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## RESUMEN DE LA TESIS EN CASTELLANO

### Antecedentes

Un avance importante en el campo de química analítica se hizo por Clark y Lyons en los años setenta. Ellos propusieron acoplar la especificidad de la enzima glucosa oxidasa con la transducción electroquímica de la señal en “biosensores”. En general, los biosensores son artefactos integrados autocontenidos, capaces de proporcionar información analítica, cuantitativa utilizando un elemento biológico de reconocimiento (receptor bioquímico) que se retiene en contacto espacial directo con un elemento de transducción. Posteriormente, los primeros biosensores de glucosa, basados en la detección amperométrica de peróxido de hidrógeno generado por glucosa oxidasa en la presencia de oxígeno fueron introducidos en el mercado por la empresa estadounidense Yellow Spring Instrument Co. (Ohio, EE. UU.) en 1975.

La respuesta de biosensores electroquímicos basados en el uso de oxígeno como cosustrato para oxidasas se ve desviada por la presencia de interferencias que pueden contribuir a la corriente. Por lo tanto la superficie de electrodo debe estar protegida por una membrana no permeable por sustancias que pueden interferir con la señal. Para evitar corrientes que perjudican la selectividad de los biosensores, el potencial aplicado puede ser aminorado usando electrocatalizadores difusionales (“mediadores”) en lugar de oxígeno, con un potencial redox controlable. Pero la respuesta de estos sensores también depende de la concentración de oxígeno porque este compete con los mediadores, para la reoxidación de las oxidasas. Un inconveniente adicional del uso de mediadores difusionales artificiales en biosensores es la baja estabilidad de los mismos debida al escape de mediadores desde la superficie del electrodo cuando esto se usa en línea. Se puede aliviar este problema creando enlaces covalentes entre los mediadores y la superficie del electrodo o usando polímeros redox que se adsorben fuertemente en la superficie del electrodo.

Una de las posibles maneras para disminuir la influencia del oxígeno a la corriente de la respuesta de biosensores es el uso de las deshidrogenasas dependientes de la pareja redox  $\text{NAD}^+/\text{NADH}$ . El potencial estándar redox de esta pareja es  $-0.56\text{ V vs. SCE}$  pero para conseguir la oxidación de NADH en la superficie de electrodos de carbono un sobrepotencial de  $+0.5\text{ V vs. SCE}$  debe aplicarse. Bajo estas condiciones los electrodos tienen tiempo de vida corto debido a la adsorción de los productos de oxidación en su superficie ya que la oxidación de NADH no es reversible químicamente. Por otro lado estos electrodos sufren por la oxidación no específica de

interferencias a estos potenciales de operación. Los electrodos modificados químicamente por mediadores pueden oxidar NADH a potenciales más bajos. Sin embargo, muchos de los mediadores mencionados en la bibliografía no son estables o/y no forman  $\text{NAD}^+$  enzimáticamente activo. Un problema adicional de los sistemas analíticos basados en deshidrogenasas dependientes de  $\text{NAD}^+$  es la necesidad de añadir este cofactor, que tiene alto coste y es inestable, en las muestras. Se puede inmovilizar  $\text{NAD}^+$  en la superficie de electrodos para producir biosensores capaces de funcionar en muestras que no contienen  $\text{NAD}^+$ , biosensores *reagentless* (sin necesidad de adición de reactivos). Los métodos descritos en la bibliografía para la fabricación de biosensores *reagentless* se basan en cinco estrategias: (1) la inmovilización en hidrogeles formados *in situ*; (2) la inmovilización por una membrana; (3) la inmovilización en películas preparadas por electropolimerización; (4) la inmovilización en una pasta de carbono; (5) la inmovilización en monocapas auto ensambladas. Sólo los electrodos preparados con la estrategia (4) son biosensores *reagentless* con estabilidad operacional relativamente alta. Las demás estrategias no resultan en biosensores con suficiente estabilidad operacional por culpa de la pérdida del mediador, de  $\text{NAD}^+$  o de la deshidrogenasa. Sin embargo la estrategia basada en electrodos de pasta de carbono no permite su aplicación a la producción de microsensors (electrodos con diámetro de menos de 10  $\mu\text{m}$ ) para su uso *in vivo*.

## Metodología

El objetivo principal de esta tesis es el desarrollo de nuevas estrategias para la fabricación de biosensores *reagentless* basados en deshidrogenasas dependientes de  $\text{NAD}^+$  con características mejoradas respecto a la densidad de la corriente, de la estabilidad operacional y de almacenamiento.

Para cumplir el objetivo se han sintetizado dos nuevos mediadores para la oxidación de NADH: un polímero insoluble en agua  $[\text{Os}(1,10\text{-fenantrolina-5,6-diona})_2(\text{PVP})_4\text{Cl}]\text{Cl}$ , (Os-fendiona-PVP) y un complejo anfifílico  $[\text{Os}(1,10\text{-fenantrolina-5,6-diona})_2 4,4'-(n\text{-C}_{18}\text{H}_{37}\text{NHCO})_2\text{bpi}](\text{PF}_6)_2$  (Os-fendiona-surfactante). El polímero Os-fendiona-PVP fue producido vía la derivatización de poli(vinilpiridina) (peso molecular 50000) con  $[\text{Os}(1,10\text{-fenantrolina-5,6-diona})_2\text{Cl}_2]$ . El estudio electroquímico de este polímero redox adsorbido en electrodos de grafito se realizó por voltametría cíclica a distintas velocidades de barrido para evaluar el número de protones y electrones que participan en la reacción redox, la influencia del pH a su potencial estándar formal, y la constante de la transferencia heterogénea del electrón  $k_s$ . Bajo bien definidas condiciones hidrodinámicas se realizaron estudios para encontrar la constante de la interacción con NADH  $k_{[\text{NADH}]=0}$ . Os-fendiona-surfactante fue producido por la complejación de  $[\text{Os}(1,10\text{-fenantrolina-5,6-diona})_2]\text{Cl}_2$  con el ligando hidrófobo octadodecilamida del ácido 2,2'-Bipiridina-4,4'-dicarboxílico. Las monocapas de Langmuir-Blodgett de Os-fendiona-surfactante y las de su análogo  $[\text{Os}(\text{bpi})_2 4,4'-(n\text{-C}_{18}\text{H}_{37}\text{NHCO})_2\text{bpi}](\text{PF}_6)_2$  fueron estudiados en un equipo de Langmuir-Blodgett.

Os-fendiona-surfactante fue aplicado a la construcción de biosensores *reagentless* del glutamato vía la inmovilización de glucosa deshidrogenasa y de  $\text{NAD}^+$  entre las bicapas en la fase lamelar formada por Os-fendiona-surfactante y el lípido 1,2-dioleilo-sn-glicerol-3-fosfatidilcolina. Dos métodos adicionales para la fabricación de los biosensores *reagentless* de glutamato y glucosa basados en deshidrogenasas fueron desarrollados. Los electrodos del grafito fueron modificados con Os-fendiona-PVP y utilizados para (a) la inmovilización de deshidrogenasa y de  $\text{NAD}^+$  en un hidrogel formado por entrecruzamiento de poli(vinilpiridina) modificado por grupos amino con el éter diglicidil de poli(etilenglicol); (b) la inmovilización por adsorción de la deshidrogenasa y del ácido alginico modificado por  $\text{NAD}^+$ . Se ha hecho un estudio de los biosensores *reagentless* para calcular sus constantes de Michaelis, el efecto del pH y de la temperatura en su respuesta y su estabilidad operacional. Además se ha comparado la estabilidad operacional a temperaturas elevadas de biosensores de la configuración (a) usando glutamato, glucosa y glucosa-6-fosfato deshidrogenasas termófilas y mesófilas. Por otro lado se han estudiado métodos nuevos para mejorar la estabilidad durante el almacenamiento de sensores de glutamato. Con este fin, se han preparado electrodos utilizando glutamato deshidrogenasa mesófila y termófila con varios estabilizadores.

## Conclusiones

1. El polímero  $[\text{Os}(\text{1,10-fenantrolina-5,6-diona})_2(\text{PVP})_4\text{Cl}]\text{Cl}$ , (Os-fendiona-PVP) para la oxidación de NADH se puede sintetizar por la complejación de  $[\text{Os}(\text{1,10-fenantrolina-5,6-diona})_2\text{Cl}_2]$  con poli(vinilpiridina). La adsorción física de este polímero sobre los electrodos de grafito desde su solución en etilenglicol resulta en la formación de una monocapa de este polímero redox en la superficie del electrodo.
2. El proceso redox de este mediador es casi-reversible e implica 4 electrones y 4 protones dentro del rango del pH de 3-6.5. El mediador pierde su estabilidad química en valores de pH más altos que 6.5. Tres ramas lineales en el diagrama de  $E^{0'}$  frente a pH con diversas pendientes se observan.
3. La constante heterogénea de la velocidad de transferencia de electrones ( $k_s$ ) de Os-fendiona-PVP es del mismo orden de magnitud que la de otros mediadores capaces de oxidar NADH mencionados en la bibliografía ( $k_s = 18 \pm 2 \text{ s}^{-1}$ ).
4. Os-fendiona-PVP es un electrocatalizador eficiente para la oxidación del NADH. La modificación de los electrodos del grafito con Os-fendiona-PVP conduce a la disminución del sobrepotencial para la oxidación electroquímica del NADH desde +0.33 V vs.  $\text{Ag/AgCl/KCl}_{\text{sat}}$  para los electrodos no modificados hasta +0.11 V vs.  $\text{Ag/AgCl/KCl}_{\text{sat}}$ . La constante cinética para la interacción del polímero redox con el NADH ( $k_{1,[\text{NADH}]=0} = (1.9 \pm 0.2) \times 10^3 \text{ s}^{-1} \text{ M}^{-1}$ ) coincide prácticamente con la de

Os-fendiona que sugiere que el número de los ligandos de fendiona en los complejos del osmio es proporcional a la corriente de la respuesta al NADH pero no afecta a las constantes cinéticas electroquímicas.

5. El mediador amfifílico para la oxidación del NADH se puede sintetizar con la complejación del complejo  $[\text{Os}(1,10\text{-fenantrolina-5,6-diona})_2\text{Cl}_2]$  con el ligando hidrofóbico octadodecilamida del ácido 2,2'-Bipiridina-4,4'-dicarboxílico.

6. El mediador amfifílico para la oxidación NADH forma monocapas de Langmuir-Blodgett estables en la interfaz de agua-aire. Multicapas de este compuesto depositado en la superficie sólida de electrodos por la técnica de Langmuir-Blodgett pierden la actividad electroquímica de 1,10-fenantrolina-5,6-diona probablemente debido a las limitaciones del transporte de protones a través de las monocapas formadas por las cadenas alifáticas hidrófobas.

7. Las características amfifílicas del análogo del compuesto antedicho, que no lleva el ligando del fenantrolina, se han estudiado también. El análogo forma monocapas estables en la interfaz de agua-aire y puede ser depositado en un electrodo sólido con la pérdida parcial de las propiedades electroquímicas del átomo del osmio, debido a la alta hidrofobicidad de las multicapas.

8. Tres nuevas configuraciones, basadas en estos mediadores, fueron desarrolladas para la fabricación de biosensores *reagentless* de glucosa y L-glutamato basados en deshidrogenasas. Las primeras dos configuraciones se basan en la adsorción de Os-fendiona-PVP en la superficie de los electrodos de grafito, seguida por la adsorción de la deshidrogenasa y del ácido algínico modificado con  $\text{NAD}^+$  (NAD-alginato) o por la inmovilización de deshidrogenasa y de  $\text{NAD}^+$  en un hidrogel formado por poli(vinilpiridina) que lleva grupos amino. La tercera configuración emplea la inmovilización de la enzima y de la coenzima en la fase lamelar creada por una mezcla de mediador amfifílico y un lípido en soluciones acuosas.

9. Se puede mejorar la estabilidad operacional de biosensores a temperaturas elevadas mediante el uso de enzimas termófilas en vez de mesófilas. Esto fue demostrado comparando el funcionamiento de los sensores de glutamato y de glucosa-6-fosfato preparados usando deshidrogenasas mesófilas y termófilas. El uso de enzimas termófilas también permite mejorar drásticamente la estabilidad de almacenamiento de biosensores. Esto se puede explicar en términos de la estabilidad intrínseca más alta de las enzimas termófilas. Algunos estabilizadores como el copolímero de vinilo-pirrolidón y de dimetilamino etil metacrilato denominado como Gafquat<sup>®</sup> HS100 pueden aumentar la estabilidad de almacenamiento de biosensores formando un complejo proteína-poli-electrolito.

## ABSTRACT

The objective of this work was the development of new configurations of reagentless biosensors based on  $\text{NAD}^+$  dependent dehydrogenases. These configurations are based on the immobilisation of enzyme, cofactor and the electrochemical catalyst used for its regeneration. In addition to being reagentless these configurations yielded biosensors with improved current density and operational stability compared to the state of the art.

To achieve the objective two new NADH oxidising mediators were synthesised: a water insoluble polymer  $[\text{Os}(1,10\text{-phenanthroline-5,6-dione})_2(\text{PVP})_4\text{Cl}]\text{Cl}$  (Os-phendione-PVP) and an amphiphilic complex  $[\text{Os}(1,10\text{-phenanthroline-5,6-dione})_2 4,4'-(\text{n-C}_{18}\text{H}_{37}\text{NHCO})_2\text{bpy}](\text{PF}_6)_2$  (Os-phendione-surfactant). The electrochemical study of Os-phendione-PVP has revealed a rate constant for the heterogeneous electron transfer of the phendione redox couple  $k_s = 25 \pm 2 \text{ s}^{-1}$ , and a second order rate constant for NADH oxidation  $k_{[\text{NADH}]=0} = (1.1 \pm 0.1) \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ . These constants are higher or of the same order of magnitude as those of previously described NADH oxidising mediators. The tensoactive mediators Os-phendione-surfactant and its analogue  $[\text{Os}(\text{bpy})_2 4,4'-(\text{n-C}_{18}\text{H}_{37}\text{NHCO})_2\text{bpy}](\text{PF}_6)_2$  (Os-bpy-surfactant) form very stable monolayers at the air-water interface collapsing at the surface pressure  $60\text{-}65 \text{ mN m}^{-1}$ .

The Os-phendione-surfactant was used for the construction of reagentless glutamate biosensors via the immobilisation of dehydrogenase and  $\text{NAD}^+$  between bilayers in lamellar phase formed by Os-phendione-surfactant and the lipid 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine. The resulting glutamate biosensors demonstrated maximum current density of  $3.5 \mu\text{A cm}^{-2}$  (RSD=25%), apparent Michaelis constant of 47 mM, and operational half life of 0.5 h. In addition graphite electrodes were modified by Os-phendione-PVP and utilised for (a) immobilisation of dehydrogenase and  $\text{NAD}^+$  in a hydrogel formed by crosslinking of poly(vinylpyridine) carrying amino groups with polyethylene glycol diglycidyl ether and (b) immobilisation of dehydrogenase and an  $\text{NAD}^+$ -alginate acid derivative by adsorption. The configuration (a) yielded glutamate sensors with maximum current density of  $8.7 \mu\text{A cm}^{-2}$  (RSD=5%), apparent Michaelis constant of 9.1 mM, operational half life of 12 h and glucose sensors with maximum current density of  $37 \mu\text{A cm}^{-2}$  (RSD=14%), apparent Michaelis constant of 4.2 mM, the operational half life of 1 h. The glutamate sensors based on the configuration (b) showed maximum current density of  $15.8 \mu\text{A/cm}^2$  (RSD=21%), apparent Michaelis constant of 17.6 mM and operational half life of 1.5 h.

Glucose, glucose-6-phosphate, and glutamate biosensors were prepared and characterised. The employment of the thermophilic enzymes helps to dramatically increase the operational stability of biosensors at elevated temperatures higher than  $60^\circ\text{C}$ . The shelf life of glutamate electrodes built with the use of thermophilic dehydrogenase was eleven times longer than this of electrodes modified with the mesophilic enzyme. The addition of the copolymer of vinyl-pyrrolidone and dimethylamino ethyl methacrylate termed as Gafquat HS100 to the enzyme also significantly improved shelf life.

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## LIST OF ABBREVIATIONS

Ag/AgCl/KCl <sub>sat</sub>	standard silver/silver chloride electrode
bpy	2,2'-bipyridine
B	base
DH	dehydrogenase
3,4-DHB	3,4-dihydroxybenzaldehyde
E	enzyme
Gafquat <sup>®</sup> HS100	copolymer of vinyl-pyrrolidone and dimethylamino ethyl methacrylate
GDH	glucose dehydrogenase
GLDH	glutamate dehydrogenase
G6P	glucose-6-phosphate
LB	Langmuir-Blodgett
M <sub>ox</sub>	oxidised form of a mediator
M <sub>red</sub>	reduced form of a mediator
NAD <sup>+</sup>	nicotinamide adenine dinucleotide
NADP <sup>+</sup>	nicotinamide adenine dinucleotide phosphate
NADH	reduced form of nicotinamide adenine dinucleotide
NADPH	reduced form of nicotinamide adenine dinucleotide phosphate
P	product
PVP	poly(4-vinylpyridine)
PVI	poly(vinylimidazole)
PQQ	pyrroloquinolinequinone
phenanthroline	1,10-phenanthroline-5,6-dione
Q	quinone
RDE	rotating disk electrode
S	substrate
SCE	standard calomel electrode
UV	ultra violet



## LIST OF SYMBOLS

$A$	the electrode surface area (cm <sup>2</sup> )
$c_{\text{NADH}}$	concentration of NADH (M)
$C$	concentration (M)
$D$	diffusion coefficient (cm <sup>2</sup> s <sup>-1</sup> )
$E^{0'}$	formal standard potential (V)
$E_{\text{pa}}$	anodic peak potential (V)
$E_{\text{pc}}$	cathodic peak potential (V)
$\Delta E_{\text{p}}$	peak-to-peak potential separation (V)
$[E_t]$ or $e_{\Sigma}$	total enzyme concentration (mM)
$I_{\text{pa}}$	anodic peak current (A)
$I_{\text{pc}}$	cathodic peak current (A)
$I_{\text{cat}}$	the current of electrocatalytical NADH oxidation at the electrodes modified with the mediator (A)
$j_{\text{el}}$	the flux (mol cm <sup>-2</sup> s <sup>-1</sup> )
$K_{\text{M}}$	Michaelis-Menten constant (mM)
$K_{\text{i}}$	formal Michaelis-Menten constant for substrate i (mM)
$K_{\text{eq}}$	equilibrium constant (mM)
$K_{\text{ij}}$	inhibition constant for substrate j (mM)
$k_{\text{S}}$	heterogeneous electron transfer rate constant (s <sup>-1</sup> )
$k_1$	the rate constant of the reaction between NADH oxidizing mediator and NADH (M <sup>-1</sup> s <sup>-1</sup> )
$k_{-1}$	the rate constant of the decomposition of the mediator-NADH complex into NADH and the oxidized mediator (s <sup>-1</sup> )
$k_2$	the rate constant of the decomposition of mediator-NADH complex into the reduced mediator and NAD <sup>+</sup> (s <sup>-1</sup> )
$k_1^{\text{'}}$ , $k_2^{\text{'}}$ , $k_3^{\text{'}}$	rate constants of the enzymatic reactions in forward direction (M <sup>-1</sup> s <sup>-1</sup> , s <sup>-1</sup> , and s <sup>-1</sup> respectively)
$k_{-1}^{\text{'}}$ , $k_{-2}^{\text{'}}$ , $k_{-3}^{\text{'}}$	rate constants of the enzymatic reactions in backward direction (s <sup>-1</sup> , s <sup>-1</sup> , and M <sup>-2</sup> s <sup>-1</sup> respectively)
$k'_{\text{i}}$	mass transfer rate coefficient for the substrate i (cm s <sup>-1</sup> )
$L$	thickness (cm)
$n$	number of electrons
$Q$	charge (C)

$t$	time (s)
$v$	overall reaction rate ( $\text{M s}^{-1}$ )
$V_1$	maximum velocity in the forward direction ( $\text{M s}^{-1}$ )
$V_2$	maximum velocity in the backward direction ( $\text{M s}^{-1}$ )

*Greek letters*

$\alpha$	transfer coefficient
$\Gamma$	surface coverage ( $\text{mol cm}^{-2}$ )
$\Pi$	surface pressure ( $\text{N m}^{-1}$ )
$\nu$	hydrodynamic viscosity ( $\text{cm}^2 \text{s}^{-1}$ )
$\omega$	rotation speed ( $\text{rad s}^{-1}$ )
$\nu$	scan rate ( $\text{V s}^{-1}$ )