Moreover, the partial or total modification of the designed device may take a long time, due to the complexity of the whole fabrication process. That is why a trend toward polymers has been observed. Polymers allow constructing more complex structures that can also include elements of different nature aimed at several analytical purposes.[6] The LTCC technology can be considered a mixture of the advantages found in both methodologies since three-dimensional structures can be constructed rapidly and at a realistic cost and different elements can be integrated and cofired at the same time resulting in a solid block without leakage problems. Additionally, the technological infrastructure needed is simple and cheaper since clean room facilities are not necessary.

In this work, the fabrication of an LTCC device integrating some of the stages needed to perform the analytical process such as sample/reagent mixing, incubation steps, and detection system is presented. To demonstrate the operational capacity of this technology, a microanalytical system for pesticide determination in water streams based on its inhibition effect on acetylcholinesterase enzymatic activity has been designed and evaluated. It is well-known that pesticides derived from carbamates and organophosphorus compounds are potent acetylcholinesterase inhibitors. This inhibition effect can be followed amperometrically using acetylthiocholine (ATCh) as substrate because thiocholine, the product of its hydrolysis, is an electroactive substance that can be oxidized at the surface of a working electrode polarized between 450 and 600 mV. Many approaches have been proposed to carry out this determination, such us the immobilization of the enzyme in different materials to produce bioreactors[7–9] or the

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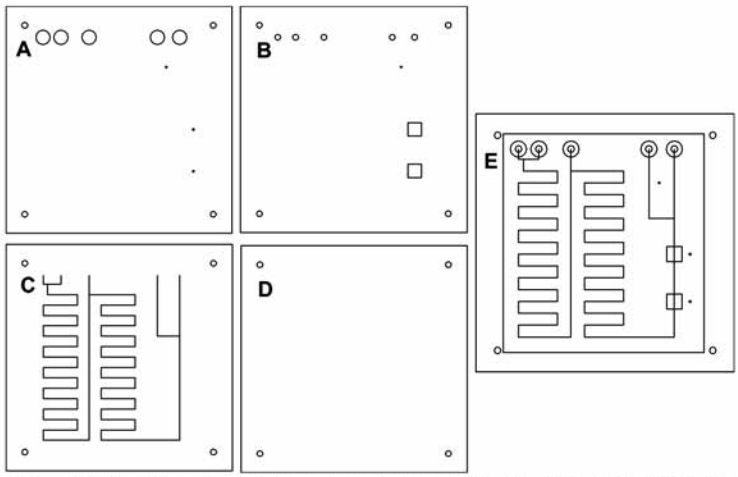

Figure 1. Layers involved in the miniaturized analyzer: (A) the top one (where the fluidic connectors are attached), (B) the layer that supports the platinum sheets, (C) the layers where the channels are mechanized, and (D) the bottom one (the base). Panel E represents all the layers once overlapped.

development of biosensors incorporating the enzyme and its use both in batch and flow systems.[10−12] To simplify the development of the microfluidic system, the enzymatic inhibition was carried out using the enzyme in solution. In this case the steps needed previous to the detection are the incubation of the inhibitor and the enzyme in a first stage and, afterward, the incubation of the inhibited enzyme with the substrate.

Some work has already been done miniaturizing devices aimed at the detection of pesticides or other toxic compounds. Some have scaled down one of the pretreatment steps needed,[13,14] and another has automated this process[15] or integrated a separation step and the detection system.[16] One of the most interesting requirements of a miniaturized analyzer is its portability so that in situ and real time analysis of samples can be performed.

The microflow system consists of two mixing reactors that will act as efficient incubation stages for the two main reactions because of their multiple 90° angles designed to enhance chaotic mixing.[17] The detection system used combines screen-printed conductors and the integration of two platinum sheets acting as counter and working electrodes, proving the versatility of this technology. The reference electrode consists of a silver-based screen-printed conductor integrated inside the LTCC structure. After the oxidation of the silver in chloride, the reference electrode potential will be kept constant by means of a flowing solution of chloride ions.[3]

As a starting point, the integrated amperometric system was characterized for sensitivity and repetitivity without enzymatic reaction. After that, the potentiality of both the reactors and the detection system to carry out the determination of a pesticide, carbofuran, by enzymatic inhibition will be tested.

EXPERIMENTAL SECTION

LTCC Device Construction. Dupont 951 LTCC tapes (thickness of 254 μm) were used. An LPKF ProtoMat C100/HF (LPKF, Germany) milling machine was used to mill the designed layers. The general fabrication procedure was described in detail elsewhere.[3] Figure 1 shows all the layers involved in the fabrication procedure that, once overlapped, turned out to be the desired device. Layer D is the bottom one, and A is the top one. A total amount of 10 layers were used: 2 × A, 2 × B, 4 × C, and 2 × D. After sintering, the height of the device was ~2.2 mm. The four holes in each corner of the layers were used to align them during the lamination process.[3]

Figure 2A shows a picture of the final device, and Figure 2B shows the inner three-dimensional structure. Once the device was burnt-out, the inner channel dimensions were 1.7 mm in width and 430 μm in height. Each reactor had a total volume of 164 μL.

The two platinum sheets (Aldrich, foil thickness 0.5 mm) of 5 × 5 mm each were used as counter and working electrodes. Their integration in the LTCC device is showed in Figure 2C. In order to compensate the shrinkage caused by the sintering process (12.3% in the $x-y$-axis and 15% in the z-axis),[18] the cavities designed to embed the platinum sheets were slightly bigger. In order to

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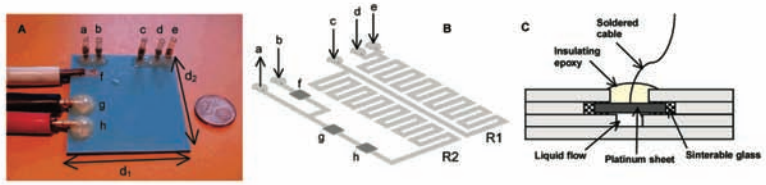

Figure 2. (A) Device picture once burnt-out. (B) Inner three-dimensional structure. (C) Integration of the platinum sheets in the LTCC device. Labels: a, waste; b, 0.1 M Cl^- solution inlet; c, acetylthiocholine (ATCh) solution in buffer A inlet; d, enzyme solution in buffer B inlet; e, water/sample inlet; f, conductor path (connected to the inner reference electrode); g, platinum counter electrode; h, platinum working electrode; d_1, 5 cm; d_2, 5 cm; R1, reactor 1; R2, reactor 2.

ensure a perfect sealing between the ceramic tapes and the metal sheets, sinterable glass (Dupont 9615) was deposited. After the sintering step, commercial connectors were attached to the conductive paths (see Figure 2A) and finally insulated with epoxy glue.

The reference electrode (indicated as f in Figure 2) consisted of an internal screen-printed Ag-based path (Dupont 6142) with its anodized surface in direct contact with the flow channel. The anodization of the Ag path to cover it with a AgCl layer was achieved by applying a voltage of 0.8 V for 20 min in the presence of a 0.2 M Cl^- solution. In order to achieve a stable reference potential, a 0.1 M Cl^- solution flowed constantly through the channel in contact with this path (indicated as b in Figure 2B). The electrical contact between the anodized path and the device surface was achieved by means of a hole filled with dense Ag paste (Dupont 6141). A solderable Ag-based path (Dupont 6146) was printed onto the device surface to provide contact between the miniaturized device and the external electronic setup.

Reagents. Carbofuran (analytical standard) was purchased from Riedel-de Haën. Pesticide standard solutions were prepared daily in double-distilled water by dilution from a 2.3×10^{-4} M stock in ethanol. Acetylthiocholine chloride (Sigma) standard solutions were prepared in 0.1 M phosphate and 0.1 M potassium chloride aqueous solutions buffered at pH 7 (buffer A). Acetylcholinesterase type VI-S from Electric Eel (Sigma) was dissolved in 0.2 M phosphate and 0.2 M potassium chloride aqueous solutions buffered at pH 7.4 (buffer B). Sodium dihydrogen phosphate, disodium hydrogen phosphate, and potassium chloride were obtained from Fluka. A solution of 0.1 M potassium chloride was used to ensure a constant concentration of chloride ions at the reference electrode.

A 0.1 M thiocholine stock solution was prepared by adding 25 units of acetylcholinesterase to 100 mL of a 0.1 M acetylthiocholine chloride solution in buffer A and allowing the hydrolysis to take place for 3 h. Thiocholine standard solutions were prepared by dilution of this stock in buffer A.

Safety Considerations. OP pesticides are highly toxic and should be handled in an extractor hood. Skin and eye contact and accidental inhalation or ingestion should be avoided.

Flow Injection Manifold. The continuous flow system setup consisted of a peristaltic pump (Minipuls 3, Gilson), a six-port distribution valve (Hamilton MVP), silicon tubing with different inner diameters (ISMATEC), and 0.8 mm i.d. Teflon tubing (SCHARLAB, S.L.).

(18) *Dupont LTCC Data Sheets.*

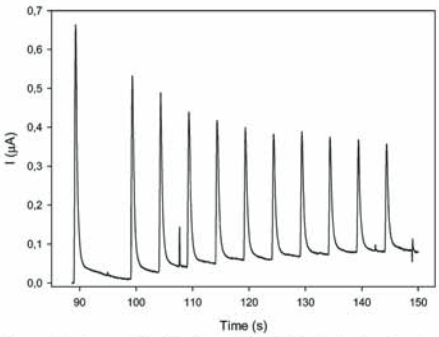

Figure 3. Consecutive injections of 0.1 M thiocholine to show the decrease of sensitivity due to the fouling effect (channel e, buffer A; channel d, buffer A/thiocholine; channel c, blocked). Flow rate: 1 $mL \cdot min^{-1}$. Volume injected: 100 μL. Applied potential: 500 mV vs Ag/AgCl.

Current intensities were measured with an LC-4C amperometric controller (Bioanalytical Systems Inc.) connected to a Duo 18 data recording system (World Precision Instruments Ltd.).

RESULTS AND DISCUSSION

The first experiments ran to characterize the miniaturized system in terms of sensitivity and repetitivity were carried out with thiocholine obtained as previously described. During the performance of these experiments, a common drawback was observed. It is well-known that the oxidation of thiocholine at the electrode surface produces a passivation (fouling) of this surface due to the electropolymerization of thiocholine.[19] The consequence of this passivation is a decrease in the sensitivity of the detection system, because the effective electrode surface is reduced. This was observed qualitatively after 10 consecutive injections of thiocholine (Figure 3). It can be seen that the peak height decreases dramatically until a certain value and, at the same time, the baseline increases.

The undesirable consequence of this passivation process is the lack of reproducibility of the system. In order to solve this problem an initial cleaning step of the electrode surface previous to each inhibition test was evaluated. This pretreatment consisted in a switch of the polarity of the potential applied to the working

(19) Barrow, G. M. *Physical Chemistry*; MC Graw-Hill: 1973; pp 703−726.

electrode, producing the solubilization of the polymerized substances on the electrode surface. Unfortunately, it was difficult to optimize the time necessary to achieve the desired results in a reproducible manner, and this cleaning procedure was discarded. As an alternative, and to further confirm that the decrease of sensitivity due to the passivation occurred only to a limit, a controlled fouling pretreatment step was tested. In this case, and previous to each inhibition test, a continuous stream (0.5 mL·min^{-1}) of a highly concentrated thiocholine solution was allowed to flow through the electrode surface polarized at 500 mV vs Ag/AgCl. The measured intensity increased up to a maximum until the extent of the passivation was high enough to keep on reducing the sensitivity. Afterward, the intensity decreased to a constant value after approximately 30 min. The current recorded at this stage was the maximum current that the electrode could measure under these controlled fouling conditions.

After this pretreatment step the reproducibility of the detection system was evaluated. Ten consecutive injections (25 μL) of a 0.5 mM thiocholine standard at a flow rate of 0.75 mL·min^{-1} were performed. The resulting RSD% was 2.1%, showing a good repetitivity and the adequacy of the pretreatment step.

Hydrodynamic voltammetries for buffer A and for 2 mM thiocholine solution were performed to determine the optimum potential to be applied to the working electrode for the amperometric detection of thiocholine. First, the background current provided by buffer A flowing at a constant rate of 0.5 mL·min at increasing applied potentials (from 0 to 700 mV and steps of 100 mV) was recorded. In a second run, the same procedure was repeated using a 2 mM thiocholine solution instead of buffer A. The maximum analyte/background current ratio was obtained at 500 mV vs Ag/AgCl, so this was selected as the potential to be applied.

A further optimization of the system performance toward the detection of thiocholine was achieved with the evaluation of the sensitivity of the electrodes. In this case, five thiocholine standard solutions were injected by triplicate through channel d by means of a six-way injection valve into a stream of buffer A. The signals measured at the working electrode, polarized at 500 mV vs Ag/AgCl, are shown in Figure 4. These values were used to construct a calibration curve (Figure 4 inset) that presents a slope of 3.239 μA·mM^{-1} and r^2 of 0.9994, showing a sensitivity good enough to detect the expected production of thiocholine with the enzymatic system.

A previous optimization of the enzymatic system parameters, such us the enzyme and substrate concentrations, was achieved by means of the construction of the pseudo-Michaelis−Menten curves (Figure 5). Each curve was constructed by recording the current intensity after the injection of 25 μL of four different standard solutions of acetylcholinesterase into a flow of ATCh at a given concentration. This was repeated for five concentrations of substrate, namely, 2.5, 5, 10, 20, and 40 mM.

From Figure 5 it can be concluded that substrate concentrations lower than 10 mM should not be used because the dependency of the rate of conversion with the substrate concentration could lead to irreproducible results. Although the initial concentration of enzyme tested was 0.5 U, to improve detection limits lower concentrations were used in subsequent experiments.

Once the detection system had been tested and the enzymatic system had been optimized, the following procedure (as described

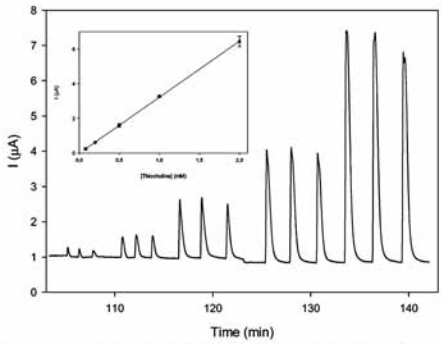

Figure 4. Registry plot and calibration curve (inset) for thiocholine standard solutions (0.08, 0.2, 0.5, 1, and 2 mM) (channel e, buffer A; channel d, buffer A/thiocholine; channel c, blocked). Flow rate: 1 mL·min^{-1}. Volume injected: 25 μL. Applied potential: 500 mV vs Ag/AgCl.

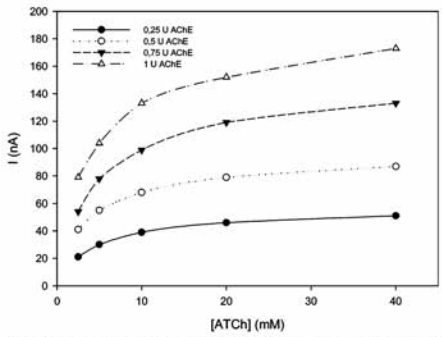

Figure 5. Michaelis−Menten curves. Flow rate: 0.5 mL·min^{-1}. Volume injected: 25 μL. Applied potential: 500 mV vs Ag/AgCl.

in Figure 2) was used to perform the inhibition test. With water and ATCh dissolved in buffer A running through channels e and c, respectively, the enzyme dissolved in buffer B was injected (25 μL) into the microfluidic system through channel d by means of the six-way injection valve. Under these conditions no inhibition should take place, so the signal obtained was considered as the maximum signal that could be reached. After that, water in channel e was replaced by a carbofuran standard solution, and the procedure was repeated in triplicate. This time, due to the inhibition of the enzyme, the signal obtained was lower than the blank signal, with a carbofuran concentration-dependent decrease. After the blank measurement, each standard took 20 min to be analyzed in triplicate, which means a frequency of 3 analysis·h^{-1}. The degree of inhibition was calculated as

$$\%I = \frac{S_{\text{blank}} - S_{\text{inhibition}}}{S_{\text{blank}}} \times 100$$

Four carbofuran standard solutions were tested (68, 113, 226, and 453 nM), and the resulting calibration plot is shown in Figure 6.

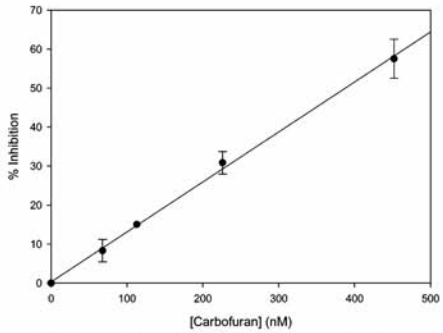

Figure 6. Calibration curve for carbofuran standard solutions (68, 113, 226, and 453 nM). Flow rate: 0.1 mL·min^{-1}. Volume of enzyme injected: 25 μL of 0.5 U·mL^{-1} stock (0.0125 U). [ATCh] = 10 mM. Applied potential: 500 mV vs Ag/AgCl.

The method showed a good correlation between the percentage of inhibition and the concentration of carbofuran (r^2 0.9983), with a sensitivity of 0.128 %I·nM^{-1}. The LOD, defined as the concentration that produces 5% of inhibition, was 36 nM carbofuran. This value can be considered as excellent compared to those values found in the literature taking into account that the inhibition takes place in continuous mode, without any preincubation of the enzyme and the inhibitor. This shows the capability of the two passive mixers to act as reactors without the need to work with stopped-flow conditions, which would decrease the amperometric system response and would lower the analysis frequency. Moreover, in spite of the common ceramic wall roughness (~5 μm), which could favor enzyme adsorption, no contamination effects due to enzyme residues along the mixers' trajectory have been observed.

CONCLUSIONS

An easy to construct and reliable LTCC-based device with amperometric detection microsystem has been constructed and evaluated by means of an enzymatic inhibition test for pesticides. The detection system, based on two platinum sheets acting as working and counter electrodes and a screen-printed Ag/AgCl path as reference electrode, provided a sensitivity of 3.239 μA·mM^{-1} toward thiocholine. The two passive mixers were proven to be effective enough to allow the inhibition of the enzyme and the hydrolization of ATCh to take place. Regarding the determination of pesticides, the system performance was evaluated, and a sensitivity of 0.128 %I·nM^{-1} with an LOD of 36 nM carbofuran were obtained. Being the system intended for in situ screening tests, these results were excellent. The device itself could be used for any other application requiring one or two mixing steps and sensitive amperometric detection.

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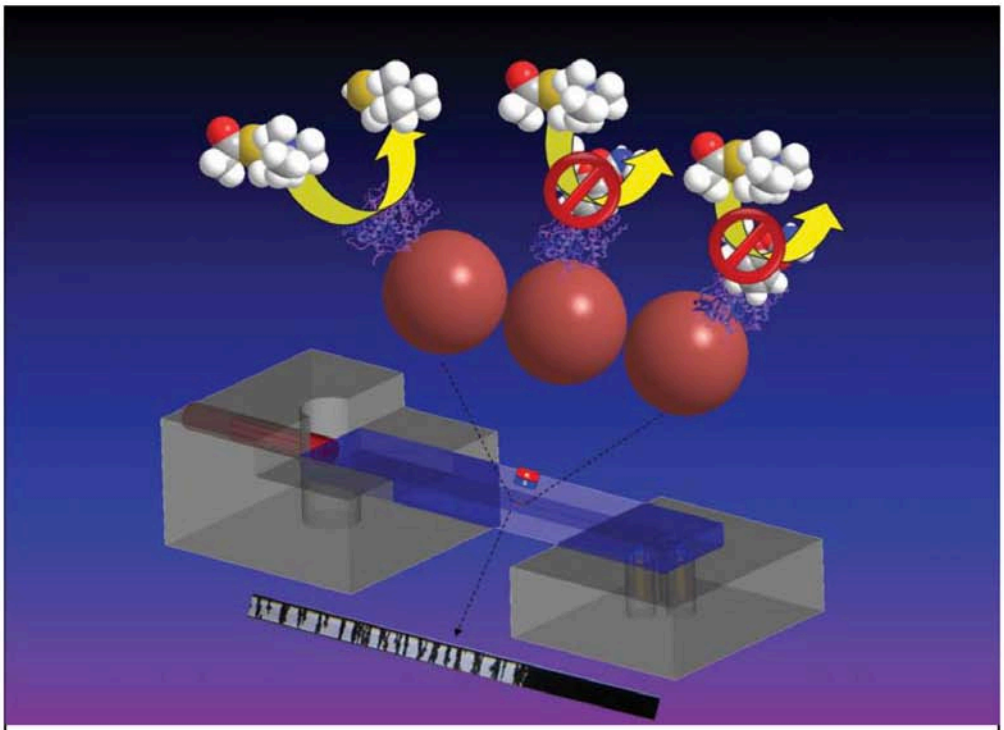

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Title: Lab-on-a-chip for ultrasensitive detection of carbofuran by enzymatic inhibition with replacement of enzyme using magnetic beads

Coupling of an enzymatic magnetobioreactor with on-chip amperometric detection can lead to extremely low limits of detection in the determination of carbofuran and other toxic substances by enzymatic inhibition.

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Lab-on-a-chip for ultrasensitive detection of carbofuran by enzymatic inhibition with replacement of enzyme using magnetic beads

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In this paper an ultrasensitive method to determine toxicity due to pesticides in a glass lab-on-a-chip by means of enzymatic inhibition of acetylcholinesterase immobilised on magnetic beads is described. The reproducible insertion of a controlled amount of enzyme-coupled magnetic beads inside the chip channel and their immobilisation in a capture region with the aid of a magnetic field has been optimised. This procedure enables the easy renewal of the biosensing material after each determination in a highly reproducible manner. Several operational parameters such as the working potential for the selective detection of thiocholine (TCh) on a platinum disc electrode, the TCh detection reproducibility and sensitivity, the electroosmotic flow driving voltage and the inhibition time were also evaluated or optimised. The detection of carbofuran (one of the most toxic carbamate pesticides) has been achieved down to the nanomolar level.

Introduction

On a global basis, more than two thousand million kilograms of conventional pesticides with agricultural, forestall and disease control applications are used every year all over the world. Within the European Union, the most used pesticides are fungicides, representing 44% of the total weight, followed by herbicides (33%), insecticides (15%) and others (8%).[1] These synthetic poisons, once released into the environment, can affect other living beings and cause unpredictable effects. Moreover, their degradation (hydrolysis, photolysis, oxidation, *etc.*) in water or foodstuffs, leads to the appearance of new compounds with similar or even higher toxicity than their precursors. European Council Directive 98/83/CE, on the quality of water intended for human consumption, sets individual maximum values for toxic substances. Thus, the maximum concentration of any individual pesticide is fixed at 0.1 ppb, and the total amount must not exceed 0.5 ppb. Carbamates and organophosphates represent the most widely used pesticides. Although they are not as persistent as the banned organochlorate compounds, they are extremely toxic due to their molecular structure, which is very similar to that of acetylcholine (ACh), so they can irreversibly bond to the active site of the enzyme acetylcholinesterase (AChE) and inhibit its activity, which is essential in the transmission of the nerve impulse.[2] Taking advantage of this particularity, carbamates and organophosphates can also be used as chemical weapons.[3] While organophosphates deactivate the enzyme by means of a phosphorylation process, carbamates block the active site by competing with the substrate.[4] The pronounced toxicity of these compounds makes it necessary for them to be detected in a sensitive, rapid and efficient way. The generally used methods are based on chromatographic techniques involving time-consuming extraction and purification procedures that are not compatible with the strict requests for in-field monitoring of contamination episodes. In the screening field a number of batch- and flow-based systems have been developed using enzymes in solution,[5] biosensors and bioreactors containing immobilised enzymes,[6–9] tissues[10] and micro-organisms[11–13] or antibodies.[14,15] Among these biological detection systems, those based on the inhibition of the activity of cholinesterases offer excellent sensitivity and specificity.

Despite the large number of advantages offered by biosensors and bioreactors based on enzyme inhibition, it is important to consider that enzymatic activity is lost after a measuring cycle. This phenomenon affects the lifetime of the biosensing device that contains a certain amount of immobilised enzymatic material. The use of special re-activators, such as 2-PAM (pralidoxime chloride) in the case of cholinesterases,[16] has been proposed to lengthen the life of the biosensor. Nevertheless these additional procedures, besides complicating the measurement protocol,[17] may increase the analysis time or induce other experimental errors. The automation of the analytical procedure by coupling the biosensor with a flow system to facilitate the regeneration steps represents another alternative.[18]

Microfluidic devices have been described for the detection of organophosphate pesticides, but the detection limits were not satisfactory. Microchip electrophoresis with direct amperometric reduction of organophosphate pesticides provided limits of detection at ppm levels.[19,20] Lab-on-chip devices with pre-column enzymatic hydrolysis of organophosphate pesticides hydrolysis and contactless conductivity detection can also achieve the detection of these harmful compounds at ppm levels.[21]

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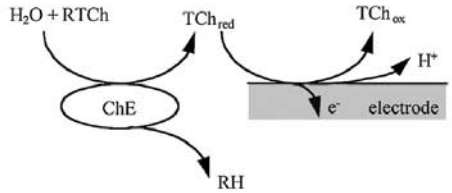

Fig. 1 Amperometric determination of cholinesterase activity. ATCh is used instead of ACh, because once it has been hydrolysed by the enzyme the resulting product, TCh_r can be oxidised in a polarised working electrode, and the resulting current can be related to the enzyme activity.

An interesting approach to overcome the regeneration problem is the use of bio-labelled magnetic beads (MBs)[22–24] that have shown attractive performance even for DNA[25] and protein[26] sensing. MBs can be introduced into a microfluidic channel and immobilised in a capture region with the aid of a magnetic field, constructing a bioreactor before the detection system or directly onto the surface of an electrochemical transducer. Once the inhibition has taken place, MBs can easily be removed and substituted with a fresh new set.

In this paper we present the use of magnetic beads as a versatile platform for an easy *in-situ* replacement of the inhibited enzyme inside a glass lab-on-a-chip[27] coupled to an amperometric detection system to detect thiocholine (TCh), which is the electrochemically active product of the AChE-catalysed hydrolysis when acetylthiocholine (ACTh) is used as substrate (Fig. 1). This approach allows sensitive detection of carbofuran (one of the most toxic carbamate pesticides) down to the nanomolar (sub-ppb) level.

Experimental section

Reagents

Sodium dihydrogenphosphate, disodium hydrogenphosphate and potassium chloride were obtained from Fluka. ATCh (Sigma) standard solutions were prepared in 10 mM phosphate and 10 mM potassium chloride aqueous solution buffered at pH 7.4 (running buffer). Carbofuran (analytical standard) was purchased from Riedel-de Haën. Pesticide standard solutions were prepared daily in double-distilled water by dilution from a 250.89 ppm stock in ethanol. AChE type VI-S from Electric Eel was purchased from Sigma. Epoxy group surface-activated magnetic beads of 2.8 μm diameter (surface area 24.63 μm^2), Dynabeads M-270 Epoxy, were purchased from Dynal BioTech. The specific surface area of these beads is 2–5 m^2/g, and the active chemical functionality is between 0.1 and 0.2 mmol/g. Once inserted and immobilised in the capture region, and assuming close packing of spherical beads, the interbead volume was around 3.33 nL (calculated from the diameter of beads, 2.8 μm, and the amount of beads inserted, around 8.25×10^5).

A 0.1 M TCh stock solution was prepared by adding 25 units of AChE to 100 mL of a 0.1 M ATCh solution and allowing the hydrolysis to take place for 3 hours. TCh standard solutions were prepared by dilution of this stock solution in running buffer.

All reagents were of analytical grade and all experiments were performed at room temperature.

Safety considerations

Carbofuran is highly toxic and should be handled in an extractor hood. Skin and eye contact as well as accidental inhalation or ingestion should be avoided.

Apparatus

The lab-on-a-chip device used in this study consisted of a glass chip fabricated by means of wet chemical etching and thermal bonding techniques, and was obtained from Micralyne (Model MC-BF4-001, Edmonton, Canada). The 88 mm × 16 mm chip consisted of a four-way injection cross, with a 74 mm main channel and side arms of 5 mm each. The original waste reservoir was cut off so the working electrode could be placed just opposite to the channel outlet, thus facilitating the end-channel amperometric detection (as described below). The channels were 50 μm wide and 20 μm deep. The microchannels of the glass chip were treated before use by rinsing with 0.1 M NaOH for 10 minutes and deionized water for another 10 minutes.

A Plexiglas holder was fabricated to hold the chip and house the detector and reservoirs as described elsewhere.[28] An NdFeB permanent magnet (cylinder-shaped, 6 mm height and 6 mm diameter) was used to handle and position the magnetic beads. The detection system consisted of a platinum wire counter, an Ag/AgCl wire reference electrode and a platinum disc working electrode (CH Instruments, Austin, TX, USA) with a diameter of 2 mm. Reference and counter electrodes were inserted through holes drilled in the Plexiglas holder. The working electrode, housed in a plastic screw, was placed opposite the channel outlet at a distance of 50 μm, measured under a microscope. The surface of the working electrode was renewed and smoothed with alumina paper (polishing strips 301044-001, Moyco Precision Abrasives Inc., Montgomeryville, PA, USA) between measurements. Amperometric detection was performed with an electrochemical analyser 621 (CH Instruments, Austin, TX, USA) connected to a personal computer. A laboratory-made high-voltage power supply with an adjustable voltage range between 0 and +5000 V was used as the driving force for the electroosmotic flow. Substrate and sample injections were performed after stabilisation of the baseline. In Fig. 2 a schematic (not in scale) drawing (A) and a picture (B) of the lab-on-a-chip used are shown.

Magnetic beads coating procedure

The immobilisation of the enzyme onto the MB surface was achieved following the coating procedure proposed by Dynal Biotech. Dry beads were washed and equilibrated three times with 0.1 M sodium phosphate buffer (PBS) at pH 7.4 for 10 minutes and resuspended in dimethyl formamide (DMF) to a final concentration of 2×10^9 MB mL^{-1}, and then stored in this organic solvent at 4 °C when not in use. 90 μL from this stock solution (1.8×10^8 MB) were washed four times with PBS and resuspended in a vial with 60 μL of this buffer. Then 60 μL of 3 M ammonium sulfate and 60 μL of enzyme solution (1500 μg × mL^{-1}) were added to the vial and the mixture was

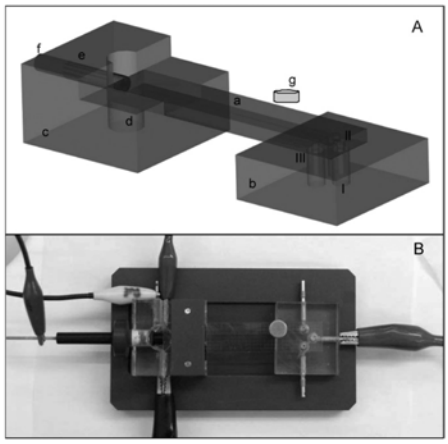

Fig. 2 (A) Schematic drawing of the lab-on-a-chip system with electrochemical detection for enzymatic inhibition determination. (a) Glass microchip, (b) Plexiglas body for reservoirs, (c) Plexiglas body for detection system, (d) detection reservoir, (e) hole for working electrode placement, (f) working electrode, (g) magnet, (I) buffer reservoir, (II) ATCh reservoir, (III) pesticide reservoir. (B) Top view of the experimental setup, showing the connections of the detection system and high voltage electrodes.

incubated for 24 hours at 37 °C with slow tilt rotation to allow the covalent bonding of the enzymes with the epoxy groups of the beads. After four washing steps with PBS, the MBs were resuspended to a concentration of 1×10^9 MB mL^{-1} with running buffer and were ready for use.

Results and discussion

Thiocholine detection

Hydrodynamic voltammograms. Hydrodynamic voltammograms (HDVs) were recorded in order to determine the optimum working potential to be applied to the platinum electrode in the determination of TCh. The HDV curve (current *vs.* applied potential) was obtained point-wise by making 100 mV changes to the applied potential from 0 to +0.9 V and measuring the produced current corresponding to TCh oxidation while injecting a fixed volume of 5 mM ATCh solution over the enzyme-labelled magnetic beads immobilised in the main channel. Results, depicted in Fig. 3, showed a rising tendency of the produced current from +0.2 V to higher potentials. The background current also showed a similar trend in terms of current but with an increase starting at +0.7 V. The potential value of +0.6 V, which offered the most favourable signal-to-noise ratio was selected as the working potential for future measurements.

Response stability towards TCh. In order to prove the reproducibility of the electrode performance towards the detection of TCh a series of repetitive injections of a 5 mM ACTh solution

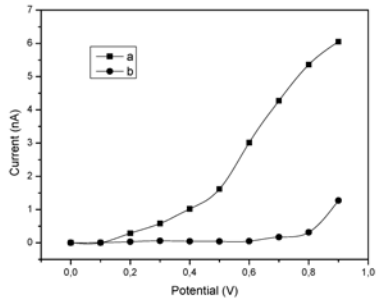

Fig. 3 Hydrodynamic voltammogram for TCh (a) and the corresponding background current obtained using running buffer (b). Conditions: ATCh concentration, 5 mM; amount of magnetic beads, approximately 8.25×10^5; electroosmotic flow voltage, 1.5 kV; injection voltage, 1.5 kV; injection time, 10 s; running buffer, 10 mM phosphate and 10 mM KCl at pH 7.4.

through a fixed amount of enzyme-labelled magnetic beads was carried out. Results for the average current response and peak time were 5.16 nA (relative standard deviation, RSD = 2.13%, n = 6) and 69.22 s (RDS = 0.84%, n = 6) respectively. Apparently, the detection of TCh does not produce fouling of the electrode surface and the substrate injection procedure is highly reproducible. Fig. 4 depicts the current peaks obtained in this characterisation.

Calibration curve for TCh. Apart from the reproducibility of the detection performance it was also important to determine the capability of the detection system to provide proportional response to different concentrations of TCh. The previous evaluation of the capability for TCh detection is crucial due to the fact that the degree of inhibition would be calculated as the relative decrease of substrate conversion caused by the reduction of the enzyme activity. This evaluation was achieved by

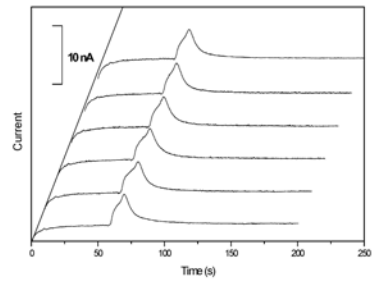

Fig. 4 Precision of repetitive injections of ATCh through the enzyme-labelled magnetic beads. Conditions: working potential, +0.6 V; ATCh concentration, 5 mM; amount of magnetic beads, approx. 8.25×10^5; electroosmotic flow voltage, 1.5 kV; injection voltage, 1.5 kV; injection time, 5 s; running buffer, 10 mM phosphate and 10 mM KCl at pH 7.4.

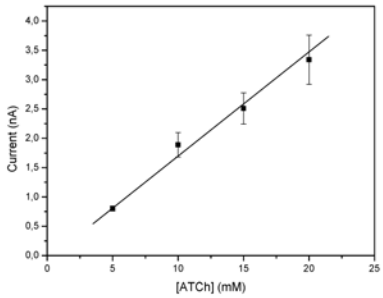

Fig. 5 Calibration curve for ATCh standard solutions (5, 10, 15 and 20 mM). Conditions: working potential, +0.6 V; amount of magnetic beads, approx. 8.25×10^5; electroosmotic flow voltage, 1.5 kV; injection voltage, 1.5 kV; injection time, 5 s; running buffer, 10 mM phosphate and 10 mM KCl at pH 7.4.

performing a calibration curve (Fig. 5) after the injection by triplicate of four ATCh standard solutions, ranging from 5 to 20 mM. The calibration measurements were carried out by consecutively replacing the substrate standard solution from reservoir II after each injection. Sensitivity (0.18 nA mM^{-1}) and correlation coefficient (0.9946) prove the linear dependence of the current response to the substrate concentration in the range from 5 to 20 mM, and the adequacy of the detection system to perform the inhibition tests.

Electroosmotic flow driving voltage. The field strength applied to produce the electroosmotic flow was also optimised. As expected, the elution time decreased almost 10 times when the applied voltage between the outlet and inlet reservoirs was increased from +500 V (t = 190.6 s) to +4000 V (t = 22.9 s). Fig. 6 shows this effect, together (as in-set) with the peak heights obtained for each applied voltage. The maximum current signal was obtained at 1500 V so this was selected as the optimum flow driving voltage for further applications. At higher flow driving voltages the flow rate was so high that the magnetic field of the permanent magnet was not strong enough to retain the magnetic beads and some of them escaped from the main plug. At 4000 V magnetic beads were completely disaggregated and short and broad double peaks were obtained.

Acetylcholinesterase inhibition. Fig. 7 schematically shows the steps (1 to 6) involved in the determination of pesticides. Initially (Step 1) a defined amount of MB-AChE was introduced and positioned in the main channel of the chip dragging the magnetic beads from reservoir I with the aid of a permanent magnet. In order to ensure the maximum reproducibility between consecutive inhibition measurements, all the magnetic beads introduced into the reservoir must be flushed through the channel. In case some beads aggregated and blocked the entrance of the channel, they could be easily disaggregated by rapid movements of the magnet. Once the magnetic plug had been correctly positioned inside the channel, a high voltage was applied between reservoir I and the detection reservoir (d) to produce the electroosmotic flow of the buffer solution and to achieve the stabilisation of the

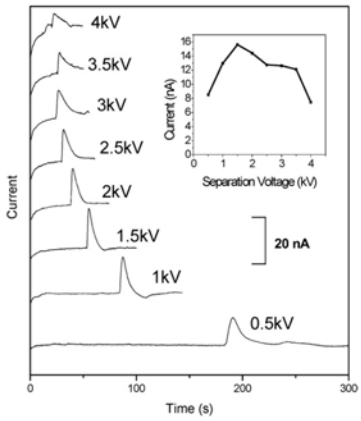

Fig. 6 Optimisation of the electroosmotic flow driving voltage. Conditions: working potential, +0.6 V; amount of beads, approx. 8.25×10^5; ATCh concentration, 10 mM; injection voltage, 1.5 kV; injection time, 5 s; running buffer, 10 mM phosphate and 10 mM KCl at pH 7.4.

background current (Step 2). Next, the application of the high voltage was switched from reservoir I to reservoir II for a fixed time (ranging from 5 to 30 s) to introduce a volume of ATCh solution and push it through the MB-AChE plug (Step 3). When the substrate ATCh passed through the magnetic bioreactor it was hydrolysed producing TCh (Fig. 1). TCh is an electroactive substance that can be detected on the electrode surface polarized at +0.6 V (optimum value), leading to the blank signal. Inhibition (Step 4) occurred when a certain amount of inhibitor solution (carbofuran) was introduced to the main channel from reservoir III. Then the injection of ATCh was repeated (Step 6) and the inhibition peak obtained. Finally the permanent magnet was removed (Step 6) in order to flush out the magnetic beads and prepare the chip for further measurements.

The degree of AChE inhibition was calculated as the relative decrease of the amperometric signal:

$$\%I = \frac{S_{blank} - S_{inhibition}}{S_{blank}} \times 100$$

where S_{blank} is the current obtained after the first injection of ATCh (Step 3) and $S_{inhibition}$ is the current obtained after inhibition of the enzyme (Step 5).

The AChE inhibition depends on the pesticide concentration as well as on the time for which the AChE is exposed (or incubated) to the pesticide. The former factor, which corresponds to the injection time of the pesticide (step 4), can be significant at low pesticide concentrations. Thus, AChE inhibition was evaluated by measuring the %I for various pesticide concentrations at two different injection times (60 and 90 seconds). The obtained %I for increasing concentrations of carbofuran ranging from 0.1 to 20 ppb were used to construct the AChE inhibition calibration curves for each of the inhibition times assayed (Fig. 8). Results showed a good linear correlation between the percentage of inhibition and the

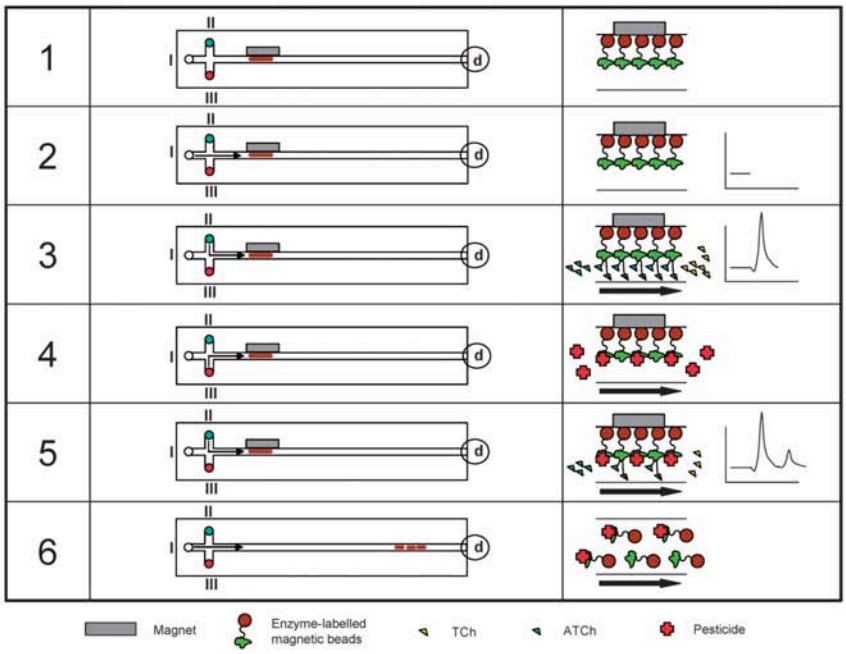

Fig. 7 Schematic drawing of the steps involved in the determination of pesticide by enzymatic inhibition using enzyme-modified magnetic beads on chip: (1) Introduction and retention of the magnetic beads, (2) injection of running buffer and obtaining of the background signal, (3) injection of substrate (ATCh) and obtaining of the blank signal, (4) injection of the inhibitor (carbofuran) and enzyme inhibition, (5) injection of substrate and obtaining of the inhibition signal, (6) removal of the magnetic field and flush out of the beads. Shown are the reservoirs for injection of MBs-AChE suspension and running buffer (I), ATCh solution (II), inhibitor solution (III) and electrochemical measurements (d).

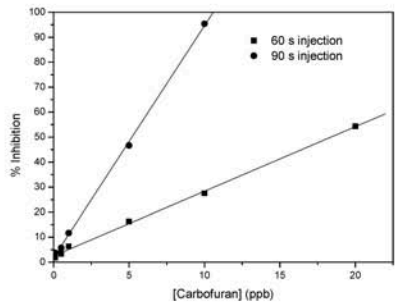

Fig. 8 Calibration curves for carbofuran standards (0.1, 0.5, 1, 5, 10 and 20 ppb) by on-chip enzymatic inhibition for 60 and 90 second injections. Conditions: working potential, +0.6 V; amount of beads, approx. 8.25×10^5; ATCh concentration, 10 mM; injection voltage, 1.5 kV; substrate injection time, 5 s; running buffer, 10 mM phosphate and 10 mM KCl at pH 7.4.

concentration of carbofuran, with a sensitivity of 2.58%I ppb^{-1} ($r = 0.9989$) for 60 seconds of injection and 9.28%I ppb^{-1} ($r = 0.9996$) for 90 seconds. The limits of detection, calculated as the concentration of carbofuran that would produce 5% inhibition, were 0.96 ppb and 0.34 ppb respectively.

Conclusions

Extremely low limits of detection (lower than those reported by liquid chromatography–mass spectrometry methods[29]) for the determination of carbofuran by enzymatic inhibition and amperometric detection in glass lab-on-a-chip devices can be achieved with the proposed method by the insertion of a low and controlled amount of magnetic beads (and, thus, a small quantity of enzyme), which leads to a high and controlled inhibited/non-inhibited enzyme ratio. This low and controlled amount of enzyme is also combined with the high sensitivity performance of the on-chip amperometric detection leading to sensitive and stable response to pesticides. This method may find applications not only in environmental analysis but equally importantly in the field of security and forensic analysis since pesticides (and

especially carbofuran as it is one of the most poisonous) can be potentially used by terrorists as "cheap" nerve agents.

The proposed magnetic bead based inhibition bioassays should lead to ultrasensitive detection of other pollutants in addition to pesticides

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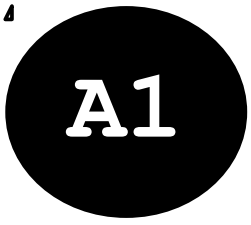

PUBLICACIONS I TREBALLS ADDICIONALS

Part del treball realitzat durant el desenvolupament d'aquesta tesi ha donat lloc a les següents publicacions i comunicacions addicionals:

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1. Zacco, E., Pividori, M. I., Llopis, X., Del Valle, M. & Alegret, S. Renewable Protein A modified graphite-epoxy composite for electrochemical immunosensing. *J. Immunol. Methods* **286**, 35–46 (2004).

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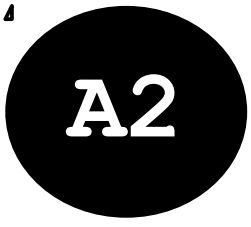
6. X. Llopis, I. Lladó, C. Sofia, L. Matía, R. Prats, S. Alegret, J. Alonso, Development of a heavy metal pollution analyser for water streams based on urease inhibition, *X Trobada Transfronterera sobre Sensors i Biosensors*, Albi (França) 2005. COMUNICACIÓ PÓSTER.



CURRICULUM VITAE

XAVIER LLOPIS

Please visit https://es.linkedin.com/in/xllopis

BUSINESS DEVELOPMENT MANAGER

- Business Development Manager (Agri-food and Health sectors) with broad experience in technology transfer and innovation management within the frame of the SMEs funding instrument of the EC (Research for the Benefit of SMEs in FP7 and SME Instrument in H2020), including opportunity identification and validation, project proposal preparation, technical coordination, reviewing with the EC and R&D consultancy. Main areas of expertise include analytical solutions for agri-food, environmental and clinical sectors.
- Horizon 2020 Authorised Consultant of ACCIÓ, Agency for Business Competitiveness of the Catalan Government.
- Assistance to the Research Executive Agency (European Commission) as Independent Expert in the evaluation of Marie Curie proposals.

PROFESSIONAL EXPERIENCE

ATEKNEA SOLUTIONS CATALONIA, S.A. (Spain) **01/14-Present**

Private research centre offering technical and scientific innovative solutions to European companies from different economic sectors (Merger of CRIC, S.A. with three other private research centres).

Business Line Manager

Responsible for two Business Lines at ATEKNEA Solutions. Activities include the identification of innovation trends in these sectors, the negotiation of collaboration agreements with academic and industrial partners and the identification and validation of new business opportunities. Participation in investment forums, networking events, and international congresses and trade fairs. SME Instrument proposal preparation.

- Healthcare and Well-being.
- Sustainable Agriculture and Food Security

AGENCY FOR BUSINESS COMPETITIVENESS,

ACCIÓ / CATALAN GOVERNMENT **10/15-Present**

ACCIÓ is the Agency for Business Competitiveness of the Catalan Government (Generalitat de Catalunya). It promotes the encouragement of innovation and internationalisation and has a network of 31 offices around the world.

Horizon 2020 Authorised Consultant

Authorised consultants provide support to the implementation of ACCIÓ programs.

CRIC, S.A. (Spain) **04/07– 12/13**

Centre de Recerca i Innovació de Catalunya. Private research centre offering technical and scientific innovative solutions to European companies from different economic sectors.

Senior Scientific Project Manager

Technical Coordination of European funded projects (Research for SMEs, KBBE, NMP) within FP6 and FP7. Research and technical tasks related to the development of biosensors and integrated analytical systems.

- Participation in the preparation of more than 10 project proposals (with budgets ranging from 1.5 – 3 Mio€).
- Scientific researcher in more than 10 funded projects, with activities related to the development of Bio-sensing Technologies.
- Coordination of a consortium of more than 20 partners, including RTD performers, SMEs and SME Associations.
- Coordination of periodic review meetings with project partners.
- Preparation of more than 30 technical reports and deliverables for project review with the EC.
- Chemistry Laboratory Manager.

RESEARCH EXECUTIVE AGENCY / EC (Belgium) **09/11, 09/12 & 09/13**

Organism of the European Commission managing large parts of EU research funding and supporting the research community to help maximise the impact of EU research programmes and foster innovation.

Independent Expert Evaluator

Evaluation work includes formulating recommendations on the proposals submitted to the REA. The work also requires acting as "rapporteur", chairperson or vice-chairperson for consensus discussions or meetings of panels of experts.

- Evaluation of 45+ Marie Curie proposals (IIF, IEF and IOF).
- Chemistry panel. Areas of expertise: biosensors and integrated analytical systems (IAS).

CORPORACIÓN MEDICHEM, S.L. (Spain) **05/06 – 03/07**

Corporación Medichem is a privately owned company engaged in the process development and manufacture of Active Pharmaceutical Ingredients (APIs) for the global pharmaceutical industry since the early 1970s.

Business Development Manager

Negotiation of generic drugs licensing contracts (in and out licensing).

- Follow-up of existing agreements (sales, budgeting, accomplishment of milestones, etc.).
- Attendance to brokerage events and negotiation of new contracts.

GSB / AUTONOMOUS UNIVERSITY OF BARCELONA (Spain) **03/03 – 05/06**

Sensors and Biosensors Group of the Chemistry Department, Autonomous University of Barcelona. Research group with more than 20 years of experience in the development of sensor and biosensors for the agr-ifood, environmental and clinical sectors.

Senior Researcher

Responsible for the development, implementation and application of environmental monitoring systems within the framework of university-industry technological transfer projects.

- Design of composites, biocomposites and nanobioconjugate materials for enzyme- and immuno-based electrochemical sensors.
- Novel FIA and Lab-on-a-Chip biosystems with electrochemical detection based on magnetic particles, biocomposites and CNTs.
- Integrated devices for automated analytical systems for environmental monitoring and process control in different fields, such as chemical and food industry.
- Construction of analytical microsystems during a research stay at Pr. Andreas Manz laboratories (July to December 2003, Imperial College of Science, Technology and Medicine, London, UK)

- Research projects for the companies Grup d'Aigües de Barcelona S.A. and Adasa Sistemas S.A: Development and evaluation of automated analysers for continuous monitoring of toxicity in water.
- Tutorship of undergraduate students from the Oporto University (ERASMUS programme).
- Participation in national and international congresses (oral and poster presentations).

Agricultural and Food Laboratory Service /

CATALAN GOVERNMENT (Spain) **09/00 – 02/01**

The Agricultural and Food Laboratory Service of the Agricultural, Livestock and Fisheries Department, Catalan Government is the official service and technical basis of the Catalan administration related to its specific analytical needs in the quality control and assessment within the agri-food sector.

Chemical laboratory technician

- Physical and chemical analysis of foods.

EDUCATION

MSc in Chemistry (Autonomous University of Barcelona) 03/03

BSc in Chemistry (Autonomous University of Barcelona) 02/00

BSc in Food Science and Technology (Autonomous University Barcelona) 02/98

Additional Education:

- Internal Quality Audits: ISO 9000 – ISO 14000. BARCELONA ACTIVA, Barcelona. 2000
- Intellectual Property and Exploitation Rights in European Research Projects, YELLOW RESEARCH, Barcelona. 2007
- Management of European Research Projects – FP7, UPF, Barcelona. 2008
- Guide for Starting up New Companies. Formación y Servicios BCN, Barcelona. 2012
- Claus per Bioempendre (Keys to Bio-entrepreneurship). ASBATEC, CataloniaBio, Fundació EE. 2015

Languages:

Catalan: mother language.

Spanish: mother language.

English: full professional proficiency level.

Computer literacy:

- Advanced user in Microsoft Office.
- Advanced user in scientific information searching tools (SciFinder, ISI Web of Knowledge).
- Intermediate user in computer aided design (AutoCad 2010, Solid Edge V20).

LEAD RESEARCHER IN EUROPEAN PROJECTS RELATED TO BIOSENSORS AND BIOANALYTICAL METHODS (CRIC, S.A.)

FP7 - RESPOC - Fast, cost-effective, user-friendly point-of-care instrument for detection of respiratory pathogens, including Streptococcus pneumoniae and Bordetella pertussis. www.respoc.eu

FP7 - VERTIGEEN - Inexpensive and reliable on-site solution for olive producers to contain verticillium wilt. www.vertigeen.eu

FP7 - VITISENS - Cost-effective hand-held device for rapid in-field detection of flavenscence doree phytoplasma in grapevines.

FP7 - CLEANHIVE - Detecting the Pathogen that Threatens European Honey Bees

FP6 - TENDERCHECK - Calpastatin biosensor for meat tenderness prediction

FP6 - OVULTEST - Improving profitability of dairy SMEs through developing, validating and promoting the use of a biosensor for ovulation detection