COSMIC: COupling Smart Molecules Into Chips

The Report 2001-03

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TARGET PROJECT ON BIOSENSORS AND BIOELECTRONICS
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THE 6TH IAEAC BIOSENSOR WORKSHOP
STAFF

Senior Scientists: R.Franconi, D.Masci, L. Mosiello, L.Nardi, R.Pilloton,
Scientists: S.Impiombato, C.Laconi, M.R.Montereali, V.Pinto, M.Tosi,
PostDocs: F.Dalla Riva, Anna Krasilnikova, J.Maly; Celine Ndong, E.Podestà, S.Timur, W.Vastarella
Students: A.Volpe, E.Magarò, Chiara Di Meo, A.Boni, L.Della Setta, S.Marino,
Technicians: A.Masci, M.De Francesco, A.Lucchi,
Biosensors and bioelectronics represent different points of view of the same technology which is intended for mass production of hybrid devices based on the so called “smart properties” of natural molecules and technological materials.

Several biomolecules were extensively studied in the past as functional and active interfaces for sensing or bioelectronic purposes. The most common example is represented by biosensors which are obtained by coupling a biomediator with a transducer. Many natural molecules were purified and used for obtaining both enzyme sensors or immunosensors, and, recently, a large spectrum of natural biomolecules were also investigated, including olfactory receptors and oligonucleotides as sensing elements.

Biosensors, biological transduction and bioelectronic information storage are the main interesting research areas which will be commercially exploited in the near future. At the moment several biomolecules are used for commercially available analytical devices, but the critical factors for their use are mainly related to their stability and optimal (oriented) immobilisation, without loss of functional properties, on electronic or optical components. Hybrid, synthetic, natural molecules, including their active fragments or modified derivatives, can be used. Genetic engineered biomolecules seem to be a new and powerful approach for obtaining simpler artificial structures with intact or improved properties (i.e. stability, sensitivity and specificity), or with additional functional groups and activities. For example, an His	extsubscript{6} tag can be used for oriented and reversible immobilization of engineered single-chain antibody fragments (scFvs), or gene fusions with enzymatic activities may allow analytical detection based on phosphatases. Not only biosensing will take advances from the availability of powerful artificial molecular structures, but also a new generation of μ−electronic devices will be certainly affected by this new approach. As a matter of fact, nano-technologies allow increased spatial resolution for electronic and bioelectronic components.

The CoSMiC project at Enea, started on January 2001, is approaching this research activity with a multidisciplinary group of scientists (chemists, physicists, molecular biologists etc.), with the aim of selection and mass production of artificial molecules which mimic the natural ones, deposition of them on the macro scale by printing techniques or LB films, and, on the μ-scale, by laser assisted μ−lithography. Oligonucleotides and carbon nanotubes are also investigated for their interesting conducting properties at the nano scale.

KEYWORDS
biosensors, bioelectronics, nanotechnologies, engineered proteins, GMMOs, synthetic molecules, thin and thick film deposition, coatings
RELEVANT PAPERS

- Suna Timur, Livia Della Seta, Nurdan Pazarlioglu, Roberto Pilloton, Azmi Telefoncu; **SCREEN PRINTED GRAPHITE BIOSENSORS BASED ON BACTERIAL CELLS**, Process Biotechnology, in press.
- Alessio Boni, Eugenia Magarò, Marina Tosi, Walter Vastarella and Roberto Pilloton; **SCREEN PRINTED ELECTROCHEMICAL BIOSENSORS BASED ON RECOMBINANT MOLECULES AND CELLS**, UNEP Reports; 2003 in press.
- Timur, Suna; Pazarlioglu, Nurdan; Pilloton, Roberto; Telefoncu, Azmi; **DETECTION OF PHENOLIC COMPOUNDS BY THICK FILM SENSORS BASED ON PSEUDOMONAS PUTIDA**, Talanta Volume: 61, Issue: 2, October 17, 2003, pp. 87 - 93.
Michal Koblizek, Jan Maly, Jirí Masojidek, Josef Komenda, Tomas Kucera, Maria T. Giardi, Autar K. Mattoo and Roberto Pilloton; A BIOSENSOR FOR THE DETECTION OF TRIAZINE AND PHENYLUREA HERBICIDES DESIGNED USING PHOTOSYSTEM II COUPLED TO A SCREEN PRINTED ELECTRODE - BIOTECHNOLOGY AND BIOENGINEERING, VOL. 78, NO. 1, APRIL 5, 2002.


• Carlo Cremisini, Roberto Pilloton, Lia Segre, Amedeo Masci; **NUOVI POLIMERI SINTETICI PER IL RICONOSCIMENTO MOLECOLARE**; Energia Ambiente e Innovazione; Bimestrale ENEA 2000, 5

• Roberto Pilloton e Franco Mazzei; **WORKSHOP ALL’ENEA SUI SENSORI CHIMICI E BIOSENSORI**; Energia Ambiente e Innovazione; Bimestrale ENEA 2000, 3

• Carlo Cremisini, Roberto Pilloton*, Lia Segre; **BIOSENSORI: UN CAMPO DI RICERCA IN CONTINUA ESPANSIONE**; Energia Ambiente e Innovazione; Bimestrale ENEA 2000, 3

• Michal Koblizek, Jiri Masojidek, Josef Komenda, Tomas Kucera, Roberto Pilloton, Autar K. Mattoo, Maria T. Giardi; **A SENSITIVE PHOTOSYSTEM II-BASED BIOSENSOR FOR DETECTION OF A CLASS OF HERBICIDES**; **BIOTECHNOLOGY AND BIOENGINEERING, VOL. 60, NO. 6, DECEMBER 20, 1998**
UPCOMING EVENTS

The 6th International Workshop on Biosensors and μ-Analytical Techniques for Environmental and Clinical Analysis

PAST EVENTS

☐ Roberto Pilloton,
  Visiting Scientist at Ege University Izmir, Bornova, Turkey
  Visiting Scientist at Pamukkale University, Delizny, Turkey
  9-20 December 2003
  • MONOLAYERS OF NATURAL AND RECOMBINANT PHOTOSYSTEM II ON GOLD ELECTRODES
  • BIOSENSORS BASED ON CHOLINESTERASE ACTIVITY INHIBITION
  • IMMOBILISATION OF PURIFIED ENZYMES, ENGINEERED MOLECULES AND ENGINEERED YEASTS ON GOLD, CARBON AND CARBON/GOLD COMPOSITES

☐ Walter Vastarella, Marina Tosi, Amedeo Masci, Roberto Pilloton,
  Screen Printed Electrochemical Biosensors: Fancy Or Valide Alternative For Environmental Analysis?
  ARG Convention
  24th-28th November 2003
  Trieste, Italy

☐ Roberto Pilloton, Walter Vastarella, Amedeo Masci,
  Screen Printed Electrodes: study of reproducibility, stability and sensitivity
  20-21 October 2003
  Barcelona, Spain

☐ International Workshop on Biosensors for Food Safety and Environmental Monitoring
  October 9-11, 2003
  Marrakech, Morocco
  • A.Boni, E.Magaró, M.Tosi, W.Vastarella and R.Pilloton; ANTICHOLINESTERASE ACTIVITY ON GRAPES BY PURIFIED ENZYMES, ENGINEERED YEASTS AND SPGE
  • J.Maly, A.Masci, J.Masojidek, M.Sugiura and R.Pilloton; MONOLAYERS OF NATURAL AND RECOMBINANT PHOTOSYSTEM II ON GOLD ELECTRODES - POTENTIALS FOR USE AS BIOSENSORS FOR DETECTION OF HERBICIDES
J. Maly, C. Di Meo, M. De Francesco, A. Masci, J. Masojidek, M. Sugiura, A. Volpe, R. Pilloton; Reversible Immobilisation of engineered molecules by Ni-NTA Chelators
XVI\textsuperscript{th} International Symposium on Bioelectrochemistry and Bioenergetics, June 19-24, 2003
Florence, Italy

Roberto Pilloton & Giulio Izzo
Gestione dell'ambiente acquatico: valutazione del rischio e della qualità ecologica dal livello di comunità al livello molecolare.
18-19 June 2003
ENEA Casaccia, Rome, Italy
- Marco Mascini (Università di Firenze) - Biosensori elettrochimici a DNA
- Roberto Pilloton (ENEA-BIOTE-MED) - Biosensori elettrochimici ad inibizione basati su molecole e cellule ricombinanti
- Aldo Roda (Università di Bologna) - Biosensori luminescenti basati su cellule ricombinanti per la determinazione di metalli e contaminanti organici in matrici acquifere
- Franco Mazzei (Università di Roma) - Sistemi elettrochimici ad inibizione: aspetti generali ed applicabilità nel controllo degli ecosistemi acquatici.
- Luigi Campanella (Università di Roma) - Il contributo della chimica al controllo della tossicità integrale dei sistemi idrici

Roberto Pilloton & Giuseppe Palleschi (Università di Tor Vergata Roma)
RoseProMilk Project MidTerm Meeting and Steering Board Meeting
16 May 2003
ENEA Casaccia, Rome, Italy
- Roberto Pilloton, Amedeo Masci, Flavia Dalla Riva, Lucia Mosiello, Celine Ndong - SPE immunosensors at Enea
- Maria Velasco-Garcia: Silsoe Research Institute, Cranfield, UK) - Specification for an automated analyser for faecal wash
- Emanuele Marconi (Università del Molise) - Validation of Aflatoxin M1 procedures and setup of reference procedures
- Laura Micheli (Università’ di Tor Vergata Roma) Aflatoxin-AP Conjugates
- Manpreet K. Khurana and Pankaj Vadgama (Queen Mary University, UK) - Development of polymeric membranes to improve the sensitivity, selectivity and stability of chlorophyll sensors
- John Hart, Roy Pemberton (University of West England, UK) - The Voltammetric Measurement of Chlorophyll using Screen-Printed Sensors
• Jeanette Pritchard GEM Technology, Cranfield UK - SPE: examples of sensor design and array @ GEM

☐ AISEM 2003
  February 2003,
  ITC-IRST, Trento, Italy
  • E.Podesta’, C.Botre’, R.Pilloton, F.Botre’, F.Mazzei; A SCREEN-PRINTED ENZYMATIC ELECTRODE FOR THE DETERMINATION OF ORGANO-PHOSPHOROUS PESTICIDES;
  • C.Di Meo, L.Della Seta, M.De Francesco, A.Masci, V.Pinto, A.Volpe, and R.Pilloton; REVERSIBLE IMMOBILISATION OF ENGINEERED MOLECULES BY NI-NTA CHELATORS;
  • L.Della Seta, S.Marino, A.Masci, R.Pilloton; SCREEN PRINTED BIOSENSORS BASED ON OXYGEN SENSING: USE OF PERM SELECTIVE MEMBRANES;

☐ Roberto Pilloton,
  COSMIC @ CEA
  October 21st, 2002
  Saclay, France

☐ Roberto Pilloton,
  Biosensing?: Why not? An informal meeting @ ENEA
  July 16th, 2002
  ENEA Casaccia, Rome, Italy
  • Roberto Pilloton - Electrochemical Biosensors Lab @ Enea
  • Jan Maly Univ. of South Bohemia, Ceské Budejovice, Czech Republic - Determination of herbicide isoproturon in soil extracts using photosystem II based biosensor - persistence and movement of herbicides
  • Miwa Sugiura - CEA Saclay - DSV/DBCM/SBE, France - Osaka Prefecture University
  Osaka, Japan - Property and structure of His-tagged PSII core complexes from Thermosynechococcus elongatus
  • Josef Komenda, Inst. Microbiol., Opatovicky mlyn, Trebon, Czech Republic - Structural and functional dynamics of the photosystem II complex in cyanobacteria
  • Jiri Masojidek, Inst. of Microbiology, Academy of Sciences, Trebon, Czech Republic - Photosynthetic biotechno-logies in the frame of the National Research Centre "Mechanisms, Ecophysiology and Biotechnology of Photosynthesis"
  • Richard A. Durst (Cornell University, Geneva, NY, USA) - Biosensors for femtomolar level detection of cholera and botulinum toxin using nano-vesicles and ganglioside receptors
  • Antje J. Baeumner (Cornell University, Ithaca, NY, USA) - Biosensors and bioanalytical microsystems for the detection of pathogenic organisms
• Elisabetta Podestà (University of Rome – La Sapienza) - A screen-printed enzymatic electrode for the determination of organophosphorous pesticides
• Laura Micheli - University of Rome – Tor Vergata - An electrochemical immunosensor for the Aflatoxin M1 determination in milk using screen printed electrodes

Timur, Suna; Pazarlioglu, Nurdan; Pilloton, Roberto; Telefoncu, Azmi;
DETECTION OF PHENOLIC COMPOUNDS BY THICK FILM SENSORS BASED ON PSEUDOMONAS PUTIDA;
The 5th Workshop on Biosensors and Analytical techniques in Environmental Monitoring (IAEAC)
June 2002
Ithaca (NYS) USA

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February 2002
Bologna, Italy
• L.Della Seta; A.Masci and R.Pilloton; A NEW LAY-OUT FOR SCREEN-PRINTED ELECTRODES: FRONT/BACK GEOMETRY;
• S.Timur, A.Telefoncu, A.Masci and R.Pilloton; OXIDIZED CARBON POWDER FOR ENZYME IMMOBILIZATION ON SCREEN PRINTED BIOSENSORS;
• L.Campanella, G.Favero, S.Marino, R.Pilloton and M.Tomassetti; DISPOSABLE SCREEN PRINTED POTENTIOMETRIC SENSORS FOR DETERMINATION OF FREE RADICALS;

Maly, J.; Illiano, E.; Sabato, M.; De Francesco, M.; Pinto, V.; Masci, A.; Masci, D.; Masojidek, J., R.franconi, R.Pilloton
IMMOBILISATION OF ENGINEERED MOLECULES ON ELECTRODES AND OPTICAL SURFACES
ECOF 8
September 2001
Otranto (Le) Italy

Roberto Pilloton & Franco Mazzei, The 2nd Workshop on Chemical sensors and Biosensors
18-19 March 1999
ENEA Casaccia, Rome, Italy
• P. Morales, L. Giulietti, L. Mosiello, R. Pilloton; F. Bordoni, G. De Gasperis, S. Sperandii, S. Santucci, E. Di Fabrizio; BIOLOGICAL AND ORGANIC MOLECULES PHOTOIONIZATION FOR BIOMOLECULAR DEVICE FABRICATION
• S. Marino, A.Masci, G. Minervini, M.R. Montereali, C. Cremisini e R. Pilloton; SCREEN PRINTING” FOR CHEMICAL SENSOR AND BIOSENSOR PRODUCTION
• L.Mosiello, C.Cremisini, L.Segre, S.Chiavarini, M.Spanò, T.Kimmel, A.Baumner, R.D.Schmid; DIPSTICK IMMUNOASSAY FORMAT FOR TERBUTHYLAZINE ANALYSIS IN WATER SAMPLES

• M.Rizzuto, C.Polcaro, C.Desiderio, M. Koblizek, R.Pilloton, M.T.Giardi ; HERBICIDE MONITORING IN SURFACE WATER SAMPLES WITH A PHOTOSYSTEM-II BASEF BIOSENSOR;

• A.Masci, Della Seta L., S.Galluppi, C.Micheli, R.Pilloton; A SMALL AND INEXPENSIVE BIOSENSOR BASED DEVICE FOR ON LINE EVALUATION OF MICROALGAE METABOLISM INHIBITION

• Giuliano Martinelli, Maria Cristina Carotta, Vincenzo Guidi, Cesare Malagù and Enrico Traversa - Thick Film Gas Sensors Based on Nanosized Semiconducting Oxides.


• M. Penza - Surface Acoustic Wave Devices for Gas Sensing Applications.

• P.Pelosi, K.Persaud - Physiological and Artificial Systems for Odour Recognition

• G. D'agostaro - Olfactory Receptors: from Cloning to Function

• M. Pardo, G. Niederjaufner, E. Comini, G. Faglia, G. Sberveglieri - Electronic Nose For Food And Other Applications


• L. Leo, G. Mele, G. Rosso, G. Stasi, L. Valli, G. Vasapollo - Deposition of Phthalocyanines and Porphirins


• T. Ferri, A. Pocia, R. Santucci - Direct Electrochemistry of Membrane-Entrapped Horseradish Peroxidase.

• D. Compagnone, D. Moscone, G. Palleschi - Amperometric Biosensors for food quality control. Determination of biogenic amines, lactulose and glycerol

• S. Tombelli, G. Marrazza, M. Mascini - Recent Advances on DNA Biosensors

• R. Franconi, A. Desiderio,P. Roggero, E. Benvenuto - Isolation of Recombinant Single-Chain Antibodies (scFvs) with Desired Specificity from a ‘Single-Scaffold’ Phage Display Library

• A.R Sprocati, V. Capuano, G.Antonini, P.D. Valenti, A.Aiello - Monitoring Water Quality in Aquaculture with a New Sensor for Microorganisms and Toxicity

• L.Tedeschi, A.Ahuwalia, C.Domenici, F.Baldini, C. Preininger, A.Mencaglia - Protein Immobilisation on Solid Substrates for the Realisation of Optical Immunosensors

• L.Campanella, G.Favero, L.Persi, M.P.Sammartino, M.Tomassetti, G.Visco - Recent Development of Environmental Sensors
• F. Mazzei, F. Botrè - Inhibition Based Biosensors: Environmental Applications
• L. Campanella, G. Favero, M.P. Sammartino, M. Tomassetti - OPEEs – What are they?
• E. Podestà, B. Silvestrini, C. Botrè - Alternative Methods to Animal Testing: the Role of Biosensors
• M. Adami, G. Martinazzo, S. Villari, M. Panza and C. Nicolini - Heavy Metals and Lactate Monitoring Systems
• C. Cantale, M. Oriolo, M. Paci, M. Sperandei - A Synthetic System Mimics the Electron Transfer in Membranes: Synthesis and Characterization of the Proteic Scaffold
• M. Pizzichini, M. Spadoni; R. Montani - Tannery Process Regulation with On Line Cr(III) Monitoring
• I. Palchetti, L. Lepore, M. Mascini - Screen-Printed Electrodes for the Detection of Heavy Metals
• S. De Vita, N. Luisetto, C. Pinelli, E. Dalla Turca - Simple and Fast Determination of Lactose and Lactulose in Raw and UHT Milk Using Differential pH-Technique
• G. Di Francia, S. La Ferrara, S. Manzo, L. Quercia, V. La Ferrara, L. Lancellotti - Feasibility of an Optical Biosensor Based on Porous Silicon
• L. Campanella, L. Persi, M. Tomassetti - Superoxide and Nitric Oxide Radicals as Modulating Agents of Enzymatic Sensor Responses.
• R. Raiteri,, S. Martinoia, M. Grattarola, H.-J. Butt - Silicon Microcantilever Based Biochemical Surface Stress Sensors
• F. Baldini, A. Falai, A. Flamini - Optical Characterization of a New Indicator Dye Covalently Bound on Controlled Pore Glasses, Potentially Suitable as Optical Transducer for Hg(II)
• A. Curulli, and G. Palleschi - Construction and Application of Highly Selective Sensors and Biosensors Using Non-Conducting Electropolymerized Films
• Alessandra Crisà, Federica Forbici, Maria Teresa Mancuso and Giacomo A.F. D'Agostaro - Cloning of Novel Seven-Transmembrane-Domain Receptors from Rat Olfactory Neuroepithelium
INTERNATIONAL FELLOWSHIPS

- Jan Maly - PHOTOSYSTEM II IMMOBILISATION OR THIN FILM DEPOSITION BY CHEMICAL PROCEDURES or - 2 months starting in May 2001.
- Suna Timur PhD - DEVELOPMENT OF MERCURY THIN FILM (MTF)-BASED SCREEN PRINTED GRAPHITE BIOSENSOR FOR THE DETECTION OF PHENOLIC COMPOUNDS - 4 months starting in September 2001
- Jan Maly - ORIENTED AND REVERSIBLE IMMOBILISATION OF ENGINEERED MOLECULES ON GOLD ELECTRODES - 4 months starting in May 2002.
- Suna Timur PhD - PHENOL BIOSENSORS BY MICRORGANIMS OR PURE ENZYMES, 2 months starting in December 2002
- Jan Maly - ELECTROCHEMICAL DEPOSITION OF SAM LAYERS ON A M-ARRAY OF GOLD ELECTRODES; 5 months starting on September 2003

- Celine Ndong
- Anna Krassilnikova
PATTERNING OF ENGINEERED MOLECULES ON BIOSENSOR MICROARRAYS (R.Pilloton, student Alessandra Volpe)

DISPOSABLE BIOSENSORS FOR FOOD, MEDICINE AND ENVIRONMENT
ELECTROCHEMICAL BIOSENSORS: APPLICATION IN FOOD, ENVIRONMENT AND AGRICULTURE
(R.Pilloton - student Eugenia Magaro', April 2003)

FUNCTIONAL COATINGS FOR PRINTED SENSORS AND BIOSENSORS (R.Pilloton - student Stefano Marino - 2001 October 21st)

PHOTOSYNTHEThIC BIOSENSORS FOR ENVIRONMENTAL MONITORING (R.Pilloton - Dr. Livia Della Seta - 2001 July 11th)

SCREEN PRINTED ELECTROCHEMICAL BIOSENSORS FOR PESTICIDES IN THE WINE INDUSTRY (R.Pilloton - student Alessio Boni, 2001 November 21st)

IMMUNOLOGICAL METHODS FOR ATRAZINE MONITORING (PhD Thesis @ Univ. of Pavia prof.M.Pesavento) - SCREEN PRINTED IMMUNOSENSORS FOR ATRAZINE (R.Pilloton student Flavia Dalla Riva, December 2001)

NEURAMINIDASE ACTIVITY DETERMINATION WITH AN ELECTROCHEMICAL LACTOSE BIOSENSOR (R.Pilloton - student Dr.M.R.Montereali - 2001 March 23rd)

IMMOBILISATION OF NATURAL AND ENGINEERED MOLECULES FOR NEW BIOSENSORS (R.Pilloton, student Dr.Chiara Di Meo, December 2002)
COSMIC (2001-06) was presented at Enea on 2000 March 15th and was evaluated from both an internal and an external commission of experts. It was considered a strategic project at ENEA on December 2000. COSMIC Project started on January 2001. The financial support is exclusively provided from external national and international projects. The following are afferent projects presented or approved.

- FISR 2003-205 “Optical and Electroptical sensors for rapid control of water body pollutants, started on January 2003 Project Leader for Enea R. Pilloton
- ROSEPROMILK 2001-04 (RObust Chemical SEnsors and biosensor for raPid on-line identification of rFreshly cOlected MILK) UE/ENEA n° QLK1-CT2001-01617 “Programma quality of life and management of living resources”; Presented to EEC 5th Framework Quality of Life, started on December 2001 Project leader for Enea R. Pilloton
- PROGRAMMA TRIENNALE DI RICERCA AGRICOLA, AGROAMBIENTALE, AGROALIMENTARE, AGROINDUSTRIALE DELLA REGIONE LAZIO 2003-2005 (2° PRAL); PRODUZIONE DI UN PROTOTIPO DI BIOSENSORE PER LA RIVELAZIONE DI ORGANO-FOSFATI, PRESENTI COME CONTAMINANTI DI PRODOTTI AGRO-INDUSTRIALI ED ESTENSIONE DELLA TECNOLOGIA ADOTTATA ALLA PRODUZIONE DI BIOSENSORI PER LA RIVELAZIONE DI MICOTOSSINE, presented on October 2003, Project Leader for Enea R. Pilloton
• Dr.F.Mazzei, University of Rome, Rome Italy
• Prof.L.Campanella, University of Rome, Rome, Italy
• Dr.J.Masojidek, Czech Accademy of Sciences, Trebon Czech Rep.
• Dr.Krejci, Krejci Engineering, Czech Republòic
• Prof.G.Augusti-Tocco Università di Roma "La Sapienza"
• Prof.C.Palleschi Università di Roma La Sapienza
• Prof.G.Palleschi Università di Roma Tor Vergata
• Dr. M. Zen ITC-irst Divisione Microsistemi (TN)
• Prof.P. Dario MiTech Labs - Scuola Superiore S. Anna (PI)
• Prof.G. Marletta Università di Catania
• Dr.G.De Bellis CNR-ITB (MI)
• Dr.P.Perlo Centro Ricerche FIAT (TO)
• Prof. E. Marconi - University of Molise
• Dr.J.Hart University of West England
• Dr.O.Lind DeLaval
• Prof.S.Alegret Universitat Autonoma de Barcelona
• Prof.P.Vadgama Queen Mary University of London
• Dr.P. Cagnasso PARMALAT
• Dr.T.Mottram Silsoe Research Institute
• Dr. Mihaela Ilie University "Politehnica" of Bucharest, Romania
• Dr.Vittorio Foglietti CNR-INF-MEMS
• Dr.Miwa Sugiura Osaka Prefecture university, (Japan)
Full-text papers
ANTICHOLINESTERASE ACTIVITY ON GRAPES BY PURIFIED ENZYMES, 
ENGINEERED YEASTS AND SPGE

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SUMMARY

ABBREVIATIONS:

1. INTRODUCTION

2. EXPERIMENTAL
   2.1- MATERIALS, CHEMICALS AND REAGENTS
   2.2- MEMBRANE PREPARATION: IMMobilisation of AChe AND ChOx
   2.3- DETECTION PROCEDURE
   2.4 PESTICIDE SOLUTIONS AND PREPARATION OF THE ARTIFICIAL SAMPLES

3. RESULTS AND DISCUSSION

4. CONCLUSIONS

ACKNOWLEDGEMENTS

REFERENCES

CAPTIONS OF FIGURES

FIGURES
SUMMARY
Engineered molecules and cells immobilized on screen printed electrodes were recently employed for biosensing purposes in environmental analysis. In this work an engineered yeast, with a genetically expressed acetyl cholinesterase activity from rat, was used for detection of organophosphorous compounds by means of screen printed electrodes. AcChE was immobilised on a membrane and placed in solution, separating the incubation step from the measurement of its residual activity. For this reason, measurements were performed in a standard solution with a choline electrochemical biosensors without any interference, avoiding the use of protecting selective membranes. The slow incubation step was simultaneously performed on several samples, thus lowering the analysis time per sample. Good LOD (0.1 ppb) for paraoxon was obtained. This procedure was applied to determine selectively AChE inhibitors directly on fruit and food by using either commercially available enzymes or a recombinant yeast.

ABBREVIATIONS:
AChE: acetyl cholinesterase; ChOx: choline oxidase; CV: coefficient of variation; GA: glutaraldehyde; ISFET: ion selective field effect transistor; LOD: limit of detection; OP: organophosphorous pesticides; PAP: polyazetidine; PB: phosphate buffer 0.1M (pH=7.0); PBS: phosphate buffer saline (NaCl 0.1M; PVC: polyvinylchloride; RE: reference electrode (Ag/AgCl); RSD: relative standard deviation; SPE: screen printed electrode; TFT: thick film technology; WE: working electrode
1. INTRODUCTION

Engineered biomolecules represent a powerful approach to obtain simpler artificial structures with new or improved properties (i.e. specificity, stability, sensitivity), useful for biosensors development. For instance, insertion of specific molecular tags can be used for oriented and reversible immobilization, preconcentration and purification, whereas, easier and sensitive analytical detection can be achieved by gene fusion with selected enzyme activities. Moreover tailored specificities can be isolated from 'phage display' antibody libraries through the 'panning' procedure[1,2]. Several engineered cells are also suitable for biosensing purposes allowing low cost continuous production of enzymes on the tip of a sensor.

In the last decades organochlorine insecticides (e.g. DDT, aldrin, lindane) were progressively replaced by organophosphorus (e.g. parathion, malathion) and derivatives of carbamic acid (e.g. carbaryl, aldicarb) insecticides, that show low persistence in the environment but represent a serious risk because of their high acute toxicity. They are now largely diffused in agriculture because of their high efficiency and relatively rapid degradation in environment, but their distribution and recycling processes result in water and soil pollution, with dangerous and acute effects on the living organisms, i.e. altering the food chain, or inhibiting the active site of fundamental enzymes, i.e. cholinesterases involved in muscle physiology and in nervous system. Main risks regard both the professional (industrial production, agricultural use) and not professional exposures (domestic use, food and fresh water contamination).

The European Drinking Water Act (1980) does not allow their concentration in drinking water to exceed individually the limit of 0.1 mg/L. Analytical monitoring of such a low level with both high sensitivity and selectivity remains a topical issue, especially when in presence of interfering compounds. HPLC, GC-MS, ELISA methods were showed to be expensive in instrumentation and/or relatively difficult, time consuming in the sample treatment, although highly sensitive[3-4] Furthermore, most of these methods do not provide any information about toxicity and effects on living organisms. These concerns have stimulated research towards development of biosensing technology as a new tool for detecting herbicide and insecticide toxicity in a simple and cost effective way. Several AcChE[5] and OPH based[6-7] biosensors were already developed to detect paraoxon, methyl-parathion e diazinon, with LOD of respectively 0.5-1.8 $10^{-4}$ M, 0.6-9.1 $10^{-4}$ M and 0.5-8.5 $10^{-3}$ M. AcChE based biosensors allow faster, cheaper and simpler screening procedures by using differential pH meters,
ISFET, Light Addressable Potentiometric Sensors, conductimetric cells, amperometric carbon modified electrodes or, as in this paper, SPGEs based on ChOx.

Residual activity of free or immobilised AcChE located on the biosensor tip was normally detected in the real sample where incubation with OP was performed. Serious problems took place due to: i) adsorbed AcChE, ii) electrochemical interferences from the sample and iii) very low lifetime of the biosensor. As previously reported[8], AcChE immobilised on a separate membrane placed in solution, provided a different protocol of analysis with separation between incubation step and final measurement in a clean standard solution allowing the biosensor to be used without any protecting membrane. A very high reproducibility for paraoxon in drinkable water, lower analysis time (270s/sample with parallel operation on 24 samples) and the possibility to use the same ChOx biosensor for several hundreds of analyses, were achieved. In this paper, residual activity of AChE immobilized onto nylon membranes was measured on SPGEs ChOx amperometric biosensors to determine AcChE inhibitors directly on fruit by using either a commercially available enzyme or a recombinant yeast[9-10] with a genetically expressed AcChE activity. Comparisons between commercial \( \text{H}_2\text{O}_2 \) electrode, as reference methodology, and SPGE were also performed.

2. EXPERIMENTAL

2.1. MATERIAL, CHEMICALS AND REAGENTS

The commercial amperometric sensor was a voltammetric cell (IDRONAUT–Brugherio, Milan), with Ag/AgCl (3 cm\(^2\)) as references electrode, and a Pt wire (0.1-1mm) as working electrode. Electrodes were printed on a PVC slide by TFT[11] acceding to several lay-outs. The screen printer was a Fleischle Gmbh machine (Brackenheim–Germany). Carbon inks for screen printed electrodes were improved by doping procedure: Pt (Aldrich 23.755-8) or Rh (Aldrich 20.616-4) absorbed on graphite powders were mixed with the carbon pastes (obtained from Acheson). An homemade flow cell, used for setting up choline measurements, consisted of two blocks where the SPGE must be inserted and fixed by an O-ring, and a three-ways inlet flow system for carrier and sample solution.

Choline Oxidase (Choline: oxygen 1-oxidoreductase, EC 1.1.3.17, extracted from Alcaligenes sp., 10 units/mg solid, lyophilised form) was purchased from SIGMA. Acetyl Cholinesterase (EC 3.1.1.7, extracted from electric eel; 1000-2000 units/mg protein, lyophilised form) as well as crystalline form of acetyl choline chloride, choline chloride, paraoxon (Diethyl p-Nitro phenyl Phosphate) were from SIGMA. Glutaraldehyde 25% aqueous solution was obtained.
from FLUKA, polyazetidine polymer from Hercules Polycup. Selective hydrogen peroxide membranes (MWCO<100 D) were prepared in laboratory; nylon Immunodyne ID-membranes were obtained from Pall Inc.-Italia (Milan). Syringe for filtration and 0.2 µm filters were purchased from Whatman Inc. Supporting electrolyte 0.1M phosphate buffer solutions were prepared from deionised water (ELGA-STAT deioniser). All other chemicals were of analytical grade and used without further purification.

2.2- Membrane Preparation: Immobilisation of AChe and ChOx

AcChE was immobilised on nylon preactivated membranes by using a 12.5% glutaraldehyde solution in PB at pH 7.0, as crosslinking agent. The treatment time consisted simply in immersing a piece of the membrane in glutaraldehyde solution for one hour, washing them in PB solution and dropping 3 µl of the enzyme solution (25 U/ml). Adsorbed AchE, not covalently linked to the membranes, was removed with sonication (1h in PBS). Twenty four AchE membranes were fixed onto the tool (fig.1) to make sequential incubation and parallel inhibition of the enzyme, with further measurements of the enzymatic activity of the membrane. Nylon membranes (0.2 µm pore size) were used for immobilisation of engineered yeast cells by siringe filtration of (AchE 75mU) of 1ml cell suspension.

ChOx in PB solution, exploiting the catalytic oxidation to betaine (reaction 1) and the production of hydrogen peroxide:

\[
\text{ChOx} \rightarrow [(\text{CH}_3)_3\text{N}^+\text{CH}_2\text{CH}_2\text{OH} + 2\text{O}_2 + \text{H}_2\text{O} \rightarrow (\text{CH}_3)_3\text{N}^+\text{CH}_2\text{COOH} + 2\text{H}_2\text{O}] \tag{1}
\]

was immobilised onto the WE with both PAP solution and a selective cellulose acetate membrane by dropping directly 10 µl on; a dialysis membrane was then placed and a pressure of 36 Kg cm\(^{-2}\) was finally applied. The preparation procedure for the commercial H\(_2\)O\(_2\) electrode is similar to the SPE but in this case the membranes were fixed onto the electrode by a neoprene O-ring. Fig.2 shows the calibration curves obtained with both the SPE and the commercial ChOx based biosensors.

2.3- Detection Procedure

The determination of OP, specifically paraoxon, was separated in different operational steps. Firstly, AcChE, which catalyzes the hydrolysis of acetylcholine according to reaction (1),

\[
(\text{CH}_3)_3\text{N}^+\text{CH}_2\text{O-C-CH}_3 + 2\text{H}_2\text{O} \rightarrow (\text{CH}_3)_3\text{N}^+\text{CH}_2\text{OH} + \text{CH}_3\cdot\text{C-O}^- + \text{H}_3\text{O}^+ \tag{1}
\]
covalently immobilized on preactivated nylon membranes, was inhibited by paraoxon. Secondly, the membranes were rinsed and incubated in $10^{-3}$M acetylcholine chloride, using the apparatus in fig.1 which enabled rapid and contemporaneous operations on 24 membranes at a time, thus lowering the time needed for each analysis. Thirdly, the residual activity of AcChE was measured under flow condition by a ChOx SPE.

2.4 PESTICIDE SOLUTIONS AND PREPARATION OF THE ARTIFICIAL SAMPLES
The AchE membranes were inhibited using different concentration of paraoxon, as reference pesticide. The application range of these membranes was between 10 and 75 ppb. Using paraoxon 37 ppb the inhibition effect was evaluated as a function of the exposure time and the best analytical results were obtained at 30 minutes.

Grapes were spray coated with the maximum amount of paraoxon allowed by the European regulation, i.e. 0.5 ppm paraoxon in ethanol solution. The preactivated nylon membrane containing AcChE was deposited directly on the contaminated grape: 10 µL of buffer solution 0.1M were dropped on the membrane to enable the absorption of the pesticide on it.

After washing and incubation of a set of 24 membranes, the choline concentration, and inversely the AcChE inhibition, were measured with high reproducibility (within 4.5% including the sample preparation) by using ChOx SPE biosensor under flow conditions.

3. RESULTS AND DISCUSSION
After immobilisation of the enzyme and sonication, AchE membranes showed different activities. Figure 3 reports the comparison of 2 sets of measurements made sequentially without the inhibition step. The real slope of the line (1.06±0.08) is nearly confident with the theoretical one (1.00). This graph shows the reproducibility of the AchE activity determination. In fig. 4A is reported the reproducibility of our procedure, starting from the sampling step on the grapes as described above. Three grapes were spray coated with paraoxon (37 ppb in ethanol) and then each grape was sampled with three different membranes. With this procedure we revealed a higher pesticide content (fig.4B) at the bottom (+15%) with respect to the top of the same grape due to the outer waterproofing layer (cuticle) of the skin, allowing the pesticide nebulized solution to slide along the fruit from top to bottom.

Genetically modified yeasts physically entrapped into membranes, were tested in order to reduce costs of analysis. The purified AcChE was thus replaced with an engineered
(Kluyveromyces lactis) yeast which expresses AcChE activity from rat into the cell wall. Only the second incubation step for developing AchE activity to be measured with ChOx biosensor required to be changed with respect to the pure enzyme based procedure. The engineered yeast required higher time (30 min) with respect to the pure enzyme (10 min) to develop the same choline concentration, as reported in fig. 5 where choline production vs incubation time due to the AchE membrane is compared for pure enzyme and engineered yeast. Cells were fixed by syringe filtration. As a result, inhibition curves are reported (fig. 6) due to paraoxon deposited on grapes and obtained with the pure enzyme (upper curve) and the engineered yeast (lower curve). The yeast-based biosensor significantly gave similar results compared to the purified enzyme biosensor under similar experimental conditions.

4. CONCLUSIONS

Nowadays, recombinant molecules or microorganisms by using TFT allow to combine the extremely high specific activity of the former with the advantages from the short time of analysis, the low production costs, and the good reproducibility of disposable SPEs. An engineered yeast with expressed AcChE activity coupled to SPE represents an economic way for preparing and immobilizing AcChE for detection of OP, i.e. paraoxon. A protocol for anticholinesterase activity on grapes was developed which well applies to the concentration range of pesticides normally on fruit and to the limit fixed by the European regulation. Comparison between H₂O₂ electrode and SPE was performed: short time of analysis (80 s/sample with parallel operation on 24 membranes) and good LOD (0.1 ppb) for paraoxon were achieved in both cases. This procedure was extended to a biosensing system based on a recombinant yeast[9-10] with a genetically expressed AcChE activity from rat. All these advantages could achieve the diffusion of reusable, in-situ, sensitive, reliable, cheap and easy-to-use sensors for environmental analytical application.

ACKNOWLEDGEMENTS

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11. White, N-M; (1994); Thick film technology; Thick film sensor (Elsevier eds.); 3-11
CAPTIONS OF FIGURES

Figure 1: Experimental setup for parallel inhibition and further measurement of the activity of AChE membranes. Initially each membrane was put on the grape as described in the text.

Figure 2: Calibration curves with 95% confidence intervals for the choline biosensors obtained 1) with the commercial hydrogen peroxide electrode, 2) with a SPE. Signals from the recorder are reported as an example.

Figure 3: Two sets of measurements of AchE activity on 24 membranes are reported with 95% confidence interval. Y=(1.06±0.08)X+(-3±2), r^2=0.89, C.V.=±7.5%, n=1

Figure 4: Detection of pesticide inhibition on grapes. A) Reproducibility of inhibited AchE membranes (n=3, three grapes). B) Pesticide inhibition at different heights on the grape.

Figure 5: The second incubation step for developing AchE activity to be measured with ChOx biosensor required higher time (30 min, upper curve) with the engineered yeast with respect to the pure enzyme (10min, lower curve).

Figure 6: Inhibition curves with 95% confidence intervals due to paraoxon deposited on grapes by spray coating different standard solutions in ethanol. Curves are obtained with the pure enzyme (upper curve) and the engineered yeast (lower curve)
AcChE on PALL membranes
$y = (2.4 \pm 0.1) \times 10^3 x + (8 \pm 6) \times 10^{-3};
R^2 = 0.9931;
C.V. = 4.2\%$

$y = (4.1 \pm 0.1) \times 10^2 x - (2 \pm 5) \times 10^{-4};
R^2 = 0.9963;
C.V. = 2.4\%$
Figure 6: Inhibition curves for paraoxon
DISPOSABLE SCREEN PRINTED POTENTIOMETRIC SENSORS FOR DETERMINATION OF FREE RADICALS

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Abstract

In this communication new screen printed potentiometric electrodes based on nitroprusside dispersed in a PVC membrane for the determination of free radicals are reported. Screen printed potentiometric sensors based on an ionophore dispersed in a PVC membrane were previously verified and optimised by using valinomycin as model molecule for potassium ion activity measurement. Valinomycin was used for finding the best deposition condition of the PVC membrane directly on the printed graphite electrodes. Valinomycin containing membranes of different thickness were obtained with “dip & dry” or “tape casting” or “screen printing” techniques by using different dilution of the PVC matrix in tetrahydrofurane (THF). Sensitivity of the valinomycin based sensors for K\textsuperscript{+} was found to be strictly related to the thickness of the membrane and the used deposition technique. A very good reproducibility was also observed by comparing the slope values of the calibration curves obtained from several electrodes. Furtherly, nitroprusside dispersed in the same PVC membrane was used for the development of disposable, screen printed potentiometric sensors for free radicals.

Keywords: screen printed potentiometric sensors, free radical determination.
1 Introduction

Screen printing technology was recently used for mass production of low cost, miniaturised and disposable chemical or biochemical sensing devices to be used on gas or liquid samples. Screen printed amperometric biosensors were extensively used for the development of new devices for environmental monitoring of waters and wastes [1-2].

Recently, low cost and rapid analytical determinations of free radicals are playing a central role in environment and food monitoring. The authors proposed in recent years different kind of sensors [3,4] and biosensors [5] for free radical analysis.

In this communication new screen printed potentiometric electrodes based on nitrone dispersed in a PVC membrane for the determination of free radicals are reported.

2 Materials and Methods

a) Electrode Printing and Lay-out: The lay-out of the screen printed electrode is reported in fig.1. The printing starts with the deposition of conducting paths and pads using Ag/Pd inks, DuPont, 5025 (B) on a 0.3mm thick PVC substrate (A). Then, the working electrode is printed using graphite inks, Du Pont 7101 (C) and finally the reference electrode is made using Ag/Pd ink (D). Finally, the conducting paths were covered by an insulator layer (E). The final layout of a single PVC graphite working and Ag/AgCl reference electrodes is reported in (F).

b) Plasticized PVC membrane deposition: Plasticized (dibutyl sebacate, 100µL) PVC membranes of different thickness containing the sensible molecules -
valinomycin (1mg, Fluka,) for potassium ion or N-t-butyl-α-phenyl-nitrone (500µg, Sigma) for free radicals - were obtained by “dip & dry”, “casting” (with a tool which allows to deposit solution of 200µm thickness) or “screen printing” techniques by using different dilution of the PVC (300-500mg) matrix in tetrahydrofurane (THF, 300µL).

c) Standard solution of superoxide radical: Due to the intrinsic unstability of free radicals, standard solutions of these species have to be prepared in situ. Several procedures can be used as well as 1) the Fenton method, based on hydroxide radical production and starting from Fe(II) and H₂O₂, or 2) the method based on xanthine / xanthine oxidase for production of superoxide radical.

d) Apparatus for flow measurements: Measurements were performed in a flow system by using a peristaltic pump (Gilson MiniPulse) and a workshop made flow cell (fig.2)

Figure 2: Scheme of the thin film flow cell for screen printed potentiometric electrodes made in our workshop.

3 Results and Discussion

Screen printed potentiometric sensors based on an ionophore dispersed in a PVC membrane were previously verified and optimised by using valinomycin as model molecule for potassium ion activity measurement. Valinomycin was used for finding the best deposition conditions of the PVC membrane directly on the printed graphite electrodes. Valinomycin containing membranes of different thickness were obtained with “dip & dry” or “tape casting” or “screen printing” techniques by using different dilution of the PVC matrix in tetrahydrofurane (THF). Screen printing of the PVC membranes gave the worse results and was abandoned. With dip & dry and casting procedures, sensitivity of the valinomicyn based sensors for
K⁺ was found to be strictly related to the thickness of the membrane and the used deposition technique. A very good reproducibility was also observed by comparing the slope values of the calibration curves obtained from several electrodes. Results of this preliminary step for obtaining the best deposition conditions of the PVC membrane are reported in table 1.

### Table 1: Features of the calibration curves obtained with valinomycin/PVC based ion selective screen printed electrodes (ISSPE) for K⁺ ions, having different thickness of the membrane and using different deposition techniques.

<table>
<thead>
<tr>
<th>Deposition technique</th>
<th>PVC (mg)</th>
<th>Sensitivity (mV/Log[K⁺])</th>
<th>C.V. (%)</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dip &amp; dry</td>
<td>247</td>
<td>63±2</td>
<td>2.9</td>
<td>0.9981</td>
</tr>
<tr>
<td></td>
<td>336</td>
<td>39±4</td>
<td>10.3</td>
<td>0.8911</td>
</tr>
<tr>
<td>Casting</td>
<td>458</td>
<td>11±3</td>
<td>27.3</td>
<td>0.8077</td>
</tr>
<tr>
<td></td>
<td>417</td>
<td>28±7</td>
<td>23.8</td>
<td>0.8988</td>
</tr>
<tr>
<td></td>
<td>336</td>
<td>34±6</td>
<td>17.6</td>
<td>0.9322</td>
</tr>
</tbody>
</table>

The development of disposable, screen printed potentiometric sensors for free radicals, was based on a nitrone species dispersed in the PVC membrane. Nitrone is able to give spin trapping reactions with free radicals and, for this reason, a membrane potential variation can be observed as previously verified [3]. PVC/nitrone coated electrodes (dip & dry) were tested in EDTA 0.02 mol/L, pH=5.16, FeCl₂ 0.02 mol/L and known amounts of H₂O₂ 0.1 mol/L were added for hydroxide radical production. A greatly over-Nernstian slope in the calibration curves was observed with high reproducibility (Fig. 3a).

![Figure 3: Calibration curves obtained for hydroxide (a) and superoxide (b) radicals with N-t-butyl-α-phenyl-nitrone, based screen printed electrodes.](image)

This effect could be due to the propagation of the radical based reaction which may result in an amplification of the signal but, at the moment, additional, interfering reactions cannot be excluded.
PVC/nitrone coated electrodes (dip & dry) were also used with the xanthine/xanthine oxidase method for superoxide radical production [3-5].

\[
\text{Xanthine + } 2\text{O}_2 + 2\text{OH} \xrightarrow{\text{xanthine oxidase}} \text{uric acid + } 2\text{O}_2^- + 2\text{H}_2\text{O}
\]

The slope of the calibration curve was again over-Nernstian but lower than in the previous experiments and nearer to the expected value (Fig. 3b).

4 Conclusions

Recently, screen printed electrodes were extensively used in the field of biosensors for obtaining mass production of low cost devices. In this frame, amperometry was the eligible technique for development of new screen printed electrochemical biosensors. In this paper, screen printed electrodes were used with the aim of demonstrating the possible development of low cost, potentiometry based, chemical sensors (ISSPE: ion selective screen printed electrode).

Interesting results were also obtained, in a preliminary stage, concerning the high sensitive determination of hydroxide and superoxide radicals with screen printed electrodes based on nitrone species dispersed in a plasticized PVC matrix.

Acknowledgement

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Carbon powders were subjected to an oxidation treatment with hydrogen peroxide in order to create surface oxygen groups (mainly carboxylic and phenolic groups) on printed electrodes for further covalent immobilisation of biomolecules. The oxidizing treatment modifies the surface chemistry of the carbon by creating surface-oxygen complexes that make it more acidic. Furthermore, the pH value of the carbon slurry was found to be changed from 6.5 to 4.4 after oxidizing step which introduced acidic groups on the surface. The porous structure of the carbonaceous support was not affected by oxidation as resulted by scanning electron microscopy before and after the treatment. Printed electrodes were fabricated by depositing several ink layers on a PVC substrate. Different oxidized carbon powders (including rhodium (10%), including dextran (25%) and lysine coupled graphite) were printed to obtain the working electrodes. Glucose oxidase was used as a model enzyme to test several immobilisation methods on the treated screen printed surfaces. Binding of the enzyme were performed on (1) EDC activated, (2) CDI activated surfaces and also, (3) Lysine coupled surfaces by using EDC activation, respectively. Moreover, storage stabilities at 4oC were investigated.

1 Introduction

Over the past few years interest has been increasing in the application of simple, rapid, inexpensive and disposable biosensors in clinical, environmental or industrial analysis. The most common disposable biosensors are those produced by thick-film technology. A thick-film biosensor configuration is normally considered to be one which comprises layers of special inks (or pastes) deposited sequentially onto an insulating support or substrate. Screen printing seems to be one of the most promising technologies allowing biosensor to be placed large-scale on the market in the near future because of advantages such as miniaturisation, versatility at low cost and also particularly the possibility of mass productions. The use of thick-film technology for the production of sensor systems is an emerging field. The most critical point in manufacturing thick film biosensors is the sensing or active
membrane and its adhesion to the transducer layer [1,2,3]. The aim of the present work is activation and derivatization of carbon powders to be used in screen printed electrode preparation. The oxidizing treatment of carbon powder with the aqueous H2O2 solution introduces mainly carboxylic and phenolic groups on the carbon surface after that as well as derivatization, it is easy to use this groups in covalent immobilization of biomolecules [4]. Glucose oxidase was used as a model enzyme to test the usage of the different covalent immobilization methods on the treated carbon surfaces.

2 Methods

Oxidizing Treatment of Carbon Powders: Carbon powders (conductive Carbon SuperP, MMM Carbon, Belgium, specific (BET) surface area 62m2/g) were subjected to an oxidation treatment with hydrogen peroxide in order to create surface oxygen groups; a given amount of the support was immersed in an aqueous solution of H2O2, 6 N (1 g of support/50 ml of solution) and the slurry so formed was stirred for 48 h at room temperature. Finally the oxidized sample was washed with distilled water to eliminate H2O2 excess and dried at room temperature [5].

Preparation of the carbon based inks for printing working electrodes: Inks for printing working electrodes were prepared by mixing a commercially available carbon ink (Du Pont 7101) with oxidized carbon and/or 10% rhodium graphite powder (Aldrich 20.616-4), or dextran (25%) or lysine coupled carbon.

Printed Electrodes: Printed electrodes were fabricated by depositing several layers of inks on a PVC substrate (Fig 1). The conducting paths and pads (Fig.1a) were deposited directly on the PVC sheets using Ag/Pd ink (DuPont, 5025). Then, an Ag/AgCl ink was deposited to obtain the reference electrode (Fig.1b). Different oxidized carbon powders were printed to obtain the working electrodes (Fig.1c). Finally, an insulator layer was placed over the conducting paths (Fig.1d). After each printing step, the paths were treated at 60°C for 60 min.

![Figure1](image)

Figure1. SPG electrodes: from right to left the lay-out of the sequentially printed layers; a) Ag/Pd counter electrode, conductive paths and pads, b) Ag/AgCl reference electrode, c) carbon based working electrode, d) insulator.
Preparation of Enzyme Electrodes. Different techniques were used for the covalent immobilisation of enzyme: binding of the enzyme were performed on (a) EDC activated, (b) CDI activated carbon surfaces and also, (c) Lysine coupled surfaces by using EDC activation, respectively. (a) Electrode surfaces were treated with EDC [1-Ethyl-3-3(dime thylaminopropyl) carbodiimide] 0.1 mol/L in NaH2PO4 (pH 4.75, 0.1 mol/L) solution for 45 min, washed with distilled water and 10 mmol/L PBS (phosphate buffer saline, pH 7.5). Then, 10 ml of enzyme solution (1 mg of enzyme in 10 ml phosphate buffer, 50 mmol/L, pH 7.5) was spread on the surface and allowed to stand at 4 oC overnight [6]. (b) The procedure was similar with the previous one except CDI (N,N’-carbonyldimidazole) was used instead of EDC for the activation of surface [7]. (c) Before printing of the oxidized carbon powder, EDC activation was performed as described before and then, instead of enzyme, carbon was treated with 0.1 M of lysine solution. After printing of the lysine coupled powder, the procedure (a) was performed.

3 Results and Discussion

The use of commercially available or home made inks for electrode printing and the wide range of possibilities with respect to the substrate material allow the development of low cost/high performances transducers which can be largely applied for biosensing in any field of analytical determinations [8].

The oxidizing treatment does modify the surface chemistry of the electrode, by creating surface-oxygen complexes that make it more acidic. Scanning electron microscopy shows in good evidence that the porous structure of the carbonaceous support was not affected (Fig 2) by the treatment. Furthermore, the pH value of the carbon slurry was found to be changed from 6.5 to 4.4 after oxidizing step because of the presence of acidic groups on the surface. Our results are well in agreement with [5]. On the other hand, as well as the covalent immobilization by using the introduced acidic groups, negatively charged functional groups on the electrode surfaces could provide useful matrices for ionic binding of proteins which could strongly interact in aqueous solution. In this study, different oxidised carbon matrices (graphite-rhodium, carbon, carbon-dextran and lysine coupled carbon) were prepared to be printed as a working electrode. Afterwards, covalent immobilization of glucose oxidase was performed. Both features of sensors towards to glucose and hydrogen peroxide are shown in Table 1.

As it can be seen in the last column of Table 1, in some cases, covalent immobilization of the enzyme directly on the electrode surfaces caused higher biosensor responses in comparison to hydrogen peroxide. This behaviour could be due to diffusion effects through the enzyme layer toward the electrode surface. This behaviour was never observed with biosensors using membranes for enzyme
immobilization because diffusion of both glucose and hydrogen peroxide is greatly controlled by the membrane properties, especially the thickness.

**Table 1.** Features of GOX immobilized on SPG with standard solutions of glucose and hydrogen peroxide.

<table>
<thead>
<tr>
<th>Linarity Range (mmol/L)</th>
<th>H$_2$O$_2$</th>
<th>Glc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sensitivity (nA L/mmol) (a)</td>
<td>C.V. (%)</td>
</tr>
<tr>
<td>1 0.25-1.50</td>
<td>25.6±1.0</td>
<td>3.90</td>
</tr>
<tr>
<td>2 0.25-2.50</td>
<td>28.5±0.6</td>
<td>2.10</td>
</tr>
<tr>
<td>3 0.10-1.50</td>
<td>69.8±3.9</td>
<td>5.59</td>
</tr>
<tr>
<td>4 0.25-2.50</td>
<td>25.4±1.3</td>
<td>5.11</td>
</tr>
<tr>
<td>5 0.25-2.50</td>
<td>24.7±1.3</td>
<td>5.26</td>
</tr>
<tr>
<td>6 0.10-1.50</td>
<td>89.8±1.0</td>
<td>1.09</td>
</tr>
</tbody>
</table>

$c=(b/a)*100$

The observed behaviour could be explained with a better diffusion of the hydrogen peroxide produced by the enzyme catalysed reaction from the active site of the enzyme toward the very close electrode surface. It was observed only with EDC, not with CDI, probably because the enzyme is bound on the electrode surface in a
different site. With the dextran-EDC matrix the effect was not observed probably
because the dextran acts as a barrier and limits diffusion of reagents and products
in the same way of a membrane.
Moreover, storage stabilities at 4 °C were investigated. GOXs both, immobilized
on carbon-dextran mixture by EDC and CDI activations completely lost their
activities after 30 days. However, activity of immobilized GOX on graphite-
rhodium by EDC and CDI activations were found to be 92.7% and 67.3%,
respectively 60 days later. GOXs immobilized on EDC activated carbon and lysine
coupled carbon were found to have 80% and 30% of their activities after 60 days.
Activation and derivatization of carbon powders to be used in screen printed
electrode preparation were performed for obtaining easier enzyme covalent
immobilization. The oxidizing treatment of carbon powder with an aqueous H2O2
solution introduced mainly carboxylic and phenolic groups on the carbon surface
which were used for further covalent immobilization of biomolecules. Glucose
oxidase was used as a model enzyme to test the usage of the different covalent
immobilization methods on the treated carbon surfaces obtaining very good
sensitivity and storage stability.

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A NEW LAYOUT FOR SCREEN-PRINTED ELECTRODES: 
FRONT/BACK GEOMETRY

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A front to back layout for screen printed electrodes has been developed to obtain µ-electrodes that can eventually be housed in hypodermic needles for in vivo measurements. Laboratory scaling and testing used such electrodes, housed in PVC disposable micropipettes tips. Discussion about lay-out, chemical and physical treatments, reproducibility of the devices as chemical sensors or biosensors by using a model enzyme as glucose oxidase is reported.

1 Introduction

Recently, screen printed electrodes (SPE) were extensively used for developing new, low cost, disposable amperometric biosensors. Several lay-outs concerning the arrangement of working, reference and auxiliary electrodes have been proposed. As a rule the working electrode, the reference electrode and the auxiliary, when needed, are printed side by side or concentrically -according to the layout used- on the same side of the substrate, being it either a ceramic or plastic one. In this paper, the global dimensions of the electrodes were decreased by applying a new front to back lay-out: the working and the reference electrodes were, in fact, printed on the opposite sides of a PVC substrate. Quasi-capillary electrodes were obtained, which could eventually be inserted in an hypodermic needle. These electrodes can be used in a two electrode configuration but an external auxiliary electrode can be easily added to the array if needed. Most of the measurements presented in this paper were obtained with a three-electrode system in which the flow cell steel outlet pipe was electrically connected to the system and acted as the auxiliary electrode.

2 Methods

Electrode Printing: Electrodes were printed on 6x6cm PVC sheets (0.3 or 0.5 mm thick) using a manual screen printer mod.HT10, Fleischle. The conductive layer of both the reference and working electrode was printed with not sinterizable Ag/Pd ink (5025 Du Pont), the AgCl reference was either printed Ag/AgCl ink (Du Pont ) or electrochemically deposed (NaCl 0.1 mol/L solution, 0.7 V vs Pt for ~ 30 minutes), and the working electrode was printed using Acheson graphite ink
drugged with a 10% w/w Pt powder supported on activated carbon (Pt 3%, Aldrich 23.755-8). All ink layers after printing were left in oven at 60°C for twenty minutes for complete drying and solvent removal before the next layer was printed. 

**Electrode assemblage:** Single electrodes were obtained from the printed PVC sheets by cutting them with a paper cutter (figure 1) and then inserting them into a micropipette disposable tip. A small quantity of epoxy resin (Araldite) had the double aim of fixing the electrodes inside the tip and acting as an insulator. Electrode width was between 0.5 and 1 mm. An electrode section as similar as possible to a square shape allows for the best fitting of the electrode inside the circular opening of a micropipette tip and avoids the risk of the resin draining onto the electrode. Thinner electrodes give bad electrical contacts because they tend to twist and wring, larger ones, on the other hand, are difficult to insert inside the micropipette tips because their sections are too different. Finished electrodes were tested both in batch and flow cells, measuring direct concentrations of hydrogen peroxide and using a reference enzyme (glucose oxidase).

**Enzyme immobilization:** Glucose oxidase was immobilized in different ways: 

a) with epoxy resin (resin and hardener 1:2) mixing equal weights of Araldite, GOD and solvent (either water or acetone were used), b) with BSA/Glutaraldehyde after treating the electrode’s surface with 10% APTES (3-amino-propyletoxyesane) in PBS. GOD was used in solution in batch measurements.

### 3 Results and Discussion

Several parameters were considered in this study. Reproducibility was one of them as a future use as disposable electrodes was to be achieved.

**Lay-outs:** Measurements reproducibility was directly related to the exact definition of the active electrode area. The hand-made manufacturing of electrodes showed immediately its limits in the “human factor” intervening in the cutting phase. A micrometric cutting device would offer a precisely defined electrode area but SEM examination of electrodes showed a further limit in the technique. When passing through the PVC sheet, while cutting, the blade takes along its edges small quantities and fragments of conductive pastes which can cause dangerous shortcuts between the two faces of the electrodes. A delicate manual scratching of the lateral PVC substrate is needed to take away all residues of paste. Moreover the cut allows the silver printed layer (figure 2) to appear from underneath the graphite layer and to enter in contact with the solution. In this way there will be an additional faradic current flowing through the conductive paste and not only through the working electrode surface. In figure 2 the peak due to this effect, can be seen around -300mV. The two voltammetry graphs are due respectively to low oxygen content solution (treatment under vacuum) and oxygen saturated solution (air bubbling).
Two different lay-outs were adopted for solving the problem. The first one was to print a shorter conductive silver layer underneath the graphite one so that, the part of the electrode in contact with the solution, will present only the graphite layer printed directly on the PVC. This new printing lay-out obviously decreases the global conductivity of the electrode. The second one was to design a new fine-toothed comb like lay-out which allowed the manufacture of single electrodes with no need of cutting the printed layers. The dimensions of the electrodes slightly increased. An insulating ink layer was printed to precisely limit the active surface of the working electrode. Two different insulating mask shapes were tried: a circular one (diameter 0.8 mm) and a rectangular window (0.6 mm wide). Both solutions increased reproducibility of electrodes.

![Figure 1: Different layers were printed on both sides of a PVC sheet (see the top of the picture); then electrodes were cut (at the right bottom) and mounted in a plastic housing (left bottom)](image1)

![Figure 2: The front/back lay-out at the SEM. Shortcuts can be observed as well as the silver path underneath the graphite layer. On the top left the resulting C.V. showing a peak of reduced silver.](image2)

**Pressing Treatment:** Pressing the electrodes resulted in a significant increase of conductivity and in an increased homogeneity of the electrodes’ surface as can be seen in the images obtained with SEM (Figure 3a and 3b).

![Figure 3: Surface of SPE at the SEM before (a) and after (b) the pressing step.](image3)
Pressure ranging from 10 to 300 kg/cm$^2$ were applied on single graphite layer prints and resistances of the printed layers, before and after the pressing treatment, were measured. Normalized data respect to the initial value are shown in Table 1. Pressing the printed sheets has the effect of increasing and stabilizing conductivity of inks layers.

<table>
<thead>
<tr>
<th>Pressure (Kg/cm$^2$)</th>
<th>Resistance decrease (Ri-R0)/R0 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>5.96</td>
</tr>
<tr>
<td>60</td>
<td>8.79</td>
</tr>
<tr>
<td>80</td>
<td>8.45</td>
</tr>
<tr>
<td>100</td>
<td>10.31</td>
</tr>
<tr>
<td>120</td>
<td>8.76</td>
</tr>
<tr>
<td>150</td>
<td>12.62</td>
</tr>
<tr>
<td>200</td>
<td>16.11</td>
</tr>
</tbody>
</table>

$y = (57\pm9) \times 10^{-5}x + 4\pm1 \ R^2 = 0.8908$

$y = (50\pm3) \times 10^{-5} x + 1.9\pm0.4 \ R^2 = 0.9766$

The limiting factor was the substrate mechanical resistance to strain. For pressures higher than 120-150 Kg/cm$^2$ in fact PVC slides got irreversibly warped. This pressure value was taken as optimal and used in consequence.

**Number of graphite layers:** Table 1 shows also the effect of the number of printed graphite layers on resistance. The regression lines have similar slopes, the only difference being the lower starting resistance value for a double passage of the squeegee. The greatest difference in resistance can be observed between the first and second passage (data not shown for number of layers>2). Increasing number of squeegee passages just flattened the effect and caused a global increase of the resistance. Electrodes were therefore always printed with a double squeegee passage in order to maximize both reproducibility and conductivity.

**Electrochemical treatments:** Electrodes were also electrochemically treated at constant and variable potential vs a Pt electrode in a buffered phosphate solution and in acid (HNO$_3$, 0.1 mol/L) or alkaline (NaOH 0.1 mol/L) solutions. The best results were obtained by pre-treating electrodes in cyclic voltammetry (-1V/1V) for 40 minutes in NaOH as can be observed in the comparison of chronoamperometry (+700mV vs Ag/AgCl) signals of two electrodes (figure 4): the upper signal belongs to a not chemically treated electrode. The signal constantly decreases, even after 40 minutes, and the noise is high. The lower signal belongs to an electrode treated in NaOH. It soon reaches a plateau, and noise and drift are significantly lower. The electrochemical treatment was performed before cutting the electrodes, so to treat at a time all the electrodes of a single printed slide (70-80 pieces)
Calibration curves for hydrogen peroxide and glucose: Bare electrodes were used for calibration with hydrogen peroxide in both flow and batch measurements. The results about reproducibility and repeatability with respect to \( \text{H}_2\text{O}_2 \) are given in table 2. In table 2 are also reported calibration curves obtained with glucose biosensors by immobilising glucose oxidase with an epoxy resin or with BSA/Glutaraldehyde.

![Stabilization curves for treated and not treated bare electrodes.](image)

Figure 4: Stabilization curves for treated and not treated bare electrodes.

4 Conclusion

A new front to back layout for SPE has been proposed allowing the preparation of needle electrodes. Laboratory scaling and testing, chemical and physical treatments, reproducibility of these devices as chemical sensors or biosensors has been reported.

5 Acknowledgements

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References

Immobilisation of engineered molecules on electrodes and optical surfaces

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Abstract

Monolayers of genetically modified proteins with an hexahistidine tag, (His)\textsubscript{6}, were obtained by using a Ni–NTA chelator synthesized on gold-sputtered surfaces (via sulphide bonds), or on gold and graphite (via sililating agents) working electrodes of screen-printed devices. Two kinds of proteins were produced and purified for this study:

(a) a recombinant antibody, derived from the ‘single-chain Fv’ (scFv) format, and
(b) a photosystem II (PSII) core complex isolated from the mutant strain CP43-H of the thermophilic cyanobacterium \textit{Synechococcus elongatus}.

An scFv previously isolated from a synthetic ‘phage display’ library was further engineered with an alkaline phosphatase activity genetically added between the carboxy-terminal of the scFvs and the (His)\textsubscript{6} to allow direct measurement of immobilisation.

Renewable specific binding of (His)\textsubscript{6} proteins to gold and graphite surfaces and fast and sensitive electrochemical or optical detection of analytes were obtained. Additionally, “on chip” protein preconcentration was conveniently achieved for biosensing purposes, starting from crude unpurified extracts and avoiding protein purification steps.

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Keywords: Biosensors; Gold; Self-assembled monolayers; Recombinant antibodies; Histidine tag; Photosystem II

1. Introduction

Biomolecules modified by genetic engineering represent a new and powerful approach for obtaining simpler artificial structures with new or improved properties (i.e. specificity, stability, sensitivity) useful for biosensors development. For instance, insertion of specific molecular tags can be used for oriented and reversible immobilization, preconcentration and purification, whereas, easier and sensitive analytical detection can be achieved by gene fusion with selected enzyme activities. Moreover, antibody-derived fragments, like the single-chain Fv antibody fragment ‘scFv’ \cite{1,3}, offer the necessary sensitivity required for a sensing element, and new desired specificities can be isolated from ‘phage display’ antibody libraries through the ‘panning’ procedure \cite{7,9}.

In this paper, an original procedure for synthesizing functional groups (Ni–NTA) suitable for immobilisation of engineered (His)\textsubscript{6} proteins on different sensor materials (gold, plastic, graphite, glass, quartz) are presented for gold and graphite surfaces, and evaluated in terms of specific binding and activity of two kinds of different engineered proteins: an scFv-derived antibody and a PSII core complex. An scFv already isolated from a synthetic phage display library \cite{2} and characterized for binding activity (Villani et al., unpublished) was further engineered at the C-terminus in order to add an alkaline phosphatase (AP) activity before the (His)\textsubscript{6} tag, obtaining the fusion protein scFv–AP–(His)\textsubscript{6}. The fusion protein was produced in the bacterial periplasm and used, rough or purified, for optimal (oriented) immobilization through the synthesized Ni–NTA chains on gold and graphite surfaces. No loss of functional properties (ability to bind the antigen) was observed, indicating suitability of recombinant antibodies as sensing molecular tools for bio-
sensors. The engineered PSII core complex, containing an (His)₆ tag, was also purified and immobilised by using the same Ni–NTA chelator on gold surfaces. The engineered PSII–(His)₆-derived molecule represent a further step for the development of new PSII-based sensing devices [5,6].

2. Materials and methods

2.1. Preparation of the scFv–AP–(His)₆

2.1.1. Preparation of the scFv–AP–(His)₆ expression plasmid

The scFv (CMV-G4) bacterial clone, expressing soluble scFv, was picked out from selection against a plant virus (cucumber mosaic virus, CMV) [2]. The scFv gene sequence was digested from the original pDN332 phagemid and subcloned as a Sfi–Not fragment in the pDAP2 expression vector (CODON Genetic System) for the production of a bifunctional protein, scFv–AP–(His)₆, with both antigen-binding activity, as well as AP activity and (His)₆ [4].

2.1.2. Expression of the scFv–AP–(His)₆ fusion protein

Competent TG1 Escherichia coli cells were transformed with the ligation product and cultured at 37 °C for 16 h on 2 × YT agar (1.5%) medium containing 100 µg/ml of ampicillin and 2% glucose (2 × YT-AG). Single colonies were subjected to PCR screening. After a mini-induction experiment (in order to assess the ability of the fusion protein to bind the antigen in ELISA and the presence of AP activity), a positive clone was picked and cultured overnight at 30 °C for maxi-induction. The culture was diluted to OD₆₀₀ = 0.05 in 1 l of 2YT-AG. The cells were transformed with the ligation product and cultured at 30 °C with vigorous shaking (250 rpm). At OD₆₀₀ of about 0.8–0.9, the cells were collected by centrifugation and resuspended in 1 l of 2 × YT containing 100 µg/ml ampicillin and 1 mmol/l IPTG (isopropyl-β-D-thiogalactoside) to induce protein expression. The cells were then cultured for 16 h at 30 °C. After centrifugation at 3000g for 20 min, the bacterial pellet was resuspended in ice-cold cell lysis buffer (Tris–HCl, pH 8.0, 0.5 mmol/l EDTA, 0.5 mol/l sucrose, 1 ml Tris, 100 ml initial culture) containing protease inhibitors (Roche). After adding water-diluted TES (1:5, 1.5 ml for 100 ml initial culture), the cells were incubated on ice for 30 min and then centrifuged at 4000g for 25 min. The supernatant (Periplasmic Extract fraction 1, EP1) was collected. Pellets were resuspended in 15 ml 5 mmol/l MgSO₄ containing protease inhibitors and shaken for additional 10 min before further extraction. The supernatant EP2 was collected after centrifugation at 20,000g for 20 min.

2.1.3. Purification of the scFv–AP–(His)₆ fusion protein

Fractions EP1 and EP2 were independently concentrated by ultrafiltration on a Diaflo YM10 membrane (Amicon) and subjected to immobilised-metal affinity chromatography (IMAC) using Ni–NTA (QIagen), following the procedure recommended by the manufacturer. Eluted fractions were collected, dialyzed against PBS and quantified by reading the absorbance at 280 nm. Protein purity was checked by SDS-PAGE (12% w/v) followed by Coomassie staining. Approximately 360 µg of purified protein was obtained from 1 l of bacterial culture.

2.2. Purification of PSII–(His)₆ core complex

Thermophilic cyanobacterial S. elongatus 43 H cells expressing psbC with an (His)₆ extension were used for purification of PSII–(His)₆ core complexes [8]. The eluate from a Ni²⁺-affinity column was diluted in 40 mmol/l MES/NaOH (pH 6.5), 100 mmol/l NaCl, 15 mmol/l CaCl₂, 15 mmol/l MgCl₂, 0.03% DM and 10% glycerol for reducing amount of imidazole, and then they were concentrated with a Centriprep-100 (Amicon, MA) to a chlorophyll concentration of 9.2 mg/ml (imidazole content was finally 12.5 mmol/l).

2.3. Immobilisation procedures

2.3.1. Deposition of thin gold layers on glass and plastic surfaces

In all experiments, thin transparent layers of gold were deposited by commercial sputter coaters. The best deposition procedure on glass was achieved as follows: 1 h washing of glass slides in cold ‘piranha’ solution (sulphuric acid/hydrogen peroxide 1:2), drying at 80 °C directly before the deposition. The gold layers deposited in this way were stable during the chelator synthesis and resistant even to nonpolar solutions (chloroform, 12 h). In the case of plastic support, gold was sputtered on both sides of the small circles (OD = 6 mm) cut from a laser printer transparent foil. The conditions for deposition were the same as in the case of glass slides, but they were not washed or chemically treated before deposition.

2.3.2. Synthesis of the Ni–NTA chelator and Ni²⁺ assay

Two types of chelators were synthesized for (His)₆ protein immobilisation. The one with the long-arm spacer created by inserting lysine molecule into the chain and the other without spacer. Graphite thick films deposited by screen printing on PVC sheets were treated (1 h at room temperature) with 10% 3-aminopropyltriethoxysilane (3-APTES) in PBS for obtaining –NH₂ groups exposed out the surface. Glass or plastic slides with sputtered gold were treated with 20 mmol/l cysteamine dissolved in ethanol for at least 12 h and resulted in a self-assembled monolayer (SAM) with the –NH₂ groups exposed out the surface. These functional groups were used for further synthesis of both chelators. The synthesis followed the common scheme: (a) 10% glutaraldehyde in PBS, (b) 5% l-lysine, (c) 10% glutaraldehyde in PBS, (d) 5% Na₂-p-bis (carboxymethyl)-l-lysine hydrate, (e) 1% NiSO₄. The chelator without spacer
was obtained by omitting steps (b) and (c). Each step of synthesis lasted 2 h. At the end of each step, samples were washed in PBS.

Then, Ni was eluted from the samples with 25% HNO₃ for determination by adsorptive cathodic stripping voltammetry (AdCSV) according to the DIN 38406E16 Standard Method for trace metals analysis. In a polarographic vessel, 0.5 ml of sample were added to 8 ml of water, 0.25 ml of DMG solution, 0.5 ml of ammonium chloride buffer, pH = 9.5 ± 0.2, and bubbled with nitrogen for 5 min. Ni content determination was carried out with three standard additions.

2.3.3. Immobilisation of scFv–AP–(His)₆ protein

The amount of fusion protein bound to the plastic and glass support was assayed after loading at 4 °C in the dark for 1 or 16 h. Then, the calibration curve of fusion enzyme activity was obtained and the maximum capacity of chelator groups determined. The saturating concentration of protein in the loading buffer was found to be 1 ng/µl. AP activity immobilised on the samples was determined at 405 nm using p-nitrophenylphosphate as a substrate. Electrochemical AP activity measurement was performed with screen-printed electrodes (Krejci Engineering) in a flow microcell as described in Fig. 1.

2.3.4. Immobilisation of PSII–(His)₆ core complex

The highest quantity of immobilised PSII–(His)₆ core complex on the surface of plastic and glass substrates was obtained by incubation in MES buffer solution (pH = 6.5) containing PSII–(His)₆ equivalent to 300 ng Chl/ml at 4 °C in complete darkness for 16 h. Chlorophyll fluorescence measurements were done on a spectrofluorometer (TRIAX 180, Jobin Yvon-Spex) at laboratory temperature. Fourier transform infrared (FTIR) spectra were also obtained (Perkin Elmer). In both cases, 40 scans were done for each measurement in at least four independent repetitions.

3. Results

The two procedures for chelator synthesis were compared in terms of quantity of Ni–NTA groups and in terms of specific and nonspecific binding capacity of (His)₆ proteins. Only data on plastic are reported because results were comparable in all experiments.

Ni²⁺ determination by AdCSV on the substrates revealed high amount of NTA groups with no significant differences between the chelator with or without lysine spacer (Table 1). Moreover, surface density of the NTA groups was higher compared to commercial Ni–NTA treated microtitre plates: 3-fold on graphite printed on PVC, 10-fold on sputtered gold (glass or plastic).

In order to perform direct measurements of immobilisation, an scFv fused to AP was used. Specific immobilisation of scFv–AP–(His)₆ through the (His)₆ was confirmed by the possibility of eluting the protein with 200 mmol/l imidazole and assuming the residual AP activity as a measure of nonspecific binding (Table 2). Again, no sig-
significant differences of the surface binding capacity were observed for both chelators.

After immobilisation and elution with imidazole, the scFv fusion protein retained binding activity toward the antigen as confirmed by ELISA on CMV-coated microtitre plates.

The possibility of direct preconcentration of (His)_6 proteins on sensor surface (“on chip” preconcentration) using rough bacterial extracts was evaluated. “On chip” preconcentration was obtained after 16 h of substrate treatment and better results (lower nonspecific binding) were obtained with the substrate with chelator having a lysine spacer (Table 2) which showed an higher binding capacity (about fourfold) than commercial (His)_6 proteins sorbing microtitre plates [even higher, sevenfold, if the unfavourable surface area ratio of about 0.6:1 (Table 2, third column) is taken into account]. Milk (5%), BSA (5%) and cytochrome c (5%) solutions were used with the aim of blocking nonspecific binding of proteins, but in all cases, specific binding was also reduced.

AP activity of the scFv fusion protein was electrochemically determined after binding to a printed gold electrode with the chelator without spacer. The calibration curve of enzyme activity as well as washing out the protein with 200 mmol/l imidazole were determined, by using ascorbate-2-phosphate as a substrate for AP (Fig. 1). “On chip” preconcentration, starting from the crude bacterial extracts containing the engineered scFv fragment, was also possible with screen-printed gold electrodes (data not shown).

The presence and activity of PSII–(His)_6 was tested by photoreduction of 2,6-dichlorophenolindophenol (DCPIP, 20 mmol/l) at 620 nm after illumination of the samples for 45 min with white light at the laboratory temperature. In all cases, the decrease in absorption of oxidised form of DCPIP due to the activity of PSII–(His)_6 was observed, whether the samples used were with or without spacer. After washing of the PSII–(His)_6 loaded samples with 200 mmol/l imidazole, photoreduction of DCPIP was measured once again. In this way, results already obtained with scFv–AP protein were confirmed. A significant decrease of activity (nearly to zero) was observed. Contrary to the antibody fragment, the engineered PSII–(His)_6 core complex was nearly completely washed out and nonspecific binding was significantly lower. Experiments were well reproducible and PSII–(His)_6 core complex was not denaturated during immobilisation since it did not lose oxygen evolving activity (Table 3). The amount of PSII–(His)_6 bound to the substrate without the spacer was higher compared to the substrate with the lysine spacer.

The presence of PSII–(His)_6 was also confirmed by measuring fluorescence in the region between 600 and 700 nm. The sample (PSII–(His)_6 on plastic or glass) was illuminated with blue light (400–530 nm) and fluorescence detected against the blank (plastic, glass with chelator without PSII–(His)_6). In all cases, the typical fluorescence

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Chemical treatment</th>
<th>Ni^{2+} surface density (ng/cm^2) ± S.D. (n=3)</th>
<th>C.V. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputtered gold on plastic</td>
<td>blank</td>
<td>4.3 ± 0.1</td>
<td>2.3</td>
</tr>
<tr>
<td>Sputtered gold on plastic</td>
<td>spacer</td>
<td>122 ± 3.0</td>
<td>2.6</td>
</tr>
<tr>
<td>Sputtered gold on plastic</td>
<td>no spacer</td>
<td>4.4 ± 0.2</td>
<td>4.5</td>
</tr>
<tr>
<td>Printed graphite ink on PVC</td>
<td>no spacer</td>
<td>114 ± 2.0</td>
<td>1.7</td>
</tr>
<tr>
<td>Commercial microtitre plates</td>
<td>–</td>
<td>10</td>
<td>–</td>
</tr>
</tbody>
</table>

DP-voltammogram at the HMDE. Deposition potential = −0.8 V; deposition time = 90 s, start potential = −0.80 V; final potential = −0.45 V; DP amplitude = −50 mV. The Ni peak potential is at −0.97 V vs. Ag/AgCl.

**Table 1**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Treatment</th>
<th>PSII–(His)_6 core complex activity measured by photoreduction of DCPIP before and after washing with imidazole</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate</td>
<td>Treatment</td>
<td>OD_{450 ± S.D.} (n=3)</td>
</tr>
<tr>
<td>Sputtered gold on plastic</td>
<td>PSII–(His)_6 core</td>
<td>0.32 ± 0.013</td>
</tr>
<tr>
<td>Sputtered gold on plastic</td>
<td>complex loading</td>
<td>0.15 ± 0.007</td>
</tr>
<tr>
<td>Sputtered gold on plastic</td>
<td>after washing with imidazole</td>
<td>0.006 ± 0.007</td>
</tr>
<tr>
<td>Sputtered gold on plastic</td>
<td>PSII–(His)_6 core</td>
<td>0.15 ± 0.007</td>
</tr>
<tr>
<td>Sputtered gold on plastic</td>
<td>complex loading</td>
<td>0.007 ± 0.006</td>
</tr>
</tbody>
</table>

*a Absolute value of OD_{450} decreases with respect to the blank sample.

**Table 2**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Treatments</th>
<th>Surface area (cm^2)</th>
<th>OD_{450 ± S.D.} (n=3)</th>
<th>C.V. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputtered gold on plastic with spacer</td>
<td>scFv loading with purified protein</td>
<td>0.6</td>
<td>0.563 ± 0.030</td>
<td>5.3</td>
</tr>
<tr>
<td></td>
<td>washing with 200 mmol/l imidazole</td>
<td>0.6</td>
<td>0.153 ± 0.010</td>
<td>6.5</td>
</tr>
<tr>
<td>Sputtered gold on plastic without spacer</td>
<td>scFv loading with purified protein</td>
<td>0.6</td>
<td>0.679 ± 0.010</td>
<td>6.8</td>
</tr>
<tr>
<td></td>
<td>washing with 200 mmol/l imidazole</td>
<td>0.6</td>
<td>0.230 ± 0.030</td>
<td>13.0</td>
</tr>
<tr>
<td>Sputtered gold on plastic with spacer</td>
<td>scFv loading with bacterial extracts</td>
<td>0.6</td>
<td>0.061 ± 0.012</td>
<td>19.7</td>
</tr>
<tr>
<td>Sputtered gold on plastic without spacer</td>
<td>(“on chip” preconcentration)</td>
<td>0.6</td>
<td>0.004 ± 0.002</td>
<td>50.0</td>
</tr>
<tr>
<td>Commercial Ni–NTA-coated microtitre plates</td>
<td>–</td>
<td>1.0</td>
<td>0.014 ± 0.003</td>
<td>21.4</td>
</tr>
</tbody>
</table>
curve of the PSII–(His)$_6$ core complex was observed (Fig. 2) with a maximum at about 685 nm and a longer-wavelength (725 nm), broad shoulder (the shape of curve was compared to the one obtained with the PSII–(His)$_6$ core complex in solution). A decrease of fluorescence was observed after washing the sample with the imidazole solution. Good reproducibility and high signal reached in this experiment confirmed the applicability of this procedure for immobilisation of PSII and preparation of optical biosensing devices (for example, using fiber optics).

PSII–(His)$_6$ immobilisation was observed with FTIR absorption in case of glass samples in reflectance mode. Sample was let to dry before measurement at laboratory temperature and difference spectra were recorded before and after PSII–(His)$_6$ loading. Typical absorption bands (amid I and amid II bands) in the 1000–2000 cm$^{-1}$ regions were obtained.

In addition, amperometric detection of PSII–(His)$_6$ activity with a screen-printed gold electrode polarised at +600 mV vs. Ag/AgCl and in presence of duroquinone as artificial electron acceptor was achieved (data not shown).

4. Conclusions

Higher Ni–NTA loading was obtained on both graphite and gold surfaces compared to commercial products. Similar results with both (His)$_6$ proteins are good proofs of possible universal use of this procedure for sensing purposes; it can be applied to several technological materials by changing the first step of chemical treatment. Imidazole can be specifically used for washing out the sensing molecule, for renewing it or replacing the biomediator depending on the analysis requirements. Slight difference in nonspecific binding and in length of the optimal chelator arm can be expected using different (His)$_6$ proteins. Finally, “on chip” protein preconcentration was conveniently achieved for biosensing purposes, starting from crude unpurified extracts and avoiding time-consuming and expensive protein purification steps.

Acknowledgements

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References

REVERSIBLE IMMOBILIZATION OF ENGINEERED MOLECULES
BY Ni-NTA CHELATORS

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Abbreviations:

AdCSV: Adsorptive Cathodic Stripping Voltammetry; AP: Alkaline Phosphatase; atrazine: 2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine; BSA: bovine serum albumin; CYS: cysteamine; CV: Cyclic Voltammetry; DM: Dodecylmaltoside; DQ: tetramethyl-p-benzoquinone; EDM: Electrochemically Deposited Multilayers; GA: Glutaraldehyde; His: histidine; LED: light-emitting diode; LOD: limit of detection; MES: 2-(N-morpholino)ethanesulfonic acid; MESB: 40mM MES, 100mM NaCl, 15mM CaCl$_2$, 15mM MgCl$_2$, 5×10$^{-7}$M chloramphenicol, 0.03% DM, pH=6.5; Ni-NTA: nickel-nitrilotriacetic acid chelator; PB: phosphate buffer 0.1M (pH=7.0); PSII: photosystem II; OCT: octadecanethiol; RE: reference electrode (Ag/AgCl); SAM: self-assembled monolayer; WE: working electrode;

Summary

Electrochemical synthesis of Ni-NTA chelators, for subsequent immobilization of (His)$_6$-tagged proteins (PSII as model molecule), on Au or Au-graphite electrodes is compared to chemical synthesis. Results show: i) higher Ni-NTA surface density, ii) shorter treatment time (1-12 min vs 16h normally needed for SAM), iii) possibility of addressing the chelator to only one Au electrode, in a sensor µ-array.

Keywords: biosensors, photosystemII, engineered proteins, (His)$_6$-tag, reversible immobilization, self assembled monolayers, conducting molecular wires

Introduction

Genetically modified molecules represent a powerful approach to artificial structures with improved properties for biosensor development. An original procedure, suitable for chemical immobilization of engineered (His)$_6$-tagged proteins on Au or graphite surfaces, was previously reported$^2$ with the result of obtaining oriented and highly specific immobilization of two engineered proteins$^1$. Renewable specific binding of (His)$_6$-proteins to sensor surfaces and fast and sensitive electrochemical or optical detection of analytes were also obtained$^3$. “On chip” protein pre-concentration was conveniently achieved for biosensing purposes, starting from crude unpurified extracts and avoiding protein purification steps$^2$. In this paper, selective electrochemical synthesis of CYS layers on Au, Pt and graphite was studied and compared, using a model molecule (PSII) with the aim of biosensors improvement

Experimental

a) Preparation of Au and Au(graphite) electrodes: Au thin films were obtained by chemical deposition on Cu paths. Cu electrodes were obtained from a commercial Cu sheet deposited on fiberglass. Cu surface was lapped with SiC sheet (4000 mesh) and diamond pastes (particle size=6 µm, then 3µm). An impermeable dye layer, screen-printed on the copper surface, was used as a mask and FeCl$_3$ dissolved the exposed Cu, leaving the desired electrode geometries under the dye (later easily removed with acetone). Chemical Au plating on Cu was obtained with Au(CN)$_2$ and Au(CN)$_3$$^-$. An Au wire was dissolved in boiling HCl/HNO$_3$ (3:1) and then dried at 70-80°C; HCl 37% was added and the solution dried again; finally, HCl 0.1M was added to have AuCl$_3$ (0.6 g/l) in solution which was mixed with KCN (10.0 g/l), Na$_2$HPO$_4$ (6.0
g/l), NaOH (1.0 g/l), Na$_2$SO$_3$ (3.0 g/l). Gold plating was obtained by sinking the samples in the plating bath for 1h at 70°C; a thin Au layer (20nm) was deposited on Cu because of the shift reaction:

$$2\text{Au(CN)}_2^{2-} + \text{Cu(s)} \rightarrow \text{Cu(CN)}_4^{3-} + 2\text{Au(s)}$$

Two series of such Au electrodes were obtained and tested (Metrohm 641, Herisau, Switzerland) in a flow-cell. Series #1, with ascorbic acid at +600 mV vs. RE, gave a sensitivity of 32.3 ± 1.2 mA/M (RSD=3.2%, n=3); series #2 showed a sensitivity of 23.3 ± 2.2 mA/M (RSD=9.8%, n=5). Real surface ($A_r$) of Au electrodes was chronocoulometrically obtained with 0.1M KFe(CN)$_6$ (FeCy) in 0.1 M KCl; The two potential steps applied were, +600mV and 0mV vs RE with 250ms pulse width. The current signal due to FeCy reduction was plotted as Q vs ($t^{1/2}$) and the slope of the line gave the real surface area of the Au electrode ($A_r$). A roughness factor of 1.13, calculated as real/geometric area ratio, was used to calculate Ni(II), CYS, and PSII surface densities reported below.

A different electrode lay-out was screen printed by overlapping deposition of Ag (conducting paths) Ag/AgCl (RE) and graphite (WE) inks from Acheson. Graphite ink was mixed with Au(graphite) particles (10%w/w) and deposited by screen printing on a PVC sheet.

c) Deposition of CYS layers and synthesis of Ni-NTA chelator: Chemical synthesis of CYS-SAM on Au was obtained in CYS 20 mM in PB for 16h$^2$. Deposition potentials of CYS on Au (0.85V) and graphite (1.2V) were obtained by CV, 0V-1.4V vs Pt in CYS 20 mM in PB (scan rate=50mV/s, step potential=10mV); The synthesis of the Ni-NTA chelator$^2$ followed the common procedure: i)glutaraldehyde 12.5% v/V in PB for 1 h, ii)N$_{\alpha}$-N$_{\alpha}$-bis(carboxymethyl)-L-Lysine Hydrate (NTA) 5% w/V in PB for 1h, iii)NiSO$_4$ 1% w/V in distilled water for 15min. Surface density of NTA chelator on the electrodes was electrochemically (AdCSV) determined as the Ni$^{2+}$ content as previously described$^2$ with blank correction (fiberglass resin only).

c) Purification and immobilization of PSII: Thermophilic cyanobacterial Synechococcus elongatus 43H cells expressing psbC with an (His)$_6$ extension were used for purification of (His)$_6$-PSII core complexes$^1$. The purified (His)$_6$-PSII core complex was immobilized on the surface of Au-CYS-NTA modified electrodes. Immobilization of PSII was obtained by incubation of electrodes in MESB containing PSII equivalent of 300 µg Chl mL$^{-1}$ at 4 °C in complete darkness for 20min. Prior to measurements, electrodes with immobilized PSII were thoroughly washed with MESB. The exact amount of (His)$_6$-PSII core complex immobilized on the electrode was determined as decrease of chlorophyll concentration$^3$ in the solution before and after immobilization (four electrodes used in one batch in order to increase the precision of the measurement) and corrected to the blank sample (clean Au electrode). All experiments were repeated four times and average number was used for subsequent calculations.

d) Amperometric measurement of PSII activity: Amperometric measurement of PSII activity on the electrodes was done in a home-made flow-cell, continuously fed with MESB (flow rate 0.25mL/min, peristaltic pump Gilson MiniPulse 3). Buffer was bubbled for 15min with N$_2$ prior to starting and all through measurements. The current intensity on the WE was registered with a potentiostat and processed by AD converter and software (Oxycorder, PSI Instruments, Czech republic). Illumination was controlled by a custom-made electronic timer, the duration was set to 1 or 5 s and light intensity about 100 µmol photons m$^{-2}$s$^{-1}$. Red and blue High Intensity LEDs were used.

Results and Discussion

Electrochemical deposition of CYS on Au or Au(graphite) electrodes: CV of 10mM CYS showed that oxidation peaks on Au and graphite electrodes decreased quickly. After few (5-10) scans the curve assumed a flat shape (Fig.1). This means CYS is rapidly oxidized till saturation of the electrode surface. The electrochemical deposition of CYS layers on Au, thus obtained potentiostatically in 20min, showed a rapid increase of current signal at the beginning of deposition (Fig2A); the shape of the 1$^{st}$ and 2$^{nd}$ derivatives (Fig.2B) shows that current intensity quickly increases till $t=3.3s$ ($f'=max$ and $f''=0$), then increases slowly till $t=6.7s$ ($f'=0$ and $f''<0$); finally current intensity decreases and steady state is reached after 30s ($f'$ and $f''=0$,
I=constant). The integral of current intensity (Q vs. t reported in fig.2C) was used to calculate surface density of CYS on Au WE, taking into account that, after Au surface was completely saturated, electrochemical reaction proceeded giving dimerisation of CYS. Ni^{2+} (513 pmol/mm^2) and NTA chelator surface density, obtained with electrochemical deposition of CYS layers in 20min resulted about 15 fold greater than that one obtained on the same material with a chemical treatment (Ni^{2+} surface density=39 pmol/mm^2 after 16h) and 30 fold greater than that one obtained with a chemical treatment of only 20min. Preliminary deposition of an His-tagged alkaline phosphatase on a µ-array of gold electrodes showed that electrochemical deposition only occurs on the chosen electrode (data not shown).

Screen printed Au-graphite or Pt-graphite particles held the same behaviour of pure Au, allowing us to deposit CYS only on metal particles dispersed in the carbon inks at 0.85V vs RE, as CYS deposition on graphite electrodes occurs at higher potential (1.2V vs RE) (fig.3).

Surface density of CYS, Ni(II) and (His)_6-PSII: As stated above, electrochemical deposition (20min) of CYS on Au, completely saturates electrode surface and, because Ni^{2+} combines with CYS-NTA with a stoichiometric ratio of 1:1, maximum CYS surface density results to be equal to the maximum Ni(II) surface density obtained after a 20min electrochemical deposition (tab.1, row E=513 pmol/mm^2, experimentally determined with AdCSV). Rows A,B,C in Tab.1 were obtained assuming moles of CYS=Q(t)/nF with (F=96484C and n=1) at t=20,300,1200s. The time (in row D) really needed for reaching the maximum of CYS surface density by electrochemical treatment, was interpolated from data in rows A,B,C and resulted to be t_{max}=711s (~12min). Our experimental data are confirmed by comparing row E (experimental data) with i)calculation by dimension of the ionic radius of Ni(II) (row F), ii)calculation by dimension of the octaedrical Ni(II) complex (row G), iii) their relative magnitude and order in the series (G>E>F). Row H was calculated by considering the dimension of the PSII core crystal and row I was spectrophotometrically obtained considering the chlorophyl content of immobilized(His)_6-PSII. Again, experimental data from row I showed the same magnitude order of row H. Again, Ni(II) combines with (His)_6-PSII with a stoichiometric ratio of 1:1 so, with an electrochemical treatment of t_{max}=12min (or higher) a ratio Ni:(His)_6-PSII~39500 was experimentally determined. This means that a single (His)_6-PSII covers ~39500 Ni(II) heads but binds only one of them through the His-tag. So, certain amount of this chelator could be substituted with other functional groups or molecules, still obtaining the same surface density of immobilized (His)_6-PSII. In our opinion, mixed layers consisting of different thiol molecules immobilized onto the surface, will improve performance of biosensors (i.e. conductive molecular wires for direct electron transfer to the electrodes or hydrophobic chains of OCT to improve diffusion of hydrophobic mediators) as reported in the following section.

Comparison of the PSII monolayer versus the crosslinked PSII in a BSA-GA matrix: The higher Ni^{2+} surface density obtained with electrochemical deposition of CYS on the electrode surface was not responsible of higher peaks respect to the chemically deposed ones as reported in Figure 4, where the reoxidation rate of the reduced form of the electron acceptor is shown for CYS-NTA-PSII (chemically and electrochemically deposed) and crosslinked PSII (e.g using the BSA-GA matrix). On the contrary, the lower peak could be explained with the redundant number of Ni^{2+} heads which does not reflect in a significant increase of PSII molecules on the electrode surface. In addition, electrochemically deposed CYS layers should be very different in structure from chemically assembled ones because of fast rate of deposition which does not allow for preliminary self assembling. In our opinion, electrochemically deposed CYS layers should be described as disordered, compact and rigid multilayers (electrochemically deposed multilayers, EDM, in contrast with self assembled monolayers, SAM) which significantly affect diffusion toward the electrode. The height of the peaks depends on the different quantity of PSII immobilized on the gold electrode, while reoxidation rate is strictly related to the ability of the mediator to diffuse through the immobilized layers. In the first case, a great amount of PSII is entrapped and cross-linked in a dense diffusion layer of BSA-GA, in the second ones, it is free in solution and linked to the electrode surface through the his-tag and the NiNTA chain.

In the case of PSII monolayer immobilized on a CYS-EDM, a rapid, nearly immediate inhibition of PSII electrode was observed directly after the addition of herbicide because of the narrow diffusion layer for reduced electron acceptor (DQ) and exposition of the active PSII out
to the buffer solution and the inhibitor. On the contrary, for BSA-GA-PSII gel matrix a stable signal of inhibited electrode is obtained after 15 min of herbicide exposition. $I_{50}$ value of 3 different type of electrodes was then compared for atrazine. Au-CYS-NTA-PSII electrode showed a slight change ($I_{50}=2\times10^{-8}M$) compared to the electrode with BSA-GA-PSII gel matrix ($I_{50}=9\times10^{-8}M$). A striking difference ($I_{50}=5\times10^{-10}M$), compared to the previous two, has been observed in the third electrode type consisting of a mixed layers (CYS+OCT) with increased hydrophobic properties. The strategy depicted at the end of the previous section, regarding the possibility to deposit mixed layers together with Ni-NTA chelators will be further investigated to obtain increased performances of this biosensor.

Conclusions

Electrochemical formation of CYS layers on Au electrode surface occurred at 0.85V vs RE and resulted in a shorter treatment time (12 min vs 16 h of chemical treatment) and in higher Ni$^{2+}$ surface density. Electrochemical deposition of CYS on Au-graphite composite electrodes occurred at 0.85V vs RE, allowing deposition of CYS only on the metal particles, because of the higher potential (1.2V) needed on graphite electrodes. Additionally, preliminary experiments with HIS-tagged AP showed the possibility to electrochemically address the synthesis of the chelator, to only one Au electrode in a sensor $\mu$-array. The difference in shape and in height of the signal, due to PSII activity in chemically and electrochemically deposed CYS layers, suggested also a difference in the structure of these layers. For this reason we suppose that our CYS film have not to be considered as a SAM but as an electrochemically deposed multilayer (EDM). The redundant number of Ni$^{2+}$ heads available for (His)$_6$-tagged proteins was then decreased and mixed layers were deposed onto the electrode surface, obtaining better performances of the biosensor in terms of substrate diffusion.

Acknowledgments

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References

Table 1: Ni²⁺ and CYS and PSII surface densities. Experimental values are compared with those obtained with theoretical calculation.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Time (s)*</th>
<th>Surface density (pmol/mm²)</th>
<th>C.V. (%)</th>
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<tr>
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<td></td>
</tr>
<tr>
<td>I</td>
<td>PSII</td>
<td>300</td>
<td>0.013</td>
<td>10.2</td>
</tr>
</tbody>
</table>

* treatment time for electrochemical deposition of CYS-SAM on Au

![Cyclic voltammetry for CYS deposition on gold (the 1st and the 10th scans)](image)

Figure 1: Cyclic voltammetry for CYS deposition on gold (the 1st and the 10th scans).

0V-1.4V vs Pt in CYS 20 mM in PB (scan rate=50mV/s, step potential=10mV); maximum of the peak is centered at 850mV vs Pt
Figure 2: Current intensity, 1st and 2nd derivatives and integral of chronoamperometric deposition of CYS on gold electrodes. A) $I(A)$ Peak vs $t(s)$ due to the CYS addition into PB to the final concentration of 20mM, B) $dI(A)/dt(s)$ (continuous line) and $d(dI(A)/dt(s))/dt(s)$ (dotted line), C) $\int I(A)dt(s)$
Figure 3: Cyclic voltammetry of 20mM CYS in PB with screen printed electrodes. Continuous line: Au-graphite WE; Dashed line: Carbon-graphite WE; Dotted line: Pt-Graphite WE.

Figure 4: Current response of the PSII on Au WE. After illumination (5s) current increases due to the reoxidation of the artificial electron acceptor (duroquinone). Data obtained with three different immobilization methods on Au screen-printed WE.
Escherichia coli detection in vegetable food by a potentiometric biosensor

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Abstract

The present work describes the application of an antibody based biosensor for the determination of Escherichia coli cells in vegetable food. The presence of E. coli as a bioindicator of bacterial contamination—faecal one in particular—was detected using the potentiometric alternating biosensing (PAB) system based on a light addressable potentiometric sensor (LAPS) transducing element, detecting pH variations due to NH₃ production by an urease–E. coli antibody conjugate.

Commercial samples of vegetable—lettuce, sliced carrots, and rucola—were washed with peptone water at pH 6.8, blended either in a stomacher or in a sonicator, to detach bacterial cells and to recover them in the liquid medium. This liquid phase was analyzed both by PAB system and conventional colony forming units (CFUs) methods. The proposed PAB system appears to be very sensitive and fast, in comparison with conventional methods: concentration of 10 cells/ml were detected in an assay time of ca. 1.5 h, showing detection time from 10 to 20 times shorter than the conventional CFU procedure.

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Keywords: Escherichia coli; Potentiometric sensor; Immunoassay biosensor; Vegetable food

1. Introduction

Demand for fresh, minimally processed vegetables has led to an increase in the amount and variety of products readily available to the consumer. Minimally processed vegetable may consist of trimmed, peeled, sliced/shredded and washed and/or disinfected vegetable. The products are packed and stored at refrigerator temperature [1]. Such “ready-to-use” (RTU) vegetable retain much of their indigenous microflora after minimal processing. Vegetables are actually contaminated by various saprophytic environmental microorganisms, the great majority of which are Gram-negative rod-shaped bacteria belonging both to the Pseudomonadaceae and Enterobacteriaceae (coliform bacteria present in soil or water); lactic bacteria, yeast and moulds are also present, but to a lesser extent. Thus, the species of microorganisms present and their quantity are dependent on the populations present in the soil, on the type of irrigation and fertilizers used and on the water utilized in cleaning the vegetable before packing [2]. Failure to implement the hazard analysis and critical control points (HACCPs) during the preparations of RTU vegetable may constitute a hazard, particularly during the warm season.

In most countries criteria has been established whereby RTU products should contain <10⁵ colony forming unit (CFU)/g or ml of mesophiles at the consumption stage and that pathogenic microorganisms or their toxins which are a risk to health should also be absent [3].

In Italy there is no specific legislation governing these kind of products; in France, the General Executive of the Concurrence of Consumption and Repression of Fraud [2] published a “Guide to good hygiene standards regarding type RTU” containing a summary of the legislation and regulations governing safety and essential hygiene standards.

To estimate food sanitary quality, the classic approach is based on the search for not only pathogenic microorganisms, but also for indicator microorganism such as faecal coliforms, whose presence indicates possible pathogens and faecal food contamination of human and/or animal origin [4]. The routine detection methods for microorganisms are based on CFU count requiring selective culture, or biochemical and serological characterizations. Although, bacterial detection by these methods is sensitive and selective, days to weeks are needed to get a results. Besides, these methods are costly and time consuming.
The growing market of fresh RTU vegetables, requires a rapid, sensitive and reliable monitoring of bacterial contamination.

The present work describes the application of an antibody based biosensor for the determination of Escherichia coli cells in vegetable food. Commercial samples were washed with peptone water to recover bacterial cells in the liquid medium. The presence of E. coli—as a bioindicator of bacterial contamination, faecal one in particular—was detected in this phase was using both conventional CFU methods and the potentiometric alternating biosensing (PAB) system, based on a transducing element (LAPS, light addressable potentiometric sensor) detecting pH variations due to NH₃ production by an urease–E. coli antibody conjugate [5].

2. Experimental

2.1. The PAB system

The transducer consists of a heterostructure made of silicon n-type, silicon dioxide and silicon nitride positioned into a measuring chamber [5]. The insulator layer is pH sensitive owing to the proton binding capacity of its surface groups over a large pH range. Redox potential measurements are obtained when the solution to be analysed reacts with the biosensor immobilized on the cover slip in contact with the silicon structure. The PAB system’s response is implemented by a lock-in technique using a modulated pulsed light from an infrared LED. The light is focused on the back of a silicon substrate and the potentiometric signal in-phase from the metallic electrode is recovered. The apparatus is computer controlled in order to obtain on-line data acquisition and recording.

The redox potential revealed by the transducer system is generated by the biosensor. The change in the redox potential is due to the production of NH₃ by a urease–E. coli antibody conjugate linked with the E. coli cells present in the water. Urea is enzymatically converted to ammonia proportionately to the amount of E. coli present in the sample. The sensitivity recorded is 59 mV per pH units.

The biological component was made by utilizing an environmental E. coli strain, DH5α, to produce rabbit polyclonal antibodies. It was chosen out of several E. coli strains, environmental, enteric and enteropathogenic, after capsule isolation and SDS-page protein analysis, because of its band pattern typical of this genus. Anti-E. coli polyclonal-IgG were purified from the serum of a New Zealand rabbit by protein-A affinity-chromatography column, after ammonium sulphate precipitation. Measurements for quantity and purity of this antibody were done spectrophotometrically at 280 nm and the anti-E. coli-IgG were identified by ELISA immunoassay.

The anti-E. coli-IgG was coupled to urease (Sigma/Aldrich) by a modified periodate coupling procedure [5,6].

The primary antibody was immobilized on a silanized glass coverslip (2 cm × 2 cm). The silanization was made by immersion on a solution containing 5% aminopropyl-triethoxysilane (APTES) and 5% water in isopropanol at 60 °C. As APTES presents NH₃ groups, a bifunctional cross-linking agent like glutaraldehyde was used to create covalent bonds with the amino-groups of the antibody. The coverslip was rinsed with a PBS solution and incubated on a 0.1% casein solution in PBS in order to block the unlinked sites so avoiding non-specific linking of the antigen.

To detect the sensitivity of the PAB system, the coverslip was washed in PBS and kept horizontally for 15 min with 1 ml of E. coli solution in PBS at different concentrations (from 10 to 10⁷ cells/ml), rinsed again with PBS and incubated with the secondary antibody (this last conjugated to urease at the concentration of 30 μg ml⁻¹) for 1 h.

Finally, the coverslip was introduced inside the measuring chamber of the PAB system, the substrate mixture (urea 50 mM) was introduced by a peristaltic pump, at flow rate of 200 μl min⁻¹ and the enzymatic activity was automatically calculated from the signals acquired during flow off periods of about 5 min. For the application described here, a micro-volume reaction chamber was utilized with a complete microchannel system near the sensitive area.

Instrument calibration was made by measuring the potential difference between pH 7 and 8 standard titration solutions (ΔVout = ΔpH × S, where S is the sensitivity).

2.2. Characteristics of the samples

Six packages of commercial RTU vegetable salad, consisting of rucoila (RC), lettuce (LT), carrots (C) and three samples of mixed salad (MS1-2-3) were purchased from chilled cabinets of local retailers 5 days after packing, over the March–June period. The products were packed under ordinary atmosphere in polypropylene bags and stored at 4 °C.

2.3. Samples treatment

Routine analyses of bacterial contamination—faecal bacteria in particular—in RTU vegetable are generally conducted by a previous sample homogenisation by a stomacher, followed by serial dilutions and plating on agarized selected media. However, the biological component of the biosensor cannot analyze samples containing plant cells such vegetable homogenates, because of their possible interference with the binding of the bacterial cell to the antibody layer. Three washing methods were set up to bypass this problem. To verify which one was the most suitable, the results of microbiological analyses of all methods were compared to those obtained by the standardized stomacher procedure: 25 g of sample were diluted in 225 ml buffered peptone water pH 6.8 (peptone 1 g/l, K₂HPO₄ 8.75 g/l, KH₂PO₄ 0.5 g/l) and homogenized for 120 s at
regular speed with a Seward Stomacher Laboratory Blender 400 [7,8].

Our sample washing methods were

(a) A 25 g of sample were diluted in 225 ml sodium-bicarbonate solution (5%) and incubated at room temperature for 2 h.

(b) A 25 g of sample were diluted in 225 ml peptone water pH 6.8 and incubated at room temperature for 2 h.

(c) A 25 g of sample were diluted in 225 ml distilled water and put in an ultrasonic Sonomatic bath (Langford Ultrasonics) for 8 min.

Washing solution of RTU vegetable were analysed at the same time by conventional microbiological methods, ELISA and by the biosensor.

2.4. Microbiological analyses

Serial dilutions of the washing solutions were made in test tubes containing 9 ml physiological solution and plated in triplicate onto the following RTU media, all purchased from Oxoid:

- Membrane endo-agar LES was utilized to count total coliforms. Plates were incubated at 37 °C for 26 h.
- Membrane faecal coliform agar [4] and violet red bile agar (VRBA) [9,10] were both used for faecal coliforms counts. Plates were incubated at 44 °C for 22 h. VRBA plates showed the best results.
- Triptone Bile X-Glu (TBX) agar was utilized for counting E. coli cells. Plates were incubated at 44 °C for 30 h [11].

2.5. Acquisition curves

Washing solutions of RTU vegetable were also analyzed by the PAB system for the presence of faecal coliforms. Samples were prepared outside the measuring chamber E. coli cells suspension in peptone water or PBS (1 ml), and PBS or peptone water without cells as a blanks, were layered on the cover slip for 30 min. Unbound cells were washed out thrice with PBS and the second antibody was added, kept for 60 min and then washed with PBS. The coverslip was put in the measuring chamber and an urea/water solution 50 mM was introduced by a peristaltic pump at a flow rate of 200 μl min⁻¹. Blanks were treated with secondary antibody and urea both together and separately. The amount of released ammonia is a function of the number of cells present in the sample and bound to the coverslip pH variation due to ammonia release is therefore a measure of cell number.

The PAB system was also utilized for the analysis of environmental water samples. Water samples were aseptically collected on sterile bottles and kept at 4 °C. Sampling was done in the following sites: Aterno river, L’Aquila upstream and downstream of the municipal sewage treatment plant, Bracciano Lake, Rome.

3. Results and discussion

3.1. Conventional microbiological analyses

Routine analyses of bacterial contamination in food are generally conducted by a previous sample homogenisation by a stomacher, followed by serial dilutions and plating on agarized selected media. However, the biological component of the biosensor cannot analyze samples containing plant cells such vegetable homogenates, because of their possible interference with the binding of the bacterial cell to the antibody layer. Three washing methods were set up to bypass this problem.

As expected, the greatest number of colonies was found in the samples treated by the stomacher (Figs. 1 and 2 and Table 1). Sodium-bicarbonate solution was found to be the less rich in CFU and this washing method was no longer
Two hours infusion in peptone water, however, gave acceptable results. The samples treated in this way were chosen to be tested with the biosensor. Cells were also tested by ELISA to check the binding with the antibodies utilized in the biosensor (data not shown). This method, however, was less sensitive because it cannot detect less than $10^3$ cells/ml.

### 3.2. Biosensor analyses

Measurements of *E. coli* cell suspension were done for the calibration curve by utilizing attenuated cells at different dilution, from $10$ to $10^7$ cells/ml [5]. The concentration of bacterial cells in the analysed liquid samples (a) washing water of RTU vegetable and (b) water from the Aterno river (Italy) is read by the instrument as pH variation responsible for the intensity of the signal. When *E. coli* is absent, the acquisition curve is almost flat (Fig. 3) instead, when bacterial cells are present, the signals variate in clear and direct connection with the *E. coli* cell number, so that one can assume that pH variation is influenced by bacterial cell number.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Vegetable</th>
<th>Stomacher</th>
<th>Peptone water</th>
<th>Ultrasonic bath</th>
</tr>
</thead>
<tbody>
<tr>
<td>RC1</td>
<td>Rucola</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>RC2</td>
<td>Rucola</td>
<td>290</td>
<td>90</td>
<td>50</td>
</tr>
<tr>
<td>LT</td>
<td>Lattuce</td>
<td>40</td>
<td>33</td>
<td>27</td>
</tr>
<tr>
<td>C</td>
<td>Carrots</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MS1</td>
<td>Mixed salad</td>
<td>16</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>MS2</td>
<td>Mixed salad</td>
<td>3</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>MS3</td>
<td>Mixed salad</td>
<td>400</td>
<td>200</td>
<td>16</td>
</tr>
</tbody>
</table>

![Fig. 2. Number of total coliform in RTU vegetable obtained by conventional microbiological analyses.](image1)

![Fig. 3. Acquisition curves of *E. coli* in washing water of mixed salad sample (top line) and blank (bottom line) in the presence of polyclonal *E. coli* antibodies. The same sample analysed by conventional methods revealed the presence of 200 *E. coli* cells/g. Blank acquisition curve obtained by flowing urea on a coverslip treated as the sample but with cell-free.](image2)
Fig. 3 shows a curve made by a sample of mix salad washing water/peptone water containing around $10^2$ cells of E. coli per ml over a blank of peptone solution. The system appears to be very sensitive: concentrations of 10 cells/ml are clearly detected over a blank of E. coli-free PBS and/or peptone water.

The PAB system was utilized to analyze environmental water samples. These samples were analyzed following the above mentioned procedure and the results obtained by the PAB were compared with the ones obtained by conventional microbiological methods and ELISA (the same antibody utilized for the biosensor). In Fig. 4 instead, is shown a curve made by a sample of water collected from the Aterno river (L’Aquila). Conventional analyses of this sample revealed the presence of 17 cells of E. coli per ml. Tap water was utilized as blank.

Bacterial cells at concentrations as low as 10 cells/ml showing detection time from 10 to 20 times shorter than the conventional CFU procedure. These preliminary results indicate PAB as an alternative profitable method to assess quality product and health care standards.

4. Conclusions

An assay for the evaluation of E. coli cells has been set up which could be useful for environmental and food control. This bacterium was chosen as typical indicator of faecal contamination. The biological component is based on a polyclonal antibody. The binding constant of the antibody is very high demonstrating that a sensing system based on the recognition antigen–antibody able to specifically detect E. coli strains in food and environmental water samples can be assembled. This system is quite flexible, manageable and easy to construct utilizing commercial electronic components although it can be improved, in particular by utilizing monoclonal antibodies.

Work is in progress to obtain a monoclonal antibody and to optimize the immobilization procedure.

Acknowledgements

The authors are grateful to Datitalia Processing, for coordinating and supporting the research by the IMI-MURST project N36/98.10/1/98 and Technobiochip for providing analytical equipment. We are particularly grateful to Prof. Marco Carmignani for helping us with the production of antibodies and to Daria Limatola for the elaboration of the figures.

References


Thick film sensors based on laccases from different sources
immobilized in polyaniline matrix

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Abstract

Thick film electrode based biosensors containing Trametes versicolor (TvL) and Aspergillus niger (AnL) laccases and Agaricus bisporus tissues (AbT) were developed for the determination of phenolic compounds and the measurement was based on oxygen consumption in relation to analyte oxidation. The electrodeposited organic polymer; polyaniline was used as a matrix for the immobilization in the preparation of thick film sensors. The systems were calibrated for different phenolic substances. A linearity was obtained in concentration range between 0.4 and 6.0 μM phenol, 0.2 and 1.0 μM catechol, 2.0 and 20.0 μM l-DOPA for TvL based biosensor; for AnL based enzyme electrode 0.4 and 4.0 μM phenol, 0.4 and 15 μM catechol, 0.4 and 6.0 μM l-DOPA; for AbT electrode 1.0 and 10 μM phenol, 0.4 and 1.6 μM catechol, 1.0 and 10 μM l-DOPA, respectively, in the response time of 300 s. Furthermore, as well as sample application and accuracy, optimum pH, temperature and thermal stabilities of the proposed systems were also detected.

Keywords: Thick film electrodes; Polyaniline film; Laccase; Phenolic compounds

1. Introduction

During the past two decades, bioelectrochemistry has received increased attention. Progress of bioelectrochemistry has been integrated into analytical applications, e.g. in biosensors working as detectors in clinical and environmental analysis [1]. The development of sensors, which are highly selective and easy to handle opens the door to the problem in analysis. On the other hand, conducting polymers have enough scope for the development of various sensors. Sensor systems based on conducting polymers also rely on sensible changes in the optical and electrical futures of this kind of materials [2–5]. Biosensors have found promising applications in various fields, such as biotechnology, food and agriculture product processing, health care, medicine, and pollution monitoring [6–12]. The combination of oxidoreductases and amperometric electrodes is by far the most commonly studied biosensor concept and through various strategies the enzyme reaction can be easily followed and sensitively measured by electrochemical means [13].

Laccases (benzenediol: oxygen oxidoreductase, E.C. 1.10.3.2) are copper containing oxidoreductases produced by higher plants and microorganisms, mainly fungi. Laccases reduce oxygen directly to water in a four-electron transfer step without intermediate formation of soluble hydrogen peroxide in expense of one-electron oxidation of a variety of substrates, e.g. phenolic compounds [1]. This kind of compounds constitutes a large group of organic pollutants, which are widely distributed throughout the environment. Phenolic compounds are used in many industrial processes, e.g. in the manufacture of plastics, paper, dyes, drugs, pesticides, and antioxidants [14,15]. Phenols are also breakdown products from natural organic compounds such as humic substances, lignins and tannins. Certain phenols and related aromatic compounds are highly toxic, carcinogenic and allergenic and due to their toxic effects, their determination and removal in the environment are of great importance. Laccases have wide substrate specificity and a great potential for the determination of phenolic compounds [16] and also show broad specificity in the process of oxidizing many compounds (mainly of phenolic type) and can be used for detoxification of a number of aquatic and terrestrial xenobiotics, industrial waste-waters, as well as for biotechnological treatment of industrial products [17]. In the presence of mediators, laccase can also play a

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role in the oxidation of non-phenolic substrates. Rather low activity and stability of plant laccases, however, limited the application of laccases based biosensors. To become practical biosensors application a reality an inexpensive source of laccases must be obtained [18–20]. In this study, different sources of laccases were used to develop thick film sensors for the determination of phenolic compounds. Moreover, characterization and sample application of these sensors were also carried out.

2. Experimental

2.1. Apparatus

Most experiments were performed using a PALM SENS electrochemical measurement system from PALM Instruments B.V. (The Netherlands). The measurements were made with thick film interdigitated sensors based on ceramic substrates with platinum working and reference electrode (BVT Technologies A.S., Czech Republic). Prior to the electropolymerization, the electrodes were cleaned by cycling the potential for 10 min between −200 and +1000 mV in 1.0 M sulphuric acid and rinsed with water.

2.2. Reagents

All chemicals were commercially available and of reagent grade. L-DOPA and catechol, were products of FLUKA AG (Switzerland). Phenol was from Merck AG (Darmstadt, Germany).

Aniline was used as a monomer for the electropolymerization and obtained from Aldrich (Gillingham, UK). Laccases from different sources were used in the present study. The first laccase Trametes versicolor (TvL) was isolated from the culture filtrates of the white-rot fungus T. versicolor (ATCC 11 235). T. versicolor was maintained at 4°C on 2% malt agar and grown in 100 ml malt extract broth (2%) for 3 days. The laccase-production medium was a nitrogen-limited medium consisting of 10 g glucose, 1 g NH₄H₂PO₄, 0.05 g MgSO₄·7H₂O, 0.01 g CaCl₂ and 0.025 g yeast extract, per litre. The cultures of T. versicolor were incubated at 26°C on a rotary shaker at 175 rpm. After 72 h cultivation, concentrated solution of phenol was added to the cultures to give 10 mg/l. The second laccase (Aspergillus niger, AnL) used in this study was a product of Novozymes A/S (Denmark), called as Denilite II and produced by genetically modified microorganisms (A. niger) and finally, Agaricus bisporus (AbT) tissues were used as a crude laccase prepare.

Laccase activity was estimated by oxidation of ABTS [21]. The protein concentration was determined by Bradford assay [22]. Final activities were 400 and 430 U/ml for T. versicolor and A. niger laccase, respectively. Mushroom tissue was also used as a biological component for the biosensor and was prepared as follows: mushroom (A. bisporus) samples were purchased from a local grocery. Firstly, tissues (1 g) were broken into pieces and then were homogenized in 1 ml of acetate buffer (0.1 M, pH 5.5). The homogenate was filtered and used for the immobilization process. Freshly prepared tissue samples were used for each day.

Synthetically concocted waste water composition; 50 g/l NaCl and 100 g/l phenol in 1.0 M HCl solution [23].

2.3. Procedures of enzyme immobilization

A three-step procedure was applied. First polyaniline (PAn) was electropolymerized for 10 min at +0.7 V from a solution containing 0.2 M aniline in 1.2 M HCl. It was followed by a 15 min cathodic polarization of the sensor at −0.5 V in 0.1 M acetate buffer, pH 5.5. After cathodic polarization, the film was immersed in 0.1 M of acetate buffer, pH 5.5 containing enzyme solution for the deposition of enzyme in polymer layer at +0.65 V for 5 min [2]. For the preparation of tissue based biosensor; instead of enzyme solution, mushroom tissue homogenate (1 g/ml in acetate buffer, pH 5.5) was used to deposit in PAn layer in the last step.

2.4. Measurements

To determine the concentration of phenolic compounds, oxygen consumption that occurred in the enzymatic reaction was detected. By using a thermostatic reaction cells, all the measurements were done at 35 °C under continuous and constant magnetic stirring and varying substrate concentrations in steady-state conditions. The working electrode was polarized at −0.7 V versus the reference electrode. On the other hand, 300 s was necessary for each analysis, a baseline was obtained in the presence of working buffer in 200 s after that, substrate solution was added and enzymatic reaction was completed in 100 s. The current changes were registered by a potentiostat. After completion of the measurement, the electrode was rinsed with distilled water and allowed to equilibrate before another measurement. 0.1 M of acetate buffer, pH 4.5 and 5.5 were used as working buffer for enzyme and tissue based biosensor, respectively.

3. Results and discussion

3.1. Immobilization in PAn matrix

The procedure with three steps was used for the immobilization in a polyaniline layer. In previous studies, the one-step formation of PAn in phosphate buffer at +1.2 V was reported and the layer was found to have very low sensitivity to hydrogen peroxide and it was not used for the entrapment of enzyme. The procedure with three steps was also tested for GOD entrapment and 15 min of deposition time for the enzyme immobilization was used and the obtained sensor was found to have an evident response to glucose [2].
In our study, prior to the optimization studies, the effect of enzyme amount for both TvL and AnL based electrodes during the deposition process at +0.65 V was tested. The deposition was performed for 15 min in the presence of various enzyme amounts (80–320 U/ml). Maximum sensor response was found in the presence of 240 U/ml of enzyme amount for both types of biosensor. A slight decrease was obtained in the presence of higher enzyme amounts (Fig. 1). Moreover, deposition time (2.5–20 min) was searched for both laccase electrodes as well as mushroom tissue based biosensor system and 5 min was found to be optimum and up to 15 min sensor response remained constant and then started to decrease slightly. Five minute of deposition time was used in further experiments in order to reduce the electrode preparation time also to protect the enzymatic activity.

3.2. Optimization of the biosensor

3.2.1. Effect of pH

According to optimization studies, the optimum pH of TvL and AnL electrodes were obtained as 4.5. In higher pH values, a sharp decrease was obtained by AnL. However, in case of using TvL, it kept half of its activity in pH 7.0 and the remaining activity value was found to be 33.3% in pH 8.0. For AbT biosensor, optimum was found to be 5.5 and in comparison to the other systems, higher activities were observed in higher pH values. Mushroom tissue is also well known tyrosinase source and this enzyme works at higher pH values than laccases. In case of AbT biosensor, the effect of tyrosinase activity could be a probable reason to obtain higher optimum pH as compared to the other systems. Fig. 2 shows the results obtained from pH optimization studies of the biosensors.

3.2.2. Effect of temperature

For the determination of temperature effect on the response of different biosensor systems varying assay temperature (15–45 °C) was examined (Fig. 3). Optimum temperature was detected as 35 °C for all three systems. According to Fig. 3, the biosensor response directly increased with temperature until 35 °C, but on the further increase in the temperature a slight decrease was observed. However, all systems kept approximately 80% of their activity even at 45 °C.

3.2.3. Thermal stability

The thermal stability experiments showed that after 8 h period only 4.6% decrease of the beginning activity of the AbT based biosensor was observed at working conditions (acetate buffer, pH 5.5, 0.1 M and 35 °C). 2.5 and 6.7% decreases were also obtained for TvL and AnL electrodes for the same period. During this period approximately 35 measurements have been made and it can be possible to make more measurements in a longer time period.
### 3.3. Analytical characteristics

#### 3.3.1. Linear range

A linearity was obtained in concentration range between 0.4 and 6.0 μM phenol, 0.2 and 1.0 μM catechol, 2.0 and 20.0 μM 1,4-DOPA for TvL based biosensor; for AnL based enzyme electrode 0.4 and 4.0 μM phenol, 0.4 and 15 μM catechol, 0.4 and 6.0 μM 1,4-DOPA; for AbT electrode 1.0 and 10 μM phenol, 0.4 and 1.6 μM catechol, 1.0 and 10 μM 1,4-DOPA. The response characteristics of to these phenolic compounds are shown in Table 1.

#### 3.3.2. Accuracy

The reproducibility of the biosensors was searched. The standard deviation (S.D.) and variation coefficient (cv) were calculated for the proposed systems and given in Table 2.

### 3.4. Sample application

The proposed biosensors were applied in waste water samples. Synthetic waste water samples prepared as described in Section 2 with known amount of phenol were used as stock substrate solutions with different dilutions by working buffer and added to the reaction cell after equilibration had occurred and then the change in current was measured. The signals obtained from the waste samples were found to be very similar with the standard phenol solutions having the same concentration (Table 3). As is seen in Table 3, the acidic nature of sample did not affect the analysis. It would be evidence that the system could be easily used for the screening of phenolic compounds in industrial waste-waters.

### 4. Conclusions

Actually biotechnological processes have been employed in several industrial productions, in biomedical applications and in environmental remediation. In this study, biosensors based on two different microbial laccases as well as mushroom tissue which is a well known laccase source were developed for determining the phenolic compounds. All data showed that the obtained biosensors could be used as simple, rapid and direct methods of determining xenobiotics in waste water samples without requiring sample pre-treatment.

In the previous work of Berka et al., it was claimed that in most microorganisms laccases are produced at levels that are too low for commercial purposes [24]. Cloning of the laccase genes followed by heterologous expression was performed to provide higher enzyme yields and to permit production of the laccases with desired properties [18]. In our cases, *T. versicolor* was used as a laccase source without requiring genetic manipulations. Extracellular fluid of the *T. versicolor* culture was used as an enzyme source without any further purification steps. Moreover, activities of another ligninolytic enzymes; manganese-peroxidase (MnP), lignin-peroxidase (LiP), arylalcohol oxidase (AAO) were also investigated in the extracellular fluid and apart from laccase, MnP was found to have higher activity than the other enzymes (unpublished data). On the other hand, as far as working conditions were concerned, in comparison with AnL, TvL based biosensor was found to keep its activity in higher pH values, up to pH 8.0. This enables us to study with TvL in higher pH values. AbT based biosensor is also stable in higher pH values as we mentioned in Section 3.2.1.

It is also clear that AbT was good alternative to obtain practical and inexpensive system having similar detection range with the others. Furthermore, it was also a novel system to obtain tissue homogenate biosensor based on conducting polymer film. As well as laccases, tyrosinase activity is also well characterized in mushroom tissues in previous works [25]. The presence of tyrosinase could contribute to analyze total phenol in different samples. All data showed that three types of systems could be used for the detection of phenolic compounds in waste water samples with acidic nature.

---

Table 1

<table>
<thead>
<tr>
<th>Type</th>
<th>Linear range (μM)</th>
<th>Slope (μA × 10⁻² M⁻¹)</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>TvL</td>
<td>Phenol</td>
<td>0.40–6.0</td>
<td>0.526</td>
</tr>
<tr>
<td></td>
<td>Catechol</td>
<td>0.20–1.0</td>
<td>1.745</td>
</tr>
<tr>
<td></td>
<td>1,4-DOPA</td>
<td>2.0–20</td>
<td>0.115</td>
</tr>
<tr>
<td>AnL</td>
<td>Phenol</td>
<td>0.40–4.0</td>
<td>0.5983</td>
</tr>
<tr>
<td></td>
<td>Catechol</td>
<td>0.4–15</td>
<td>0.4357</td>
</tr>
<tr>
<td></td>
<td>1,4-DOPA</td>
<td>0.4–6.0</td>
<td>0.4099</td>
</tr>
<tr>
<td>AbT</td>
<td>Phenol</td>
<td>1.0–10</td>
<td>0.1605</td>
</tr>
<tr>
<td></td>
<td>Catechol</td>
<td>0.1–6.0</td>
<td>0.5189</td>
</tr>
<tr>
<td></td>
<td>1,4-DOPA</td>
<td>1.0–10</td>
<td>0.1892</td>
</tr>
</tbody>
</table>

Table 2

<table>
<thead>
<tr>
<th>Type</th>
<th>Phenol concentration (μM)</th>
<th>Standard deviation (S.D.)</th>
<th>Variation coefficient (cv) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TvL</td>
<td>2.0</td>
<td>±0.074</td>
<td>3.67</td>
</tr>
<tr>
<td>AnL</td>
<td>2.0</td>
<td>±0.02</td>
<td>1.0</td>
</tr>
<tr>
<td>AbT</td>
<td>4.0</td>
<td>±0.033</td>
<td>1.6</td>
</tr>
</tbody>
</table>

Table 3

<table>
<thead>
<tr>
<th>Phenol concentration in waste water sample (μM)</th>
<th>Detected amount (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
<td>2.02 ± 0.09</td>
</tr>
<tr>
<td>4.0</td>
<td>4.10 ± 0.20</td>
</tr>
</tbody>
</table>

Results are expressed as ±S.D., n = 5.
Electrochemical deposition of enzymes on the polymer film provided to have wired electrodes with sufficient activity in comparison to conventional procedures for the immobilization [5,26]. At the moment, a comparison of laccases from different sources was made by using polyaniline film as a good gas membrane, with good permeability for oxygen with respect to other gases. In further experiments, the conducting ability of the polymer will be used to obtain faster signal and higher sensitivity at lower cathodic potentials.

References

A biosensor for *Escherichia coli* based on a potentiometric alternating biosensing (PAB) transducer

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**Abstract**

In the present paper, we describe a biosensor for the detection of bacterial contamination in drinking water based on an immunoassay test. The biological component was made by a rabbit polyclonal antibody obtained utilising an environmental strain of *Escherichia coli*, DH5α. This strain, showed a capsular protein pattern typical of this species. Cells present in water samples were detected by a complete potentiometric alternating biosensor (PAB). The PAB system consists of a measuring chamber, acquisition and driving electronics and the appropriate software: the apparatus is computer controlled in order to obtain on-line acquisitions and recording of data. The transducer principle is based on a light addressable potentiometric sensor (LAPS) technology which, in our case, reveals the production of NH₃ by a urease–*E. coli* antibody conjugate. The proposed system appears to be very sensitive and fast, in comparison with conventional methods: concentrations of 10 cells per ml were detected in an assay time of ca. 1.5 h. No signals were detected from other potential water polluting bacteria, such as *Pseudomonas marina* and *Sphaerotilus natans* and also from bacteria such as *Klebsiella oxytoca* phylogenetically related to *E. coli*. © 2002 Elsevier Science B.V. All rights reserved.

**Keywords:** Biosensor; *E. coli*; Water pollution; Light addressable potentiometric sensor (LAPS); Immunoassay

1. **Introduction**

The rapid, sensitive and reliable screening for bacterial contamination in drinking water is fundamental for the prevention of infections. The routine detection methods for microorganisms are based on colony forming unit (CFU) count and require selective culture, biochemical and serological characterisations. Although, bacterial detection by these methods is sensitive and selective, days to weeks are needed to get a result. Besides, these methods are costly and time consuming.

Despite the progress achieved in recent years, there is still no practical sensor for microbial detection which can satisfy market requirements, such as short analysis time, cost-effective instrumentation, high sensitivity and aptitude to on-line monitoring of industrial processes and environmental surveying [1].

Immunoassays are known to be competitive with conventional analytical techniques because they can detect trace amount of analyte substances in a short time and at lower cost. Gerhing et al. [4] describe a system based on a sensor utilising LAPS for the detection of *Escherichia coli*, directed towards the detection of pathogen strains on food. The system, utilising polyclonal antibodies, was able to detect about 10⁵ cells per ml.

The present work describes the properties of a biosensor for the determination of *E. coli* cell number in water samples. The potentiometric alternating biosensing (PAB) system utilised is based on a transducing element (light addressable potentiometric sensor (LAPS)) [2–4] detecting pH variations due to NH₃ production by an urease–*E. coli* antibody-conjugate.

2. **Experimental**

2.1. **The transducer**

The transducer consists of a heterostructure made of silicon n-type, silicon dioxide and silicon nitride positioned into a measuring chamber (top of Fig. 1). The insulator layer is pH sensitive owing to the proton binding capacity of its surface groups (essentially Si–O and Si–NH₂) over a large
pH range (2–12), with a theoretical Nernstian response (if hysteresis and drift phenomena are not considered). Redox potential measurements are obtained when the solution to be analysed reacts with the biosensor immobilised on the cover slip in contact with the silicon structure. Redox pairs produced, change the silicon nitride potential towards a value stated by the Nernst equation. This process is the result of the initial differences in electron affinities of the two phases in contact (Si3N4 layer and the solution) producing an electron exchange between the silicon and the solution which continues up to the equilibrium.

When the sinusoidally modulated infrared LED illuminates the back side of the silicon structure, it produces an alternating photocurrent with a characteristic ($I$, $V$) shape [3] changing according to the concentration of the reacting analyte. The PAB system’s response is implemented by a lock-in technique using a modulated pulsed light from an infrared LED. The light is focused on the back of the silicon substrate and the potentiometric signal in-phase from the metallic electrode is recovered. In the bottom of the Fig. 1, a block diagram of our conditioning and acquisition electronic is shown. The apparatus is computer controlled in order to obtain on-line data acquisition and recording.

2.2. The biosensor

The redox potential revealed by the transducer system is generated by the biosensor whose principle is schematically described in Fig. 2. The change in the redox potential is due to the production of NH3 by a urease–E. coli antibody-conjugate linked with the E. coli cells present in the water. Urea is enzymatically converted to ammonia proportionately to the amount of E. coli present in the sample. The sensitivity recorded is 59 mV per pH units.

2.3. Bacterial culture and sampling procedures

An environmental E. coli strain, DH5α, was utilised to produce rabbit polyclonal antibodies. It was chosen out of several E. coli strains, environmental, enteric and enteropathogenic, after capsule isolation and SDS-page protein analysis, because of its band pattern typical of this genus.

The strain was grown in LB broth at 30 °C in a rotary shaker (New Brunswick G25) for 1 day at 200 rpm. For solid media, 2% agar was added. Bacterial cells were removed from liquid culture by centrifugation at 4000 × g at 4 °C for 20 min, washed twice and resuspended in phosphate buffer saline (PBS). The concentration was adjusted to 10⁶ cells per ml. Cells were attenuated by heating at 60 °C for 30 min (three cycles). Cell attenuation was estimated by viable cell counts. Capsule presence also was estimated by a contrast dyeing [5]. Aliquots of fresh culture were stored at −20 °C for up to 4 months, while solid cultures were stored at +4 °C for up to 1 month.

The PAB system was also utilised for the analysis of environmental water samples. Water samples were aseptically collected on sterile bottles and kept at 4 °C. Sampling was done in the following sites: Aterno River, L’Aquila upstream and downstream of the municipal sewage treatment plant, Bracciano Lake, Rome, and a sea water from the beach of Ladispoli, Rome. Samples were analysed at the same time by conventional microbiological methods (FM disks [7]), ELISA (the same antibody utilised for the biosensor) and by the biosensor. The sample collected from the

Fig. 1. Block diagram of the transducer system. On top a detailed drawing of the measuring chamber is reported with the sensing electrode made of silicon, silicon dioxide and silicon nitride. In the bottom, the signal measurements performed by a synchronous demodulation techniques and the conditioning electronics blocks are shown.
Lake of Bracciano was also spiked with an *E. coli* cells suspension, 1 ml in PBS per litre, to a concentration of \(1 \times 10^3\) cells per ml.

2.4. Preparation and purification of rabbit antiserum

A New Zealand rabbit was given several small subcutaneous injections of an attenuated *E. coli* cells suspension in the Freund’s complete adjuvant (FCA) for a total of \(1 \times 10^3\) cells. The animal received two s.c. injections of \(10^2-10^3\) *E. coli* cells in FCA after 15 and 30 days and after 40 days it was bled under anaesthesia by a cut of the ascellary artery.

Immuneserum with high titre was cleared by centrifugation (\(8000 \times g\), 15 min). Anti-*E. coli* polyclonal-IgG were purified from the serum by protein-A affinity-chromatography column, after ammonium sulphate precipitation. Measurements for quantity and purity of this antibody were done spectrophotometrically at 280 nm and the anti-*E. coli*-IgG were identified by ELISA immunoassay.

2.5. Preparation of immunochemicals

The anti-*E. coli* IgG was coupled to urease (Sigma–Aldrich) by a modified periodate coupling procedure [6] as follows: 0.5 mg urease was resuspend in 1.2 ml water. A total of 1.2 ml urease solution was added to 0.3 ml of 0.1 M Na-periodate suspended in phosphate buffer 10 mM—pH 7.00, and it was incubate at room temperature for 20 min.; the urease solution was dialysed versus 1 mM sodium acetate overnight at 4 °C; 0.5 ml of antibody solution (1 mg ml\(^{-1}\)) were added and the mixture was incubated at room temperature for 2 h and dialysed versus PBS. Final concentration of the anti-*E. coli*–urease was 1 mg ml\(^{-1}\).

2.6. Immobilisation procedures

The primary antibody was immobilised on a glass cover slip (2 cm × 2 cm) previously cleaned with hot sulfochromic mixture and successively silanised. The silanisation was made by immersion on a solution containing 5% aminopropyltriethoxysilane (APTES) and 5% water in isopropanol at 60 °C for 1 h, followed by drying at 130 °C for 1 h. As APTES presents NH\(_3\) groups, a bifunctional crosslinking agent like glutaraldehyde was used to create covalent bonds with the amino-groups of the antibody; the cover slip was immersed in a 5% glutaraldehyde:water solution at room temperature for 30 min, and then an antibody solution (60 \(\mu g\) ml\(^{-1}\)) was laid on the cover slip and kept overnight at room temperature.

The cover slip was rinsed with a PBS solution to remove unbound antibodies and incubated for 1.5 h on a 0.1% casein solution in PBS in order to block the unlinked sites so avoiding non-specific linking of the antigen.

To detect the sensitivity of the PAB system, the cover slip was washed in PBS and kept horizontally for 15 min with 1 ml of *E. coli* solution in PBS at different concentrations (from 10 to \(10^7\) cells per ml), rinsed again with PBS and incubated with the secondary antibody (this last conjugated to urease at the concentration of 30 \(\mu g\) ml\(^{-1}\)) for 1 h.

Finally, the cover slip was introduced inside the measuring chamber (Fig. 2) of the PAB system, the substrate mixture (urea-50 mM) was introduced by a peristaltic pump, at flow rate of 200 µl min\(^{-1}\) and the enzymatic activity was automatically calculated from the signals acquired during flow off periods of about 5 min. For the application described here, a microvolume reaction chamber was utilised with a complete microchannel system near the sensitive area. A glass support was used, facing the transducer at a distance of about 300 µm, and an area of 75 mm\(^2\) was delimited by a rubber spacer, yielding a volume of 20 µl (see Fig. 1).

Preliminary trials were also done to verify how to manage with the fluxing procedure needed for the on line use of the biosensor (data are not shown).

2.7. Acquisition curves

Instrument calibration is a prerequisite for the reliability of the data and it was made by measuring the potential difference between pH 7 and pH 8 standard titration solutions (\(AV_{out} = \Delta PH \times S\), where \(S\) is the sensitivity). Measurements of bacterial suspension were done utilising attenuated cells at different dilution, from 10 to \(10^7\) cells per ml for *E. coli* and \(10^6\) cells per ml for *Klebsiella oxytoca*, *Pseudomonas marina* and *Sphaerotilus natans*. Samples were prepared outside the measuring chamber. Cell suspensions in PBS (1 ml), and PBS without cell as a blank, were layered on the cover slip for 30 min. Unbound cells were thrice washed out with PBS and the second antibody was added, kept for 60 min and then washed with PBS. The cover slip was finally put in the measuring chamber, an urea/water solution 50 mM was introduced by a peristaltic pump at flow rate of 200 µl min\(^{-1}\) and the release of ammonia was read as pH variation. Blanks were treated with secondary antibody and urea both together and separately. The amount of released ammonia is a function of the number of cells present in the sample and bound to the cover slip. The pH variation due to ammonia release is therefore a measure of cell number.

3. Results

The concentration of bacterial cells in the analysed samples was read by the instrument as a pH variation proportional to signal response. The presence of *E. coli* cells induce a potential variation (Fig. 3) proportional to the cell number (Fig. 4). The system appears to be very sensitive: concentrations of 10 cells per ml are clearly detected over a blank of *E. coli*–free PBS. The calculated limit (intercept for \(V = 0\) in Fig. 4) of the limit of detection of the instrument (LOD) appears to be 7.5 cells per ml. The background noise was not influential on the instrument sensitivity. No signals were
detected when bacteria other than *E. coli* were present and the same type of acquisition curves were given both by bacteria frequently polluting drinking water such as *P. marina* and *S. natans*, and by bacteria phyletically related to *E. coli* such as *K. oxytoca* (Figs. 5 and 6).

The PAB system was also utilised to analyse environmental water samples. These samples were analysed following the above mentioned procedure and the results obtained by the PAB were compared with the ones obtained by conventional microbiological methods and ELISA (the same antibody utilised for the biosensor). All analysed samples were found *E. coli*-free. We also spiked the Bracciano Lake water sample by adding 1 ml of *E. coli* cells, (1 × 10³ ml⁻¹), to check for a possible background interference when treating environmental waters. No background interference was recorded as shown in Fig. 7.
4. Discussion

In this work, we demonstrate how an immunoassay-based PAB sensing system was able to specifically detect \textit{E. coli} strains in water. The system can be connected to a signal-transducer in order to have a calibration curve giving the interrelationship between the registered answer and the number of \textit{E. coli} cells present in the water.

This system is quite flexible, manageable and easy to construct utilising commercial electronic components and it can be further improved. At present, we are improving the system by improving LOD (sensitivity of the instrument below 10 cells per ml) and by developing a monoclonal antibody—to increase the reproducibility—and, in particular, by setting up regeneration procedures for the immunoaffinity layer to lower the costs and to speed up the reading of the samples. The possibility of measuring microbial pollution in lakes, rivers and ground water—being \textit{E. coli} a typical indicator of faecal contamination—with a cheap, quick, compact and easy to use equipment, is attractive and should promote a larger diffusion of environmental monitoring.

Work is in progress to insert this biosensor system in a network monitoring city water distribution lines and to apply it to other water contaminants.

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References

Time-resolved absorption as optical method for herbicide detection

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Abstract

A new sensing system for the detection of photosynthetic herbicides in water has been developed, based on the use of a trans-membrane protein complex, the reaction centre (RC) isolated from Rhodobacter sphaeroides. The stationary and excited state of this protein are characterised by different absorption properties. The path followed by the protein to return to the stationary state is influenced by the presence of photosynthetic herbicides. Therefore the concentration of herbicides could be measured by monitoring the temporal changes of absorption following optical excitation. For this purpose, an optoelectronic system has been realised. It makes use of a 860 nm light emitting diode and a hybrid photodetector and is coupled to a 5 cm-long optical cell containing the RC solution through optical fibres. The system was tested with atrazine and a limit of detection of 10 nM was obtained.

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Keywords: Time; Absorption; Optical method; Biosensors; Herbicides; Reaction centre

1. Introduction

Herbicides are commonly used in agriculture to control weeds. These chemicals and the products of their degradation can contaminate runoff and well waters, giving rise to serious damage to the environment.

A class of herbicides widely used is that comprising the so-called photosynthetic herbicides [1]. These compounds act by blocking the photosynthetic process by interrupting the electron transfer chain which is at the basis of photosynthesis. In fact they replace a plastoquinone, i.e. one of the quinones involved in electron transport in chloroplast during photosynthesis of plants, algae and bacteria. This replacement prevents the formation of adenosintriphosphate (ATP) and, consequently, the formation of the energy necessary for the vital function of the photosynthetic organism, causing its death.

Consolidated techniques for the detection of herbicides are high performance liquid chromatography (HPLC) and gas chromatography (GC) coupled to mass spectrometry. Although very selective, these techniques do not allow fast detection and data processing becomes very complex in the presence of samples containing different herbicides. Moreover they necessitate specialised operators and expensive instrumentation in a professional laboratory environment.

Another approach is to use immunochemical techniques based on polyclonal and monoclonal antibodies: test kits based on enzyme-linked immunosorbent assay (ELISA) are commercially available for several pesticides.

On the other hand, it should be stressed that these kits are specific for only one pesticide, and also that their use often requires particular care in the interpretation of the collected data. Moreover, in environmental analysis there is a growing demand for systems capable of carrying out rapid in situ analysis at very low cost.

Optical fibre sensors have been proposed to solve these problems, based on immunosensing and using, as optical transduction, surface plasmon resonance [2,3], evanescent wave alone or coupled with interferometric methods [4–8].

As in the case of the ELISA tests, the above mentioned optical fibre approaches are very selective. Although in many cases this high selectivity is desirable, multianalyte assays, capable of detecting the presence of different pesticides, would be preferable in many cases [8,9].

This is particularly true in in situ analysis where the generic identification of the presence of pesticides should be the first step before going through further highly selective and expensive analyses.
Optical enzyme-based sensors, in which pesticides inhibit the activity of the enzyme, have been proposed [10–12]. Since the enzyme is not characterised by optical properties, the optical sensor is designed so as to detect one of the reagents or of the products of the enzymatic reaction. In the case of acetylcholinesterase (AchE), the enzymatic reaction which takes place is given by:

\[
\text{acetylcholine} + \text{H}_2\text{O} \rightarrow \text{acetic acid} + \text{choline}
\]

The subsequent production of acetic acid gives rise to a decrease in pH. In the presence of pesticides, the activity of the enzyme decreases and a smaller pH change takes place. Therefore pesticides can be detected by means of an optical pH sensor.

In the present paper an optical sensing system is proposed for the detection of photosynthetic herbicides based on the use of the reaction centre (RC) isolated from *Rhodobacter sphaeroides*, a micro-organism belonging to the class of purple bacteria [13].

### 2. Working principle

RC is a trans-membrane protein complex, formed by three subunits with different weights, low, medium and high, generally denoted as L, M and H subunits. It plays a fundamental role in the photosynthesis of micro-organisms: almost all the photons (>90%) absorbed by the light-harvesting complexes of the bacterium are transferred through several pigments to the RC in few hundreds of picoseconds. Then a charge separation takes place and a sequence of reactions guarantees the production of a large trans-membrane proton gradient. This gradient is exploited for the production of ATP which is used by the organism as energy for the synthesis of carbohydrates [13,14]. The RC is the smallest unit capable of producing charge separation after light absorption. It contains non-proteic cofactors: two bacteriochlorophylls, which form a dimer, two accessory bacteriochlorophylls, two bacteriopheophytins, two ubiquinones and one non-heme Fe²⁺. These cofactors are located in the L and M protein subunits, forming the so-called A and B branches of the RC, respectively, and are directly responsible for the protein’s activity.

As schematised in Fig. 1, the absorption of a photon promotes the primary electron donor, i.e. the bacteriochlorophyll dimer (D), to its excited state. An electron is preferentially transferred through the A branch, firstly to the accessory bacteriochlorophyll (Bₐ) and, then, to the molecule of bacteriopheophytin (Φₐ). The unidirectionality of the charge separation across the A branch originates from the combination of the structural engineering and specific environmental effects [15]. Lastly the electron is transferred to the first ubiquinone electron acceptor (Qₐ), which is located in a hydrophobic pocket of the protein [16].

In the presence of the secondary ubiquinone molecule Qₐ, the electron is quickly transferred (about 100 μs) from Qₐ to Qₐ and the return to the stationary state then takes place with a charge recombination rate of about 1 s (dashed arrow). On the other hand, Qₐ is loosely bound to its pocket, and can be displaced from its binding site by competitive inhibitors, such as herbicides. If the Qₐ site is empty or occupied because of binding by herbicides, the only possible recombination path is directly from Qₐ (solid arrow), with a lifetime of about 100 ms.

This different recombination rate together with the absorption properties of the RC is the basis of the working principle for the detection of herbicides. Fig. 2 shows the absorption spectrum, in the visible range, of the RC complex solution in 20 mM Tris–HCl and 0.08% lauryl-dimethyl-amine oxide (LDAO). Six main bands can be identified. The
accessory bacteriochlorophylls (B) are responsible of the two bands centred at 600 and 800 nm, whereas the bacteriochlorophyll dimer (D) is responsible of the absorption centred at 860 nm. Both the bacteriochlorophyll and its dimer are also characterised by a large absorption band centred at 380 nm. The two bands centred at 540 and 760 nm are due to the bacteriopheophytins (F).

The prominent aspect is that the intensity of the absorption band centred at 860 nm due to the bacteriochlorophyll dimer changes when the protein passes from the fundamental to the excited state. More precisely RC, if excited, undergoes a decrease in absorption at this wavelength. The charge recombination kinetics, i.e. the kinetics scheme followed to return to the fundamental state, will be dependent on the availability of the secondary ubiquinone. Since herbicides can replace the secondary ubiquinone, it is possible to monitor them by monitoring the time-resolved absorption subsequent to an excitation. Generally the excitation of the RC is carried out with a flash lamp or a laser and the subsequent time-resolved absorption is monitored [17].

The use of compact optoelectronic components is desirable in view of the realisation of a portable sensing system [18].

3. Experimental

3.1. Materials

3.1.1. Bacteria growth and reaction centre preparation

R. sphaeroides strain R-26 was kindly provided by Professor H. Scheer of the University of Munich (Germany). The bacteria was grown anaerobically in parafilm-sealed bottles (1 l volume) filled with Codgell medium and exposed to a tungsten-lamp for approximately 6 days.

After this time the optical absorbance at 660 nm is about 1 and the cell suspensions are harvested in the stationary phase by centrifugation for 10 min at 5000 × g (Beckman J2-21).

RC was isolated by a modified procedure after Feher and Okamura [19], that consists in repeated solubilisation of chromatophores with LDAO in the presence of 10 mM Tris buffer, pH = 8 at 4 °C. Then, RC is purified by column chromatography on diethyl-amino-ethyl-cellulose.

The purity of the RC centre was checked before storage at −20 °C by measuring the ratio of the absorbance A at 280 nm to that at 802 nm. In fact, SDS-PAGE electrophoresis indicates that when A_{280}/A_{802} = 1.21 ± 0.03 the purity of the protein is at least 95%.

3.1.2. Reconstitution of Q_B

The reconstitution of Q_B is essential for the correct evaluation of herbicide content because Q_B is only lightly bound to RC and after purification RC is partially depleted in Q_B. The reconstitution of Q_B was accomplished with the natural ubiquinone (UQ_{10}) by incubating for 4 h at 4 °C 1 ml of RCs (A_{564} = 1, Tris–HCl 20 mM, pH = 8, 0.08% LDAO) with UQ_{10}.

Because of the large isoprenic chain, UQ_{10} must be added in solution of EtOH/DMSO and therefore the v/v ratio must be not greater than 3%, in order to avoid protein degradation. After incubation, the solution must be centrifugated for 15 min at 4 °C (Hermle Z233) at 6000 × g.

Fig. 2. Absorption spectrum of RC solution. The absorption bands due to the accessory bacteriochlorophylls (B), the bacteriochlorophyll dimer (D), and the bacteriopheophytins (Φ) are perfectly visible. The large absorption band centred at 380 nm is due to both the bacteriochlorophylls and the dimer.
3.2. The optoelectronic unit

An optoelectronic unit was realised which makes use of a single wavelength both to excite the RC complex as well as to determine the subsequent time-dependent absorption. The scheme of the system is shown in Fig. 3. A light emitting diode at 860 nm and a hybrid photodetector are used as optical source and detector, respectively. Two optical fibres (200 μm) enable the connection between a 5-cm-long optical cell, which contains the RC solution, and the optoelectronic device. Two GRIN lenses are used in order to guarantee good coupling efficiency between the two fibres and the measurement cell. The optoelectronic unit was connected with a laptop via a National Instrument DAQ card 1200 data acquisition board. The software which drives the optoelectronic unit and processes all the collected data was implemented in LabView.

4. Results and discussion

The LED is turned on for 2.5 s and then is turned off for 2.5 s, which can be considered a sufficient time to allow the RC to come back to the fundamental state. The intensity of light at the beginning of the pulse (t = 0) is the \( I_0 \) value considered for the calculation of the quantity \( A_t = \log(I_0/I) \), hereafter defined as transient absorbance, which gives the change in absorbance with respect to its value in the fundamental state. The cycle is continuously repeated and the time-resolved absorption is evaluated considering the average over 50 pulses. With this approach a very good signal/noise ratio is obtained and changes up to 0.0005 in absorbance units can be detected.

The excitation of RC complex takes place in the presence of 2.5 s pulse, and an equilibrium between the excited and the steady state is then established. The manner in which this equilibrium is reached and the absorbance value at equilibrium depend on the concentration of herbicides.

The feasibility of the system was tested with atrazine in a 3 μM RC solution. In Fig. 4, the transient absorbance during the 2.5 s pulse is shown. The curve denoted as “RC no \( Q_B \)” is the curve obtained by using the RC solution after purification, which contributes to the removal of the secondary quinone because of its lightly bound to RC. As mentioned in the Section 3.1, the removal of the secondary quinone during the purification is only partial, so that proteins with and without the secondary quinone are present in the RC solution. The curve denoted as “RC + \( Q_B \)” is the curve obtained using the solution prepared after \( Q_B \) reconstitution process, in which all the secondary quinone should be in place. The concentration of atrazine is indicated for the other curves. The \( A_t \) value at equilibrium increases with an increase in the atrazine concentration, thus testifying to a progressive replacement of \( Q_B \) with the herbicide.

Fig. 3. Scheme of the optoelectronic system used for the measurement of time-resolved absorption.

Fig. 4. Transient absorption in a 3 μM RC solution at different atrazine concentrations (from 0.01 to 10 μM) between the “RC + \( Q_B \)” and “RC no \( Q_B \)” solutions in which the secondary quinone is in place or removed, respectively.
By inspection, the time dependence of the transient absorbance can be represented by the sum of two-exponentials model:

\[ A_t = A_1 (e^{-t/\tau_1} - 1) + A_2 (e^{-t/\tau_2} - 1) \]

where \( \tau_1 \) and \( \tau_2 \) are the time constants from the excited state to the stationary state through the secondary and primary quinone, respectively, and \( A_1 \) and \( A_2 \) are related to the RC concentration with the secondary \( Q_B \) present or absent, respectively, in the hydrophobic pocket.

The experimental curves in Fig. 4 were fitted with the two-exponential function. The fitting showed that “RC + \( Q_B \)” is a two exponential curve (\( \tau_1 = 748 \) ms, \( \tau_2 = 91 \) ms, correlation coefficient = 0.9998), testifying a non-complete reconstitution of the secondary \( Q_B \) RC proteins with and without the secondary quinone are present in the solution and both recombination paths are observed. It should be mentioned that the time constants coming out from the fitting for the different curves are comparable and their mean values are \( \tau_{1m} = 748 \pm 34 \) ms, \( \tau_{2m} = 93 \pm 6 \) ms, respectively. Furthermore, in Fig. 5, the \( A_1 \) values obtained from the fitting of the curves in Fig. 4 are plotted as a function of atrazine concentration, showing its direct dependence on the herbicide content in the solution.

5. Conclusions

The RC protein can be used as chemical transducer for the detection of photosynthetic herbicides. A detection limit of 10 nM atrazine was obtained and further steps will be devoted to the determination of the sensitivity of RC towards other herbicides, as diuron or terbutryn. The detection limit obtained is still not satisfactory for environmental application: the limit established for atrazine by the European Directive 80/778/CEE is 0.46 nM. On the other hand, improvements could be attained either by increasing the concentration of the RC in solution or by immobilising it on a solid support with a proper density. Future efforts will be aimed in this direction. The optoelectronic system makes use of a single LED at 860 nm for both excitation of the protein and detection of the absorption during the charge recombination. This should allow the realisation of a compact and portable unit to be utilised in situ, as required in environmental applications.

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References

Biographies

Francesco Baldini He graduated with a magna cum laude degree in physics from the University of Florence in 1986. Since 1986, he has been involved in optical-fibre research and spectrophotometric analysis at IROE, now named Institute of Applied Physics. His research activity is mainly devoted to optical fibre sensors for chemical parameters and optical methods used for the restoration of paintings and frescoes. He is the author of more than forty publications on this subject in international journals, in scientific books and in International Conference Proceedings as an invited speaker.

Claudio Domenici He received the Laurea degree in physics from the University of Pisa, Italy, in 1977. In 1980 he received the DEA degree from the University of Grenoble, France. He also holds an Italian Research Doctorate degree (PhD) in material engineering, awarded in 1987. From 1977 to 1979 he worked at the Institute of Physics, University of Pisa. From 1979 to 1981 he was a Research Fellow at L.E.T.I.-C.E.A., Grenoble, France. Since 1981 he has been involved in the research activities of Centro “E. Piaggio”, University of Pisa, and at the Institute of Clinical Physiology, Pisa, Italy, where he is currently a staff member of the Italian National Research Council. His research interest and scientific activity include electrical and mechanical properties of polymeric materials and their interaction with biological systems. He is also engaged in research on sensors and transducers for application in the biomedical and environmental fields.

Ambra Giannetti She received the Laurea degree in pharmaceutical chemistry and technology from the University of Pisa, Italy, in 2001. From then, she cooperates in the common research activities on biosensors and biosurfaces of Centro “E. Piaggio”, University of Pisa, and CNR Institute of Clinical Physiology, Pisa, where at present is PhD student. Her scientific experience and interests in the fields of chemistry and biochemistry include protein treatment for preparation of active surfaces in biosensors development.

Domenica Masci She was graduated in chemistry in 1982 training experience in the field of spectroscopy and of physical chemistry of surfaces and interfaces. From 1982 to 1985 she was awarded a 3-year post-doc fellowship at Max Planck Institute for Quantum Optics in Munich, Germany, in the laboratory of Laser Photochemistry, where she worked on metal thin film growth using multiphoton photodissociation by UV laser sources. From the beginning of 1990s she worked at ENEA within the research group in Photosynthesis and Bioelectronics on the subject of biomolecular spectroscopy and biomolecular deposition by UV photoionisation. At present her scientific interest is focused on the study of photosynthetic proteins and their reconstruction in model membranes for applications as sensitive elements of bioelectronic and biosensor devices.

Andrea Azelio Mencaglia He received his degree in physics from the University of Florence in 1987. He was with CNR, at the Institute of Research on Electromagnetic Waves (IROE) since 1987 until April 1996, first awarded with fellowships and then as researcher. In 1996, he was Research Fellow at the University of Strathclyde, Glasgow, UK, for six months within the Human Capital Mobility program. From January 1997 to 2001, he was responsible for research activity at Prodotec. Since January 2002 he is scientist at the Institute of Applied Physics of CNR. His activity is mainly concentrated in the area of optical fibres sensors and systems.
REVERSIBLE IMMOBILISATION OF ENGINEERED MOLECULES
BY Ni-NTA CHELATORS

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In this paper graphite or gold working electrodes obtained by several
deposition techniques were used as substrates for electrochemical synthesis of
Ni-NTA chelators. obtaining higher immobilization yields (increased 50 fold),
lower treatment time (5 min) with respect to the chemical treatment (12h), and
ability to address the synthesis on only one electrode in a sensor µ-array.

1. Introduction

Biomolecules modified by genetic engineering represent a new and powerful
approach to obtain simpler artificial structures with new or improved properties
(i.e. specificity, stability, sensitivity), useful for biosensors development. For
instance, insertion of specific molecular tags can be used for oriented and
reversible immobilization, preconcentration and purification, whereas, easier
and sensitive analytical detection can be achieved by gene fusion with selected
enzyme activities. Moreover new desired specificities can be isolated from
'phage display' antibody libraries through the 'panning' procedure1,2. An original
procedure for synthesizing groups suitable for immobilisation of engineered
(His)₆-proteins on different materials (gold, plastic, graphite, glass, quartz) was
previously reported3 for gold and graphite surfaces obtaining optimal (oriented
and highly specific) immobilization of two engineered molecules (an scFv and a
Photosystem II4). Renewable specific binding of (His)₆-proteins to gold and
graphite surfaces and fast and sensitive electrochemical or optical detection of
analytes were also obtained4. Additionally, “on chip” protein pre-concentration
was conveniently achieved for biosensing purposes, starting from crude
unpurified extracts and avoiding protein purification steps5. In this paper gold
working electrodes were obtained using several deposition techniques such as
sputtering, screen-printing and chemical gold plating on copper paths. NH₂
groups exposed out the surface were obtained by self assembled monolayers of:
cysteamine on sputtered gold and chemically plated gold on copper.

The synthesis of the Ni-NTA chelator followed the scheme:
a)glutaraldehyde, b)lysine, c)glutaraldehyde, d)Na-α-βis(carboxymethyl)-L-
Lysine Hydrate, eNiSO\textsubscript{4}; also a shorter chelator without lysine/glutaraldehyde spacer was obtained by omitting steps (b) and (c). In this paper the electrochemical synthesis of the SAM was studied, obtaining higher immobilization yields (increased 50 fold) and lower treatment time (5 min) with respect to the chemical treatment (12h), and the ability to address the synthesis on only one electrode in a sensor \( \mu \)-array.

2. Experimental

2.1. Chemical gold plating on copper paths.

For preparing copper electrodes template a commercial copper sheet deposited on fiberglass was used. The copper surface was carefully lapped using a lapping-machine with SiC sheet 4000 mesh followed by sheet with a diamond paste (particle size = 6 \( \mu \)m, then 3 \( \mu \)m). An impermeable dye layer was screen-printed on the lapped copper surface obtaining the positive electrodes lay-out; the following treatment with FeCl\(_3\) dissolved the exposed copper leaving the copper electrodes template under the dye (removed with acetone).

Chemical gold plating on copper was obtained using a bath gold cyanides (Au(CN)\textsuperscript{2-} and Au(CN)\textsuperscript{4-}) based. Gold plating bath was prepared with the following procedure: first, a solution of AuCl\(_3\) was obtained dissolving a gold wire in boiling HCl/HNO\(_3\) 3:1 and then dried at 70-80°C; HCl 37% was added and then the solution was dried again; finally, HCl 0.1M was added to have AuCl\(_3\) (0.6 g/l) in the solution which was mixed with KCN (10.0 g/l), Na\(_2\)HPO\(_4\) (6.0 g/l), NaOH (1.0 g/l), Na\(_2\)SO\(_3\) (3.0 g/l). Gold plating was done by sinking the samples in the plating bath for 1 h at 70°C; a thin gold layer (20nm) was deposited on copper because of the shift reaction:

\[
2\text{Au(CN)}\text{\textsuperscript{2-}}_{\text{aq}} + \text{Cu(s)} \rightarrow \text{Cu(CN)}\text{\textsuperscript{4-}}_{\text{aq}} + 2\text{Au(s)}.
\]

Two series of home-made golden electrodes were obtained with the described procedure. The behaviour of electrodes was tested with ascorbic acid at +600mV vs. Ag/AgCl in a flow-cell using a METHROM 641 VA-DETECTOR with CHROMATOPAC C-R6A recorder.

2.2. Determination of roughness factor

The real surface (\( A_r \)) of gold electrodes was obtained chronocoulometrically (AUTOLAB PGSTAT 10). The gold electrode was sunked in 0.1mM KFe(CN)\(_4\) (FeCy) in 0.1 M KCl; two potential steps starting from +600 mV and ending at 0mV vs Ag/AgCl with 250 ms pulse width were applied. The FeCy reduction was measured and the current signal was plotted as Q (t\(^{1/2}\)). The slope of the linear curve gave the \( A_r \) value according to the following relationship:

\[
k = 2nA_rFCD^{1/2}/\pi^{1/2} \quad (A_r = \text{electrode area, } D = \text{diffusion coefficient for FeCy}=7.6\times10^{-6} \text{ cm}^2\text{s}^{-1}, F=\text{Faraday’s constant, } C = \text{FeCy concentration, } n = 1)\]
The roughness factor was calculated as real area / geometrical area ratio. The real surface of deposited gold was measured as described and the calculated roughness factor was 1.13. (Ally M.R). This value was subsequently used to determine surface density of Ni-NTA chelator synthesized.

2.3. Deposition of cysteamine SAM and synthesis of Ni-NTA chelator

Chemical synthesis of cysteamine SAM on gold was obtained in Cys 20 mM in PBS 0.1M solution (pH=7) for 16 h. Deposition potential of Cys on gold was obtained by cyclic voltammetry (CV) 0V↔1.4V vs Pt in Cys 20 mM in PBS 0.1M solution (pH=7, scan rate = 0.05 V/s, step potential = 0.01V); electrochemical oxidation on golden electrodes was so obtained amperometrically at 850 mV vs Pt in Cys 20 mM solution for 20 minutes. The synthesis of the Ni-NTA chelator followed the common scheme: a)glutaraldehyde 12.5% v/V in PBS 0.1M solution (pH=7) for 1 h, b)Nα-Nα-bis(carboxymethyl)-L-Lysine Hydrate 5% w/V in PBS for 1h, c)NiSO4 1% w/V in distilled water for 15 minutes; all reactions were performed under stirring conditions.

2.4. Determination of the surface density of nickel

Surface density of NTA chelator on the surface of electrodes was determined as the Ni²⁺ content. Ni²⁺ was eluted from NTA groups on samples with HNO₃ 0.7M for assay by AdCSV (Adsorptive Cathodic Stripping Voltammetry) according to the DIN 38406 E16 Standard Method for trace metals analysis. In a polarographic vessel, 0.5 ml of the sample was added to the mixture of 8 ml of water, 0.25 ml of DMG solution, 0.5 ml of ammonium chloride buffer (pH=9.5±0.2) and bubbled with nitrogen for 5 minutes. The DP-Voltammogram at the HMDE was recorded according to the following parameters: potential=-0.8V, deposition time = 90 s, start potential = -0.80V, final potential =-1.2V, DP amplitude=-50mV. Ni²⁺ content determination was carried out using three standard additions. The Ni peak potential is at –0.97 V vs. SCE.

3. Results

Home-made gold electrodes tested with ascorbic acid showed good sensitivity and reproducibility, giving acceptable RSD. Series #1 tested with ascorbic acid 3*10⁻⁶M at +600 mV vs. Ag/AgCl gave a sensitivity of 32.3±1.2 nA/µM (RSD=3.2%, n=3); very high current background was recorded when copper was not completely covered by gold plating. In the same experimental conditions, series #2 showed a sensitivity of 23.3±2.2 nA/µM (RSD=9.8 %, n=5). In fig.1 calibration curves for two electrodes from the different series are reported.
Figure 1. Calibration curves for ascorbic acid at +600mV vs Ag/AgCl: A) batch #1.5; B) batch #2.1.

Figure 2. CV of cysteamine 20mM. The 1st scan show a peak at 750mV while very low current is recorded at the 6th scan.

Cyclic voltammetry done with gold working electrode in the Cys 20 mM vs. Ag/AgCl showed an oxidation peak of Cysteamine at about 750 mV. This peak decreases quickly in few minutes and after 6 scans the curve assumes the flat shape reported in the figure. This probably means cysteamine is oxidised rapidly on gold till the saturation of electrode surface. The electrochemical formation of Cysteamine SAM obtained amperometrically at 850 mV vs Pt for 20 min. showed a rapid increase of current signal at the beginning of deposition; the shape of the first and second derivatives (fig.3) shows that current quickly increases till t=3.3s after the potential application, where $f^{\prime}=\text{max}$ and $f^{\prime\prime}=0$, then increases slowly till $t=6.7s$, where $f^{\prime}=0$ and $f^{\prime\prime}<0$; then, current decreases and steady state is reached after 30s, $f^{\prime}$ and $f^{\prime\prime}=0$ (I=constant). The surface density of deposited Cysteamine was deduced from the integral (Q vs. t) of current intensity signal acquired during the electrochemical deposition process (fig.3); the charge increasing is proportional to the amount of Cysteamine oxidized that was normalized by the surface area of gold electrode. After electrochemical deposition of SAM, the synthesis of Ni-NTA chelator was followed up as described before. The resulting surface density of Ni$^{2+}$ was very high, about 513 pmol/mm$^2$. This value was corrected from the blank (treatment on fiberglass resin only) and was calculated considering the roughness factor of the gold surface. The surface density of Ni-NTA chelator achieved with electrochemical formation of cysteamine SAM for 20min. resulted about 15 fold greater than that obtained on the same material with chemical treatment for 16 h (39 pmol/mm$^2$ of Ni$^{2+}$) and 30 fold greater than that one obtained with a chemical treatment of 20min.
Figure 3. The 1st and the 2nd derivatives (a) and the integral (b) of the current signal during cysteamine deposition on gold at 850mV vs Pt calculated.

Experimental values of Ni²⁺ and Cys surface densities are compared with those obtained with theoretical calculus in table 1. As stated above, electrochemical deposition (20min) of cysteamine on gold, completely saturates electrode surface and because Ni²⁺ combines with cysteamine with a ratio 1:1, the maximum of cysteamine surface density is equal to the maximum of Ni surface density obtained after a 20' electrochemical deposition (row E=513 pmol/mm²). In table 1 row E was experimentally determined.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>time (s)</th>
<th>Surface density (pmol/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Cysteamine</td>
<td>20  100,4</td>
</tr>
<tr>
<td>B</td>
<td>Cysteamine</td>
<td>300  245,7</td>
</tr>
<tr>
<td>C</td>
<td>Cysteamine</td>
<td>1200  1305,8</td>
</tr>
<tr>
<td>D</td>
<td>Cysteamine</td>
<td>711  513</td>
</tr>
<tr>
<td>E</td>
<td>Ni²⁺</td>
<td>1200  513</td>
</tr>
<tr>
<td>F</td>
<td>Ni²⁺</td>
<td>-  400</td>
</tr>
<tr>
<td>G</td>
<td>Ni²⁺</td>
<td>-  767</td>
</tr>
<tr>
<td>H</td>
<td>PSII</td>
<td>-  0,088</td>
</tr>
<tr>
<td>I</td>
<td>PSII</td>
<td>300  0,013</td>
</tr>
</tbody>
</table>
with AdCSV, by using the real surface of the gold electrode (roughness factor=1.13). Rows A, B, C were obtained assuming moles of cysteamine=Q(t)/nF (fig.3b) with (F=96484C and n=1) at t=20,300,1200s. The time (in row D) really needed for reaching the maximum of cysteamine surface density by electrochemical treatment, can be interpolated from data in rows A, B, C. This time is equal to 711s (about 12 min). In addition, order of magnitude of row E (experimental data) is the same of row F (calculation by dimension of the ionic radius of Ni(II)) and row G (calculation by dimension of the octaedrical Ni(II) complex). Row H was calculated by considering the dimension of the PSII core crystal and row I was spectrometrically obtained considering the chlorophyll content of immobilised his-tagged PSII. With an electrochemical treatment of 12’ (or higher) a ratio Ni/(his-tagged protein)=2800 was experimentally determined. This means that a single his-tagged protein (in our case his-tag-PSII) cover 2800 Ni(II) heads and only one of them is responsible of the immobilisation mediated by his-tag.

4. Conclusions

The electrochemical formation of cysteamine SAM on gold electrodes surface results a good method to obtain lower treatment time (12 min with respect to 12h of the chemical treatment), and the ability to address the synthesis on only one electrode in a sensor µ-array. Decreasing the number of Ni heads, can be used to put different molecules (i.e. polyaniline conducting films or mixed hydrophobic SAMs) on the gold surface, hopefully obtaining better performances of the biosensor in terms of substrate diffusion or direct electron transfer onto the electrode.

Acknowledgments

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References

Different membranes were tested on screen printed electrodes to improve signal quality when sensing oxygen at a –700 mV potential (vs Ag/AgCl). The use of a cellulose acetate membrane, drugged with quaternary ammonium salts to provide the necessary electrolyte, allows for a sensible reduction of noise for measurements at negative potentials.

1. Introduction

Biosensors which use thick or thin film electrodes carrying the biological mediator directly immobilized in various ways on their surface are very common in literature. This approach takes little care of the great number of interfering species which are often present in real samples. All the same the incidence of interfering species can be of great relevance on the measured signal. Some species can increase or decrease the signal if respectively discharge on the electrode or they're able to deplete oxygen concentration on the electrode surface. Some other interfering species do not affect directly the signal by a reduction reaction but may poison the electrode surface - phenols for example reduce themselves on the graphite electrode with a radicalic mechanism covering the electrode surface with a thin film which reduces oxygen diffusion, other species such as proteins can be adsorbed on the electrode with similar effect. The quality of signals improves even in use with standard solutions and in absence of interfering species with a membrane covering the electrode, as in absence of membranes oxygen can saturate the electrodes surface.

The possibility of covering μ-electrodes and capillary electrodes with a suitable membrane would be a great advantage for improving biosensors signal even with standard solutions and in absence of interfering species.

Different membranes were tested on screen printed graphite electrodes to improve signal quality when sensing oxygen depletion due to enzyme reactions in presence of suitable substrates. Both planar electrodes and front to back geometry μ-electrodes were tested using GOD as a model biomolecule, having in this way the possibility of studying the electrode behavior at both positive
and negative potentials, ranging from hydrogen peroxide oxidation potential (+700 mV vs. Ag/AgCl) to dissolved oxygen reduction potential (-700 mV vs. Ag/AgCl). Teflon, cellulose acetate and Araldite® membranes were tested even drugged with quaternary ammonium salts obtaining stabler signals, improved signal to noise ratio and, in some cases, extended linearity range for glucose.

2. Experimental

Electrodes - µ−electrodes were printed, assembled and pre-treated for stabilization, as stated in former work [1], while planar electrodes were printed using a classical circular layout.

Cellulose Acetate Membrane: 3.96 g Cellulose acetate (Fluka) and 40 mg Polyvinyl acetate (AW 51000 droplets - BDH) were put in a solution of 20 ml tetra-hydrofurane (THF) and 30 ml acetone, and stirred continuously to complete dissolution. The solution so obtained was perfectly sealed and stored at 4°C until use. Quaternary ammonium salts in a percentage of 0.1%w/w were added to the solution before casting the membrane on the electrodes. Membranes were cast by dipping on µ-electrodes while in the case of planar electrodes, a drop of membrane solution was simply put on the electrode surface and used as a convenient “glue” to fix a Pall membrane to be used for enzyme immobilization in a second time. To make both membranes tightly adhere to the surface electrodes were pressed at 30Kg/cm².

Araldite® Membrane: (CIBA- resin :bisphenolA- epichlorohydrine, hardener: N(3-di-methyl-amino-propyl)-1,3-propylen-diamine) and solvent (either water or acetone). The paste obtained was casted on the electrode and eventually pressed.

Enzyme immobilization: Glucose oxidase (EC1.1.3.4 from Aspergillus Niger, activity180U/mg Fluka) was immobilized in different ways:

a) with epoxy resin (resin and hardener 1:2) mixing equal weights of Araldite, and solvent (either water or acetone were used),
b) with PAP (polyazetidine Hercules Polycup 172, 12% solids in water, from M. Delaney-New York 10010) dissolving directly the enzyme with the least quantity of PAP needed,
c) with BSA (FLUKA)/Glutaraldehyde (25% aqueous solution Fluka) after treating the electrode’s surface with APTES (3-amino-propyl-ethoxyesane 98% Sigma),
d) with the amphiphilic Pall membrane previously fixed on the electrode (20µl GOD solution – 20mg/ml).

Free GOD was also used in solution in batch measurements.
3. Results and discussion

Very high currents and a time lasting drift were obtained when bare electrodes coupled with GOD were based on oxygen depletion measurements (figure 1a). The very high partial pressure of the gas which reaches the bare electrode surface, negatively affected the sensor responses to glucose giving as a result noisy and unstable signals.

A barrier to limit the diffusion of the gas and to lower the current on the electrode surface [14] with similar approach of commercial Clark electrodes, was adopted, putting a membrane onto the screen printed working electrode. As in this case a membrane could not be strictly fixed onto the tip of the electrode with an o-ring, it was cast close to the working electrode surface. Different membrane solutions were deposited with different techniques ranging from Teflon dispersed in aqueous solution, to silicon and cellulose acetate by dip and dry or screen printing or spin coating or casting (unpublished data). The best result, in terms of film adhesion, integrity and mechanical resistance, was obtained by casting a solution of CA (see materials and methods above) as shown in figure 1b where a stable baseline, a linear correlation of the current with glucose concentration, stable steady states for each glucose addition/concentration and higher signal to noise ratio, were obtained.

Figure 1. Glucose biosensor by oxygen detection (-700mV vs Ag/AgCl) with (b) and without (a) CA membrane casted onto the surface of screen printed graphite electrodes. Arrows report time and final concentration of the glucose addition. In (a) the recordings from two glucose biosensors without CA membrane are reported.

To assure the electrolyte needed for amperometric measurements and to keep the membrane closely adherent to the electrode surface without using a polyelectrolyte gel,
quaternary ammonium salts were added directly to the CA solution (0.1% w/v). Different salts were tested (Tetra-ethyl-ammonium perchlorate (TEAP), Tetra-ethyl-ammonium Bromide, Tetra-methyl-ammonium Bromide, Tetra-n-butyl-ammonium Bromide (TBAB)) and the ones with the best solubility in the CA solution (TEAP and TBAB) were chosen. Results obtained with different salts added to CA and different immobilization (simple physical adsorption on a Pall membrane or chemical immobilization using PAP) can be compared in table 1.

Table 1. Sensitivity of glucose biosensors by chronoamperometry at –700mV vs Ag/AgCl with different ammonium quaternary salts added to CA membrane. GOD immobilized on Pall membrane by adsorption or PAP procedures.

<table>
<thead>
<tr>
<th>Mem Electrolyte</th>
<th>GOD imm.</th>
<th>Detected Species</th>
<th>Flow/Batch</th>
<th>Electrode lay-out</th>
<th>Sensitivity (µA/mM cm²)</th>
<th>C.V.</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>No No No</td>
<td>H₂O₂</td>
<td>batch</td>
<td>Front/back</td>
<td>1.42±0.03</td>
<td>2.1%</td>
<td>0.9981</td>
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<tr>
<td>No No No</td>
<td>H₂O₂</td>
<td>batch</td>
<td>Front/back</td>
<td>1.76±0.10</td>
<td>5.6%</td>
<td>0.9998</td>
<td></td>
</tr>
<tr>
<td>No No No</td>
<td>H₂O₂</td>
<td>batch</td>
<td>Front/back</td>
<td>1.76±0.14</td>
<td>8.0%</td>
<td>0.9995</td>
<td></td>
</tr>
<tr>
<td>CA TBAB No</td>
<td>H₂O₂</td>
<td>batch</td>
<td>Front/back</td>
<td>12.45±0.71</td>
<td>5.7%</td>
<td>0.9740</td>
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<tr>
<td>CA TEAP No</td>
<td>H₂O₂</td>
<td>batch</td>
<td>Front/back</td>
<td>7.80±0.60</td>
<td>7.3%</td>
<td>0.9739</td>
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</tr>
<tr>
<td>CA TEAP No</td>
<td>H₂O₂</td>
<td>batch</td>
<td>Front/back</td>
<td>13.90±0.30</td>
<td>2.0%</td>
<td>0.9974</td>
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</tr>
<tr>
<td>CA No Free</td>
<td>O₂</td>
<td>batch</td>
<td>Front/back</td>
<td>11.34±1.42</td>
<td>12.5%</td>
<td>0.9941</td>
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<tr>
<td>CA TBAB Free</td>
<td>O₂</td>
<td>batch</td>
<td>Front/back</td>
<td>7.38±0.42</td>
<td>5.8%</td>
<td>0.9723</td>
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<tr>
<td>CA TEAP Free</td>
<td>O₂</td>
<td>batch</td>
<td>Front/back</td>
<td>4.5±0.28</td>
<td>7.4%</td>
<td>0.9677</td>
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</tr>
<tr>
<td>CA TEAP Free</td>
<td>O₂</td>
<td>batch</td>
<td>Front/back</td>
<td>4.82±0.28</td>
<td>5.9%</td>
<td>0.9606</td>
<td></td>
</tr>
<tr>
<td>CA TEAP PAP</td>
<td>O₂</td>
<td>flow</td>
<td>Front/back</td>
<td>5.25±0.60</td>
<td>11.4%</td>
<td>0.9633</td>
<td></td>
</tr>
<tr>
<td>CA TEAP PAP</td>
<td>O₂</td>
<td>flow</td>
<td>Front/back</td>
<td>4.23±0.14</td>
<td>3.3%</td>
<td>0.9905</td>
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<tr>
<td>CA TEAP PAP</td>
<td>O₂</td>
<td>flow</td>
<td>Front/back</td>
<td>3.57±0.04</td>
<td>1.2%</td>
<td>0.9964</td>
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<tr>
<td>CA TEAP PAP</td>
<td>O₂</td>
<td>flow</td>
<td>Front/back</td>
<td>4.70±0.60</td>
<td>11.4%</td>
<td>0.9611</td>
<td></td>
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<tr>
<td>CA No PALL</td>
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<td>batch</td>
<td>planar</td>
<td>6.70±0.30</td>
<td>4.7%</td>
<td>0.9890</td>
<td></td>
</tr>
<tr>
<td>CA TBAB PALL</td>
<td>O₂</td>
<td>batch</td>
<td>planar</td>
<td>7.80±0.10</td>
<td>1.3%</td>
<td>0.9991</td>
<td></td>
</tr>
<tr>
<td>CA TBAB PAP</td>
<td>O₂</td>
<td>batch</td>
<td>planar</td>
<td>11.40±0.20</td>
<td>2.3%</td>
<td>0.9979</td>
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</tr>
<tr>
<td>CA TEAP PALL</td>
<td>O₂</td>
<td>batch</td>
<td>planar</td>
<td>13.00±2.00</td>
<td>13.0%</td>
<td>0.9346</td>
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<tr>
<td>CA TEAP PAP</td>
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<td>batch</td>
<td>planar</td>
<td>6.85±0.04</td>
<td>0.6%</td>
<td>0.9999</td>
<td></td>
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</table>

In fig. 2 chronoamperometry at –700mV vs Ag/AgCl are reported for different quaternary salts (1% w/v) added to CA membrane with GOD immobilized on the Pall membrane (20µl solution GOD 20mg/ml).

Epoxy used as a membrane with entrapped glucose oxidase, showed a large limiting effect on glucose diffusion. With epoxy resins we were able to obtain an extended linearity to glucose till 50-100mM. In figure 3 examples of extended linearity obtained with epoxy membranes of different thickness are reported.

4. Conclusions

Membranes (CA with ammonium quaternary salts as TBAB and TEAP or or epoxy resins) were cast on screen printed graphite electrodes obtaining better
performances of the graphite screen printed electrodes in terms of stability, signal to noise ratio, selectivity and extended linearity when oxygen detection at -700mV vs Ag/AgCl is used with oxidases.

Figure 2: Chronoamperometry at -700mV vs Ag/AgCl comparing different quaternary salts (1%w/v) added to CA membrane. GOD was immobilized on the Pall membrane (20µl solution GOD 20mg/ml)

Figure 3: Calibration curves for glucose obtained with several thickness of the epoxy membrane

Acknowledgments

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References


A SCREEN-PRINTED ENZYMATIC ELECTRODE FOR THE DETERMINATION OF ORGANO-PHOSPHOROUS PESTICIDES

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Evolution of a previously reported bienzymatic biosensor for the determination of organo-phosphorous pesticides are reported. This class of pesticides are detected thanks to their high reversible inhibition power towards acid phosphatase (AcP). The catalytic activity of AcP was detected by means of a screen-printed electrode, in presence of the substrate ascorbic acid 2-phosphate (A2P). The current change due to the electrochemical oxidation of the ascorbic acid as reaction product was monitored. Particularly we focused our attention to the determination of the widely diffused pesticides, i.e. Malathion. Results obtained show a high sensitivity, with a lower detection limit of about 5 ppb.

1. Introduction

Most of the biosensors, either electrochemical, optical or piezoelectric, which are used for pesticides determinations, are based on the detection of the catalytic activity of several enzymes in the presence of pesticides [1-8]. The enzymes which have been most extensively employed for the realization of pesticide-sensitive biosensors are the cholinesterases, especially acylcholinesterases (primarily among them butyrylcholinesterase and acetyl-cholinesterase) owing to the strong and mostly irreversible inhibition of their catalytic activity operated by different classes of pesticides [9-12]. The major drawback of those
biosensors is due to the fact that the irreversible inhibition of the enzyme activity leads to a rapid decrease in the sensitivity and performance of most of the cholinesterase based biosensors, so that each biocatalytic membrane can be used only for few assays. To overcome this limitation of cholinesterase-based biosensors, and on the basis of the same approach that has already been followed in the past for the realization of an organophosphorous pesticide biosensor, we are proposing an improvement of this pesticide-selective sensor, based on the reversible inhibition of acid phosphatase (AcP) [1] coupling with the screen printing technology [13-18]. The method here proposed is based on the AcP catalyzed reaction which produces ascorbic acid amperometrically detected. The determination of Malathion was carried out by measurements of its inhibition values of the catalytic activity of AcP by means of the A2P-selective sensor.

2. Experimental

2.1. Materials

L-Ascorbic Acid 2-phosphate, Acid Phosphatase (Orthophosphoric-monoester phosphohydrolase; EC 3.1.3.2 from potato), Malathion were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The reference solution was obtained from BAS (Bioanalytical Systems Inc. IN, USA). Carbon paste, silver conductive inks and dielectric polymer ink were purchased from Acheson.

2.2. Methods

Screen-printing was performed with a manual printing machine (Fleischle). Screen-printed graphite (SPG) electrodes were designed for use in batch and flow cell measuring systems. Electrodes were supported on a PVC layer. Silver ink acting as conductive medium was printed and cured at 70°C for 15 min. Carbon paste ink was printed and cured at the same temperature. An insulator layer was finally applied to cover the body of the electrode as reported in the figure 1. Electrodes were cut from the printed sheet Figure 1. Screen printed electrodes: from right to left the lay-out of the sequentially printed layers; a) Ag/Pd counter electrode, conductive paths and pads, b) Ag/AgCl reference electrode, c) carbon based working electrode, d) insulator shield.
and placed in 10 mL of 0.1M NaOH. Voltammetric cycles were carried out between -1500 and +1500 mV (vs. Ag/AgCl) at a scan rate of 100 mV/s for 40 minutes. Amperometric measurements were carried out by connecting the previously described electrodes to a potentiostat at a constant potential of +400mV vs. Ag/AgCl. Experiments were carried out in a glass cell, by ensuring an uniformity of solution by a constant magnetic stirring, in 2.5 mL of citrate buffer 0.1 M, pH=5.5 and KCl 0.01M. The electrode was employed both in the absence of the enzyme, to the direct determination of ascorbic acid concentration, and in the presence of AcP in the buffer solution, for the determination of A2P concentration. All the assays were performed under the same experimental conditions as described for the calibration experiments. The measurements were performed by dipping the sensor in citrate buffer 0.1 M, pH=5.5 and KCl 0.01M containing AcP and A2P at concentrations of respectively 0.132 U/mL and 26 M. After stabilization of the current signal, increasing quantities of the pesticide solution were added under constant stirring to calibrate the system. The current decrease, proportional to the lower ascorbic acid production due to the AcP inhibition, was recorded for 30 minutes. It represents the best compromise between the optimal response of the electrode and short time of analysis. In this way the calibration curve gives the decrease of current as a function of the concentration of added inhibitor in an easy and reliable way.

3. Results

The calibration graph, obtained with the standard solution of ascorbic acid without AcP, is reported in figure 2 together with calibration curve performed in the presence of AcP and carried out on standard solution of A2P. Figure 3 shows measurements carried out in the presence of several concentrations of pesticide.

Table 1 summarizes the main electroanalytical features of the screen-printed electrode referred to the inhibition assays. Figure 4 shows the amperometric recording in a typical inhibition experiment performed by means of the enzyme sensor here described. The first arrow refers to the addition of A2P, to a final concentration of 26 µM, the following arrow to the addition of a malathion solution to a final concentration of 50 ppb.

4. Conclusions and future studies

The performance of the AcP based screen printed electrode proves to be a close correlation with previously obtained analogous bienzymatic biosensor, with the advantage of the requirement of only a single enzyme. Another advantage of
the AcP based sensor with respect to the cholinesterase based sensor is that the inhibition of AcP by organophosphorous pesticides is almost completely reversible, so that no reactivating treatment is required. The reversibility of the inhibition of AcP, in the case of the immobilized enzyme biosensor, could be also responsible for the relatively long shelf life of the sensor, leading to a drastic reduction of the overall costs of operation. Additional experiments carried out to study the appropriate enzyme immobilization procedure for the optimization of the electroanalytical features of the screen-printed biosensor, are currently in progress.

Table 1. Data summarize the main electroanalytical features of the screen-printed electrode referred to the inhibition assays

| pH: | 5.5 |
| Buffer: | Citrate 0.1M |
| Response time: | 30 min |
| Equation of calibration curve: Y = $\Delta I$ (nA); X = Malathion (ppb) | Y = 0.69 + 0.057 X |
| Linearity range (ppb): | 10-100 |
| Correlation coefficient: | 0.9908 |
| Lower detection limit (ppb): | 5 |
| Reproducibility of measurements: | 2.5 % |

(as “pooled standard deviation” in the linearity range)
Acknowledgments

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References


Screen Printed Electrochemical Biosensors Based on Recombinant Molecules and Cells

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Summary

Engineered biomolecules and cells immobilised on screen printed electrodes were employed for biosensing purposes in environmental analysis. Examples from “Electrochemical Biosensor Lab @ ENEA” are reviewed:

- an engineered yeast (*Kluyveromices Lactis*), which express acetyl cholinesterase activity from rat, used for detection of organophosphorous compounds and
- an engineered photosystem II from *Synechococcus elongatus* used for herbicides detection by means of screen printed electrodes.

Abbreviations:
- *atrazine*: 2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine;
- *BSA*: bovine serum albumin;
- *ChOx*: choline oxidase;
- *CYS*: cysteamine;
- *CV*: Cyclic Voltammetry;
- *DQ*: tetramethyl-p-benzoquinone;
- *GA*: Glutaraldehyde;
- *His*: histidine;
- *ISFET*: Ion selective Field Effect Transistor;
- *LED*: light-emitting diode;
- *LOD*: limit of detection;
- *Ni-NTA*: nickel(II)-nitrilotriacetic acid;
- *OP*: organophosphorous pesticides;
- *OPH*: organophosphorous hydrolase;
- *PAN*: polyaniline;
- *PAp*: polyazetidine;
- *PB*: phosphate buffer 0.1M (pH=7.0);
- *PSII*: photosystem II;
- *PVC*: polyvinylchloride;
- *OCT*: octadecanethiol;
- *RE*: reference electrode (Ag/AgCl);
- *RSD*: relative standard deviation;
- *SAM*: self-assembled monolayer;
- *SEM*: Scanning electron microscopy;
- *SPE*: Screen Printed Electrode;
- *SPGE*: screen printed graphite electrode;
- *TF*: Thick Film;
- *TFT*: TF Technology;
- *WE*: working electrode;

INTRODUCTION

Engineered biomolecules represent a new and powerful approach to obtain simpler artificial structures with new or improved properties (i.e. specificity, stability, sensitivity), useful for biosensors development. For instance, insertion of specific molecular tags can be used for oriented and reversible immobilization, preconcentration and purification, whereas, easier and sensitive analytical detection can be achieved by gene fusion with selected enzyme activities. Moreover new desired specificities can be isolated from ‘phage display’ antibody libraries through the ‘panning’ procedure. Several engineered cells are also suitable for biosensing purposes allowing low cost continuous production of enzymes on the tip of a sensor. Examples of engineered molecules and cells for development of biosensors based on both AChE and PSII inhibition are reviewed for detection of classes of pesticides and herbicides.

As a matter of fact, pesticides are largely diffused in agriculture because of their high efficiency and relatively rapid degradation in environment, but their distribution and recycling processes result in water and soil pollution, with dangerous and acute effects on the living organisms, i.e. altering the food chain, or inhibiting the active site of fundamental enzymes, as well as cholinesterases involved in muscle physiology and in nervous system. On the other hand triazine herbicides, which continue to be used every year in large amounts, inhibit the photosynthetic activity of plants and show dangerous carcinogenic effects on mammalians. Some of these compounds, like dinoseb and atrazine, were banned in most countries for their certified toxicity, whilst the European Drinking Water Act (1980) does not allow their concentration in drinking water to exceed individually the limit of 0.1 mg/L.

Analytical monitoring of such a low level with both high sensitivity and selectivity remains a topical issue, especially when in presence of interfering compounds. HPLC, GC-MS, ELISA methods were showed to be expensive in instrumentation and/or relatively difficult, time consuming in the sample treatment, although highly sensitive. Furthermore, most of these methods do not provide any information about toxicity and effects on living organisms. These concerns have stimulated research
towards development of biosensing technology as a new tool for detecting herbicide and insecticide toxicity in a simple and cost effective way by simply measuring the residual activity of several enzymes after the exposure to several classes of pollutants. Several AChE\textsuperscript{8} and OPH based\textsuperscript{9,10} biosensors were already developed to detect paraoxon, methyl-paration and diazinon, with LOD of respectively 0.5-1.8 $10^{-4}$ M, 0.6-9.1 $10^{-4}$ M and 0.46-8.5 $10^{-3}$ M.

In this paper, residual activity of AChE immobilized onto nylon membranes was measured with a previously described procedure\textsuperscript{11} based on ChOx amperometric biosensors and extended to SPEs. Comparisons between commercial H$_2$O$_2$ electrode, as reference methodology, and SPGE were performed. Short time of analysis (80s/sample with parallel operation on 24 samples) and good LOD (0.1ppb) were achieved in both cases. This procedure was applied directly on fruit by using commercial enzyme or a recombinant yeast\textsuperscript{12} with a genetically expressed AChE activity from rat, in order to detect the enzyme inhibition due to OP, and to extend the procedure in a easy to be used and reliable biosensor.

Moreover, a stable and sensitive semi-automated biosensor for detection of triazine-type herbicides was recently developed, using isolated PSII particles immobilized on a Clark electrode. This biosensor exhibited a good stability as well as high sensitivity, achieving a LOD=5x$10^{-10}$ M for diuron\textsuperscript{13}. However the use of Clark electrodes prevents mass production, thus printing technology, useful in batch production of low price electrochemical devices, was initially adopted with the purified wild-type PSII\textsuperscript{14}. Printed electrodes and recombinant PSII\textsuperscript{5,16} were finally coupled with several advantages concerning sensitivity, stability, life time and response time. An original procedure for oriented and highly specific immobilization of a (His)$_6$-PSII on gold and graphite surfaces allowed: i) renewable (by using imidazole) ii) specific binding, iii) sensitive detection of analytes, iv) “on chip” protein pre-concentration starting from crude unpurified bacterial extracts and avoiding protein purification steps\textsuperscript{15}.

### 1. SCREEN PRINTED ELECTRODES

Technological advances and miniaturized systems enabled to reduce costs of biosensor production and increased their potential for large scale diffusion only in the last years. TFT\textsuperscript{17}, largely adopted in printed circuits, was showed also to be efficient in production of disposable sensors with several lay-outs and configurations. In our lab, electrodes are printed on a PVC slide by TFT with several lay-outs. Some examples are reported in fig.1.

The method consisted in placing the paste containing the electrode material on the opposite side of a screen with a well defined mesh sizes and forcing the inks to pass through and deposit on the PVC substrate. The layout drawn on the open mesh of the screen is thus transferred onto the substrate. Normally Pt, Ag/Pd, metal-doped-carbon (Rh or Pt) or simple graphite pastes are printed by sequential deposition of multi-layered TFs as conducting or electrode materials together with dielectric insulators and polymer pastes on the PVC substrate. In order to optimize the electrochemical response and the reproducibility of the electrodes, four different treatments are adopted in our lab: ink doping, chemical treatments, physical treatments and membrane coating.

a) Ink doping: metals as well as Pt or Rh adsorbed on graphite particles can be added to the graphite paste used for printing the WE. It was showed that sensitivity to H$_2$O$_2$ increases by increasing the metal content in the graphite up to a maximum value (0.5%). Moreover, carbon inks can be doped with several electrochemical mediators as well as ferrocene derivatives or prussian blue or, in some cases, even with enzymes.
b) Chemical treatment is used to improve reproducibility of the signal and reduce the stabilization time. Polishing treatments were tested on several SPEs, both at constant potential and under CV in the range $-1V \div +1V$ vs RE, in acid or basic solutions.

c) Physical treatment increases the conductivity of SPEs. A mechanical pressure onto the SPGE of 100 Kg cm$^{-2}$ increased the conductivity up to 40%. SEM pictures in fig.2 show the electrode surface before and after this treatment.

d) Membrane deposition: selective membranes, extensively used in the first generation biosensors for increasing selectivity, are not normally used on SPE because of difficult deposition and low mechanical stability. As shown in fig.3, in the absence of a membrane onto the surface of an oxygen SPE with immobilized glucose oxidase, long stabilization time, high background current and drift occurred, probably due to the very high oxygen partial pressure. On the other hand, after a deposition of a cellulose acetate membrane (15-20 µm thick) onto the electrode surface, higher signal reproducibility and a good correlation between current and concentration were achieved. The same effect was obtained with molecular layers of CYS and/or OCT deposited onto the electrode surface.

![Figure 2: SEM pictures of not pressed (left side) and pressed (right) graphite electrode surface](image)

![Figure 3: Glucose detection by an oxygen SPE with (b) and without (a) cellulose acetate membranes. Vertical arrows represent the glucose addition as final concentration](image)

2. PESTICIDE DETECTION: PURIFIED AChE vs GENETICALLY MODIFIED YEASTS

AChE based biosensors allow faster, cheaper and simpler screening procedures by using several sensors: differential pH meters, ISFET, Light Addressable Potentiometric Sensors, conductimetric cells, amperometric carbon modified electrodes or, as in this paper, SPGEs based on ChOx.
Residual activity of free or immobilised AChE located on the biosensor tip was normally detected in the real sample where incubation with OP was performed. Serious problems took place due to: i) adsorbed AChE, ii) electrochemical interferences from the sample and iii) very low lifetime of the biosensor. As previously reported, AChE immobilised on a separate membrane placed in solution, provided a different protocol of analysis with separation between incubation step and final measurement in a clean standard solution allowing the biosensor to be used without any protecting membrane. A very good LOD (0.1 ppb) for paraoxon in drinkable water, higher reproducibility, lower analysis time (4.5'/sample with parallel operation on 24 samples) and the possibility to use the same ChOx biosensor for several hundreds analyses, were thus obtained with this two step procedure. In this paper, the procedure was applied to determine AChE inhibitors directly on fruit by using either a commercially available enzyme or a recombinant yeast (Kluyveromyces Lactis) with a genetically expressed AChE activity from rat. According with the above procedure the determination of OP was made as reported below:

1) AChE, which catalyzes the hydrolysis of acetylcholine according to reaction (1), was covalently immobilized on preactivated nylon membranes (Pall Inc.) and the enzymatic activity was inhibited by paraoxon. The membranes were inhibited using different concentration of paraoxon, as reference pesticide. The application range of these membranes was between 10 and 75 ppb. Using paraoxon 37 ppb the inhibition effect was evaluated vs exposure time and the best analytical results were obtained at around 30 minutes.

\[
\begin{align*}
&O \\
&\text{AChE} \\
&(CH_3)_3N^+-(CH_2)_6-C-\text{OH} + 2H_2O \rightarrow (CH_3)_3N^+-(CH_2)_6-CO- + H_2O\tag{1}\n&\text{reaction (1)}
\end{align*}
\]

2) the nylon membranes were then rinsed and incubated in 10^{-3}M acetylcholine chloride (Sigma) in PB, using a relatively simple apparatus (fig.4) which enable rapid and contemporaneous operations on 24 membranes at a time, thus lowering the time needed for each analysis;

3) the residual activity of AChE was measured under flow condition by a ChOx SPE, exploiting the catalytic oxidation to betaine (reaction 2) and the production of hydrogen peroxide at the electrode.

\[
\begin{align*}
&\text{ChOx} \\
&(CH_3)_3N^+-(CH_2)_6-C-\text{OH} + 2\text{O}_2 + H_2O \rightarrow (CH_3)_3N^+-(CH_2)_6-COOH + 2\text{H}_2\text{O}_2\tag{2}
\end{align*}
\]

ChOx SPE was obtained by coating the WE with a cellulose acetate membrane (MWCO<100D), ChOx with PAP (Hercules Polycup) was dropped directly on; a dialysis membrane was then placed and a pressure of 36 Kg cm^{-2} was finally applied. An example of calibration curves for choline obtained with a commercial Pt electrode and our SPE is reported in fig.5: SPGE based biosensor shows higher sensitivity and lower RSD. After setting up the best working conditions for SPE biosensors, direct measurements on artificially contaminated grapes (by spray coating the maximum amount of paraoxon allowed by the European regulation, 0.5 ppm paraoxon in ethanol solution), were performed. As shown in fig.6, the Pall preactivated nylon membrane containing AChE was deposited directly on the contaminated grape: 10 \mu L of 0.1M of buffer solution were dropped on the membrane to enable the absorption of the pesticide on it.
a) $y=(2.4 \pm 0.1) \times 10^3 x + (8 \pm 6) \times 10^{-3}; R^2=0.9931; \text{RSD}=4.2\%$

b) $y=(4.1 \pm 0.1) \times 10^2 x - (2 \pm 5) \times 10^{-4}; R^2=0.9963; \text{RSD}=2.4\%$

**Figure 5:** Calibration curves and confidence intervals (95%) for choline with a) a Pt electrode and b) an SPGE.

After several washing and incubation of the set of 24 membranes, the choline concentration, and inversely the AChE inhibition, were measured with high reproducibility (within 4.5% including the sample preparation) by using ChOx SPE biosensor in a flow cell (fig.7). With this procedure we revealed a higher pesticide content at the bottom (+15%) with respect to the top of the same grape due to the outer waterproofing layer (cuticle) of the skin, allowing the pesticide nebulized solution to slide along the fruit from top to bottom.

Tests with genetically modified yeast physically entrapped on membranes, were also performed in order to reduce costs of analysis. The purified AChE was thus replaced with an engineered *Kluyveromices Lactis* yeast which expresses AChE activity onto the cell wall. Yeast cells were fixed by syringe filtration (Whatman 0.2 µm filters). Acetylcholine chloride ($10^{-3}$ M) solution was pumped through the filters for 30 minutes, and the produced choline was measured under flow condition. The electrochemical response due to the choline oxidation was also investigated as a function of the incubation time: the best results were accomplished at around 30 minutes of incubation, as a further increase of the incubation time did not produce significant improvements. The yeast-based biosensor significantly gave very similar results compared to the purified enzyme biosensor using the same experimental conditions.
3. HERBICIDE DETECTION: WILD-TYPE PSII AND ENGINEERED (His)<sub>6</sub>-PSII

*Biosensors based on wild-type PSII particles:* The wild-type PSII was isolated from the thermophilic cyanobacterium *Synechococcus elongatus*<sup>13,14</sup>. In order to assure a firm coupling of PSII particles to the electrode surface, several immobilisation techniques were tested, such as either entrapping PSII particles into agarose, calcium alginate, which were showed to be poor supports, or into gelatin, either crosslinking into BSA-GA matrix<sup>13,14</sup>. Only the latter technique resulted in a relatively long biosensor stability, in presence of 0.2 mM DQ as artificial electron acceptor, a good reproducibility and sensitivity towards classical herbicides were obtained. The electrode was polarised at 620 mV vs. RE using a potentiostat Metrohm 641 (Herisau, Switzerland). The PSII activity measurements was based on potentiostatic registration of the reoxidation current of the reduced form of DQ. The biosensor was mounted in a home made flow cell (fig.8) with a timer-controlled LED (ultrabright, peak wavelength at 650 nm) for illuminating the electrode. Under illumination, the PSII complex split water, released oxygen and reduced DQ. Then the reduced form was re-oxidized on the WE surface. The resulting increase in current was proportional to PSII activity.

The presence of herbicides in solution was detected as a decrease of the biosensor signal compared to the activity without the herbicides. In fig.9 typical plots of residual activity against several inhibitor concentration are reported.

![Figure 8: The flow cell used for measurements of photosynthetic activity of PSII](image)

![Figure 9: Residual PSII activity as a function of the main herbicides concentration. 100 % of the signal response indicates the activity in the total absence of inhibitor](image)
and architecture of the quinone pocket and mostly on the immobilization technique of PSII system. In order to assure high PSII particles concentration in the microenvironment around the electrode for the maximal sensitivity, improvements in immobilization technique were required. This issue opened the way to a new development in PSII-based sensing devices, the use of an engineered (His)$_6$-PSII.

**Engineered (His)$_6$-PSII**

Engineered (His)$_6$-PSII based biosensors: an original synthetic procedure involving a CYS-SAM and a Ni-NTA group suitable for immobilizing (His)$_6$PSII onto gold or graphite WEs, was set up. NH$_2$ groups exposed out the surface were obtained by electrochemically deposited monolayers of CYS. Ni-NTA group was synthesized along with a long-arm spacer, which was created inserting lysine molecule into the chain, according a well experimented scheme (fig. 10): (a) 10% GA in PB, (b) 5% lysine, (c) 10% GA in PB, (d) 5% N$_\alpha$-N$_\alpha$-bis (carboxymethyl)-L-lysine hydrate, (e) 1% NiSO$_4$. Arms without spacer did not required steps (b) and (c). Each step lasted 1h, at the end of which PB washing was made.

Photosynthetic activity on the electrode surface was found and confirmed with both amperometric and fluorescence detection under flow condition. One of the possible advantages expected to have oriented (His)$_6$-PSII monolayers on the WE is improved sensitivity and velocity of the response to the presence of inhibitors, properties not usually met in the case of crosslinked wild-type PSII (e.g using the BSA-GA matrix$^{13,14}$). In the case of (His)$_6$-PSII monolayer, a rapid, nearly immediate inhibition was observed directly after the addition of herbicide because of the narrow diffusion layer for reduced electron acceptor (DQ) and direct exposure of the Q$_B$ site out to the buffer solution and the inhibitor. On the contrary, for BSA-GA-PSII gel matrix a stable signal of inhibited electrode was obtained after 15' of herbicide exposure. The diffusion of herbicide and of the reduced electron acceptor in the BSA-GA gel matrix is a slow process compared to results obtained with monolayers and this fact had an important practical impact also on sensitivity and LOD. $I_{50}$ value decreased ($I_{50} = 2 \times 10^{-8}$M) with respect to the crosslinked BSA-GA-PSII gel matrix ($I_{50} = 9 \times 10^{-8}$M). A striking difference ($I_{50} = 5 \times 10^{-10}$M), compared to the previous two, has been observed when a mixed SAM layer of CYS and OCT was used, due to the increased hydrophobicity and disarranged structure of the monolayer onto the electrode surface (best diffusion).

**Figure 10**: Synthesis of Ni-NTA arms with(III) or without (II) spacers in the case of gold electrodes. The 1$^{st}$ step with CYS was indifferently obtained on gold or graphite WEs by electrochemical deposition at +750mV vs RE.

A direct electrical communication between the redox protein and the electrode surface could solve the complications with the mediator diffusion and also could improve the overall performance of the biosensor as was already shown in case of other proteins (e.g. glucose oxidase, horse radish peroxidase etc.). A PAN coating was thus performed onto the electrode surface prior of the (His)$_6$-$
PSII immobilisation, obtaining direct electron transfer without using the artificial electron acceptor DQ and again increasing the biosensor performances in terms of sensitivity and rapidity.

CONCLUDING REMARKS

Nowadays, coupling recombinant molecules or microrganisms by using TFT permit to combine the extremely high specific activity of the former with the advantages from the short time of analysis, the low production costs, and the good reproducibility of disposable SPEs.

In this paper, examples of engineered microrganisms and biomolecules for SPE inhibition biosensors were showed, with specific applications with respect to the detection of pesticides and herbicides.

1) A protocol for anticholinesterase activity on grapes was developed which well applies to the concentration range of pesticides normally on fruit and to the limit fixed from the European regulation. An engineered yeast with expressed AcChE activity coupled to SPE biosensor represents an economic way for preparing and immobilizing AcChE for detection of OP. LOD=0.1ppb (paraoxon) and time of analysis=4.5'/sample, because of parallel operation on 24 membranes, were achieved.

2) PSII-based SPE biosensors for triazine-type compounds were set up with LOD=10^{-10}M (diuron). Immobilization of genetically modified (His)$_6$-PSII, via Ni-NTA arms and a CYS-SAM, onto the electrode surface were carried out, obtaining:
   i. higher Ni-NTA loading on gold or graphite or gold/graphite electrodes compared to similar commercial products used for purification of his-tagged proteins and
   ii. ability to address the immobilization on only one electrode in a sensor µ-array because of the 1$^{st}$ electrochemical step.

These results open the possibility to extend this synthetic procedure to other materials and biomolecules (especially molecular libraries), depending on the specific sensing purpose. All these advantages could achieve the diffusion of reusable, in-situ sensitive, reliable, cheap and easy-to-use sensor systems for environmental analytical application.

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MONOLAYERS OF NATURAL AND RECOMBINANT PHOTOSYSTEM II ON GOLD ELECTRODES - POTENTIALS FOR USE AS BIOSENSORS FOR DETECTION OF HERBICIDES

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SUMMARY

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SUMMARY

Two methods for monolayer immobilisation of photosystem II isolated from thermophilic cyanobacteria *Synechococcus elongatus* and prospects for its use as a biosensor for detection of herbicides are reported:

(i) Immobilisation based on recombinant (His)$_6$-tagged PSII coupled with Ni-NTA chelator monolayer on Au electrode.

(ii) Immobilisation based on the natural PSII coupled with the protein A - anti-D1 antibody modified Au electrode.

Here, CYS-SAM-NTA-PSII monolayers were compared with traditional BSA-GA-PSII cross-linked gel matrix and better performances of the derived electrochemical biosensors were pointed out. Better diffusion of inhibitors and mediators resulted in improved sensitivity, velocity of the response and lower $I_{50}$ for herbicides.

ABBREVIATIONS:

AP: alkaline phosphatase; atrazine: 2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine; BSA: bovine serum albumin; CYS: cysteamine; CV: Cyclic Voltammetry; DM: Dodecylmaltoside; DCMU: 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DPC: 1,5-Diphenylcarbazide; DQ: tetramethyl-p-benzoquinone; GA: Glutaraldehyde; His: histidine; LED: light-emitting diode; LOD: limit of detection; MES: 2-(N-morpholino)ethanesulfonic acid; MESB: 40mM MES, 100mM NaCl, 15mM CaCl$_2$, 15mM MgCl$_2$, $5 \times 10^{-5}$M chloramphenicol, pH=6.5, 0.03%DM; Ni-NTA: nickel-nitrilotriacetic acid chelator; PB: phosphate buffer 0.1M (pH=7.0); PSII: photosystem
II; OCT: octadecanethiol; RE: reference electrode (Ag/AgCl); SAM: self-assembled monolayer; WE: working electrode.

1. INTRODUCTION
The herbicides inhibiting PSII still represent a basic tool of weed control. Their overuse brings serious environmental and health risks. Since chromatographic methods and immunoassays are not suitable for prescreening assay, we have recently designed a biosensor based on isolated PSII from thermophilic cyanobacteria *Synechococcus elongatus* [1,2]. The performance of this set-up is restricted by cross-linking the PSII in a gel matrix of bovine serum albumine (BSA) and GA. Gel entrapment resulted in slow mediator diffusion and decreased sensitivity to herbicides. An oriented monolayer of PSII could bring entirely new properties such as fast mediator transport, increased sensitivity and spatially controlled immobilisation. Here we present the results of two methods of monolayer immobilisation of PSII and prospects for its use as a biosensor for detection of herbicides:

(i) Immobilisation based on the recombinant (His)$_6$-tagged PSII[3,4]. Genetically modified biomolecules represent a powerful approach for artificial structures with improved properties for biosensor development. An original procedure for immobilisation of engineered (His)$_6$-proteins was previously reported[3] for Au and graphite surfaces. We achieved an oriented and reversible immobilisation of two engineered proteins and “on chip” protein pre-concentration starting from crude unpurified bacterial extracts.

(ii) Immobilisation based on the protein A. Monolayer of protein A on a CYS-SAM on Au electrode was deposited. An antibody against the N-terminal part of D1 protein of PSII was selectively immobilised on protein A monolayer. Later on, the PSII from wild type of *Synechococcus elongatus* was immobilised on it by means of anti-D1 antibody specific reaction.

2. EXPERIMENTAL

2.1 DEPOSITION OF CYS-SAM AND SYNTHESIS OF Ni-NTA CHELATOR
The Au screen printed electrodes (Krejci engineering ltd., Brno, Czech republic) were used. The electrodes were cleaned with Piranha solution (H$_2$O$_2$;H$_2$SO$_4$,1:3) for 1 minute and then cycled using the CV in 0.1 M H$_2$SO$_4$ till the stable repeatable voltammograms were obtained. Chemical synthesis of CYS-SAM on Au was obtained in CYS 20 mM in
PB solution (0.1M, pH=7) for 16h[3]. Electrochemical deposition of CYS was also achieved at the 0.85V vs Pt. from the same solution of CYS for 20'. Deposition potential of CYS on Au (0.85V) were obtained by CV 0V-1.4V vs Pt in CYS 20 mM in PB 0.1M solution (pH=7.0, scan rate=50mV/s, step potential=10mV). After chemical or electrochemical deposition of CYS, the synthesis of the Ni-NTA chelator followed the common scheme as previously described in detail [3]: i) GA 12.5% v/V in PB 0.1M solution (pH=7) for 1 h, ii) (name copied from producer) Nα-Nα-bis(carboxymethyl)-L-Lysine Hydrate (NTA) 5% w/V in PB for 1h, iii) NiSO₄ 1% w/V in distilled water for 15 min.

2.2 IMMOBILISATION OF (His)₆-PSII ON Ni-NTA CHELATOR AND NATURAL PSII IN GA-BSA GEL MATRIX
Thermophilic cyanobacterial Synechococcus elongatus 43H cells expressing psbC with an (His)₆ extension[4] were used for purification of (His)₆-PSII core complexes[4]. The purified (His)₆-PSII core complex was immobilised on the surface of Au-CYS-SAM-NTA modified electrodes. Immobilisation of PSII was obtained by incubation of electrodes in 40mM MESB containing PSII equivalent of 300 µg Chl mL⁻¹ at 4 °C in complete darkness for 20 minutes. Prior to the measurements, electrodes with immobilised PSII were thoroughly washed with clean MESB. In case of GA-BSA gel entrapment, the procedure described by[1] was followed. At first, 10% solution of BSA in the measuring buffer was mixed with equal volume of the PSII preparation. Then 10% solution of GA in PB was added to final concentration of 0.3%. The suspension was mixed well and approximately 5 µl of the suspension was placed on the surface of the working electrode. Finally, the electrodes were stored in a freezer until the use.

2.3 IMMOBILISATION OF PSII BY PROTEIN A MODIFIED AU ELECTRODES
Protein A coated electrodes were obtained using Au screen printed electrodes (Krejci engineering ltd, Brno, Czech republic) cleaned with Piranha solution (H₂O₂:H₂SO₄,1:3) for 1 minute and than cycled using the CV in 0,1 M H₂SO₄ till the stable repeatable voltammograms were obtained. Then, CYS was deposited chemically or electrochemically on the surface according the procedure described above. Further on, electrodes were modified by GA (10% solution in PB, 20 minutes) obtaining the aldehyde reactive groups at the surface of CYS layer. Electrode was then immersed into the protein A (Sigma) solution (500µg/ml in PB buffer) for 30'. After washing in PB, the resting reactive aldehyde groups were blocked in the glycine solution (100mM in
PB) for 20 min in order to decrease the non-specific binding of antibodies. Quality of the protein A monolayer formation and its specific antibody capture activity was determined using the AP conjugated with anti-rabbit antibody molecules (Sigma). The protein A covered electrode as well as a blank (pure Au and Au with SAM of cysteamine) electrode was immersed in to the antibody solution (100µg in PB) for 1 hour. Then, the electrodes were rinsed in the pure PB with 1% Triton detergent for 30' in order to wash out the nonspecifically bounded antibodies. Presence of bound antibody was detected using the AP activity and p-nitrophenyl phosphate as the substrate (i) optically and (ii) amperometrically at the +850 mV in N₂ saturated solution (pH=9.8, 1M diethanolamine buffer). A N-terminal anti-D1 specific antibodies were diluted 1:30 000 in PB and attached to protein A modified electrode using the same procedure as described above. Then, electrode was immersed in to the solution of PSII (300 µg Chl mL⁻¹, 40mM MESB) at 4 °C in complete darkness for 1 hour. Each electrode was properly washed in pure MES prior to use.

2.4 Amperometric measurement of PSII activity
Amperometric measurement of PSII activity on the electrode were done in a home-made flow-cell continuously fed with MESB (flow rate 0.25 mL/min, peristaltic pump Gilson MiniPulse 3). The reoxidation of DQ, the artificial electron acceptor of PSII, was detected at +650mV vs RE after the short illumination. In some cases, the artificial electron donor DPC was used in order to increase the PSII generated photocurrent. The current intensity on the WE was registered with a potentiostat and processed by AD converter and software (Oxycorder, PSI Instruments, Czech republic). The illumination of electrode was controlled by a custom-made electronic timer, the duration was set to 1 or 5 s and the light intensity to about 100 µmol photons m⁻²s⁻¹. Red (650 nm) and blue high intensity LEDs were used.

3. RESULTS AND DISCUSSION

3.1 CYS-SAM-NTA-PSII MONOLAYER VS CROSSLINKED PSII IN A BSA-GA MATRIX
One of the possible advantages expected to have PSII monolayers on the WE is improved sensitivity and velocity of the response to the presence of inhibitors - properties not usually met in case of crosslinked PSII (e.g using the BSA and GA matrix[1,2]). Fig.1 and its 1st derivative (not shown) reports the disproportion between
the velocity of reoxidation of the reduced form of the electron acceptor (DQ) in the case of BSA-GA-PSII electrode[1,2] and CYS-SAM-NTA-PSII. In case of chemically deposited SAM layer of chelator, fast increase of signal comparable to signal rise of PSII entrapped in the BSA-GA gel can be seen after illumination of electrodes. Typical initial value of peak maximum was about 250pA. The number of DQ molecules reoxidised on the electrode surface was calculated to be $n=1.09 \times 10^{-14}$ moles (moles of DQ=$Q(t)/nF$ with $F=96484 \text{ C}$ and $n=2$). In case of electrochemically deposited SAM layer of chelator, initial signal intensity is lower, typically about 100 pA. Signal response to the illumination is still fast and also comparable to kinetics of signal rise of PSII entrapped in GA-BSA matrix. Again, number of transferred and reoxidised molecules of DQ is $n=9.65 \times 10^{-15}$. In our previous work [5] we have shown that there is a certain surplus of the NTA molecules on the SAM layers available for immobilisation of PSII in case of chemically deposited SAM of chelator (in case of electrochemical deposition even 30-fold higher indicating densely packed layer). Comparing the values of electrical charge transport of PSII in each case and assuming the same amount of PSII bound to the electrode in both cases (due to the surplus of NTA available for PSII) we can presume that densely packed SAM layer of chelator act as the one of potential barrier lowering DQ diffusion to some extent towards the electrode surface. As seen in the Fig.1 it is clear, that there is the disproportion between the velocity of the reoxidation in the case of BSA-GA-PSII electrode and PSII-NTA-SAM. In the case of BSA-GA-PSII electrode, the fast increase of the electric current is visible within the initial 500ms of illumination and after this, a slow increase follow even if the red light is off. Later on, long 30s period of slow decrease follows till the initial signal is restored. Contrary, PSII-NTA-SAM electrode act differently. The fast increase of signal is finished instantly when the red light is turned off and rapid decrease of electric current follows within the next 500ms. Than, slow decrease of activity is visible during the additional 10s till the initial baseline signal is restored. The disproportion can be explained on the basis of different double layer appearance of the two presented types of electrodes and different diffusion coefficient of oxidised and reduced form of DQ in the surrounding enviroment of PSII and electrode. In both cases, the initial 500 ms fast increase could be ascribed to the reducing activity of the PSII centres in the close proximity of electrodes, fully supplied by oxidised DQ from solution during this initial period. In the case of monolayer, this demand for oxidised substrate can be fulfilled easily because the narrow diffusion layer and its fast supply from solution or from electrode after its reoxidation. In contrast, in the case of BSA-
GA-PSII gel matrix, the process of substrate diffusion is limited by its different (lower) diffusion coefficient in gel (assumed from the long time of stabilisation of electrodes). Hence, the reduced DQ accumulated in the upper layers of gel could be reoxidised even if the light is off. This process is probably responsible for slow reoxidation kinetics observable in Fig.1. Because of lack of additional upper layers of PSII in the case of PSII-NTA-SAM electrode, all reduced DQ is reoxidised almost instantly on the electrode when the light is turned off.

3.2 Sensitivity and inhibition kinetics of PSII monolayer by herbicide

Expected advantages of protein monolayers are improved sensitivity and velocity of the response to the presence of inhibitors - properties not usually met in case of cross-linked (e.g using the BSA and GA matrix) bulk quantities of proteins immobilised in various types of matrix. These two presumptions were confirmed by our studies of inhibition of PSII activity using the herbicides DCMU (Fig.2) and atrazine (data not shown).

In case of monolayer, a rapid, nearly instant inhibition of PSII electrode is observable directly after the addition of herbicide. This process is permitted due to the narrow diffusion layer for reduced electron acceptor (short distance between the all active centres and surface of electrode) and direct exposition of the active PSII out to the buffer solution (and thus fast diffusion of inhibitor to the active centre of PSII, contrary to the gel entrapment immobilisation mechanisms). For comparison, stable signal of PSII-BSA-GA electrode exposed to the $10^{-7}$ M concentration of DCMU is obtained after 15 minutes to herbicide exposition [2]. This time is needed for setting up the dynamic equilibrium between the gel and surrounding buffer with herbicide. The diffusion of herbicide in the gel is a slow process compared to results obtained with monolayers and this fact could have an important practical impact also on sensitivity and LOD.

$I_{50}$ value of 3 different type of electrodes were compared for atrazine. Au-CYS-SAM-NTA-PSII electrode has shown a slight change ($I_{50}=2x10^{-8}$ mol/L) compared to the electrode with BSA-GA-PSII gel matrix ($I_{50}=9x10^{-8}$ mol/L). A striking difference ($I_{50}=5x10^{-10}$ mol/L), compared to the previous two, has been observed in the third electrode type consisting of a mixed SAM layer (CYS + OCT) with increased hydrophobic properties. The two order magnitude lower $I_{50}$ in this case could be caused due to the local pre-concentration of herbicide at the interface between hydrophilic-hydrophobic environment around the electrode surface.
3.3 Working Life-time of the Monolayer PSII Electrodes

One of the most important properties of biosensors determining their practical use is their working life-time. As we have shown [2] in the case of PSII-GA-BSA electrode, the measured half-time is about 24 hours. This makes it feasible for practical measurement. In case of the Au-CYS-SAM-NTA-PSII electrode (Fig.3) only presence of 0.03 % detergent DM in the measuring buffer significantly prolonged the life-time to the values comparable to PSII-GA-BSA electrode. Without DM, the activity of the electrode decreases very fast (half-time = 1 hour). The stabilisation is possible just only in the detergent presence, because it surrounds PSII particles and protect it against the denaturation in the hydrophilic environment. Contrary, BSA-GA-PSII was stable also without detergent in the measuring buffer, probably because of the stabilisation of PSII provided by cross-linking.

3.4 Characterization of the PSII-antiD1-Protein A Modified Au Electrodes

Anti-D1 polyclonal antibodies were raised against the sequence of the 20 aminoacids of the N-terminal part of D1 (courtesy Dr.J.Komenda, MBU AVCR Trebon, Czech rep.). The binding activity of the antibody against the D1 in non-denaturated native form of PSII were tested using the electrophoresis under non-denaturating conditions (data not shown). At first, the monolayer of protein A on Au electrode was characterized in term of its binding activity to the AP-conjugated anti-rabbit antibodies. Fig.4 shows the response of the electrode to the addition of p-nitrophenyl phosphate as the substrate into the measuring solution. An increase of the signal due to the oxidation of the p-nitrophenyl produced by AP was observed. No significant response was observable for the blank samples under same conditions. Results were also confirmed using the optical detection of p-nitrophenyl product, where the same response (in terms of increase of absorption at $\lambda=540$nm) was observable after addition of the substrate. Results confirmed the formation of the protein A monolayer and also active selective binding of antibodies.

Protein A coated Au electrodes were then immersed in 1:30000 diluted antibodies against D1 in PB for 1 hour and finally washed in PB with 1% Triton detergent for 30 minutes in order to deplete of non-specifically bound antibody. Further on, electrodes were immersed into the solution of PSII (300 µg Chl mL$^{-1}$ in MESB, pH=6.8 ) for 1 hour. The activity of PSII was measured amperometrically in the presence of 0.5mM of artificial electron donor DPC using the modified method described for Au-CYS-SAM-NTA-PSII electrode. A stable photocurrent of PSII loaded electrodes measured as the
rate of DQ reoxidation was obtained after illumination with red light indicating the presence of non-denatured PSII on electrode surface (Fig.5). The decrease of PSII generated photocurrent was observable in the presence of herbicide.

4. CONCLUSION

Two methods for immobilisation of PSII monolayers on Au electrodes were presented in this paper. They are based on the common preparation of a CYS-SAM on the Au surface of the electrodes followed by a chemical treatment with GA. Last step before PSII immobilisation was the treatments with i) an NTA derivative or ii) with protein A-anti-D1 antibody conjugate. Ni-NTA monolayers, as previously reported [3], were used for immobilisation of recombinant (His)$_6$-tagged PSII from Synechococcus elongatus. Here, CYS-SAM-NTA-PSII monolayers were compared with traditional BSA-GA-PSII crosslinked gel matrix and better performances of the derived electrochemical biosensors were pointed out. Better diffusion of inhibitors and mediators resulted in improved sensitivity, velocity of the response and lower $I_{50}$ for herbicides.

Protein A based immobilisation was obtained with an anti-D1 polyclonal antibody against the sequence of the 20 aminoacids of the N-terminal part of D1 protein of PSII. Later on, the immobilisation of non-denatured PSII from wild type of Synechococcus elongatus was verified and its sensitivity towards the photosynthetic herbicides observed.

ACKNOWLEDGEMENTS

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REFERENCES


CAPTIONS OF FIGURES

Figure 1: Current response of the PSII on Au WE. After illumination (5s) current increases due to the reoxidation of the artificial electron acceptor (DQ). Data were obtained with three different immobilisation methods on Au screen-printed WE (Krejci Eng. ltd.).

Figure 2: An inhibition curve of PSII-NTA-SAM screen printed electrode (Krejci production) using the herbicide DCMU (5*10^{-7} M). [A] buffer without the herbicide [B] buffer containing the DCMU [C] wash out the herbicide using the same buffer as in A. Data from the interval A-B were fitted with the exponential decay curve in order to see a possible reconstitution of the PSII activity after washing step. Measurement was done without the presence of DM in the solution.

Figure 3: Life-time of the PSII-NTA-SAM electrodes can be significantly prolonged by detergent DM in the measuring buffer. Life-time observed in this case is comparable to the life-time of the PSII immobilised in BSA and GA matrix[1,2]

Figure 4: Activity of AP conjugated with anti-rabbit antibodies immobilised on the surface of protein A coated Au electrode. [A] solution of p-nitrophenyl phosphate was added [B] pure buffer.

Figure 5: Measurement of the activity of the proteinA-antiD1-PSII electrode under continuous light illumination in the presence of artificial electrode donor DPC. [A] red light was switched on [B] DCMU added to the solution (1*10^{-8} M panel A, 1*10^{-7} M panel B) [C] light was switched off.
Screen printed graphite biosensors based on bacterial cells

Suna Timur a,*, Livia Della Seta b, Nurdan Pazarlioğlu a, Roberto Pilloton b, Azmi Telefoncu a

Abstract

A microbial biosensor was developed for the determination of phenolic compounds and the measurement was based on oxygen consumption in relation to analyte oxidation. Induced cells of Pseudomonas putida DSM 50026 were immobilised on the surface of SPG electrodes covered with cellulose acetate membrane by means of gelatine which was then cross linked with glutaraldehyde. The systems were calibrated for different phenolic substances. Detection ranges were 0.1–1.0 $\mu$M for phenol and 0.05–1.0 $\mu$M for l-tyrosine and l-DOPA, respectively, with a response time of 3 min. Furthermore, phenol detection was performed in the presence of synthetic wastewater samples.

Keywords: Microbial biosensor; Pseudomonas putida; Screen printed graphite electrode; Phenolic compounds

1. Introduction

Phenols are highly toxic and are chiefly derived from industrial waste water, effluents, municipal sewage and cooking plant effluents. Phenol is used in the production of a large variety of aromatic compounds, e.g. explosives, pharmaceuticals, fertiliser, paint, paint removers, textiles, drugs, bake-lite and plastics, at the same time, the compounds have high toxicity to humans when present above certain concentration limits [1,2]. Chemical analysis of environmental samples for these compounds are expensive, their cost prohibiting systematic surveys or monitoring activities. An inexpensive analytical tool for sample screening is needed. Use of biosensors for routine determinations of environmental pollutants has been suggested as a possibility [3]. The application of thick-film technology to the construction of sensors is well-documented [4,5]. Compared to other technologies that are available for manufacturing electrodes, such as thin-film, thick-film electrodes are relatively inexpensive, simple to fabricate and are congruent for mass production. For these reasons, the scientific and technical communities regard thick-film electrodes as disposable [6]. In addition to these very attractive advantages, the technology enables biomolecules, such as enzymes, etc. and ion-selective membranes to be deposited onto the electrode surface in a straight-forward manner without tedious preparation steps which may compromise the sensing molecule integrity [7]. The fabrication of phenolic biosensors may be a suitable alternative method of analysis, which may circumvent the problems associated with ‘traditional’ analytical methods.

On the other hand, microbes have a number of advantages as biological sensing materials in the fabrication of biosensors. They are present ubiquitously and are able to metabolise a wide range of chemical compounds. Micro-organisms have a great capacity to adapt to adverse conditions and to develop the ability to degrade new molecules with time. Microbes are also amenable for genetic modifications through mutation or through recombinant DNA technology and serve as an economical source of intracellular enzymes. Purified enzymes have been most commonly used in the construction of biosensors due to their high specific activities as well as high analytical specificity. Purified enzymes are, however, expensive and unstable, thus limiting their applications in the field of biosensors. As judged by their sensitivity, time of response and stability of signals, microbial sensors are similar to enzyme-based sensors but are less selective. This may be due to the complexity of the elements of the enzymic apparatus of cells. Insignificant amount of biomass as well as high stability make the use of microbial sensors preferable.
2. Material and methods

2.1. Reagents

All chemicals were commercially available and of reagent grade. L-DOPA and L-tyrosine were products of FLUKA AG (Switzerland). Phenol was from Merck AG (Darmstadt, Germany). Mineral salts medium (MSM) with the following composition [9] was used as a growth medium; 0.1% NH₄NO₃, 0.05% (NH₄)₂SO₄, 0.05% NaCl, 0.05% MgSO₄·7H₂O, 0.15% K₂HPO₄, 0.05% KH₂PO₄, 0.0014% CaCl₂·2H₂O, 0.001% FeSO₄·7H₂O and trace element solution (1 ml/l).

The pH of the medium is 6.9. Trace element solution was prepared according to [10].

2.2. Biological material

Pseudomonas putida DSM 50026 were sub-cultured on Nutrient Agar. Adaptation of the cells to phenol (250 mg/l) was performed with the following steps; the organism was inoculated in to MSM medium containing gradually increasing phenol and decreasing glucose concentrations by daily inoculations until the medium contained 250 mg/l phenol. After adaptation was completed, a cellular paste was obtained. After 24 h, when cells were grown, the biomass was harvested by centrifugation at 10,000 × g, suspended in MSM and then re-centrifuged. The supernatant was removed and the cellular paste was used for making biosensor. Cell growth was followed spectrophotometrically by measuring optical density at 560 nm. The relationships between optical density and living cells were also investigated [12].

2.3. Apparatus

Preparation of the carbon-based inks for printing working electrodes: inks for printing working electrodes were prepared by mixing a commercially available carbon ink (Du Pont 7101) with rhodium (Aldrich 20.616-4) or platinum (Aldrich 23.755-8) adsorbed on graphite particles.
Electrodes are washed repeatedly in phosphate buffer before use.

2.5. Measurement of the biosensor response

All measurements were carried out at 28 °C under continuous and constant magnetic stirring. After each run, the electrode was washed with distilled water and MSM and kept in the same buffered solution at room temperature for 10 min. The microbial sensor was initially equilibrated in MSM solution for 30 min. Phenolic substrates were then added individually to the reaction cell. After that, phenol oxidation takes place, which caused a decrease of oxygen concentration in the bioactive layer sensed as a decrease in the current. The current changes were registered with a potentiostat at −0.7 V.

3. Results and discussion

Oxygen detection with the screen printed electrodes was previously optimised by adding Pt or Rh adsorbed onto graphite particles to the commercial carbon ink during the printing step of the working electrode. With Rh powders, higher sensitivity to hydrogen peroxide, ascorbic acid and oxygen were obtained and a 10% mixture of these metal activated particles was finally used for the best performances of the electrodes (data not shown).

A glucose biosensor, the most simple and common biosensor, was also used for testing the transducers. The biosensor was obtained by immobilising glucose oxidase enzyme onto the working electrode surface by BSA-glutaraldehyde procedure. Oxygen consumption due to glucose in the presence of the immobilised glucose oxidase was recorded.

A very high current and a long time drift were obtained (Fig. 2a). The very high partial pressure of the gas which reaches the bare electrode surface, negatively affected the sensor responses to glucose giving as a result noisy and unstable signals.

A barrier to limit diffusion of the gas and to lower the current on the electrode surface has been adopted for commercial Clark electrodes [15]. A similar scheme was adopted here, putting a membrane onto the screen printed working electrode. In this case it could not be fixed onto the tip of the electrode with an o-ring and it was cast close to the working electrode surface. Different membrane solutions were deposited with different techniques ranging from Teflon dispersed in aqueous solution, to silicon and cellulose acetate by dip and dry screen printing, spin coating and casting (unpublished data). The best result, in terms of film adhesion, integrity and mechanical resistance, was obtained by casting a solution of CA as shown in Fig. 2b where a stable baseline, a linear correlation of the current with glucose concentration, stable steady states for each glucose addition/concentration and higher signal to noise ratio, were obtained.

The sensor response to phenol was then tested in the presence of potassium phosphate buffer (50 mM, pH 6.9) and MSM (pH 6.9) by adapted cells immobilised on a Clark electrode and a higher signal was observed in MSM in previous studies (unpublished data). MSM was chosen as a working buffer solution in these experiments. All measurements were performed at 28 °C which is the growth temperature for the micro-organism. Furthermore, in these experimental conditions, the response time of the microbial electrode was found to be 3 min. During the experiments, daily inoculated cells were used for the preparation of each microbial electrode in order to obtain uniform and repeat-

Fig. 2. Glucose biosensor by oxygen detection (−700 mV vs. Ag/AgCl) with (b) and without (a) CA membrane casted onto the surface of screen printed graphite electrodes. Arrows report time and final concentration of the glucose addition. In part (a) the recordings from two glucose biosensors without CA membrane are reported.
able results. In previous work, *Rhodotorula* species yeast was chosen as biological material and immobilised on to the surface of an oxygen electrode by means of a dialysis membrane. Optimum conditions were pH 7.0 and 25 °C and a linear range of 10.0–100 μM for the phenol detection was observed [16]. In a previous study, *P. putida* cells were immobilised on both Clark electrode and modified thick film electrode surface by mixing Na-alginate which was then, treated with CaCl₂ solution. In this system, the optimum pH and temperature were found as 7.0 and 32 °C. The detection range for phenol was 1.0–56.25 μM for the Clark electrode-based system and 6.3–90 μM for the thick film sensor [17]. The present working conditions were similar and the lower detection range was obtained as 0.1–1.0 μM for phenol and 0.05–1.0 μM for the l-tyrosine and l-DOPA. At higher concentration of these compounds, the sensor response remained steady. Analytical features are given in Table 1. The proposed sensor seems to be more sensitive in comparison to others previously described. Preliminary experiments showed that immobilisation on the working electrode surface without a CA membrane caused signals with background noise. The controlled diffusion of oxygen by the CA membrane seems to be the main reason that enabled better results to be obtained.

The next part of the study was to test the application of the microbial biosensor in waste water samples. In these experiments, waste water samples with appropriate dilutions were added to the reaction cell instead of substrate, then the signal was recorded and concentrations were calculated from the calibration curve for phenol. No matrix effect was observed as is seen in Table 2. These data provide evidence that the system could be easily and usefully addressed to the screening of phenolic compounds in industrial waste water samples of an acidic nature. The proposed system would be useful as disposable arrays for field use. The immobilisation method is also practical and provides the mild conditions to continue microbial activity. The advantages of both screen printed disposable electrodes [18] and micro-organism enable economical and easy to use systems for multi-analyte detection to be produced in combination with engineered microbial strains with different substrate specificities and screen printed sensor arrays.

### Table 1
Analytical features of the proposed microbial biosensor

<table>
<thead>
<tr>
<th>Phenol</th>
<th>l-Tyrosine</th>
<th>l-DOPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equation of the calibration graph&lt;sup&gt;a&lt;/sup&gt;</td>
<td>( y = 19.58x + 0.01 )</td>
<td>( y = 112.88x - 1.74 )</td>
</tr>
<tr>
<td>Linearity range (μM)</td>
<td>0.1–1</td>
<td>0.05–1</td>
</tr>
<tr>
<td>Correlation coefficient ((r^2))</td>
<td>0.9976</td>
<td>0.9977</td>
</tr>
<tr>
<td>Standard deviation (S.D.)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>±0.008 ((n = 7))</td>
<td>±0.002 ((n = 5))</td>
</tr>
</tbody>
</table>

<sup>a</sup> Slope is given in nA/μM, intercept in nA.

<sup>b</sup> Repeatability measurements and S.D. calculation were performed with \( n \) replies of a 0.25 μM solution containing the phenolic compounds.

### References


A Sensitive Photosystem II-Based Biosensor for Detection of a Class of Herbicides

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Abstract: We have developed a biosensor for the detection of residual triazine-, urea- and phenolic-type herbicides, using isolated photosystem II (PSII) particles from the thermophilic cyanobacterium, Synechococcus elongatus, as biosensing elements. The herbicide detection was based on the fact that, in the presence of artificial electron acceptors, the light-induced electron transfer through isolated PSII particles is accompanied by the release of oxygen, which is inhibited by the herbicide in a concentration-dependent manner. The PSII particles were immobilized between dialysis membrane and the Teflon membrane of the Clark oxygen electrode mounted in a flow cell that was illuminated. Inclusion of the antibiotic chloramphenicol in the reaction mixtures prolonged, by 50%, the lifetime of the biosensor. The use of highly active PSII particles in combination with the flow system resulted in a reusable herbicide biosensor with good stability (50% of initial activity was still remaining after 35-h use at 25°C) and high sensitivity (detection limit for diuron was $5 \times 10^{-10}$ M). © 1998 John Wiley & Sons, Inc. Biotechnol Bioeng 60: 664–669, 1998.

Keywords: herbicides; photosystem II; thermophilic cyanobacteria; biosensor

INTRODUCTION

During the last several decades, herbicide application in agriculture has markedly increased, resulting in mass production and herbicide pollution of soil and water. Because herbicides can be highly toxic to human and animal health, their indiscriminate use has serious environmental implications. Understandably, therefore, dinoseb was banned in the U.S. and in most other countries for its high toxicity (U.S. Federal Register, 1986), while atrazine, a possible human carcinogen, was also banned (U.S. EPA, 1988). The European Union introduced the European Drinking Water Act of 1980, which does not allow concentrations of pesticides in drinking water to exceed 0.1 mg/L of an individual pesticide or 0.5 mg/L of total pesticides. To monitor such low residual herbicide levels a need has arisen for developing sensitive and reliable detection methods.

Currently, three methods are generally employed to determine the presence of most herbicides, HPLC, GC-MS, and ELISA. HPLC and GC-MS represent reliable and routine methods, but their disadvantages are that they require expensive equipment, organic solvents, and purification of samples prior to assay, thus limiting the number of samples that can be analyzed (Pacakova et al., 1996). A recently developed, immunochemical method (ELISA) has high sensitivity ($2 \times 10^{-10}$ M for diuron; $1 \times 10^{-10}$ M for atrazine) (Giersch, 1993; Schneider and Hammock, 1992; Schneider et al., 1994), but involves preparation of monoclonal antibodies, which is difficult, time-consuming, and expensive. Furthermore, the antibodies generated are specific either to one compound or a few of its structural analogs.

About one half of the herbicides presently used in agriculture inhibit the light reactions in photosynthesis, mostly by targeting the photosystem II (PSII) complex (Draber et al., 1991). PSII is a pigment–protein membrane complex made up of the reaction center D1/D2 heterodimer carrying the main functional groups of PSII, the chlorophyll (Chl) proteins CP47 and CP43 acting as inner antennae, α- and β-subunits of cytochrome b559, and the oxygen-evolving complex (Mattoo et al., 1989). The D1 protein is the main target of herbicides that inhibit photosynthesis. Based on chemical structure and binding properties, PSII herbicides fall into two main groups: (1) phenylureas and triazines and (2) phenols (Trebst and Draber, 1979). Although both classes replace the Q_B acceptor on the D1 protein, (Mattoo et
al., 1981; Pfister et al., 1981), they interact with different amino acid residues on D1 (Draber et al., 1991). The high binding affinity of these herbicides to D1 offers a unique opportunity to use the PSII complex for herbicide detection.

Interestingly, in the 1950s, selection for the most effective herbicides took advantage of the fact that herbicides could inhibit the Hill reaction in isolated chloroplasts (Good, 1961; Wessels and Van der Veen, 1956). Based on a similar strategy, isolated chloroplasts and thylakoids have been used, conversely, to detect herbicides, by testing inhibition of the Hill reaction (Loranger and Carpentier, 1994; Rouillon et al., 1994), inhibition of DCPIP photoreduction (Brewster and Lightfield, 1993; Brewster et al., 1995), or change in Chl fluorescence (Conrad et al., 1993; Merz et al., 1996). These observations have initiated interest in developing biological sensors to detect low levels of herbicides in water and soil using PSII. So far, the practical use of herbicide biosensors based on isolated PS II preparations has been limited by their instability, particularly upon illumination. Here we present data showing that, by manipulating PSII particles from the thermophilic cyanobacterium *Synechococcus elongatus*, it is possible to construct a stable and sensitive PSII-based biosensor for detecting herbicides.

**MATERIALS AND METHODS**

**Preparation of PSII Particles**

Mature plants of potato (*Solanum tuberosum*), pea (*Pisum sativum*), and broad bean (*Vicia faba*), grown in a greenhouse, were used for isolation of thylakoids and of PSII particles by the method of Berthold et al. (1981) using a Triton X-100:Chl ratio of 15 for broad bean and 20 for potato and pea (Giardi et al., 1994). Chl content was determined in 80% (v/v) acetone extracts of tissue (Lichtenthaler et al., 1995). Hill activity for PSII particles from various photosynthetic organisms. As expected, the activity was measured spectrophotometrically, with DCPIP as electron acceptor. We found that the DCPIP reduction by PSII preparations from higher plants was inactivated with a half-life of about 10 h, whereas that of the SB12 particles from the thermophilic *Synechococcus* had a half-life of ~20 h (Table I). Interestingly, photoreduction of DCPIP was most stable with *Synechococcus* thylakoids solubilized by the nonionic detergent HTG (HTG particles), with a half-life of ~24 h (Table I). Photochemical activity of PSI particles from cya-
nobacteria declined by only 2% to 5% after 10 h of dark treatment at 25°C, whereas that of PSII particles from higher plants decreased by 44% to 58% (Table I). After 20 h of incubation, ~50% of the activity of cyanobacterial preparation was lost compared with 65% to 82% from the higher plant material. From these data, it was apparent that the PSII preparations most appropriate for biosensor construction would be those from the thermophilic cyanobacterium.

**Description of Biosensor and Flow-Cell System**

To design the biosensor, we entrapped about 1 μg of Chl equivalent of either the SB12 particles or the HTG extract of *Synechococcus* thylakoids between the dialysis membrane and Teflon membrane of the Clark electrode (Oxyliquid Model 22 Idronian, Brugherio, MI, Pt-electrode diameter 1 mm; inner gas permeable Teflon membrane, No. 670597, Beckman). A potentiostat (Metrohm 641, Herisau SW) was used to maintain the correct potential (~−700 mV for Ag/AgCl) and record the electrical current on the platinum electrode. The flow cell (Fig. 1d, e) connected to the electrode had a “dead” volume of about 3 μL. The HTG PSII particles immobilized on the electrode were continuously washed with the reaction medium containing 15 mM MES (pH 6.5), 0.5 M-mannitol, 0.1 M NaCl, 5 mM MgCl₂, and an artificial electron acceptor (Fig. 1a), at a flow rate of 0.25 mL/min. A test herbicide was dissolved in the reaction medium and introduced into sample reservoir (Fig. 1b). A valve was used to switch between the reaction medium reservoir and the test (herbicide) solution reservoir (Fig. 1c).

Oxygen evolution was measured upon 20-sec illumination of the biosensor with a cluster of seven red light-emitting diodes (LEDs, ultrabright type, wavelength maximum at 660 nm, 150 μmol m⁻² s⁻¹ (Fig. 1m). The light was controlled by a custom-made control box (Fig. 1n). The light source and the valve were switched either manually or by a computer using a modified Gilson HPLC system. The signal usually ranged between 2 and 20 nA.

**Optimization of Biosensor Activity**

We measured the time-dependent (at intervals of 20 min) decline in the oxygen-evolving activity of the biosensor with immobilized SB12 and HTG particles in the presence of either *p*-benzoquinone (BQ), duroquinone (DQ), or ferricyanide (FeCy). These three electron acceptors were chosen due to their low affinity for the Q₈ niche on D1 and thus they do not compete with herbicide binding (Satoh et al., 1995). The SB12 biosensor showed good initial activity with BQ and FeCy but not with DQ (not shown). On the other hand, the HTG-biosensor exhibited high activity with all the three acceptors. The SB12 particles are likely depleted of the bound Q₈ and, therefore, DQ cannot act as the electron acceptor. This could be explained by the model of Satoh and coworkers (Kashino et al., 1996; Satoh et al., 1995) who proposed that DQ can accept electrons only via plastocynone bound in the Q₈ niche. Therefore, for the remaining experiments, we used only the HTG biosensor. Figure 2 shows the stability of HTG particles at 25°C for the three acceptors. Addition of the antibiotic chloramphenicol (CAP; 5 × 10⁻⁵ M final concentration), an inhibitor of protein synthesis on 70S ribosomes, greatly improved the biosensor stability. In the presence of quinones, a consistent increase (30% to 40%) in oxygen-evolving activity was observed within the first 2 h of the assay (Fig. 2). The reason for this observation is not fully understood, but the absence of such an activation in isolated thylakoids (not shown) and

![Figure 1. Scheme of the biosensor flow system: (a) buffer reservoir; (b) herbicide solution reservoir; (c) three-way valve; (d) flow cell; (e) biosensor; (f) peristaltic pump; (g) waste; (h) electrochemical detector; (i) chart recorder; (l) magnetic stirrer; (m) LEDs cluster; and (n) timer and power supply. (Insert) Mediator entrapped on the surface of the electrode.](image1)

![Figure 2. Time dependence of stability of the biosensor using HTG-particles at 25°C in the presence of 10⁻³ M BQ, 2 × 10⁻⁴ M DQ, or 10⁻³ M FeCy in the measuring buffer. CAP was present in all samples at the concentration 5 × 10⁻⁵ M. A 100% value represents the maximal oxygen evolution activity obtained in each measurement.](image2)
in the PSII particles in the presence of FeCy suggest that some rearrangement of the acceptor side may take place during detergent solubilization.

The stability of the HTG biosensor was tested under continuous flow conditions also at 15°C, 25°C, and 35°C, as shown in Figure 3. At higher temperatures, a significant reduction in the stability of the preparation was observed, the half-life of oxygen-evolving capacity being longer at 15°C than at 35°C. Moreover, at the three temperatures tested, the half-life of the biosensor activity with BQ was significantly shorter compared with DQ and FeCy (Fig. 3). At 25°C, the half-lives of the biosensor activity were 42, 36, and 8 h, respectively, in the presence of FeCy, DQ, and BQ (Fig. 3). These half-life measurements were reasonable and intermediate between 15°C and 35°C; therefore, it was desirable to use the laboratory temperature, 25°C.

Sensitive Detection of Herbicides

Considering the positive and negative effects of these acceptors on stability and herbicide activity (Figs. 2 and 3)—BQ destabilized the PSII (Fig. 3) and interfered with herbicide-binding activity, by reducing the inhibitory effect of DCMU (not shown)—we tested DQ or FeCy as electron acceptors for the remaining experiments.

Calibration curves for six herbicides (three urea/triazine type herbicides — DCMU, atrazine, simazine; and three phenolic type herbicides—dinoseb, ioxynil, and bromoxynil) were determined in the presence of $2 \times 10^{-4} M$ DQ. Initially, the activity of the biosensor was recorded in the absence of the herbicides, then 5 mL of herbicide solution was loaded from the sample reservoir and the activity was recorded again. The ratio between these two measurements was determined. After each measurement, the herbicide was removed by washing the system with reaction medium for 15 min. The biosensor was then tested with another concentration of the standard herbicide solution. The repeated use of the biosensor did not affect the reproducibility of the measurement. Each concentration was used in two to four independent measurements, and calibration curves were constructed for each herbicide (Fig. 4).

We found that the sensitivity of detection did not vary during the lifetime of the biosensor. The results with DQ as an electron acceptor are shown in Figure 4; similar results were obtained when FeCy was used, but measurements with FeCy were not always reproducible (not shown). The poor reproducibility with FeCy is perhaps due to slow oxidation of nonheme iron (II) by ferricyanide, resulting in the reduction of binding affinity of herbicides to the Q_B site, as discussed by Diner and Petrouleas (1987) and Satoh et al. (1992). It is also possible that FeCy accepts electrons from the PSII reaction centers that do not bind plastoquinone.

The $I_{50}$ values (herbicide concentration causing 50% inhibition of the initial activity) for herbicides and their detection limits were calculated and are summarized in Table II. The detection limit (LOD) was calculated using the graphical method described by Meier and Zund (1993).

According to this method, the limit of detection (LOD) is possible at the 99% confidence level. The standard error in our measurements was below 5%, due mainly to low signal to noise ratio. The biosensor was most sensitive for the detection of DCMU and atrazine, whereas the detection limits for the other herbicides were higher by one or two orders of magnitude.

![Figure 3. Temperature dependence of half-life ($t_{1/2}$ in hours) of the oxygen evolving activity of the biosensor (HTG particles) using the indicated electron acceptors. The measurements were done at the indicated temperatures. CAP was present in all samples at the concentration $5 \times 10^{-5} M$. Hill reaction activity for HTG-particles was about 860 μmol O₂ mg⁻¹ Chl h⁻¹ (35°C, in the presence of FeCy + BQ as electron acceptors), respectively.](image)

![Figure 4. Calibration curves to determine the limit of detection (Meier and Zund 1993) and $I_{50}$ dose for six herbicides, DCMU (diuron), atrazine, simazine, ioxynil, bromoxynil and dinoseb. The calculated values are summarized in Table II. Biosensor activity was measured in the presence of $2 \times 10^{-4} M$ DQ and $5 \times 10^{-3} M$ CAP. A 100% value represents the oxygen evolution activity of the biosensor measured at the beginning of the experiment in the absence of herbicide. Measurements at each concentration of herbicide were made in two to four replicate experiments.](image)
Table II. $I_{50}$ dose and limit of detection (LOD) determined for various herbicides.a

<table>
<thead>
<tr>
<th>Herbicide</th>
<th>$I_{50}$ (M)</th>
<th>LOD (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diuron (DCMU)</td>
<td>$8 \times 10^{-8}$</td>
<td>$5 \times 10^{-10}$</td>
</tr>
<tr>
<td>Atrazine</td>
<td>$3 \times 10^{-7}$</td>
<td>$2 \times 10^{-9}$</td>
</tr>
<tr>
<td>Simazine</td>
<td>$8 \times 10^{-8}$</td>
<td>$1 \times 10^{-9}$</td>
</tr>
<tr>
<td>Ioxynil</td>
<td>$4 \times 10^{-7}$</td>
<td>$9 \times 10^{-9}$</td>
</tr>
<tr>
<td>Bromoxynil</td>
<td>$8 \times 10^{-8}$</td>
<td>$2 \times 10^{-7}$</td>
</tr>
<tr>
<td>Dinoeb</td>
<td>$8 \times 10^{-7}$</td>
<td>$6 \times 10^{-8}$</td>
</tr>
</tbody>
</table>

a Oxygen evolution activity of the HTG-particle-based biosensor was measured with $2 \times 10^{-4}$ M DQ, in the absence and presence of various concentrations of each herbicide, as described in the Materials and Methods. $I_{50}$ represents the herbicide concentration causing 50% inhibition of the initial biosensor activity. Hill reaction activity for HTG particles was about 860 $\mu$mol O$_2$ mg$^{-1}$ Chl h$^{-1}$ (35°C, in the presence of BQ + FeCy as electron acceptors). The detection limit was calculated on the basis of a 99% confidence level using a graphical method reported by Meier and Zund (1995).

**DISCUSSION**

We have constructed a stable, highly sensitive, user-friendly PSII-based biosensor. Contributing factors to the success in building the biosensor were: the stable nature of the PSII complex source (the thermophilic cyanobacterium, *Synchococcus elongatus*); use of an isolated PSII complex instead of thylakoids for immobilization; and incorporation of a flow cell system. Compared with the previous herbicide detection systems that employed PSII as a biosensing element, sensitivity of the biosensor reported here is greater than that reported for amperometric biosensors (Loranger and Carpentier, 1994; Rouillon et al., 1995a, b). A detection limit of $5 \times 10^{-10}$ M for diuron is similar to that obtained using ELISA or Chl fluorescence detection methods (Merz et al., 1996).

The advantages of the system developed here include: requirement of small amounts of biological material: very low detection limit for selected herbicides — for diuron comparable with the most sensitive ELISA tests; higher stability of the biosensor at room temperature; reusability and regeneration of the biosensor because the test herbicide can be easily washed off; and the possibility of automating the measurements. Interestingly, the observed half-life of the biosensor at 25°C is greater than the half-life of the original material; that is, the “free” HTG extract (compare data in Table I and Fig. 3). The reason could be that the HTG particles are surrounded by many detergent molecules that considerably influence the activity of the PSII complex. When the material is used in a flow system, as in the biosensor, the detergent is removed by dialysis after several minutes, and the activity is stabilized. This is supported by the fact that, in the present study, the material became insoluble and formed a lipid-like film on the Teflon membrane of the electrode.

The general sensitivity of the PSII-based biosensor is given by the binding constant of a herbicide. At low concentrations of herbicide the decrease in activity is minimal, and to record this minimal response, it is necessary to maximize the signal to noise ratio. In our system, this was achieved by using PSII particles that enabled high specific activity of oxygen evolution on Chl and protein bases (compared with chloroplasts or thylakoids), and immobilization of PSII particles on a small surface area of the Clark electrode, which enabled a high concentration of the PSII complex in the microenvironment of the measuring probe. The signal to noise ratio was further increased by using an optimized flow cell with minimal dead volume. The flow system ensured the signal stability because the herbicide concentration in the solution does not decrease, as in batch systems, where herbicide is in equilibrium with the PSII complex. In addition, the flow system significantly minimized the heterogeneity of the sample compared with the mixed batch sample.

In summary, we have developed a relatively stable and sensitive semiautomated biosensor for the detection of residual triazine-, urea-, and phenolic-type herbicides, using isolated PSII particles from a thermophilic cyanobacterium. The data presented can be used as a basis for developing a commercial, miniaturized biosensor for rapid monitoring of PSII herbicides.

M.T.G. thanks Dr. G. Angelini for allowing the use of his laboratory facilities.

**References**


Komenda, J., Masojidek, J., Setlikova, E. 1992. Structure of the cyanobacterium, *Synchococcus elongatus*; use of an isolated PSII complex instead of thylakoids for immobilization; and incorporation of a flow cell system. Compared with the previous herbicide detection systems that employed PSII as a biosensing element, sensitivity of the biosensor reported here is greater than that reported for amperometric biosensors (Loranger and Carpentier, 1994; Rouillon et al., 1995a, b). A detection limit of $5 \times 10^{-10}$ M for diuron is similar to that obtained using ELISA or Chl fluorescence detection methods (Merz et al., 1996).

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U.S. Federal Register, October 14, 1986. 51 FR 36634.
A Biosensor for the Detection of Triazine and Phenylurea Herbicides Designed Using Photosystem II Coupled to a Screen-Printed Electrode

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Abstract: A biosensor for the detection of triazine- and phenylurea-type herbicides was constructed using isolated Photosystem II (PS II) complexes as a biosensing element. PSII isolated from the thermophilic cyanobacterium Synechococcus elongatus was immobilized on the surface of a screen-printed sensor composed of a graphite working electrode and Ag/AgCl reference electrode deposited on a polymeric substrate. The biosensor was mounted in a flow microcell with illumination. The principle of the detection was based on the fact that herbicides selectively block PSII electron transport activity in a concentration-dependent manner. Changes of the activity were registered amperometrically as the rate of photoreduction of an artificial electron acceptor. The setup resulted in a reusable herbicide biosensor with a good stability (half-life of 24 h) and limit of detection of approximately $10^{-9}$ M for diuron, atrazine and simazine.

INTRODUCTION

Massive use of herbicides in agriculture over the last several decades has become a serious environmental problem. The pollution of soil and water in many agricultural areas represents an important risk factor as a result of the toxicity or carcinogenic nature of some of these compounds.

Herbicides inhibiting photosynthesis via targeting Photosystem II (PSII) function still represent the basic means of weed control. This group consists of several classes of chemicals such as triazines (e.g., atrazine [2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine], simazine [2-chloro-4,6-bis(ethylamino)-1,3,5-triazine], cyanazine), phenylureas (linuron, diuron), or phenols (e.g., ioxynil, bromoxynil, Draber et al., 1991). Triazine herbicides continue to be used every year in large quantities. For example, in the United States, approximately about $35 \times 10^6$ kg of atrazine, $9 \times 10^6$ kg of cyanazine, and $3 \times 10^6$ kg of simazine are applied every year (U.S. Environmental Protection Agency http://www.epa.gov/oppbeadl/pestsales/97pestsales/table8.htm).

This practice frequently leads to soil contamination and the subsequent pollution of surface and ground water. Triazines persist in water and represent some of the most frequently detected pesticides in ground water.

These concerns have stimulated research towards development of sensitive methods and technology to detect pesticide residues. Chromatographic methods high-performance liquid chromatography, gas chromatography...
and gas chromatography/mass spectrometry are reliable and are most commonly used to monitor the presence of herbicides (Pacáková et al., 1996). The requirement for expensive equipment, organic solvents, and laborious sample preparation hampers the use of chromatography for rapid screening of a large number of samples. For this reason, attention has been focused on immunochemical methods for herbicide detection (Bushway et al., 1991) offering high sensitivity and specificity of detection (Giersch, 1993; Schneider et al., 1994; Schneider and Hammock, 1992). The disadvantage of the immunological methods, however, is that the antibodies bind specifically to only one compound or a few structural analogues. Moreover, their preparation is difficult.

Recognition of the fact that a large number of herbicides inhibit PSII activity (Morelând, 1992) has resulted in its use as an analytical tool (bioassay) for designing of new compounds (Good, 1961; Wessels and Van der Veen, 1956; or detecting herbicides (Brewster and Lightfield, 1993; Conrad et al., 1993; Giardi et al., 2000; Loranger and Carpentier, 1994; Merz et al., 1996; Rouillon et al., 1995; Soukupová et al., 1999). Biosensors are analytical devices possessing a biological component that provides high sensitivity and/or specificity and a physicochemical transducer recording the signal (Turner, 2000). Biosensor technology has created new ways for simple analysis in a cost-effective manner.

Previously, we constructed a biosensor by immobilization of PSII complex on a Clark oxygen electrode. The system exhibited a good stability at laboratory temperature as well as high sensitivity to herbicides (Koblížek et al., 1998). However, the use of Clark electrode as the transducer prevents simple and potentially mass production of this system. For this reason, screen printing was chosen as suitable technology offering batch production of electrochemical biosensors with high reproducibility at low price. The electrochemical sensors are made by sequential, multi-layer deposition of metal conductors (Pt, Au, Ag, Pd, Rh, Ru, Ti), dielectric insulators (Al or Zn oxide powders), and polymeric pastes on inorganic (Al2O3 ceramic) or polymeric [polyvinylchloride (PVC)] substrates. The layout drawn on the open mesh of a screen is transferred onto a substrate, providing two-dimensional microcircuits and electrodes (Karlb erg and Pacey, 1989; Prudenziati, 1994; Scheller and Schubert, 1992).

In this article, we present a herbicide biosensor based on isolated PSII particles immobilized to a graphite-Ag/AgCl screen-printed electrode. The system exhibits selective sensitivity to phenylurea and triazine herbicides, whereas phenolic herbicides are not registered. The use of screen-printed electrodes allows a cheap, large-scale production of these devices.

**MATERIAL AND METHODS**

**Printed Electrodes**

Printed electrodes were fabricated by depositing several layers of inks on a PVC substrate (Fig. 1). The conducting paths and pads were deposited directly on the PVC sheets using the Ag/Pd ink (DuPont, 5025). The second layer was printed with graphite (DuPont 7101, Acheson 423S) to obtain the working electrode. For the reference electrode, the metallic layer was printed using the same Ag/Pd ink as used for the conducting paths. Finally, an insulator layer was placed over the conducting paths. After each printing step, the pastes were left overnight at laboratory temperature or treated at 80°C for 20 min.

**Preparation and Immobilization of PSII Particles**

The PSII particles were prepared from the thermophilic cyanobacterium *Synechococcus elongatus* (cells grown at 56°C) as described previously (Koblížek et al., 1998; Šetlíková et al., 1999). After cells were digested with lysozyme, the protoplasts were disrupted by osmotic shock. Thylakoid membranes were solubilized with the non-ionic detergent heptylthioglucoside using a detergent/chlorophyll (Chl) ratio of 8. After ultracentrifugation at 250,000 g for 30 min, the supernatant of the PSII particles usually contained between 200 and 400 mg Chl L⁻¹.

To assure a firm coupling of the PSII particles to the electrode surface, several immobilization techniques were tested, the first of which was entrapment into gelatin. Gelatin (10%) in the measuring buffer [15 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 6.5, containing 0.5 M mannitol, 0.1 M NaCl, 5 mM MgCl₂, and 5 × 10⁻⁵ M chloramphenicol] was left for several hours to swell. The mixture was warmed up to about 50°C and intensively mixed with an equal volume of the PSII preparation. Then, about 5 μL of the mixture was placed on the surface of the working electrode. The second technique used was entrapment into agarose.

**Figure 1.** Scheme of a screen-printed electrode preparation. The printing starts with the deposition of conducting paths and pads using Ag/Pd inks (B) on the PVC substrate (A). Then, the working electrode is printed using graphite inks (C) and the reference electrode is made using Ag/Pd ink (D). Finally, the conducting paths were covered by an insulator layer (E). The final layout of a single PVC graphite working and Ag/AgCl reference electrode (F).
The procedure was the same as the previous one, except that 3% agarose (low melting point; Sigma Chemical Co., St. Louis, MO.) was used instead of gelatin. The third technique used was entrapment into calcium alginate. The immobilization procedure was similar to the one described by Synkova´ et al. (1990) and Brewster et al. (1995). A 3.5% solution of alginate was dissolved in the measuring buffer and mixed with an equal volume of the PSII preparation. About 5 µL of the mixture was placed on the surface of the working electrode. Then, 5 µL of 50 mM CaCl₂ was added to solidify the mixture. In the case of calcium alginate immobilization, 20 mM CaCl₂ was included into the measuring buffer. The last technique used was crosslinking into bovine serum albumin (BSA)–glutaraldehyde matrix. The immobilization procedure was similar as described for chloroplasts and thylakoid membranes (Loranger and Carpentier, 1994; Synková and Šesták, 1991; Thomasset et al., 1988). A 10% solution of BSA in the measuring buffer was mixed with equal volume of the PSII preparation. Then, 10% glutaraldehyde was added to a final concentration of 0.3%. The suspension was mixed and approximately 5 µL was placed on the surface of the working electrode. In all the cases, the prepared sensors were stored in a freezer (–20°C) until used.

Biosensor Assembly, Flow Cell and Apparatus

The biosensor was prepared by immobilizing the PSII particles (0.5 to 1 µg Chl, ~10⁻¹¹ mol PSII) on the graphite (working) electrode surface as described above. The graphite electrode was polarized at 0.62 V for duroquinone (DQ; 0.2 mM) or 0.36 V for ferricyanide (FeCy; 1 mM) to the reference Ag/AgCl electrode. Changes of the current intensity on the graphite electrode were registered by a potentiostat (Metrohm 641, Herisau, Switzerland). Impurities were removed during the initial stabilization under the constant potential (20–40 min). No other special (electrochemical) treatment of the electrode surface was used.

The biosensor was mounted into a custom-made flow cell with the illumination provided by a single light-emitting diode (ultrabright, peak wavelength at 650 nm) controlled by an electronic timer. The duration of light pulses was set to 5 s at the light intensity of about 100 µmol photons m⁻²·s⁻¹. The volume of the flow-cell was adjusted by a silicon spacer (0.5 mm) as shown in Figure 2. The electrode was continuously washed with the measuring buffer (see above) plus an electron acceptor. The flow of the buffer (0.25 mL·min⁻¹) was driven by a peristaltic pump (type MiniPulse 3, Gilson).

RESULTS

Method of Measurement

The measurement of the PSII activity was based on potentiostatic (amperometric) registration of the reduced form of artificial electron acceptors, DQ, or FeCy. The acceptor was present in the measuring buffer in its oxidized form. Thus, there was only a small background signal in the dark (about 20 nA), which was more stable (low noise) as compared to the biosensor based on Clark oxygen electrode previously described (Kobližek et al., 1998). Under illumination, the immobilized PSII complex splits water, releases oxygen, and reduces the artificial electron acceptor. The reduced electron acceptor (DQ, FeCy) was re-oxidized on the surface of the graphite working electrode. The resulting increase of the current was proportional to PSII activity. Response signals were about 5 nA when using 0.2 mM DQ or 15 nA for 1 mM FeCy used as electron acceptors.

Immobilization and Stability Optimization

Immobilization ensures the spatial coupling of the PSII preparation with the transduction system. We tested four immobilization techniques: entrapment in agarose, alginate, or gelatin gels and cross-linking into the BSA–glutaraldehyde matrix. The immobilization techniques were first characterized by the half-life of the constructed biosensor. The entrapments in agarose and alginate failed in this test because these gels exhibited only poor adhesion to the electrode surface and were therefore washed out rapidly. Better results were obtained...
when the PSII particles were entrapped in gelatin or cross-linked into the BSA–glutaraldehyde matrix. Both the latter techniques provided biosensors with a half-life of about 8 h in the presence of DQ (Table I). On the other hand, the half-lives of the biosensors were much shorter than 30 h for the PSII preparation itself (Koblížek et al., 1998). The lower stability of the sensors was probably caused by Ag\(^+\) ions released from the reference electrode, which are toxic for the PSII activity. This assumption was verified by spatial separation of the working and reference electrodes, placing the latter one down-stream to the flow cell. Thus, the Ag\(^+\) ions released from the reference electrode do not come into the contact with the PSII particles immobilized on the working electrode. Indeed, when set-up this way, the half-life of the biosensor was extended to 24 h, which was similar to that of the PSII preparation (Table I).

### Detection of Herbicides

The presence of herbicides in a solution was detected as a decrease of the biosensor signal in the presence of DQ compared to the activity in the absence of the herbicide. Using a three-way valve, it was possible to switch among the buffer and the sample/standard reservoirs. When the sample (about 5 mL) containing herbicide \{diuron\-[3-(3,4-dichlorophenyl)-1,1-dimethylurea], atrazine, simazine, ioxynil [4-hydroxy-3,5-diiodobezonitrile], bromoxynil [3,5-dibromo-4-hydroxybenzonitrile], or dinoseb [2-sec-butyl-4,6-dinitrophenol]\} was loaded into the cell, the resulting light-induced activity was measured after 20 min (see Fig. 3). The ratio of the signals (expressed in percent) in the presence and absence of herbicide was plotted against herbicide content in the sample (Figs. 4 and 5). The measured value was corrected for about 1% decline in the sensor activity over the 20 min measurement due to its natural decomposition (24 h half-life). Then, the herbicide was washed out using the measuring buffer and the biosensor used for a new measurement. The repeated use of the biosensor did not affect the reproducibility of the measurement when the BSA–glutaraldehyde immobilization was used. This, however, was not possible in case of the immobilization into gelatin (see Table I). Therefore, only the BSA–glutaraldehyde immobilization was used for further measurements.

### Calibration Curves

Calibration curves for each herbicide were analyzed using the Langmuir adsorption isotherm:

\[
act = \frac{100}{C_0} - \frac{100}{C_0 + \frac{[H]}{I_{50} + [H]}}
\]

where \(act\) is residual activity of the biosensor (in percent) after addition of the herbicide, \([H]\) is concentration of the herbicide in the solution, and \(I_{50}\) is the concentration causing a 50% inhibition of the activity (equal to the PSII–herbicide complex dissociation constant). The limit of the detection (LOD) was determined on the basis of 99% confidence interval, which, assuming the normal distribution, corresponds to \(2.6 \times \) standard error of the measurement (\(\sigma\)). Then, using the modified relationship for the Langmuir adsorption isotherm LOD was calculated as

\[
LOD = 2.6 \times \sigma \times I_{50}/(100 - 2.6 \times \sigma)
\]

### Table I. Comparison of immobilization techniques.

<table>
<thead>
<tr>
<th>Immobilization method</th>
<th>Half-life (h)</th>
<th>Herbicide detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alginate (coupled electrodes)</td>
<td>&lt;1</td>
<td>Not tested</td>
</tr>
<tr>
<td>Agarose (coupled electrodes)</td>
<td>&lt;1</td>
<td>Not tested</td>
</tr>
<tr>
<td>Gelatin (coupled electrodes)</td>
<td>7.5</td>
<td>Not reproducible</td>
</tr>
<tr>
<td>BSA–glutaraldehyde (coupled electrodes)</td>
<td>8</td>
<td>Reproducible</td>
</tr>
<tr>
<td>BSA–glutaraldehyde (separated electrodes)</td>
<td>24</td>
<td>Reproducible</td>
</tr>
</tbody>
</table>

Stability was characterized as half-life of the biosensor activity at laboratory temperature. The measurement was performed at 25°C in the presence of 0.2 mM DQ. The reproducibility of the detection was tested by sequential measurements of a sample containing 10\(^{-7}\) M of diuron.
The results of the analysis are shown in Table II.

Using the BSA–glutaraldehyde immobilization system, an important observation was made. The system was found sensitive to classical herbicides (diuron, atrazine, simazine) but it had only low sensitivity to phenolic-type herbicides (dinoseb, ioxynil, bromoxynil). The detection limit for phenolic herbicides was more than an order of magnitude higher when compared with the system using the physical entrapment of the PSII particles (Table II; Kobližek et al., 1998).

The herbicide detection was also tested in the presence of FeCy as an artificial electron acceptor. In this case, the biosensor exhibited about three times higher signals as compared to the signals obtained with DQ. The calibration curve for atrazine showed that about 50% of the biosensor activity could not be blocked even at very high herbicide concentrations. It was found that one half of the activity was herbicide-sensitive and could be characterized by the Langmuir isotherm ($I_{50} = 10^{-7} \text{M}$), and the other part of the activity was herbicide insensitive (Fig. 5). Because this feature reduced the amplitude of the signal measured and, in this way affected the sensitivity of the assay, only DQ was considered well suited for the measurements.

**DISCUSSION**

We have developed a sensitive biosensor for detection of classical photosynthetic herbicides. The biosensor was constructed using screen-printed electrodes with immobilized PSII particles as the biosensing element. The use of PSII preparation from *S. elongatus* assured higher stability of the biological part of the biosensor (Kobližek et al., 1998). The PSII activity was measured as a rate of the photoreduction of an artificial electron acceptor.

![Figure 4. Calibration curves for diuron and simazine in the presence of 0.2 mM DQ. The residual activity (in %) was calculated as the ratio of signals in the presence/absence of the herbicide. The experimental points were fitted using the Langmuir adsorption isotherm yielding the $I_{50}$ values of $7 \times 10^{-8} \text{M}$ for diuron ($R^2 = 0.991$) and of $2 \times 10^{-7} \text{M}$ for simazine ($R^2 = 0.993$).](image1)

![Figure 5. Calibration curves for atrazine in the presence of 0.2 mM DQ (left-hand panel) and of 1 mM FeCy (right-hand panel). The residual activity (in %) of the biosensor was calculated as the ratio of the signal obtained in the presence and absence of the herbicide. The experimental points were fitted using the Langmuir adsorption isotherm yielding the $I_{50}$ dose of $9 \times 10^{-9} \text{M}$ in the presence of DQ ($R^2 = 0.995$). In the presence of FeCy about 50% of the activity was herbicide-insensitive and $I_{50}$ was $1 \times 10^{-7} \text{M}$ ($R^2 = 0.980$).](image2)

<table>
<thead>
<tr>
<th>Herbicide</th>
<th>Screen-printed sensor</th>
<th>Clark electrode$^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$I_{50}$ (M)</td>
<td>LOD (M)</td>
</tr>
<tr>
<td>Classical</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diuron</td>
<td>$7 \times 10^{-8}$</td>
<td>$1 \times 10^{-9}$</td>
</tr>
<tr>
<td>Atrazine</td>
<td>$9 \times 10^{-8}$</td>
<td>$2 \times 10^{-9}$</td>
</tr>
<tr>
<td>Simazine</td>
<td>$2 \times 10^{-7}$</td>
<td>$4 \times 10^{-9}$</td>
</tr>
<tr>
<td>Phenolic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ioxynil</td>
<td>n.d.</td>
<td>~$10^{-7}$</td>
</tr>
<tr>
<td>Bromoxynil</td>
<td>n.d.</td>
<td>~$10^{-6}$</td>
</tr>
<tr>
<td>Dinoseb</td>
<td>n.d.</td>
<td>~$10^{-6}$</td>
</tr>
</tbody>
</table>

$^*$Data from Kobližek et al. 1998.

n.d. = not determined.

The biosensor activity was measured in the presence of 0.2 mM DQ at 25°C. $I_{50}$ represents a herbicide concentration causing a 50% inhibition of the initial biosensor activity (in the absence of herbicide). The data for the screen-printed sensor (this work) were obtained by fitting the experimental points using the Langmuir adsorption isotherm. In this model, the $I_{50}$ value is equal to the dissociation constant of herbicide. The detection limits were determined on basis of the 99% confidence interval calculated from the standard error of the measurement. For comparison, the data reported previously for the Clark electrode sensor are shown in the right part of the table (Kobližek et al., 1998).
(DQ), which was registered as an increase in the response to light pulses.

The close coupling of the biosensing element (PSII) and the transducer (electrode) improved the measured signal and required smaller amount of the biological material in comparison with standard batch detection systems. The use of the flow-through set-up made it possible to re-use the biosensor for several analyses after washing out the inhibitor. Among the four immobilization techniques tested, only cross-linking into the BSA–glutaraldehyde matrix provided a convenient system in terms of stability and reproducibility of the measurement. The system displayed good sensitivity to classical herbicides (diuron, atrazine and simazine) but significantly reduced sensitivity to phenolic herbicides (ioxynil, bromoxynil, and dinoseb). This feature was likely caused by a very low diffusion rate of the phenolic herbicides in the BSA–glutaraldehyde matrix or a steric hindrance and modified specificity of the cross-linked PSII. When the BSA–glutaraldehyde cross-linking was replaced by the gelatin entrapment, the inhibitory effect of phenolic herbicides was restored (data not shown). On the basis of different sensitivity, the classical and phenolic herbicides can be distinguished by this biosensor system.

In general, the sensitivity and the detection limit of the biosensor are given by the binding constant of the herbicide which is determined by its chemical structure and by the architecture of the Qb pocket (Sobolev and Edelman, 1995; Trebst, 1986). This major factor was, in our case, further modulated by the characteristics of the immobilization system. At low concentrations of herbicides the recorded decrease of the PSII activity is minimal. Thus, the maximal signal-to-noise ratio is necessary. In the system described here, this goal was achieved by two ways. First, we used isolated PSII particles with higher specific activity compared to more frequently used chloroplasts or thylakoid membranes. Second, the PSII particles were immobilized on the surface of the transducer (electrode). This set-up assures a high concentration of PSII complexes in the micro-environment around the electrode, which in turn, results in a good, stable signal. This approach also made it possible to use a very low amount of PSII (~10^{-11} mol). The Langmuir adsorption isotherm assumes an excess of the free compound with respect to the number of binding places. At very low herbicide concentrations this assumption is not always met and the number of herbicide molecules in the sample starts to be limiting (5 mL of 1 nM herbicide corresponds to 5 × 10^{-12} mol). In this case, the calibration curve deviates from the model and the signal underestimates the true herbicide concentration. Thus, the amount of PSII used in the assay is critical for detection of very low herbicide concentrations.

Other substances (e.g., heavy metals) can also inhibit the activity of the PSII biosensor; however, their effect is usually found at much higher concentrations than those typical for herbicides. For instance, copper, one of the most phytotoxic heavy metals (Küpper et al., 1996), starts to inhibit the biosensor activity at concentrations above 10^{-6} M. This concentration is by two to three orders of magnitude higher than the limit of detection found for herbicides. Moreover, contrary to herbicides, the inhibition of the PSII activity by Cu^{2+} ions is irreversible and therefore, it can be distinguished by its distinct kinetic behavior (Rizzuto and Koblížek, unpublished data).

An interesting finding was the incomplete inhibition of PSII activity of the biosensor by atrazine observed in the presence of FeCy (Fig. 5). Because the binding constant (I_{50}) remained practically unchanged, it seems that the affinity of PSII towards atrazine did not change. Similar phenomenon was observed earlier with isolated thylakoid membranes from a thermophilic cyanobacterium (Yamaoka et al., 1978). A possible explanation is, that, in the presence of FeCy, there exists an alternative herbicide-insensitive pathway of PSII reoxidation bypassing the Qb pocket (herbicide binding site) and operating at 50% of the rate in the absence of herbicides.

In conclusion, the developed biosensor offers the sensitive detection method for triazine and phenylurea herbicides. In comparison to other analytical techniques, such as chromatography and immunological methods, the PSII-based biosensors have opened a possibility to be used for rapid field detection of herbicides, which in addition allows assessment of their biological effect (phytotoxicity). They can be successfully applied for prescreening environmental samples, indicating which samples require detailed analysis by other analytical techniques.

References

KOBLIŽEK ET AL.: PHOTOSYSTEM II-BASED BIOSENSOR 115


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Detection of phenolic compounds by thick film sensors based on *Pseudomonas putida*

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**Abstract**

Amperometric biosensors using bacterial cells were developed for the determination of phenolic compounds and the measurement was based on the respiratory activity of the cells. For this purpose, *Pseudomonas putida* DSM 50026 which is one of the well-known phenol degrading organisms, was used as a biological component. The cells were grown in the presence of phenol as the sole source of organic carbon. As well as phenol adapted cells, the bacterium which used the glucose as the major carbon source, was also used to obtain another type of biosensor for the comparison of the responses and specificities towards different xenobiotics. The commercial oxygen electrode was used as a transducer to test the sensor responses for both induced and non-induced cells. Our results showed that the adaptation step enable us to obtain biosensor devices with different substrate specificity. Moreover, *P. putida* was immobilized on the surface of thick film working electrodes made of gold by using gelatin membrane cross-linked with glutaraldehyde. The biosensors were calibrated for different phenolic substances. Furthermore, phenol detection was performed in synthetic wastewater samples.

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**Keywords:** Microbial biosensor; Thick film electrodes; *Pseudomonas putida*

1. Introduction

The intensive increase in the release of a diverse range of hazardous compounds into the environment has made their detection of paramount importance. In particular, this is necessary for successful clean-up of pollution from the environment and timely elimination of the consequences or a reduction of the scale of hazardous release [1]. Phenolic compounds are one of the major pollutants of industrial waste waters and, at the same time, the compounds have high toxicity to the human organism when present above certain concentration limits this require rapid, easy to operate and low-cost toxicity screening procedures. The trend towards simplification and automation of the analysis methods used in modern
chemical and biochemical laboratories or in quality control of some industrial biosynthesis processes has led to setting up some electrometric procedures for determining phenol, based on biosensors [2–4]. The use of microbial biosensors to determine the concentrations of substances is based on the presence of specific enzyme systems in microorganisms which transform certain chemical compounds. The transformation processes can be accompanied by the appearance of electrochemically active products or utilization of reaction co-substrates, which enable the use of standard electrochemical techniques—amperometry or potentiometry [5]. As judged by their sensitivity, time of response and stability of signals, microbial sensors are similar to enzyme based sensors but are less selective. This may be due to the complexity of the elements of the enzyme apparatus of cells. Insignificant amount of biomass as well as high stability make the use of microbial sensors preferable in some cases compared to enzyme sensors. This is especially true in the detection of a pool of toxic compounds showing similar composition, or the assessment of comprehensive indices of the condition of the environment as, for instance, biological oxygen demand (BOD) [6]. In this study, amperometric biosensors using bacterial cells were developed for the detection of phenolic compounds. P. putida DSM 50026 was used as a biological component and the measurement was based on the respiratory activity of the cells. For this purpose, the cells were grown in the presence of phenol as the sole source of organic carbon. As well as phenol adapted cells, the bacterium which used the glucose as the major carbon source, was also used to obtain another type of biosensor for the comparison of the responses and specificities towards to different xenobiotics.

2. Experimental

2.1. Reagents

All chemicals were commercially available and of reagent grade. L-DOPA, L-tyrosine, syringic acid, caffeic acid, resorcin, picric acid, hydroquinon, 2,6-dihydroxybenzoic acid, benzoic acid were products of FLUKA AG (Switzerland). Phenol was from Merck AG (Darmstadt, Germany).

Mineral salts medium (MSM) with the following composition [7] was used as a growth medium: 0.1% NH₄NO₃, 0.05% (NH₄)₂SO₄, 0.05% NaCl, 0.05% MgSO₄·7H₂O, 0.15% K₂HPO₄, 0.05% KH₂PO₄, 0.0014% CaCl₂·2H₂O, 0.001% FeSO₄·7H₂O and trace element solution (1 ml/l). The pH of the medium is 6.9. Trace element solution was prepared according to Ref. [8].

Synthetically concocted waste water compositions: Sample type 1 composed of (NH₄)₂SO₄ (0.5 g), MgSO₄ (0.1 g), MnSO₄ (0.01 g), FeSO₄ (0.0005 g) and known amounts of phenol in tap water (100 ml), pH 7.2 [9]. Sample type 2 included NaCl (50 g/l) and phenol (100 g/l) in 1 M HCl solution [10].

2.2. Microorganism and culturing conditions

P. putida DSM 50026, which is a gram negative, polar, flagelled unicellular bacteria was sub-cultured on Nutrient Agar.

To obtain non-adapted, control cells which use glucose as a carbon source, the microorganism was inoculated into 50 ml of MSM containing 250 mg/l glucose and incubated at 28°C on an orbital shaker at 150 rpm. After 24 h, when cells were grown, the biomass was harvested by centrifugation at 10,000 rpm and suspended in MSM and then re-centrifuged. The supernatant was removed and the cellular paste was used for making biosensor.

Induction of the cells to phenol (250 mg/l) was performed with the following steps; the wild type microorganism was inoculated to the MSM medium containing gradually increasing phenol and decreasing glucose concentrations by daily inoculations until the medium reached 250 mg/l of phenol. After adaptation was completed, the cellular paste was obtained as described above.

Cell growth was followed spectrophotometrically by measuring optical density at 560 nm and the relationship between optical density and the living cells was also investigated [11]. In all experiments, log-phase bacterial cells (OD₅60 = 0.450) were used.
2.3. Apparatus

Clark electrode (Oxiliquid mod.22 IDRO-NAUT, Brugherio MI, Pt diameter; 1 mm) covered with a Teflon membrane (Beckman, no 670597) and ceramic thick film sensors (BVT Technologies a.s., Czech Republic) with gold working, Ag/AgCl reference and Pt auxiliary electrodes were used, respectively. The changes in current were measured with a Potentiostat (Metrohm 641, Herisau, Sweden).

2.4. Electrode preparation

*P. putida* cells which have $1.8 \times 10^9$ cell titer (50 μl) and 225 bloom gelatin (10 mg) were mixed at 38 °C in MSM (250 μl). 50 μl of mixed solution was spread over the membrane of the oxygen electrode and allowed to dry at 4 °C for 1 h. Finally, it was immersed in 2.5% glutaraldehyde in 50 mM phosphate buffer (pH 7.5) for 5 min [12]. Moreover, the procedure belong to thick film sensors was the same as the previous one, except 2.5 μl of mixed solution was placed on gold working electrode of ceramic thick films. Daily prepared enzyme electrodes including fresh cells in logarithmic phase were used in all experimental steps.

2.5. Measurements of the respiratory activity by the microbial electrodes

All measurements were carried out at 28 °C which is the growth temperature of the bacteria. Various phenolic substrates were added individually to the reaction cell. When a respirable substrate was added, the oxygen concentration in the bioactive layer decreased until new steady state was reached within 5–10 min. The Working electrodes of both, commercial Clark and screen printed ceramic electrode, were polarized at −700 mV vs Ag/AgCl. The current changes were recorded. After completion of the measurement in the 5 min of reaction time, the electrode was rinsed with distilled water and allowed to equilibrate before another measurement. MSM, pH 6.9 was used as a working buffer.

3. Results and discussion

3.1. Optimization of the sensor response

The sensor response to phenol was tested in the presence of potassium phosphate buffer (50 mM, pH 6.9) and MSM (pH 6.9), respectively by adapted cells immobilized on Clark electrode (Fig. 1). The higher signal was observed in MSM which was also used as a growth medium for the bacteria and then, MSM was chosen as a working buffer solution in further experiments. During the measurements, to protect metabolic activity was the most important point. For this reason the other buffer system with different pH values and also different temperatures were not used. On the other hand, working temperature and pH were also similar with the previous works [13,14]. In these experimental conditions, the response time of microbial sensor based on Clark electrode and thick film were found to be 10 and 5 min, respectively.

10, 25, 50 and 100 μl of bacterial cell which have the same cell titer given in section 2.4 were also

![Graph](image-url)
used to prepare the immobilization mixture and the sensor responses were measured in the presence of 1.0 mM phenol concentration by biosensor based on Clark electrode. Maximum response was found to be in 50 µl cell amount and higher amounts caused to decrease in signal because of the diffusion problem.

### 3.2. Substrate specificities

The substrate specificities of adapted and control cells to different phenolic compounds were tested by the oxygen electrode as a transducer and given in Table 1. As can be seen, both adapted and control cells showed the response towards phenol, 2,6-dihydroxybenzoic acid and benzoic acid. However, apart from these compounds, while adapted cells are giving the response to DOPA, tyrosine, syringic acid, resorcin, picric acid, methyl-parathion, no detectable signal was observed for these compounds by the biosensor including control cells. This circumstance can be explained by the adaptation effect that caused different substrate specificities in bacterial cells. In *P. putida*, phenol degradation depends on producing the phenol hydroxylase and further metabolic enzymes, produced only in the presence of their substrates. While phenol adapted cells can use phenol as a sole carbon source and metabolize it at high levels, non-adapted cells were exposed to phenol at quite low concentrations, their response to phenol can be more rapid than phenol adapted cells.

### 3.3. Thick film sensors

In this part of the study, thick films with gold working electrode were used as a basic sensor for the determination of detection limits of phenolic compounds. Bacterial cell which are in same age were directly immobilized by means of gelatin cross linked with glutaraldehyde. Decrease of the current because of the oxygen consumption was easily detected without requiring the gas permeable selective membrane. Instead of gold surface we also used screen printed graphite electrode to test the sensor response. However, in this case surface modification with the cellulose acetate membrane was necessary to obtain better signals without noise (unpublished data).

The calibration curves for phenol, catechol, hydroquinone, benzoic acid and 1,2-dihydroxynaphthalene were obtained by control cells immobilized on gold working electrode on ceramic substrates (Table 2). For these compounds linear detection ranges were found (Table 2) and above these levels, inhibition was observed. On the other hand, adapted cells were used to obtain calibration graphs for phenol, L-tyrosine, L-DOPA, L-syringic acid and methyl-parathion (Table 2). Detection ranges for phenol and parathion-methyl were found to be 0.5–6.0 µM and 0.3–2.5 µM. Moreover, L-tyrosine, L-DOPA, L-syringic acid were determined in the same range (0.02–0.2 µM). In the higher concentrations of these compounds, sensor response remained steady. This could be due to the adaptation process which caused the higher resistance towards the xenobiotics in bacterial cells [15].

<table>
<thead>
<tr>
<th>Phenolic compound</th>
<th>Signal, current (I, nA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenol</td>
<td>6.7 ± 0.3</td>
</tr>
<tr>
<td>Catechol</td>
<td>23.6 ± 0.3</td>
</tr>
<tr>
<td>L-DOPA</td>
<td>30.3 ± 0.3</td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>38.2 ± 0.3</td>
</tr>
<tr>
<td>L-Syringic acid</td>
<td>34.2 ± 0.6</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>112.1 ± 0.2</td>
</tr>
<tr>
<td>Hydroquinone</td>
<td>27.8 ± 0.3</td>
</tr>
<tr>
<td>Adrenaline</td>
<td>20.9 ± 0.3</td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>38.4 ± 0.3</td>
</tr>
<tr>
<td>Resorcin</td>
<td>15.5 ± 0.4</td>
</tr>
<tr>
<td>Picric acid</td>
<td>3-Hydroxytyramine</td>
</tr>
<tr>
<td></td>
<td>0.62 ± 0.01</td>
</tr>
<tr>
<td>2,6-Dihydroxybenzoic acid</td>
<td>10.3 ± 0.3</td>
</tr>
<tr>
<td>2,3-Dihydroxynaphthalene</td>
<td>27.8 ± 0.3</td>
</tr>
<tr>
<td>Toluen</td>
<td>18.2 ± 0.3</td>
</tr>
<tr>
<td>Methyl-parathion</td>
<td>11.4 ± 0.3</td>
</tr>
</tbody>
</table>

* Results are expressed as mean ± S.D, n = 4.
* The concentration of substances in reaction mixture; 1 mM.
Operational lifetime of thick film sensors were also tested in MSM, 28°C. In these conditions, during 6 h, 25 measurements could be performed without any decrease in sensor response. After that, reduced signals were observed. Because of this reason, to obtain similar results, each electrode was prepared daily.

The reproducibility of the thick films based on adapted and control cell were also searched. For adapted cell, 2.0 mM (n=7) phenol concentration was tested and the S.D. and variation coefficient (C.V) were calculated as ±0.09 mM and 4.4%. Moreover, these values were found as ±0.02 mM and 3.9% for control cell in the presence of 0.5 mM (n=7) phenol concentration, respectively.

### 3.4. Sample application

The microbial biosensors were applied in waste water samples. For this aim, two different types of synthetic waste water sample were prepared as described in material and methods. Both types of sample which included known amount of phenol were used as stock substrate solutions with different dilutions by working buffer and added to the reaction cell after equilibration and then the change in current was measured. The signals obtained from the waste samples were found to be very similar with the standard phenol solutions having the same concentration (Table 3). The results showed that, no sample matrix effect interfered in our measurements. Both type of cell could be used the presence of these kinds of waste water samples without requiring sample pretreatment.

Enzymes with a broad substrate spectrum, i.e. laccase, tyrosinase, polyphenol oxidase and peroxidase as well as specific enzymes, e.g. phenol hydroxylase and catechol oxidase, may be used for construction of phenol biosensors [16,17]. The tyrosinase electrodes are often unstable due to fouling of electrodes by accumulation of polymerization products formed by the resulting quinones. Another reason may be inactivation of enzymes by

### Table 2

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Linear range (µM)</th>
<th>Slope (mA·M⁻¹)</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenol</td>
<td>0.10–1.00</td>
<td>16.54</td>
<td>0.9986</td>
</tr>
<tr>
<td>Catechol</td>
<td>0.50–5.00</td>
<td>5.07</td>
<td>0.9971</td>
</tr>
<tr>
<td>Hydroquinone</td>
<td>0.25–1.00</td>
<td>15.20</td>
<td>0.9972</td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>0.10–0.75</td>
<td>15.82</td>
<td>0.9981</td>
</tr>
<tr>
<td>1,2-Dihydroxynaphthalene</td>
<td>0.25–2.50</td>
<td>8.01</td>
<td>0.9992</td>
</tr>
<tr>
<td>Adapted cells</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Phenol</td>
<td>0.50–6.00</td>
<td>5.03</td>
<td>0.9995</td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>0.02–0.20</td>
<td>87.08</td>
<td>0.9988</td>
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<td>L-Syringic acid</td>
<td>0.02–0.20</td>
<td>37.01</td>
<td>0.9852</td>
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<tr>
<td>L-DOPA</td>
<td>0.02–0.20</td>
<td>61.88</td>
<td>0.9989</td>
</tr>
<tr>
<td>Methyl-parathion</td>
<td>0.30–2.50</td>
<td>3.64</td>
<td>0.9917</td>
</tr>
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</table>

### Table 3

<table>
<thead>
<tr>
<th>Phenol concentration (µM)</th>
<th>Detected amount (µM)</th>
<th>Adapted cell</th>
<th>Control cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample type 1</td>
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<td></td>
</tr>
<tr>
<td>0.25</td>
<td>0.24±0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.50</td>
<td>0.52±0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>1.01±0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>1.98±0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample type 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.25</td>
<td>0.25±0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.50</td>
<td>0.51±0.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>1.01±0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>2.02±0.03</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Results are expressed as mean±S.D, n=7.
these reactive products [18]. In spite of this fact, most biosensors for detection of phenols employ tyrosinase as biorecognition material. Another biosensor for phenol detection was based on phenol hydroxylase entrapped in a sol/gel matrix linked to a Clark-type oxygen electrode. The limit for detection of phenol demonstrated by the sensor was 2.5 mM and the injection of cofactor to the medium was required. Alternatively, whole cells and plant tissues have been combined with various electrodes. Microbial sensors are inexpensive and easily produced, possess the sensitivity and stability comparable with those of enzyme sensors and no additional efforts are needed for purification of enzyme [19].

From the practical point of view, to characterize the substrate selectivity of sensors is very important. The selectivity of microbial sensors is known to be generally rather low, and researchers are looking for new ways to improve it [20]. In our case, biosensors with different selectivity for detection of phenol based on whole cells were obtained by adaptation process. Our findings showed that, adaptation process affected to substrate specificity but, higher sensitivity was not observed by this way. In further studies, adaptation will be performed with different amounts of xenobiotics to obtain biosensors having different selectivity.

4. Conclusions

Actually biotechnological processes have been employed in several industrial productions, in biomedical applications and in environmental remediation. Interest was focused on biomediators as purified enzymes or metabolic pathways of cells, tissues etc. with specific biochemical properties for analytical and either production or degradation applications [21]. In this study, development of P. putida based biosensors for determining the phenolic compounds. Bacterial cells with and without adaptation steps enable us to get biosensor systems with different substrate specificities, thus it could be possible to analyze different phenolic substances individually in the mixed samples by using these sensors. On the other hand, in some cases adaptation may eliminate the necessity of using genetically manipulating microorganisms.

In previous studies, different types of microorganisms were used in biosensor systems [22–25]. In comparison to the others [20], our biosensors have higher sensitivity as microbial sensor. Furthermore, immobilization of microorganisms instead of pure enzymes on the thick film electrodes provided economic and practical disposable biosensors. The immobilization method was also useful for the protection of microbial activity. All our data showed that the obtained biosensors could be used as simple, rapid and direct methods of determining phenol in various media such as waste water samples with different compositions.

References

Lesigenze del mercato globale e dello sviluppo sostenibile richiedono un sensibile sforzo per incrementare le attuali potenzialità di misura nei campi della qualità dei prodotti, dei controlli ambientali e della protezione sanitaria. La sensoristica rappresenta uno strumento potente e sempre più necessario per rispondere a queste esigenze, consentendo la riduzione dei tempi e dei costi rispetto alle analisi tradizionali. In questo quadro negli ultimi due decenni c'è stato un notevole incremento di attività di ricerca e sviluppo sui sensori.

L'interesse per queste tematiche ha determinato l'avvio di progetti comunitari (IV e V Programmi quadro) mentre anche a livello nazionale si ricordano rilevanti iniziative quali:

- Progetti finanziati dal MURST (ad esempio l'intesa con l'ENEA per lo sviluppo di un Centro Nazionale Ricerca e Sviluppo Materiali ed in particolare la linea "Rivestimenti funzionali: sviluppo di sensori chimici innovativi basati su tecnologie avanzate di produzione")
- Progetti Finalizzati del CNR (ad esempio quello Biotecnologico e quello sui Materiali).

In questo quadro di crescente interesse si è svolto, per iniziativa comune dell’ Enea, dell’ Universita’ di Roma "La Sapienza" e dell’Area di ricerca di Roma del CNR, il 18 e 19 Marzo nella sala conferenze del centro Enea della Casaccia, il Workshop: Sensori chimici e Biosensori.

Il Workshop ha visto la partecipazione di numerosi gruppi provenienti da tutta Italia per consolidare le collaborazioni interne ed esterne anche in vista del V Programma Quadro. Si sono avuti circa 130 partecipanti provenienti da 19 città italiane e da alcuni Paesi europei, dal mondo accademico, dalle istituzioni di ricerca pubbliche (CNR ed ENEA), dalla Agenzia Nazionale per l'Ambiente, e dalla piccola, la media e grande industria (quali ad esempio la Barilla e la Parmalat).

Il programma delle due giornate prevedeva la presentazione di 42 lavori, tra comunicazioni orali e poster, suddivisi in 8 sessioni: Sensori per i gas, Naso elettronico e riconoscimento degli odori, Biosensori, Biosensori fotosintetici, Biosensori per i metalli pesanti, Applicazioni ambientali, Sensori per i metalli pesanti, Produzione su vasta scala di sensori e biosensori.

Nella Sessione Sensori per i gas, sono state affrontate le tematiche riguardanti la rivelazione chimica dei gas con diversi trasduttori di segnale basati su differenti principi di funzionamento (conducibilità, propagazione delle onde acustiche superficiali).

Particolare attenzione è stata posta ai materiali impiegati per la preparazione dei sensori (ossidi metallici semiconduttori, silicio) ed alle loro caratteristiche (polveri di dimensioni nanometriche e materiali nano porosi). La sensibilità a differenti gas, ad esempio, dipende dalla scelta dell'ossido metallico e di eventuali droganti, quindi dalla struttura del metallo e dalla temperatura operativa. Questi sensori, impiegati per la rivelazione degli inquinanti urbani (emissioni da riscaldamento e traffico urbano, consentiranno di controllare la qualità dell'aria nelle nostre città in modo più capillare.

Combinando un certo numero di sensori, anche limitatamente selettivi, e ricorrendo alle tecniche di pattern recognition (principal component analysis, PCA o artificial neural network, ANN) potranno essere analizzate misture complesse di gas. Queste tecniche
sono inoltre alla base dei cosiddetti "nasi elettronici" a cui i media hanno dato notevole risalto in questi ultimi tempi, in alcuni casi confondendo i problemi che realisticamente possono affrontare con la fantascienza.

Nella sessione dedicata al **naso elettronico** ed al "riconoscimento degli odori", che rappresenta un esempio di multidisciplinarità, si è avuto un interessante incontro-scontro tra ricercatori provenienti da differenti culture. Così, accanto alla fisiologia della percezione olfattiva, ai meccanismi cerebrali del riconoscimento e all'isolamento dei recettori, erano presenti i sistemi a matrice di sensori basati sul riconoscimento di un pattern con meccanismi più semplici ma del tutto analoghi a quelli presenti in natura.

In quest'ottica è risultato molto interessante l'intervento che il Prof. P. Pelosi (Università di Pisa) ha condotto insieme al Prof. K. Persaud (Università di Manchester), durante il quale è stata presentata la fisiologia e la biochimica della percezione olfattiva, accanto ai risultati ottenuti in anni di sperimentazione con il primo dispositivo commerciale (AROMASCAN), disponibile già dagli anni '80, basato su matrici di 32 sensori.

Il sistema olfattivo influenza profundamente la comunicazione e molte funzioni importanti, incluse il gusto e la riproduzione. Negli animali, inclusi pesci e insetti, le ciglia olfattive contengono recettori per alcune specifiche molecole, e possono distinguere migliaia di odori differenti.

Recentemente è stata identificata nel ratto una larga famiglia di geni che codificano i recettori olfattivi. Questa famiglia di geni è una delle più vaste, poiché programma da 500 a 1000 differenti tipi di recettori olfattivi. È ragionevole pensare che un gruppo così vasto e differenziato di geni sia responsabile della larga varietà di odori che gli animali sono in grado di percepire con elevata sensibilità e specificità. Il pattern attivato, a livello dei glomeruli olfattivi, da uno specifico odore forma un codice che il cervello riconosce come unico. Geni molto simili a quelli che codificano i recettori olfattivi sembrano controllare altri tipi di riconoscimento chimico, ad esempio l'abilità dello spermatozoo a raggiungere e localizzare l'ovulo o la percezione di altri segnali chimici come i feromoni che regolano il rilascio di ormoni e l'accoppiamento negli animali e negli uomini.

L'incontro tra queste linee di ricerca, prettamente biologiche, con altre culture potrebbe favorire la realizzazione di nasi bio-elettronici basati sull'accoppiamento di recettori olfattivi isolati con trasduttori chimico-fisici del segnale.

Al momento, il naso elettronico, basato su "sensor array", cerca di riprodurre in modo molto semplice e con un numero limitato di sensori (da 6 a 32), quel che avviene in natura, avvalendosi dell'acquisizione del segnale e di sistemi di **pattern recognition**.

Risultati interessanti sono stati riscontrati nella identificazione di indici di qualità alimentare (oli, vini, formaggi). Con un naso elettronico sarà possibile controllare l'appartenenza di un olio ad una particolare zona geografica o l'uso di olive provenienti da uliveti selezionati. Questi sensori si discostano dalle tecniche di analisi tradizionali (gascromatografia e spettrometria di massa); infatti sono sistemi qualitativi che richiedono un processo di "addestramento" più o meno complesso. La proprietà di un naso elettronico nel riconoscere determinati odori deriva dalla capacità delle reti neurali di immagazzinare le "esperienze olfattive" precedenti. Sono sistemi che non misurano la concentrazione di una specie chimica, ma l'effetto (percezione, olfatto, sensazione) di un insieme di molecole (odore) e rappresentano un importante risultato dello sforzo compiuto dalla comunità scientifica nel rendere l'analisi sensoriale oggettivamente quantificabile. Allo stato attuale
infatti, questo tipo di analisi è condotto con panel di esperti e con metodi statistici proprio per minimizzare la componente soggettiva nella percezione di odori, sapori ecc.

Le sessioni dedicate ai Biosensori, (Biosensori, Biosensori Fotosintetici, Biosensori per i metalli pesanti, Biosensori ad Inibizione) hanno ospitato in larga maggioranza lavori indirizzati alle analisi ambientali. Un'interessante applicazione ambientale di biosensori basati sull'impiego di oligonucleotidi (sDNA) è stato presentato dal Prof. Mascini (Università di Firenze).

La misura del grado di inibizione di vari tipi di materiale biocatalitico quali: enzimi (ad esempio l'acetilcolinesterasi e la fosfatasi alcalina), complessi enzimatici (fotosistema II) o organismi viventi (alghe); in presenza di inquinanti ambientali mediante l’ausilio di sistemi elettrochimici, ha permesso la realizzazione di interessanti dispositivi di misura in grado di raggiungere limiti di rivelabilità in linea con la normativa vigente.

Con questi sistemi è possibile realizzare uno screening preliminare dei campioni direttamente in campo e a costi molto contenuti, destinando alle analisi chimiche tradizionali solo i campioni sospetti (generalmente in un numero pari al 5-10% della totalità dei campioni prelevati).

Con questo tipo di biosensori è stato possibile realizzare sistemi di misura alternativi e correlabili ai test di tossicità che normalmente richiedono l'impiego di organismi viventi, in primo luogo animali, e tempi di analisi decisamente più lunghi.

Altri lavori hanno riportato i vantaggi dei biosensori nella valutazione della qualità degli alimenti come il latte e il vino, l’impiego di tecnologie laser per la realizzazione di dispositivibioelettronici su scala nanometrica, le tecniche di immobilizzazione di biomediatori.

Sul versante degli immunosensori, interessante l'esempio di un dispositivo ottico portatile per la determinazione di erbicidi triazinici, e la disponibilità di anticorpi della desiderata sensibilità, impiegando libbre di frammenti sintetici di anticorpi espressi su fago.

Prospettive per i biosensori si stanno aprendo nel mondo della medicina sportiva, in particolare nel campo della rivelazione del doping negli atleti. In questo campo e’ stata molto interessante la comunicazione del Dr. F. Botrè dell'Università di Roma La Sapienza e responsabile chimico del Laboratorio Antidoping della AMSI del CONI.

Infine, alcuni lavori hanno affrontato l'impiego di tecniche a film spesso, come quella serigrafica, per la produzione di sensori chimici e biosensori su vasta scala, in modo da ridurre i costi di produzione e raggiungere, in taluni casi, l'obiettivo dell'"usa e getta".

La partecipazione è stata numerosa ed interessata ai diversi aspetti del workshop. Dei 42 lavori presentati, 15 coinvolgevano ricercatori o gruppi dell’ENEA dei Centri di Ricerca della Casaccia e di Portici, dimostrando una interessante capacità di collaborazione, spesso non formalizzata a livello istituzionale, con altri gruppi di ricerca in Italia e all’estero.

Il Workshop ha alimentato e dato vita a una serie di seminari di approfondimento anche attraverso la costituzione di gruppi di lavoro ristretti su temi specifici. Notizie sulla sua evoluzione sono presenti sui siti web:

HTTP://inn7201.Casaccia.Enea.it/workshop.htm
HTTP://www.uniroma1.it/bioprobe/workshop.htm
Biosensors: an ever expanding research field

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Abstract: Over the past two decades, enormous R&D activity has taken place in the field of sensor technology. Biosensors, in particular, have attracted considerable attention because of their extraordinary sensitivities and specificities. They are able to reveal analytical concentration and/or biochemical variables in real samples using biological components interfaced with signal transduction systems. Sensitivity and selectivity, simplified procedures, versatility, small size, low cost and presumably low environmental impact, enable analysis and monitoring of chemicals in solutions or solids substrates, even in traces. Immobilised enzymes, antibodies and whole cells have been coupled with transducers for a variety of biosensors with the additional advantage of reduced pre-treatments of the sample. Actual research trends, market and perspectives in the field of biosensors, with the target of environmental analysis, mass production technologies and miniaturization have been critically discussed through several examples of application.

Biosensori: un campo di ricerca in continua espansione

I biosensori incorporano un elemento biologicamente attivo in intimo contatto con un trasduttore di segnale fisico-chimico. Lo scopo di questa combinazione è di utilizzare l'alta selettività dei mediatori biologici per propositi analitici in vari campi della ricerca applicata, della tecnologia, della produzione industriale e della protezione ambientale.

INTRODUZIONE

Le biotecnologie, con i grandi progressi della biologia molecolare moderna, hanno conosciuto una enorme crescita negli ultimi decenni. L'ingegneria genetica, le tecniche immunologiche e l'ingegneria proteica hanno dato nuovo impulso alle ricerche sui biosensori che appaiono come uno degli argomenti trainanti delle biotecnologie sia nel campo della ricerca che nelle applicazioni pratiche.

La determinazione in continuo di un gran numero di sostanze è di enorme rilevanza scientifica e tecnologica, nel controllo di processo, nelle industrie chimiche ed alimentari, nel monitoraggio ambientale. La necessità di metodi analitici sempre più sensibili e selettivi è continuamente soddisfatta dai progressi costanti nella strumentazione analitica (gas cromatografia, HPLC, spettrometria di massa, spettroscopia di assorbimento atomico etc). Tuttavia per il loro elevato costo, queste potenti tecniche strumentali sono impiegate solo in laboratori specializzati e, in generale, non sono adatte alle determinazioni in linea o in campo. Per questo motivo lo sviluppo di sensori altamente selettivi, sensibili, facili da usare, da introdurre in un qualsiasi ambiente anche notevolmente complesso (aria, acqua, fumi, sangue, urina, saliva, scarichi industriali o anche brodi di fermentazione) suscita un crescente interesse.

Negli ultimi anni è aumentata considerevolmente la richiesta di dati sull'ambiente, con particolare riguardo alle indagini sui livelli di concentrazione o di tossicità di un numero sempre crescente di inquinanti nelle varie matrici ambientali. Le attività di monitoraggio ambientale richiedono disponibilità di dispositivi analitici affidabili, di facile impiego, di costo relativamente contenuto, automatici ed utilizzabili in campo. La ricerca nel campo dei biosensori ha proposto diversi "dispositivi analitici" per la determinazione di analiti importanti per la stima della "qualità dell'ambiente".

I biosensori sono dispositivi analitici che incorporano un elemento biologicamente attivo, anche vivente, immobilizzato secondo particolari procedure ed accoppiato ad idonei trasduttori di segnale per la determinazione selettiva e reversibile della concentrazione o dell'attività di specie chimiche in una matrice biologica o ambientale.
Il meccanismo di funzionamento di un biosensore è relativamente semplice: il mediatore biologico immobilizzato sulla superficie del sensore prende parte ad uno o più processi che determinano la variazione di un parametro chimico o fisico che viene prontamente rivelato dal trasduttore che lo converte in un segnale elettrico.

Il **mediatore biologico** o elemento di riconoscimento molecolare è responsabile della selettività; nel caso più semplice può essere un enzima purificato o un sistema più complesso con un certo numero di enzimi che lavorano in cascata, organelli sub cellulari, cellule o batteri, o infine tessuti animali o vegetali specializzati contenenti una o più molecole proteiche specifiche, fattori di protezione ecc. È questo il caso dei cosiddetti "Enzyme Based Biosensors", che utilizzano la specificità di una o più reazioni enzimatiche. I "Receptor Based Biosensors" impiegano invece anticorpi monoclonali, recettori endogeni o di membrana o ancora enzimi. In quest'ultimo caso gli enzimi non espongono la loro attività sull'analita ma è piuttosto l'analita a modulare l'attività interagendo con il biomediatore (biosensori ad inibizione).

Il **trasduttore** è l'elemento che traduce il segnale di riconoscimento biologico in un segnale elettrico (può essere un semplice dispositivo elettrochimico, una fibra ottica, un analizzatore di onda acustica, un cristallo piezoelettrico ecc.).

Le **membrane** assumono notevole importanza come supporti per realizzare l'accoppiamento tra trasduttore e mediatore biologico. Sono note numerose tecniche di immobilizzazione dei mediatori biologici per realizzare l'accoppiamento con il trasduttore. La tecnica utilizzata è scelta in base alle caratteristiche del biomediatore per evitare la modifica strutturale, e quindi delle caratteristiche e del comportamento catalitico con riduzione o perdita dell'attività. A titolo di esempio la figura 2 riporta differenti curve di calibrazione di biosensori ottenuti con lo stesso enzima (colina ossidasi) immobilizzato con tecniche diverse.

Sono disponibili procedure di **immobilizzazione fisica e chimica**. Nel primo caso l'enzima è semplicemente trattenuto dal supporto (impiego di membrane da dialisi, intrappolamento in gel di acrilamidse per citare solo alcuni esempi); nel secondo è legato covalentemente ad esso. L'immobilizzazione fisica è operativamente più semplice, preserva con maggiore efficienza le caratteristiche dell'enzima, ma questo risulta più esposto agli agenti denaturanti (pH, forza ionica, temperatura). L'enzima intrappolato fisicamente può essere utilizzato in genere solamente per alcune centinaia di analisi, rispetto alle diverse migliaia che possono essere realizzate con un procedimento di immobilizzazione chimica. Negli ultimi anni le tecniche di immobilizzazione hanno assunto notevole importanza anche per orientare spazialmente il biomediatore ottenendo film (Langmuir-Blodgett) ordinati in modo da espornere i siti attivi alla interazione con il campione.

Le **membrane** giocano un ruolo decisivo non solo nell'immobilizzazione del catalizzatore ma anche nella selettività del biosensore all'interfaccia biomediatore-trasduttore e a quella biosensore-ambiente-campione-analita.

Gli elementi appena citati ed altri ancora, testimoniano il grado di complessità dell'argomento, il suo carattere interdisciplinare, il successo, l'interesse e l'attenzione che attualmente riscuotono i biosensori.

I biosensori sono classificati in base alla natura del mediatore biologico o al tipo di trasduzione impiegata. In accordo con il primo criterio i biosensori possono essere:
- biosensori biocatalitici o sensori enzimatici;
- biosensori chemorecettoriali;
- immunosensori, ossia biosensori basati sulle interazioni antigene -anticorpo, mentre in base al tipo di trasduttore di segnale si può operare una distinzione tra:
  - biosensori elettrochimici o bioelettrodi;
  - biosensori ottici o bio-optrodi;
  - biosensori calorimetrici o biotermistori;
  - biosensori acustici

**LA RICERCA SUI BIOSENSORI ED IL MERCATO**

I biosensori sono attualmente studiati per la soluzione di molti problemi analitici: dalla rivelazione di inquinanti negli scarichi industriali, al monitoraggio delle droghe in vivo.

Fino a pochi anni fa, il numero dei ricercatori che si dedicava allo studio dei biosensori era decisamente limitato. Attualmente, invece, sia in Italia che in Europa e nel resto del mondo si assiste ad un crescente interesse per questa linea di ricerca e per le applicazioni insieme alla dilatazione della complessità e della varietà dei temi ed alla diversificazione delle tecnologie applicate. Attualmente i biosensori sono inseriti in numerosi Progetti di Ricerca in differenti aree (Biotecnologie, Medicina, Ambiente, Tecnologie Alimentari). La ricerca sui biosensori è relativamente giovane e richiede la necessaria cooperazione fra ricercatori di discipline differenti. In alcuni paesi questo è stato realizzato con centri specializzati (Cranfield nel Regno Unito, GBF in Germania). Questi centri hanno saputo produrre nell’ultimo decennio non solo ricerca di punta nel campo dei biosensori ma anche dispositivi analitici commerciali: dalla “Glucose pen” per la misura della glicemia ai sistemi automatici per l’analisi dei pesticidi organofosforici e dei fenoli.
In Italia la gran mole di ricerche compiute e i risultati acquisiti nel campo dei biosensori, per la maggior parte a livello accademico, stentano a trovare sbocchi industriali e a concretizzarsi in prodotti commerciali. Le linee di ricerca attuali in Europa, come la miniaturizzazione dei sensori, l’impiego di nuovi enzimi o la loro sintesi con tecniche di ingegneria proteica, sono finalizzate al prodotto commerciale.

Nel campo della medicina l’interesse è focalizzato soprattutto sui biosensori per il diabete e il monitoraggio “in vivo”, ma anche a biosensori per la diagnosi di malattie infettive come AIDS o malattie cardiovascolari.

Nel campo delle industrie alimentari (latte, vino, pasta, olio), cresce l’esigenza di biosensori rapidi, semplici da usare e di costo contenuto per il monitoraggio della qualità o della freschezza del prodotto alimentare o il controllo dei processi. Nella ricerca ambientale rivestono particolare importanza i biosensori per analisi di parametri relativi alla potabilità e alla tossicità delle acque, biosensori per nitrati, fosfati in acqua di mare, tossine algali.

Particolare attenzione è rivolta alle tecnologie di trasduzione superficiali (onde acustiche, risonanza di plasmon superficiale) e alle interazioni interfacciali, orientamento spaziale, ingombri sterici fra la superficie del trasduttore il mediatore biologico e l’analita.

Le ricerche nel campo tecnologie di stabilizzazione dei mediatori biologici cercano soluzioni e risposte ai problemi di stoccaggio e conservazione dei biosensori che rappresentano un serio limite alla produzione su vasta scala ed alla commercializzazione.

Fino ad oggi la ricerca sui biosensori per le misure in campo ambientale è stata prevalentemente mirata alla ideazione di nuovi sistemi per i quali veniva verificata essenzialmente la potenzialità applicativa; oggi bisogna lavorare più a fondo sulla affidabilità dei sistemi proposti e sulla loro validazione. Nell’ultimo programma quadro, ad esempio, la Comunità Europea ha promosso preferibilmente quei progetti che prevedevano una fase finale di validazione dei biosensori mediante il confronto con metodiche analitiche standard. Ci sono due ampie aree di applicazione in cui i biosensori sembrano particolarmente efficaci e per le quali è possibile prevedere nel medio periodo un mercato di dimensioni interessanti:

- indicatori on-line, in situ, immediati, nelle industrie alimentari, nel monitoraggio ambientale, e in altri processi industriali
- monitoraggio dei pazienti in diagnostica medica.

Nel passato il passaggio dalla sperimentazione alla produzione su vasta scala di dispositivi che in laboratorio sembravano promettenti, ha comunque trovato una serie di difficoltà. La prima è che il mercato prevede elevati volumi di produzione per realizzare l’abbassamento dei costi di produzione, mentre i biosensori hanno fino ad ora richiesto processi manuali ed individuali di fabbricazione. Negli ultimi anni la disponibilità di tecnologie che consentono la produzione su vasta scala ha contribuito notevolmente ad abbassare il costo di produzione dei biosensori raggioungendo, in alcuni casi, l’obbiettivo del biosensore monouso.

La Tecnologia a Film Spesso (Thick Film Technology) largamente utilizzata nell'industria elettronica per la produzione di circuiti miniaturizzati, si sta affermando come tecnologia matura ed efficace nella produzione su larga scala di biosensori, miniaturizzati, a basso costo, con elevata riproducibilità.

Ma accanto a questo traguardo bisogna considerare la conservazione dei componenti biologici del sensore che richiedono un accurato immagazzinaggio, spesso la refrigerazione, non tollerano alte temperature e sono poco stabili in ambienti umidi.

Nelle aree non mediche (ambiente e industria alimentare principalmente) il mercato risulta ancora molto frammentato, anche se nel medio e lungo termine questi settori sono destinati ad acquisire sempre più importanza non solo nel campo dei biosensori ma delle biotecnologie in genere.

Le caratteristiche ideali di un biosensore per le misure in campo ambientale sono prioritariamente di tipo analitico come l’accuratezza, la precisione, la specificità e/o la selettività, il limite di rivelabilità e la sensibilità adeguati alle normative in campo ambientale; ma anche altri requisiti come l’economicità, i tempi ridotti di analisi, l’utilizzabilità "in campo" giocano un ruolo di notevole importanza.

Considerando in termini generali il livello di sviluppo attuale dei biosensori, questi sono oggi in molti casi proponibili per analisi di "screening" in campo (avendo verificato prioritariamente la possibilità di escludere risultati “falsi negativi”). Questo utilizzo consentirebbe di ridurre sensibilmente il numero di campioni da sottoporre alla necessaria verifica in laboratorio con procedure analitiche standard che richiedono strumentazione spesso complessa e costosa e personale esperto.

**BIOSENSORI AD INIBIZIONE**

Nella classe dei biosensori ad inibizione, gli enzimi non vengono impiegati per trasformare l’analita in una molecola facilmente rivelabile come nei casi precedenti, ma viene misurato l’effetto che un composto o classe di composti ha sull’attività del biomediatore. I “biosensori ad inibizione” permettono di verificare la presenza in un campione di composti caratterizzati da un effetto tossico specifico quali ad esempio i pesticidi organofosforici e carbammici o erbicidi (triazine, feniluree e fenoli). Il meccanismo di tossicità dei composti all’interno delle singole classi è il medesimo ma l’effetto può essere quantitativamente differente.
L’accoppiamento di due enzimi come l’acetilcolinesterasi e la colina ossidasi con un sensore elettrochimico per l’acqua ossigenata consente di ottenere un biosensore specifico per l’effetto anticolinesterasico di una larga classe di inquinanti. Nel caso dei pesticidi organofosforici viene sfruttata l’inibizione delle colinesterasi: l’attività di questo enzima viene misurata con un biosensore a colina prima e dopo il contatto con il campione (figura 3).

Nel caso dei biosensori fotosintetici viene misurato, con un elettrodo di Clark, l’ossigeno sviluppato da alghe, ciano-batteri o sistemi fotosintetici isolati e purificati, in seguito ad illuminazione. L’evoluzione di ossigeno dovuta alla fotosintesi (reazione di Hill) e’ inibita dai polifenoli, dai metalli pesanti, dagli erbicidi triazinici e dalle fenil uree.

Con un semplice dispositivo basato sull’accensione intermittente di un led rosso ad alta intensita’, e sul fotosistema il isolato da un ciano batterio termofilo, purificato e immobilizzato su un elettrodo di Clark e’ possibile risalire al grado di tossicita’ per l’acqua ossigenata consente di ottenere un biosensore specifico per l’effetto anticolinesterasico di una larga classe di inquinanti. Nel caso dei pesticidi organofosforici viene sfruttata l’inibizione delle colinesterasi: l’attività di questo enzima viene misurata con un biosensore a colina prima e dopo il contatto con il campione (figura 3).

La figura 4 riporta le curve di calibrazione per diverse classi di erbicidi. Con questo sistema elettrochimico sono state ottenute misure di erbicidi con una sensibilita’ ed un limite di rivelabilita’ uguale ed in alcuni casi superiore a sistemi piu’ complessi basati sulla misura della fluorescenza (triazine al livello di 0.1 ppb).

I biosensori di tossicità misurano il grado di inibizione di enzimi (acetil colinesterasi 23,24) o complessi enzimatici (PSII 25) e quindi l’effetto tossico globale di intere classi di composti, piu’ che la concentrazione analitica di ogni contaminante.

Questa informazione puo’ essere di notevole interesse per la valutazione del rischio per l’ambiente e la salute e puo’ essere considerata preliminare e complementare a quella ottenibile con i metodi chimici tradizionali (tecniche cromatografiche). Infatti, pesticidi ed erbicidi sono spesso presenti nei campioni ambientali come “cocktail” la cui composizione dipende dalla formulazione del prodotto commerciale, dalla sua degradazione, dalle differenti e successive applicazioni alle colture agricole, condizioni metereologiche ecc.. Per queste motivi, risulta difficile prevedere gli effetti sulla salute della loro combinazione, a volte sinergiche basandosi solo sui dati di concentrazione analitica.

I biosensori ad inibizione sono in grado di fornire una valida indicazione sul livello di tossicità specifica di campioni ambientali nei confronti della flora (impiego di un mediatore fotosintetico) e della fauna ivi compreso l’uomo (impiego di un mediatore della trasmissione degli impulsi nervosi alle sinapsi).

La risposta del biosensore anticolinesterasico, riportata a scopo esemplificativo nella figura 5, sembra essere correlata con la tossicità acuta espressa come LD50 (sommenistrazione orale nel ratto) e suggerisce la possibilita’ (da confrontare con un numero maggiore di anticolinesterasici) di usare il biosensore per valutare la tossicità di nuovi pesticidi di sintesi o di altri prodotti.

Con questi sistemi e’ possibile realizzare uno screening preliminare dei campioni direttamente in campo e a costi molto contenuti, destinando alle analisi chimiche tradizionali solo i campioni sospetti (generalmente in un numero pari al 5-10% della totalita’ dei campioni prelevati).

IL TRASDUTTORE, LA SCALA DI PRODUZIONE E LA MINIATURIZZAZIONE

Nel futuro prossimo, le intuizioni del presente circa lo sviluppo con i requisiti della sostenibilita’, troveranno attuazione se saranno possibile controllare, con sensori a basso costo e che non prevedono l’uso di reagenti e solventi con relativi problemi di smaltimento, tutte o quasi le attivita’ quotidiane, dai grandi processi produttivi all’elettrodomicostico, dalla qualita’ dell’aria urbana a quella “indoor”, dall’efficienza dei motori alla loro compatibilita’ con l’ambiente, per citare solo alcuni esempi vicini all’uomo comune.

Negli ultimi anni, accanto alle tematiche fin’ora descritte, ha acquisito predominante interesse il trasduttore, i materiali che lo compongono e le tecnologie (avanzate e non) capaci di raggiungere la produzione di massa. Il limite alla diffusione dei sensori e dei biosensori, infatti, e’ stato da sempre costituito dalla difficolta’ di raggiungere la produzione su larga scala dovuta all’impiego di sistemi di preparazione spesse volte artigianali e per questo motivo difficilmente riproducibili industrialmente.

In questa direzione, il trasferimento di una “tecnologia matura” come la ”screen printing”, gia’ utilmente impiegata nell’industria elettronica per la stampa dei circuiti, bene si presta alla produzione su vasta scala di sensori e biosensori a basso costo ma indispensabili e necessari per il monitoraggio di ambiente (suolo e acque), salute (settore diagnostico e farmaceutico) e alimenti (produzione e qualità).

Il trasduttore viene quindi preparato con tecniche serigrafiche secondo lo schema riportato nella figura 6. Nel campo della miniaturizzazione, la deposizione laser di strati metallici e di componenti biologici ha gia’ raggiunto risultati di eccellenza con il primo µ-biosensore a glucosio. Questa tecnologia permetterà, ad esempio, di aspirare all’interno di un agio ipodermico decine di sensori e biosensori per il monitoraggio in vivo di numerosi metaboliti di interesse diagnostico (dispositivi sottocutanei o localizzati direttamente in vena) o per le analisi della freschezza di alimenti, del loro contenuto vitaminico e/o del grado di tossicità dovuto ad erbicidi o pesticidi (carne, pesce, prodotti ortofrutticoli).

SVILUPPI FUTURI
Un approccio differente, nell’ambito del progetto MIMICS (Molecularly Imprinted Materials for Integrated Chemical Sensors, BE [95] 1745) finanziato dalla comunità europea, ha visto la partecipazione, tra gli altri, di Thomson (FR) e EEV (UK), e riguarda l’impiego di materiali polimerici selettivi.

La natura delle interazioni con l’analita, la sensibilità e la selettività del materiale polimero ricordano quelle ottenute con gli immunosensores, ma si registrano, insieme alla migliore stabilità nel tempo, la resistenza ai solventi organici ed alle alte temperature. I materiali plasici (metacrilato) sono polimerizzati in presenza della molecola d’interesse (atrazina). Dopo la polimerizzazione, la molecola target (atrazina) è allontanata con adatti solventi (E1Oh) e all’interno del polimero rimangono le cavità modellate sulla struttura dell’analita, una sorta di impronta altamente specifica della struttura e delle interazioni operanti nel riconoscimento. L’ENEA ha partecipato al progetto con un trasduttore elettrochimico serigrafato. Le competenze presenti all’ENEA hanno dimostrato nel passato notevole flessibilità attorno a specifici progetti comunitari. Così, nel campo dei sensori chimici e biologici, le aggregazioni che potrebbero verificarsi nel futuro per affrontare il V° Programma Quadro della Comunità Europea spaziano su tematiche che rappresentano l’ultima generazione dei dispositivi bioanalitici in diverse direzioni:

- µ-DEVICE ottenuti per DEPOSIZIONE LASER di mediatori biologici, ad esempio di oligonucleotidi per realizzare chip bioelettronici ad altissima integrazione per screening e diagnostica nelle analisi del genoma. L’immobilizzazione di matrici di oligonucleotidi (1000x1000=10^6 molecole) su chip di 2x2 cm (1pixel/2µm) consentirà nel futuro di eseguire in un giorno le analisi che attualmente si effettuano in 27 anni con piastre a 96 pozzetti contribuendo al risparmio dei reattivi, del personale specializzato e ad una crescita esponenziale delle conoscenze.

- Naso Elettronico: la misura degli odori con approcci differenti che impiegano, da un lato la sensoristica a stato solido (sensor array), l’analisi combinatoriale e le reti neurali e dall’altro, l’isolamento di recettori olfattivi che attivano sensibilmente (10^{-15} mol/L) e selettivamente le complesse vie metaboliche cellulari in risposta a un odore specifico.

CONCLUSIONI

La disponibilità di nuovi dispositivi analitici affidabili, di facile impiego, di costo relativamente contenuto, automatici ed utilizzabili in campo rappresenta una necessità improrogabile nel monitoraggio ambientale. I biosensori sono stati a lungo studiati per la determinazione di analiti importanti per la stima della “qualità dell’ambiente” ma fino ad oggi la ricerca, condotta a livello accademico, è stata prevalentemente mirata alla ideazione di nuovi sistemi per i quali è stata verificata essenzialmente la potenzialità applicativa. La necessità di investigare più a fondo l’affidabilità dei sistemi proposti e la loro validazione scaturisce dalla esigenza di nuovi metodi analitici più economici, versatili e operativamente semplici. Mentre in Europa e specialmente nel Regno Unito ed in Germania la creazione di centri biotecnologici specializzati ha prodotto, in quest’ultimo decennio, dispositivi commerciali basati su biosensori per la diagnostica medica, per il settore alimentare e per quello ambientale, in Italia, la gran mole di ricerche compiute e i risultati acquisiti nel campo dei biosensori stentano a trovare sbocchi industriali e commerciali. Nonostante questo ritardo, il campo ambientale si presenta ancora come un’area di mercato dove l’attesa rivoluzione dei biosensori deve ancora trovare collocazione e spazi, in Italia e all’estero.

La diffusione dei biosensori e la loro applicazione ai problemi ambientali sono legati a considerazioni di natura economica, e gli sforzi si sono concentrati, negli ultimi anni, sulle tecnologie che consentendo la produzione su vasta scala, conciliano le caratteristiche analitiche con l’esigenza dell’economicità del prodotto. La Thick Film Technology, largamente utilizzata nell’industria per la produzione di circuiti elettronici, si sta affermando come tecnologia maturo ed efficace nella produzione su larga scala di biosensori, miniaturizzati, a basso costo, con elevate prestazioni e riproducibilità. Considerando il livello di sviluppo attuale dei biosensori nell’area biomedicale, questi sono oggi in molti casi proponibili per analisi di “screening” in campo (avendo verificato prioritariamente la possibilità di escludere risultati “ falsi negativi”). Questo consente in alcuni casi di ridurre sensibilmente il numero di campioni da sottoporre alla necessaria verifica in laboratorio con procedure analitiche standard che richiedono strumentazione complessa e costosa e personale specializzato. Con queste premesse è facile capire che il confronto delle caratteristiche analitiche dei metodi tradizionali con quelle dei biosensori non è pertinenti: i biosensori non si pongono, infatti, come alternativa ai metodi tradizionali ma come complemento capace di incrementare il numero di analisi per il monitoraggio ambientale abbassando di pari passo il loro costo e il tempo richiesto per le analisi.

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I BIOSENSORS ALL’ENEA

L’attività ENEA sui Biosensori Elettrochimici nasce nel 1988 con il monitoraggio di un processo di depurazione del siero di latte\textsuperscript{21}. Il biosensore\textsuperscript{12}, basato su una tecnica analitica ad impulso in flusso continuo (Flow Injection Analysis, FIA), che consente di monitorare in tempo reale il glucosio prodotto da un bioreattore lattasco a membrana, muove i primi passi da una esperienza pregessa con un pancreas artificiale commerciale (β-Like, Ansaldo Elettronica Biomedicale\textsuperscript{2}). Il β-Like fu impiegato per il monitoraggio ospedaliero della glicemia e per la determinazione dell’acido lattico in atleti durante lo sforzo (maratoneti). Il sistema sviluppato dall’ENEA\textsuperscript{12}, differente dal β-Like nelle applicazioni, risultava più versatile ed evoluto poiché consentiva la misura continua del glucosio in matrici complesse e ad elevatissima concentrazione (2g/l). La tecnica a flusso impiegata (FIA), una novità in quegli anni prima del massiccio sviluppo degli anni ’90, era inoltre perfezionata da un micro-bioreattore (catalasi e glucosio ossidasi immobilizzati). Questo dispositivo consentiva la rigenerazione delle soluzioni di alimentazione con la conseguente riduzione delle soluzioni esauste e degli interventi manuali (il biosensore funzionava per circa tre mesi senza alcuna manutenzione e ricambio delle soluzioni).

Nella medesima direzione fu sviluppato un bioreattore a tessuto vegetale per la degradazione dei fenoli e dei polifenoli nelle acque di vegetazione provenienti dalla spremitura delle olive\textsuperscript{22}. Un biosensore per i polifenoli basato sull’enzima Polifenolo ossidasi (PPO) ed ancora sulla FIA, consentiva il monitoraggio continuo ed in tempo reale dello stadio enzimatico del processo.

All’ENEA esiste una stretta collaborazione tra i Dipartimenti Ambiente ed Innovazione per lo sviluppo, la validazione e la promozione dei biosensori. La realizzazione di biosensori per il controllo di processi biotecnologici\textsuperscript{12} e lo sviluppo di materiali e biomateriali, sono i presupposti per ottenere dispositivi elettrochimici (Screen Printing, Tecnologie di microdeposizione Laser\textsuperscript{19} etc.) dotati di caratteristiche interessanti non solo dal punto di vista analitico ma anche da quello della produzione su vasta scala e per la realizzazione di immunoassistenti\textsuperscript{20}. Sono stati sviluppati immunoassistenti per il dosaggio di erbicidi che utilizzano anticorpi monoclonali prodotti presso i laboratori ENEA mediante la tecnologia dell’ibridoma, ed associati a sistemi di trasduzione ottica basati sulla determinazione della fluorescenza indotta da un’onda evanescente e funzionanti con sorgenti luminose miniaturizzate, quali diodi Laser. Sempre nel campo ambientale sono stati sviluppati biosensori elettrochimici ad inibizione basati sull’impiego del fotosistema o degli enzimi colinesterasici.

La vasta esperienza dell’ENEA nel monitoraggio ambientale mediante metodi tradizionali di analisi (gas-chromatografia con diversi rivelatori - ECD, FPD, NPD, mass - HPLC etc.) contribuisce alla fondamentale fase di valutazione e successiva validazione dei metodi basati su biosensori. La cooperazione tra i gruppi nei due dipartimenti e’ stretta e consolidata da collaborazioni comuni con altri gruppi internazionali (Università di Stoccarda, Università di Monaco, GBF a Braunschweig, Università di Lund, Università di Delft, Thomson e Gec-Marconi) nell’ambito di progetti comunitari su sensori e biosensori per l’ambiente.
Le tecnologie serigrafiche per la produzione su vasta scala di biosensori

La diffusione dei biosensori e la loro applicazione ai problemi reali sono fattori sicuramente legati a considerazioni di natura economica. Per questo motivo negli ultimi anni gli sforzi si sono concentrati su particolari tecnologie che consentono la produzione di biosensori su vasta scala conciliando in tal modo le esigenze di sensibilità e selettività con le caratteristiche di riproducibilità ed economicità.

La Tecnologia a Film Spesso ("Thick Film Technology"), largamente utilizzata nell'industria elettronica per la produzione di circuiti stampati, si sta affermando come tecnologia matura ed efficace nella produzione su larga scala di biosensori, miniaturizzati, a basso costo, con elevata riproducibilità.

La Tecnologia a Film Spesso si basa sull’uso di particolari paste a film spesso che vengono deposte in vari strati su un substrato isolante. Le fasi principali del processo sono:

- la deposizione ("screen printing"), l’asciugatura (drying cycle) e la sinterizzazione (firing cycle) di paste o inchiostri contenenti il materiale attivo.

Il processo di deposizione, essenzialmente identico a quello impiegato per la serigrafia, consiste nel forzare l’inchiostro attraverso uno stampo mediante una spatola di silicone. Lo stampo presenta un disegno aperto che definisce ciò che verrà deposto o stampato sul supporto.

Dopo la deposizione il film è asciugato all’aria e in forno a muffola a 100°C (drying cycle) e successivamente viene sinterizzato (firing cycle), ad una temperatura compresa tra 500 e 1000 °C.

In tal modo si ottiene un film duro, aderente al substrato con le proprietà elettriche e meccaniche desiderate. I profili di temperatura necessari per la sinterizzazione sono ottenuti utilizzando fornì contenenti natri trasportatori a zone multiple (di solito 6 o più zone) a diverse temperature, o fornì programmabili. Generalmente gli spessori dei film spessi sono di 20-30 µm dopo l’asciugatura e di 10-15µm dopo la sinterizzazione. La minima risoluzione raggiungibile in un processo di produzione è di circa 50µm. I materiali più comuni per i substrati sono i materiali ceramici come allumina, magnesia, zirconia, berillia. Molto usato è l’ossido di alluminio, Al2O3, ad elevata purezza (generalmente 96%).

Le paste utilizzate in questo processo differiscono in composizione chimica e in conducibilità elettrica e sono classificate come paste conduttrici, dielettriche, resistive e saldabili.

I principali costituenti delle paste a film spesso sono:
- materiale attivo
- vetro poroso (legante inorganico)
- legante organico.

Le paste conduttrici contengono come materiale attivo un metallo nobile (Ag, Pt, Au, Pd) o grafite, le paste resistive ossidi di metalli come biossido di rutenio (RuO2) [Wang J., 1994; Wang J., 1994] e le paste dielettriche polveri ceramiche (allumina e zirconia) come materiali attivi.

Il ruolo del vetro poroso è quello di legare le particelle del materiale attivo fra loro ed al substrato; a tale scopo vengono spesso usati vetri di borosilicato di piombo.

Per alcune applicazioni sono anche usati supporti polimerici; in tal caso le paste utilizzate differiscono da quelle tradizionali in quanto contengono come legante un polimero, anziché vetro, e quindi non è necessario sinterizzare nella fase finale del processo.

Anche l’immobilizzazione del biomediatore può essere condotta con la tecnica serigrafica al fine di semplificare le operazioni necessarie per ottenere un biosensore e consentirne la produzione su vasta scala. A questo proposito sono impiegate paste polimerizzabili con irraggiamento UV che consentono l’intrappolamento del biocatalizzatore in uno strato reticolato deposto sull’elettrodo stampato, oppure gel ad alto sviluppo superficiale che consentono l’assorbimento di elevate quantità di biomediatore e, più importante, la semplice preparazione del biosensore al momento dell’uso.
Figura 1: il mediatore biologico è l'elemento centrale che riconosce l'analita, il trasduttore trasforma il segnale biochimico in un segnale elettrico misurabile.
Figura 2: curve di calibrazione per la colina (intensità di corrente vs concentrazione di colina) ottenute con diverse procedure di immobilizzazione dell'enzima colina ossidasi
Figura 3: Le reazioni catalizzate da acetilcolisterasi (AChE) e colina ossidasi (ChOx) impiegate per la realizzazione di biosensori per pesticidi organofosforici e carbammici.

Acetilcolina → AChE → Acido Acetico $\xrightleftharpoons{H_2O} CH_3COO^- + F^-$

Colina

Betaina

ChOx (rid)

FAD

ChOx (ox)
Figura 4: Un esempio di curve di inibizione del PSII dovute a diversi erbicidi commerciali.
Figura 5: Risposta relativa al Paraoxon di pesticidi organofosforici e correlazione con la tossicità acuta espressa come LD50 (orale, ratto)
Figura 6: Le fasi di una stampa serigrafica di elettrodi con inchiostri contenenti polveri disperse del materiale elettrodico in mezzi organici (Pt, grafite, Ag)
Molecular Imprinting: un nuovo approccio per il riconoscimento molecolare

- Introduzione
- Preparazione dei polimeri
- Caratteristiche dei polimeri
- Applicazioni dei polimeri come:
  1. materiali per separazioni
  2. sostituti di anticorpi e recettori
  3. sostituti di enzimi in funzioni cataliche
  4. elementi di riconoscimento nei biosensori
- Conclusione
- Bibliografia
Introduzione

Il concetto di interazione molecolare ha origini molto antiche ma comincia ad emergere alla fine del XIX° secolo quando van der Waals compie i suoi studi sull’interazione tra gli atomi in fase gassosa e nel 1894 Fischer presenta il modello chiave-serratura per spiegare il funzionamento enzimatico. Negli anni trenta Pauling propose la teoria “istruttiva” per la determinazione della diversità anticorpale, secondo la quale la struttura tridimensionale (III°) dell’anticorpo si formerebbe solo in seguito all’incontro con l’antigene che viene a rappresentare uno stampo intorno al quale la proteina si modella modificando la propria configurazione tridimensionale per formare quante più interazioni possibili con gli epitopi dell’antigene e presentare poi siti di riconoscimento altamente specifici. Si comprese solo qualche anno dopo che la presenza di un antigene induce una “selezione” tra i linfociti B e questi, stimolati a dividersi in cellule della memoria e a differenziarsi in plasmacellule per produrre grandi quantità di anticorpi, danno origine ad un clone di cellule identiche altamente reattive e specifiche (teoria della “selezione clonale”).

Nonostante “l’infondatezza” immunologica, la teoria di Pauling fu sorgiva di grande interesse nel campo del riconoscimento molecolare e costituisce le fondamenta della tecnologia definita molecular imprinting. L’idea di sviluppare una tecnologia basata sul riconoscimento molecolare e sulla capacità di mimare le proprietà naturali di legame delle molecole biologiche, ha stimolato la curiosità scientifica di molti ricercatori.

Negli anni ‘70 Wulff (Wulff et al., 1973) sintetizzò polimeri sintetici con cavità specifiche in grado di separare le forme enantiomeriche di alcuni zuccheri. L’approccio (pre-organised) di Wulff e collaboratori si basa su legami covalenti reversibili tra la molecola stampo e i monomeri prima della polimerizzazione. I tipi di legami più comuni sono esteri degli acidi carbossilici e borici, imine (basi di Schiff) e ketali per la loro rapida cinetica di formazione e rottura in confronto ad altri legami covalenti. Mosbach e collaboratori (Arshady e Mosbach, 1981) hanno sviluppato un approccio diverso (self-assembly) stimolati dall’osservazione che la maggior parte delle interazioni tra molecole biologiche si basa su forze non covalenti.

Approcci di polimerizzazione
pre-organised approach

La procedura di sintesi di polimeri “stampati” (MIP) con questo sistema (fig.1) richiede la
scelta di una molecola di interesse in grado di formare legami covalenti reversibili con i monomeri
prima della polimerizzazione. Questo riduce il numero degli stampi potenziali nonché le possibili
strategie operative. Una volta formatosi il derivato polimerizzabile, si aggiunge il crosslinker e si
procede alla polimerizzazione. Alla formazione del polimero segue l’estrazione della molecola con la
rottura di questi legami covalenti mediante idrolisi (si ottiene un’estrazione al massimo del 90% ma
generalmente i valori sono inferiori). Il successivo legame della molecola target con il polimero
comporterà il formarsi degli stessi legami covalenti nelle cavità specifiche rimaste libere.

Self-assembly approach

Con l’approccio non covalente, si sfruttano solo legami deboli (interazioni tipiche in questo
caso sono legami idrogeno, interazioni ioniche, π–π, e idrofobiche) e interazioni con metalli di
coordinazione. I monomeri funzionali formano in soluzione interazioni deboli diverse con la molecola
e solo la successiva aggiunta di crosslinkers porta alla co-polimerizzazione del complesso.

Il processo di polimerizzazione avviene in solventi organici come il toluene o il cloroformio,
data l’influenza che la polarità del solvente esercita su questo tipo di legami. Dopo la rimozione della
molecola usata come stampo mediante estrazioni blande, si ottiene una matrice sintetica relativamente
porosa dotata di memoria, con un’architettura macromolecolare complementare alla forma della
molecola e alle sue funzioni.

Associazione e dissociazione della molecola con i siti artificiali creati, avvengono per semplice
diffusione dentro e fuori il polimero senza formazione o rottura di alcun legame covalente. Il sistema
non covalente risulta più versatile ed è più facile identificare un cocktail di monomeri che permetta la
realizzazione di un polimero con determinate caratteristiche.

In una procedura tipica (fig.2) si usa metacrilato acido (MAA) come monomero, Ethylen
glycoldimetacrilato (EGDMA) come crosslinkers, cloroformio o toluene come porogeno, 2,2’-
azobisisobutynitrile (AIBN) come iniziatore della polimerizzazione che può essere determinata da
raggi UV o termica. Il polimero (MIP) che si ottiene è sotto forma di un bulk che viene rotto, macinato
finemente in un mortaio in particelle omogenee, setacciato prima del recupero della molecola stampo. Alternativamente si può depositare la miscela di polimerizzazione tra un “silicon wafer” e un vetrino in modo che il successivo processo di polimerizzazione porti ad una membrana polimerica (sandwich).

I polimeri possono essere rigenerati con estrazioni che portano al recupero fino al 99% della molecola stampo.

Caratteristiche dei polimeri

I polimeri (MIPs) che si ottengono con questa tecnica hanno caratteristiche chimico-fisiche interessanti e vantaggiose rispetto alla componente biologica che sostituiscono: sono infatti altamente stabili rispetto a stress meccanici, resistenti a temperature e pressioni relativamente elevate, nonché a trattamenti con acidi, basi, ioni metallici; si possono conservare a temperatura ambiente per lunghi periodi e riutilizzare diverse volte senza perdita dell’effetto memoria.

Bisogna tenere in considerazione anche alcuni aspetti svantaggiosi. Un primo problema è rappresentato dalla necessità di disporre di quantità discrete della molecola di partenza, che può essere di per sé costosa o di difficile reperibilità (da 50 a 500 µmol/g di polimero secco). La natura stessa del processo di polimerizzazione comporta inoltre la presenza nel polimero finale di siti di legame con diversa affinità. Questa eterogeneità può essere paragonata alla policlonalità degli anticorpi prodotti in vivo da animali da laboratorio in risposta all’immunizzazione e si riflette nella distribuzione della forza di legame del polimero che, rappresentata sotto forma di un’analisi Scatchard, mostra un andamento non lineare, passando da siti ad alta affinità a siti con affinità nulla. Un grosso ostacolo rimane inoltre la polimerizzazione con le proteine quali molecole stampo: essendo di grandi dimensioni è difficile realizzare una rete polimerica con sufficienti legami crociati e rimuovere poi la proteina dal polimero e farla diffondere attraverso il polimero durante le procedure di analisi sembra ancora poco probabile; le proteine sono inoltre insolubili e denaturabili in solventi organici. Alcuni risultati sono stati ottenuti usando interazioni forti tipo boronato-estere (Glad et al., 1985) o metalli-istidine (Kempe et al., 1995).

- Applicazioni dei polimeri come:
1. materiali per separazioni

La prima area di applicazione dei MIPs è stata la cromatografia. Il molecular imprinting è una tecnica che si adatta molto bene alle separazioni permettendo la preparazione di supporti fatti su misura con una certa specificità.

Di particolare interesse sono le separazioni chirali in cui l’ordine di eluizione dipende dall’enantiomero che si è utilizzato per la costruzione del polimero. Con le colonne classiche infatti la separazione dipende da scambi ionici e esclusione molecolare e gli enantiomeri avrebbero lo stesso tempo di ritenzione. Le tradizionali fasi stazionarie chirali commerciali (CSPs) utilizzano composti chirali immobilizzati (da piccole molecole organiche a proteine intere) che formano complessi diastereomerici con l’analita da separare ma con questa tecnica generalmente è necessario un range di CSPs per trovare il sistema adatto alla specifica separazione e le molecole biologiche che vengono usate hanno una stabilità chimica, termica e meccanica significativamente minore dei MIPs. Ci sono oggi più di 500 molecole di interesse farmaceutico otticamente attive e la risoluzione delle soluzioni racemiche è una delle principali possibili applicazioni.

Il primo MIP in questo campo fu realizzato per il Timolol (Fischer et al., 1991), repressore dei recettori β-adrenergici, seguito dal Naproxen (Kempe and Mosbach, 1994), agente antinfiammatorio non steroideo e dall’efedrina, agonista adrenergico, (Ramström et al., 1996). Altri polimeri per separazioni chirali sono stati preparati per analiti importanti nella diagnostica medica e nei processi biotecnologici: aminoacidi, derivati di aminoacidi e peptidi, (Kempe and Mosbach, 1995), carboidrati (Mayes et al., 1994; Nilsson et al., 1995; Wulff and Haarer, 1991) e ormoni (Andersson et al., 1995).

E’ stata verificata la possibilità di sfruttare la tecnologia dell’imprinting molecolare per estrazioni in fase solida per la purificazione di estratti. Nella prima applicazione in questo settore sono stati prodotti MIP specifici per atrazina da utilizzare su estratti di fegato di bue (Muldoon and Stanker, 1997). Questo tipo di sistema, il “Molecularly Imprinted Solid Phase Extraction” (MISPE), permette così di separare analiti da campioni biologicamente complessi e potrebbe offrire una valida alternativa nelle fasi di pretrattamento per composti lipofili.

Oltre alle tradizionali colonne cromatografiche sono stati sviluppati con i MIPs altri sistemi di separazione come la cromatografia a strato sottile (L-phenilalanina anilide, Kriz et al., 1994) e
l’elettroforesi capillare (Nilsson et al., 1994). Una delle applicazioni in questo senso è stata la polimerizzazione con la pentamidina (un farmaco usato nel trattamento delle affezioni AIDS-correlate, presente in concentrazioni fisiologiche nelle urine) quale molecola stampo (Sellergren, 1994).

2. sostituti di anticorpi e recettori

Gli anticorpi per la loro alta specificità e affinità vengono largamente impiegati per realizzare saggi immunologici per scopi diagnostici in campo biom edico e negli ultimi anni per la rilevazione qualitativa e quantitativa di contaminanti in matrici di interesse ambientale.

La possibilità di preparare anticorpi (Henricksen and Martin, 1996) e recettori artificiali potrebbe rappresentare un’alternativa interessante viste le caratteristiche chimico-fisiche dei polimeri rispetto a molecole proteiche quali gli anticorpi. Sono stati portati avanti diversi progetti per verificare se i MIPs possano rappresentare una valida alternativa all’uso di anticorpi come elementi di riconoscimento negli immunoassagi.

I “molecularly imprinted sorbent assays” (fig.3) fino ad ora realizzati, sono saggi competitivi in cui l’analita da rilevare compete, per i siti chimicamente e stericamente specifici nel polimero, con l’analogo radioattivo (protocollo simile ai RIA). La misura della radioattività nel supernatante sarà direttamente proporzionale alla concentrazione di analita non marcato presente nel campione.

Sono stati prodotti polimeri per il riconoscimento del farmaco broncodilatatore, teofillina e del tranquillante diazepam (Vlatakis et al., 1993). Un saggio competitivo tipo-RIA è stato utilizzato per determinare la concentrazione di teofillina nel siero dei pazienti e i risultati ottenuti mostrano una buona correlazione con quelli avuti con i kits immunoenzimatici commerciali. I valori di cross-reattività dei polimeri stampati con teofillina e diazepam sono inoltre molto vicini a quelli riportati per gli anticorpi monoclonali e il polimero “anti-teofillina” è risultato in grado di distinguere questa molecola dalla caffeina, che differisce per un solo gruppo metile. Altre molecole di interesse biomedico utilizzate per la polimerizzazione di MIPs sono state la morfina e il neuropeptide endogeno leu-encefalina (Andersson et al., 1995-1996).

Negli ultimi anni ha avuto particolare attenzione lo sviluppo di nuovi sistemi che permettano il riconoscimento qualitativo e quantitativo anche di molecole di rilevanza ambientale. Allo scopo di verificare l’utilità dei MIP in questo settore sono stati ad esempio costruiti polimeri per la triazina...
molecola base degli erbicidi s-triazinici) utilizzati in sistemi di rilevazione ottica con triazina fluorescente (Piletsky et al., 1997): il saggio è di tipo competitivo tra l’erbicida libero e lo stesso coniugato ad una molecola fluorescente e la misura viene fatta sulla fluorescenza residua nel supernatante. Sono stati anche prodotti MIP per il riconoscimento specifico dell’atrazina la cui presenza e concentrazione viene poi rilevata con saggi di tipo competitivo tra l’erbicida libero e l’atrazina marcata, in questi casi, radioattivamente (Muldoon and Stanker, 1995; Siemann et al., 1996).

Un vantaggio nella scelta dell’uso di polimeri in sostituzione agli anticorpi o ai recettori risiede, oltre che nelle già citate proprietà chimico-fisiche, nella possibilità di ottenere, facilmente e a basso costo, polimeri specifici per molecole contro le quali sarebbe artificioso e costoso produrre anticorpi monoclonali. Esempi sono le molecole a basso peso molecolare difficilmente immunogeniche quali gli apteni che devono essere coniugati con una proteina carrier prima dell’immunizzazione o gli antibiotici come l’eritromicina o molecole immunosoppressive come le ciclosporine (Senholdt et al., 1997).

3. sostituti di enzimi in funzioni catalitiche

Un’applicazione interessante è l’uso dei MIP come sostituti di enzimi in reazioni catalitiche. Sono state adottate diverse strategie che prevedono l’uso come stampo di: 1) analoghi dello stato di transizione come. Altri approcci sfruttano 2) analoghi dei coenzimi per sviluppare un sistema con una determinata attività catalitica; 3) composti di coordinazione per mediare la reazione catalitica e infine strategie “bait and switch” per la corretta organizzazione del gruppo catalitico nel sito.

Nel primo caso (fig.4), per esempio, lo stato di transizione dell’idrolisi dell’estere carbossilico può essere mimato da derivati fosfonati (Robinson e Mosbach, 1989). E’ stato preparato un polimero usando come stampo il p-nitrofenilmetilfosfonato, un analogo dello stato di transizione dell’idrolisi del p-nitrofenilacetato e il MIP ottenuto lega preferenzialmente l’analogo dello stato di transizione inducendo anche un certo aumento del tasso di idrolisi del p-nitrofenilacetato a p-nitrofenolo e acetato (Fig.). Studi più recenti si sono concentrati sull’idrolisi di esteri di aminoacidi e sulla preparazione di polimeri enantioselettivi cataliticamente attivi (Ohkubo et al., 1995; Sellergren and Shea, 1994).
Nel secondo caso Andersson and Mosbach (1989) hanno provato ad usare come stampo l’analogo del complesso coenzima-substrato N-piridossil-L-fenilalanina anilide e hanno osservato che la capacità di catalizzare la formazione dell’addotto piridossale libero e fenilalanina anilide è aumentata di 8 volte rispetto al controllo.

Per quanto riguarda i composti di coordinazione metallica è stato eseguito un esperimento con la classe II delle aldolasi (Matsui et al., 1996). Anche in questo caso si è avuto un incremento dell’attività catalitica.

Nel caso della strategia del bait-and-switch (fig.5) sono state fatte alcune prove (Müller et al., 1993; Beach and Shea, 1994) con la reazione di β-eliminazione coinvolta nella deidroalgenazione dei β-fluorochetoni, un sistema già utilizzato con gli anticorpi catalitici.

4. elementi di riconoscimento nei biosensori

Una delle applicazioni più interessanti è l’uso dei polimeri come elementi di riconoscimento in sistemi tipo biosensori.

I biosensori sono dispositivi in grado di identificare da un punto di vista qualitativo e quantitativo un determinato analita presente in un campione e sono costituiti da una componente biologica, cui è deputata la specificità, in intimo contatto con un trasduttore atto alla rilevazione ed amplificazione del segnale. L’elemento biologico che può essere un enzima, un recettore o un anticorpo, costituisce la componente sensibile in grado di riconoscere selettivamente e con alta affinità la molecola target e viene posto all’interfaccia tra il sensore e il campione contenente l’analita di interesse. L’interazione della molecola target con l’elemento biologico determina un cambiamento in uno o più parametri chimico-fisici come la produzione di ioni, elettroni, gas, calore, cambiamenti di massa o luce che il trasduttore, converte in un segnale elettrico che viene poi amplificato e convertito nella forma interpretabile.

Le caratteristiche importanti di un biosensore sono la selettività, sensibilità, stabilità e possibilità di rigenerazione. La selettività è determinata dalla componente biologica deputata al riconoscimento di una determinata molecola in una miscela contenente anche eventuali altri composti con struttura chimica simile. La sensibilità dipende sia dalla componente biologica che dal trasduttore ed essendo in relazione al rapporto segnale/rumore (S/N), ulteriori passaggi di amplificazione possono incrementare la sensibilità e abbassare il limite della minima concentrazione rilevabile.
L’uso dei MIPs come sostituti delle molecole biologiche quale elemento sensibile permette di conservare le caratteristiche di specificità e selettività essendo le cavità di questi complementari in forma e funzionalità alla molecola intorno alla quale è stato realizzato il polimero. La scelta dei MIPs come alternativa alle molecole proteiche offre molti vantaggi sia per le caratteristiche chimico-fisiche proprie di questi polimeri sia per la possibilità di usare come stampo molecole contro le quali sarebbe molto difficile e costoso produrre degli anticorpi nonché per la semplicità della procedura e il basso costo di questa tecnica.

L’approccio che più si avvicina al concetto di biosensore (con l’elemento sensibile in prossimità del trasduttore) prevede l’uso di polimeri in forma particelle ottenute dalla macinazione del bulk o di membrane ottenute con la tecnica del sandwich ed il cambiamento che avviene nel polimero in seguito al legame dell’analita viene rilevato dal trasduttore.

Uno dei primi lavori nello sviluppo di questi nuovi sensori rientra nell’ambito dei sensori elettrochimici capacitativi (Field Effect Capacitors): membrane polimeriche realizzate con la tecnica del sandwich, contenenti siti di riconoscimento specifici per la L-fenilalanina anilide utilizzata come molecola stampo, sono accoppiate ad un sistema di trasduzione che, misurando la variazione di capacità in funzione del voltaggio applicato), rivela il legame della molecola target sotto forma di una diminuzione della capacità applicando lo stesso range di voltaggio (Hedborg et al., 1993). Si può pensare di applicare lo stesso tipo di procedura con trasduttori field-effects transistors ed in questo caso sono interessanti anche le dimensioni ridotte, il basso costo di fabbricazione e la possibilità di integrarli nella microelettronica.

Altri tipi di trasduttori elettrochimici conduttimetrici e amperometrici sono stati accoppiati con la tecnologia dell’imprinting molecolare. E’ stato realizzato un sensore basato su un sistema di rilevazione conduttimetrica da Kriz (Kriz et al., 1996) per un analita carico, lo ione benzyltriphenylphosphonium: il legame dell’analita carico ai siti specificatamente reattivi del polimero porta ad un significativo aumento di conducibilità opportunamente rilevato. Sempre nell’ambito dei trasduttori conduttimetrici si trova il sensore per la rilevazione di atrazina (Piletsky et al., 1995); in questo caso il legame dell’atrazina provoca una diminuzione della conducibilità. Utilizzando come trasduttore un sistema amperometrico è stato sviluppato un sensore competitivo per la misura di morfina (Kriz and Mosbach, 1995). La procedura prevede due steps: il legame della morfina con il polimero e successivamente il distacco da questo per l’azione competitiva della codeina elettroinattiva; si misura poi elettrochimicamente la morfina tornata in soluzione.
Nell’ambito dei sensori ottici è stato realizzato un sistema a fibre ottiche in fluorescenza (Kriz et al., 1995) basato su MIPs polimerizzati in presenza del derivato di un aminoacido marcato fluorescente (Dansyl-L-fenilalanina anilide) la cui rilevazione avviene in maniera diretta.

Un significativo miglioramento potrebbe venire dai polimeri che mostrano contemporaneamente riconoscimento molecolare e conduttività elettrica (Kriz et al., 1995). Un esperimento in questo senso è stato eseguito preparando polimeri specifici per il riconoscimento della morfina (MIPs). Le particelle ottenute dall’macinazione del bulk sono state saturate con monomeri di pirrolo; un successivo passaggio di ossidazione in soluzione acida del pirrolo a polipirrolo ha portato a particelle dotate di memoria per la morfina e di elettroconducibilità (M-MIP-PPy), interessanti per lo sviluppo della tecnologia dei sensori elettrochimici.

Un ulteriore metodo di rilevazione che dia una risposta immediata è rappresentato dai sensori piezoelettrici, tra i quali si annoverano gli acustici, che si basano su misure del cambiamento della frequenza di risonanza del cristallo piezoelettrico come risultato di un cambiamento di massa sulla sua superficie (Okahata et al., 1994).

In un altro approccio il trasduttore è separato dalla soluzione contenente il campione da una membrana selettivamente permeabile alla molecola stampo (Piletsky et al., 1994) la cui presenza viene rilevata con misure elettrochimiche. In questo caso l’elemento di riconoscimento non si trova in prossimità del trasduttore ed è più corretto parlare di sistemi di misura più che di sensori.

- **Prospettive future**

L’idea di utilizzare i MIP come elemento di riconoscimento per lo sviluppo di nuovi sensori ha stimolato molti gruppi di ricerca ed è l’obiettivo intorno al quale è stato concepito il progetto europeo MIMICS (Molecularly Imprinted Materials for Integrated Chemical Sensors) al quale partecipano centri e istituti di ricerca di chiara e riconosciuta fama tecnico-scientifica a livello internazionale, non solo in questo settore. Al fine di realizzare un sensore chimico innovativo per misure real time sono stati prodotti, con la tecnologia del Molecularly Imprinted Materials, dei polimeri, in forma di bulk, specifici per determinati analiti di interesse ambientale. Per la realizzazione del prototipo finale sono attualmente in corso attività di ricerca finalizzate allo sviluppo di un metodo per la produzione di MIP sotto forma di film sottili. Per facilitare lo scambio di informazioni scientifiche, stimolare ricerche che portino ad una più approfondita conoscenza dei diversi aspetti riguardanti le tecniche di
polimerizzazione, la caratterizzazione dei polimeri, le possibili applicazioni dei MIP, è stato creata sulla rete internet una pagina web dove si può trovare l’elenco delle pubblicazioni on line, con i links verso siti che si occupano di argomenti in stretta relazione (http://inn7201.casaccia.enea.it/index.html).

Fig. 1  Preorganized approach:
I gruppi diolo del D-mannitolo vengono prima esterificati con il monomero acido vinilbenzyl-boronico per formare la molecola stampo. La successiva polimerizzazione con il crosslinker porta al polimero con siti specifici per la molecola stampo
La molecola stampo (S-Timolol) forma in soluzione interazioni non-covalenti con i monomeri funzionali di metacrilato (MAA) prima della successiva co-polimerizzazione con il crosslinkers etilen glicol dimetacrilato (EGDMA).

La rimozione della molecola stampo, mediante estrazioni blande, lascia un polimero con cavità specifiche per la molecola target.

Fig. 2 "Self-assembly approach":
La molecola stampo (S-Timolol) forma in soluzione interazioni non-covalenti con i monomeri funzionali di metacrilato (MAA) prima della successiva co-polimerizzazione con il crosslinkers etilen glicol dimetacrilato (EGDMA). La rimozione della molecola stampo, mediante estrazioni blande, lascia un polimero con cavità specifiche per la molecola target.
Fig. 3 rappresentazione schematica di un "molecularly imprinted sorbent assay"
Fig. 4 analoghi dello stato di transizione

A) Il paranitrofenilmetilfosfonato viene usato come molecola stampo per la polimerizzazione di MIPs. 
B) I MIPs, presentando le cavità in grado di riconoscere e stabilizzare lo stato di transizione della reazione, mostrano attività catalitica e favoriscono l'idrolisi del paranitrofenilacetato.
Posters
A NEW LAYOUT FOR SCREEN PRINTED ELECTRODES: FRONT/BACK GEOMETRY

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Introduction
Traditional screen printed electrodes show as a rule the working electrode, the reference and the auxiliary, when needed, printed side by side (1) or concentrically (2), according to the layout used, on the same side of the substrate, being it either a ceramic or plastic one. To decrease the global dimensions of electrodes a new front to back layout (3) has been developed to obtain capillary sensors or biosensors that can eventually be housed in hypodermic needles for in vivo measurements.

Results and discussion
Reproducibility was directly related to the exact definition of the active electrode area. SEM examination of electrodes shows a further limit in the technique. When cutting, the blade takes along its edges small quantities and fragments of conductive pastes which can cause dangerous shortcuts between the two faces of the electrode. Moreover the cut allows the silver printed layer (5) to appear from underneath the graphite layer. In this way an additional faradic current flowing through the conductive paste and not only through the working electrode surface was recorded. Two different solutions were found to the problem. The first one was to print a shorter conductive silver layer underneath the graphite one so that, the part of the electrode in contact with the solution, will present the graphite printed directly on the PVC. This printing layout obviously decreases the global conductivity of the electrode. The second one was to design a new layout which allowed the manufacture of single electrodes with no need of cutting them (4). An insulating ink layer was printed to precisely limit the active surface of the working electrode. Both solutions increased reproducibility of electrodes. Physical and chemical pre-treatments were tested to increase signal stability and reproducibility and to increase the conductivity of the printed layers. Pressures ranging from 10 to 300 kg/cm² were applied on graphite layers and resistance was measured. Pressing the electrodes resulted in a significant increase of conductivity and in an increased homogeneity of the electrode’s surface as can be seen in the images obtained with SEM (6). Electrodes were also chemically treated at constant and variable potential in buffered, acid (HNO₃ 0.1 N) and alkaline (KOH 0.1 M) solutions. Best results were obtained by pre-treating electrodes in KOH with a cyclic voltammetry between -1V and 1V for 40 minutes. After the treatment stabilization of the signal reached a plateau very soon and the noise was significantly lower than the untreated electrodes (7).

Materials, methods, experimental
Electrodes were printed on 6 cm x 6 cm PVC sheets (3) using a manual screen printer mod.HT10, Fleischle. The conductive layer of both the reference and working electrode was printed with not sinterizable Ag/Pd ink (5025 Du Pont), the AgCl reference was either printed Ag/AgCl ink (Du Pont) or electrochemically deposited (NaCl 0.1 M/L solution, 0.7 V vs Pt for about 30 minutes), and the working electrode was printed using Acheson graphite ink drugged with a 10% w/w Pt powder supported on activated carbon (Pt 3%, Aldrich 23.755-8). All ink layers after printing were left in oven at 60°C for twenty minutes for complete drying and solvent removal before the next layer was printed. Single electrodes were obtained from the printed PVC sheets by cutting them with a paper cutter (3) and then inserting them into a micropipette disposable tip with a small quantity of epoxy resin (Araldite ®). Dimension of electrodes’ width was between 0.5 and 1 mm. Finished electrodes were tested both in batch and in flow cells measuring hydrogen peroxide (8) and glucose (9) with a reference enzyme (Gox).

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OXIDIZED CARBON POWDERS FOR ENZYME IMMOBILIZATION ON SCREEN PRINTED BIOSENSORS

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Introduction
Over the past few years interest has been increasing in the application of simple, rapid, inexpensive and disposable biosensors in clinical, environmental or industrial analysis. The most common disposable biosensors are those produced by thick-film technology. A thick-film biosensor configuration is normally considered to be one which comprises layers of special inks (or pastes) deposited sequentially onto an insulating support or substrate. Screen printing seems to be one of the most promising technologies allowing biosensor to be placed large-scale on the market in the near future because of advantages such as miniaturisation, versatility at low cost and also particularly the possibility of mass productions. The use of thick-film technology for the production of sensor systems is an emerging field. The most critical point in manufacturing thick film biosensors is the sensing or active membrane and its adhesion to the transducer layer [5].

The aim of the present work is activation and derivatization of carbon powders to be used in screen printed electrode preparation. The oxidizing treatment of carbon powder with the aqueous H2O2 solution introduces mainly carboxylic and phenolic groups on the carbon surface after that as well as derivatization, it is easy to use this groups in covalent immobilization of biomolecules [4]. Glucose oxidase was used as a model enzyme to test the usage of the different covalent immobilization methods on the treated carbon surfaces.

Material and Methods
(1) Thixo-oxidation of Carbon Powders: Carbon powders were subjected to an oxidation treatment with hydrogen peroxide in order to create surface oxygen groups; a given amount of the support was immersed in an aqueous solution of H2O2. 6 N (1 g of support/60 ml of solution) and the slurry so formed was stirred for 48 h at room temperature. Finally the oxidized sample was washed with distilled water to eliminate H2O2 excess and dried at room temperature [5].

Preparation of the carbon based inks for printing working electrodes: Inks for printing working electrodes were prepared by mixing a commercially available carbon ink (Du Pont 71B), with oxidized carbon and/or 10% rhodium graphite powder (Aldrich 20,616-4), or dextran (25%) or lysine coupled carbon.

Printed Electrodes: Printed electrodes were fabricated by depositing several layers of inks on a PVC substrate (Fig 1). The conducting paths and pads (Fig. 1a) were deposited directly on the PVC sheets using Ag/Pd ink (DuPont, 502S). Then, an Ag/AgCl ink was deposited to obtain the reference electrode (Fig. 1b). Different oxidized carbon powders were printed to obtain the working electrodes (Fig. 1c). Finally, an insulator layer was placed over the conducting paths (Fig. 1d). After each printing step, the paths were treated at 60°C for 60 min.

Figure 1. SPG electrodes: from right to left the layout of the sequentially printed layers; a) Ag/Pd counter electrode, conductive pads and paths, b)Ag/AgCl reference electrode, c) carbon based working electrode, d) insulator.

Preparation of Enzyme Electrodes Different techniques were used for the covalent immobilization of enzyme: binding of the enzyme were performed on (1) EDC activated, (2) CDI activated carbon surfaces and also, (3) Lysine coupled surfaces by using EDC activation, respectively.
(1) Electrode surfaces were treated with EDC [1-Ethyl-3(3-dimethylaminopropyl) carbodiimide] 0.1 mol/L in NaHPO4 (pH 4.75, 0.1 mol/L) solution for 45 min, washed with distilled water and 10 mmol/L PBS (phosphate buffer saline, pH 7.5). Then, 10 μl of enzyme solution (1 mg of enzyme in 10 μl phosphate buffer, 50 mmol/L, pH 7.5) was spread on the surface and allowed to stand at 4°C overnight [8].

(2) Before printing of the oxidized carbon powder, EDC activation was performed as described before and then, instead of enzyme, carbon was treated with 0.1 M of lysine solution. After printing of the lysine coupled powder, the procedure 1 was performed.

Results and Discussion
The use of commercially available or home made inks for electrode printing and the wide range of possibilities with respect to the substrate material allow the development of low cost/high performances transducers which can be largely applied for biosensing in any field of analytical determinations [8].

The oxidizing treatment does modify the surface chemistry of the electrode, by creating surface-oxygen complexes that make it more acidic. Scanning electron microscopy shows in good evidence that the porous structure of the carbonaceous support was not affected (Fig 2) by the treatment.

Furthermore, the pH value of the carbon slurry was found to be changed from 6.5 to 4.4 after oxidizing step because of the presence of acidic groups on the surface. Our results are well in agreement with [5]. On the other hand, as well as the coating immobilization by using the introduced acidic groups, negatively charged functional groups on the electrode surfaces could provide useful matrices for ionic binding of proteins which could strongly interact in aqueous solution.

In this study, different oxidized carbon matrices (graphite-rhodium, carbon, carbon-dextran and lysine coupled carbon) were prepared to be printed as a working electrode. Afterwards, covalent immobilization of glucose oxidase was performed. Both features of sensors towards to glucose and hydrogen peroxide are shown in Table 1.

Table 1. Features of GOX immobilized on SPG with standard solutions of glucose and hydrogen peroxide. (1)EDC and (2)CDI activated graphite-rhodium mixture, (3) EDC activated carbon, (4)EDC and (5)CDI activated carbon-dextran mixtures and (6)lysine coupled graphite surface

<table>
<thead>
<tr>
<th>Linear Range (mM)</th>
<th>Sensitivity (kA/mM)</th>
<th>CV (%)</th>
<th>Correlation coefficient</th>
<th>Sensitivity (kA/mM)</th>
<th>CV (%)</th>
<th>Correlation coefficient</th>
<th>Ratio of C0/Ca*100</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1)</td>
<td>0.25-1.50</td>
<td>25.6±0.90</td>
<td>3.90</td>
<td>0.9862</td>
<td>38.2±1.4</td>
<td>3.39</td>
<td>0.9869</td>
</tr>
<tr>
<td>(2)</td>
<td>0.25-2.50</td>
<td>25.6±0.90</td>
<td>2.10</td>
<td>0.9900</td>
<td>21.8±0.71</td>
<td>3.39</td>
<td>0.9869</td>
</tr>
<tr>
<td>(3)</td>
<td>0.10-1.50</td>
<td>46.8±3.9</td>
<td>5.99</td>
<td>0.9875</td>
<td>23.7±3.0</td>
<td>4.01</td>
<td>0.9903</td>
</tr>
<tr>
<td>(4)</td>
<td>0.25-2.50</td>
<td>24.8±3.5</td>
<td>5.11</td>
<td>0.9895</td>
<td>20.2±2.2</td>
<td>3.00</td>
<td>1.0000</td>
</tr>
<tr>
<td>(5)</td>
<td>0.25-2.50</td>
<td>24.7±3.5</td>
<td>5.26</td>
<td>0.9895</td>
<td>20.2±2.2</td>
<td>3.00</td>
<td>1.0000</td>
</tr>
<tr>
<td>(6)</td>
<td>0.25-2.50</td>
<td>24.1±3.5</td>
<td>9.89</td>
<td>0.9895</td>
<td>14.6±0.9</td>
<td>2.63</td>
<td>0.9973</td>
</tr>
</tbody>
</table>

As it can be seen in the last column of Table 1, in some cases, covalent immobilization of the enzyme directly on the electrode surfaces caused higher biosensor responses in comparison to hydrogen peroxide. This behavior could be due to diffusion effects through the enzyme layer toward the electrode surface. This behavior was never observed with biosensors using membranes for enzyme immobilization because diffusion of both glucose and hydrogen peroxide is greatly controlled by the membrane properties, especially the thickness.

The observed behavior could be explained with a better diffusion of the hydrogen peroxide produced by the enzyme catalysis reaction from the active site of the enzyme toward the very close electrode surface. It was observed only with EDC, not with CDI, probably because the enzyme is bound on the electrode surface in a different site. With the dextran-EDC matrix the effect was not observed probably because the dextran acts as a barrier and limits the reagents and products in the same way as a membrane.

Moreover, storage stabilities at 4°C were investigated. GOXs both, immobilized on carbon-dextran mixture by EDC and CDI activations completely lost their activities after 30 days. However, activity of immobilized GOX on graphite-rhodium by EDC and CDI activations were found to be 92.7% and 67.3%, respectively 60 days later. GOX immobilized on EDC activated carbon and lysine coupled carbon were found to have 83% and 30% of their activities after 60 days.

Conclusions
Activation and derivatization of carbon powders to be used in screen printed electrode preparation were performed for obtaining easier enzyme covalent immobilization. The oxidizing treatment of carbon powder with an aqueous H2O2 solution introduced mainly carboxylic and phenolic groups on the carbon surface which were used for further covalent immobilization of biomolecules. Glucose oxidase was used as a model enzyme to test the usage of the different covalent immobilization methods on the treated carbon surfaces obtaining very good sensitivity and storage stability.

References

Acknowledgements
This research was supported by the ENEA Target Project COSMIC (Coupling Smart Molecules Into Chips, http://biosensing.net/cosmic.htm) and by the ENEA fellowship to S.Timur.
DISPOSABLE SCREEN PRINTED POTENTIOMETRIC SENSORS
FOR DETERMINATION OF FREE RADICALS

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Abstract
In this communication new screen printed potentiometric electrodes based on nitroene dispersed in a PVC membrane for the determination of free radicals are reported. Screen printed potentiometric sensors based on an ionophore dispersed in a PVC membrane were previously verified and optimised by using valinomycin as model molecule after potassium ion activity measurement. Valinomycin was used for finding the best deposition condition of the PVC membrane directly on the printed graphite electrodes. Valinomycin containing membranes of different thickness were obtained with "dip & dry" or "tape casting" or "screen printing" techniques by using different dilution of the PVC matrix in tetrahydrofuran (THF). Sensitivity of the valinomycin based sensors for K⁺ was found to be strictly related to the thickness of the membrane and the used deposition technique. A very good reproducibility was also observed by comparing the slope values of the calibration curves obtained from several electrodes.

Furthermore, nitroene dispersed in the same PVC membrane was used for the development of disposables, screen printed potentiometric sensors for free radicals.

Keywords: screen printed potentiometric sensors, free radical determination.

Introduction
Screen printing technology was recently used for mass production of low cost, miniaturised and disposable chemical or biochemical sensing devices to be used on gas or liquid samples. Screen printed amperometric biosensors were extensively used for environmental monitoring of waters and wastes. Recently, low cost and rapid analytical determinations of free radicals are playing a central role in environment and food monitoring. Present authors proposed in recent years different kind of sensors [1] and biosensors [2,3] for free radical analysis. In this communication new screen printed potentiometric electrodes based on nitroene dispersed in a PVC membrane for the determination of free radicals are reported.

Results and Discussion
Screen printed potentiometric sensors based on an ionophore dispersed in a PVC membrane were previously verified and optimised by using valinomycin as model molecule in order to verify the potassium ion activity measurement. Valinomycin was used for finding the best deposition condition of the PVC membrane directly on the printed graphite electrodes. Valinomycin containing membranes of different thickness were obtained with "dip & dry" or "tape casting" or "screen printing" techniques by using different dilution of the PVC matrix in tetrahydrofuran (THF). Screen printing of the PVC membranes gave the worse results and was abandoned. With dip & dry and casting procedures, sensitivity of the valinomycin based sensors for K⁺ was found to be strictly related to the thickness of the membrane and the used deposition technique. A very good reproducibility was also observed by comparing the slope values of the calibration curves obtained from several electrodes. Results of this preliminary step for obtaining the best deposition condition of the PVC membrane are reported in table 1.

Table 1: Features of the calibration curves obtained with valinomycin/PVC based ion selective screen printed electrodes (ISSPE) for K⁺ ions, having different thickness of the membrane and using different deposition techniques.

<table>
<thead>
<tr>
<th>PVC</th>
<th>Sensitivity (mV/log K⁺)</th>
<th>C.V. (%)</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dip &amp; dry</td>
<td>247</td>
<td>63±2</td>
<td>2.9</td>
</tr>
<tr>
<td>Casting</td>
<td>458</td>
<td>11±3</td>
<td>27.3</td>
</tr>
<tr>
<td>Casting</td>
<td>417</td>
<td>28±7</td>
<td>23.8</td>
</tr>
<tr>
<td>Casting</td>
<td>336</td>
<td>34±6</td>
<td>17.6</td>
</tr>
</tbody>
</table>

The development of disposable, screen printed potentiometric sensors for free radicals, based on a nitrone species dispersed in the PVC membrane. Nitrone is able to give spin trapping reactions with free radicals and, for this reason, a membrane potential variation can be observed as previously verified [1]. PVC/nitroene coated electrodes (dip & dry) were tested in EDTA 0.02 mol/L, pH=5.16, FeCl₃ 0.02 mol/L and known amounts of H₂O₂ 0.1 mol/L were added for hydroxide radical production. A greatly over-Nernstian slope of the calibration curve was observed with high reproducibility (Fig. 3). This effect could be due to the propagation of the radical based reaction which may result in an amplification of the signal but, at the moment, additional interfering reactions cannot be excluded. PVC/nitroene coated electrodes (dip & dry) were used with the enzyme based method for superoxide radical production (xanthine/xanthine oxidase). The slope of the calibration curve was again over-Nernstian but lower than in the previous experiments and nearer to the expected value (Fig. 4).

Conclusions
Recently, screen printed electrodes were extensively used in the field of biosensors for obtaining mass production of low cost devices. In this frame, amperometry was the eligible technique for development of new screen printed electrochemical biosensors. In this paper, screen printed electrodes were used with the aim of demonstrating the possible development of low cost, potentiometry based, chemical sensors (ISSPE). Interesting results were also obtained, in a preliminary stage, concerning the high sensitivity determination of hydroxide and superoxide radicals with screen printed electrodes based on a nitrone species dispersed in a plasticized PVC matrix.

Acknowledgement
This work was financially supported by Consiglio Nazionale delle Ricerche (CNR) of Italy, Targeted Project "MADESS" and by the ENEA Target Project COSMIC (http://www.bioensing.net/cosmic.htm).

References

Figure 1: Scheme of a screen-printed electrode preparation. The printing sequence for obtaining multi-layered electrodes.

Figure 2: Scheme of the thin film flow cell for screen printed potentiometric electrodes made in our workshop.
SCREEN PRINTED BIOSENSORS BASED ON OXYGEN SENSING: USE OF PERM SELECTIVE MEMBRANES

ABSTRACT
Different membranes kinds were tested on screen printed electrodes to improve signal quality when sensing oxygen at a -700 mV potential (vs Ag/AgCl reference). The use of a cellulose acetate membrane, drugged with quaternary ammonium salts to provide the necessary electrolyte, allows a sensible reduction of noise for measurements at negative potentials.

INTRODUCTION
Biosensors which use thick or thin film electrodes carrying the biological mediator directly immobilized in various ways on their surface are very common in literature. This approach takes little care of the great number of interfering species which are often present in real samples. All the same the incidence of interfering species can be of great relevance on the measured signal. Some species can increase the signal if they discharge on the electrode or decrease the signal if they're able deplete oxygen concentration on the electrode surface. Some other interfering species do not affect directly the signal by an electrochemical reaction but may poison the electrode surface (phenols, proteins). Even in use with standard solutions and in absence of interfering species the presence of a membrane improves the quality of the signal. In absence of membranes, oxygen can saturate electrode. The possibility of covering µ-electrodes and capillary electrodes with a suitable membrane would be a great advantage for improving biosensors signal even with standard solutions and in absence of interfering species. Different membranes were tested on screen printed graphite electrodes to improve signal quality when sensing oxygen depletion due to enzyme reactions in presence of suitable substrates. Both planar electrodes and front to back geometry µ-electrodes were tested using GOD as a model biomolecule, having in this way the possibility of studying the electrode behavior at both positive and negative potentials, ranging from hydrogen peroxide oxidation potential (+700 mV vs. Ag/AgCl) to dissolved oxygen reduction potential (-700 mV vs. Ag/AgCl). Teflon, cellulose acetate and Araldite® membranes were tested even drugged with quaternary ammonium salts added directly to the CA solution (0.1%w/v). Different salts were tested (Tetra-ethyl-ammonium perchlorate (TEAP), Tetra-ethyl-ammonium Bromide, Tetra-methyl-ammonium Bromide, Tetra-n-butyl-ammonium Bromide(TBAB)) and the ones with the best solubility in the CA solution (TEAP and TBAB) were chosen. Results obtained with different salts added to CA and different immobilization procedures can be compared in table 1.

METHODS
Electrodes - µ-electrodes were printed, assembled and pre-treated for stabilization, as stated in former work [1], while planar electrodes were printed using a classical circular layout. Cellulose Acetate Membrane 3.95 g Cellulose acetate (Fluka) and 40 mg Polyvinyl acetate (MW 510000 droplets - RE-9) were put in a solution of 20 ml tetrahydrofuran (THF) and 30 ml acetone, and stirred continuously to complete dissolution. The solution so obtained was perfectly sealed and stored at 4°C until use. Quaternary ammonium salts in a percentage of 0.1%w were added to the solution before casting the membrane on the electrodes. Membranes were cast by dipping in m-electrodes while in the case of planar electrodes a drop of membrane solution was simply put on the electrode surface and used as a natural "glue" to fix a Pal membrane to be used for enzyme immobilization in a second time. To make both membranes tightly adhering to the surface electrodes were pressed at 30Kg/cm. Araldite® Membrane: (CBA: resin styrhenedi-acrylamide, hardener N3,4-di-methyl-amino-propyl)-1,3-propylen-diamine and solvent (either water or acetone were used), 2) with PAP (polyaniline) Harleco Polyacryl 173, 12% solids in water, from M. Delaunay-New York 10010) dissolving directly the enzyme with the least quantity of PAP needed, 3) with BPA (FLUKA)/Glutaredide (25% aqueous solution FLUKA) after treating the electrode's surface with aPTES (3-aminopropyl-triethoxysilane 98% Sigma), 4) with the amphoteric Pal membrane previously fixed on the electrode (20µl GOD solution – 20mg/ml). Free GOD was also used in solution in batch measurements.

RESULTS AND DISCUSSION
Very high current and a long time drift were obtained when bare electrodes coupled with GOD were based on oxygen depletion measurements (figure 1a). The very high partial pressure of the gas which reaches the bare electrode surface, negatively affected the sensor responses to glucose giving as a result noisy and unstable signals. A barrier to limit diffusion of the gas and to lower the current on the electrode surface [14] with similar approach of the commercial Clark electrodes, was adopted, putting a membrane onto the screen printed working electrode. Because in this case it could not be strictly fixed onto the tip of the electrode with an o-ring, it was cast close to the working electrode surface. Different membrane solutions were deposited with different techniques ranging from Teflon dispersed in aqueous solution, to silicon and cellulose acetate by dip and dry or screen printing or spin coating or casting (unpublished data). The best result, in terms of film adhesion, integrity and mechanical resistance, was obtained by casting a solution of CA (see materials and methods above) as shown in figure 1b were a stable baseline, a linear correlation of the current with glucose concentration, stable steady states for each glucose addition/concentration and higher signal to noise ratio, were obtained. To assure the electrode needed for potentiometric measurements and to keep the membrane closely adherent to the electrode surface without using a polyelectrolyte gel, quaternary ammonium salts were added directly to the CA solution (0,1%w/v). Different salts were tested (Tetra-ethyl-ammonium perchlorate (TEAP), Tetra-ethyl-ammonium Bromide, Tetra-methyl-ammonium Bromide, Tetra-n-butyl-ammonium Bromide(TBAB)) and the ones with the best solubility in the CA solution (TEAP and TBAB) were chosen. Results obtained with different salts added to CA and different immobilization procedures can be compared in table 1.

Epoxy resin used as a membrane with entrapped glucose oxidase, showed a large effect on glucose and oxygen diffusion. With epoxy resins we were able to obtain an extended linearity to glucose till 50-100mM. In figure 3 examples of extended linearity obtained with epoxy membranes of different thickness are reported.

CONCLUSIONS
Membranes were cast on screen printed graphite electrodes obtaining better performances in terms of stability, signal to noise ratio, selectivity and extended linearity when oxygen detection at -700mv vs Ag/AgCl is used with oxidases.

Acknowledgements: This research was supported by the EC (RoseProMilk Project, the 5thFW, Quality of Life) and by ENEA Target Project COSMIC (Coupling Smart Molecules Into Chips, http://biosensing.net/cosmic.htm)
A SCREEN-PRINTED ENZYME ELECTRODE FOR THE DETERMINATION OF ORGANO-PHOSPHOROUS PESTICIDES

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ABSTRACT

An improvement of pesticide-selective sensors, based on the reversible inhibition of acid phosphatase1 (AcP) coupled with screen printing technology. The catalytic activity of AcP was detected by means of a screen-printed graphite electrode, in the presence of the substrate ascorbic acid 2-phosphate (A2P). The corresponding current change due to the electrochemical oxidation of the ascorbic acid as reaction product was monitored. Particularly we focused our attention to the determination of the widely diffused pesticides, i.e. Malathion. Results show a high sensitivity, with a lower detection limit of about 5 ppb.

INTRODUCTION

Most of the biosensors, either electrochemical, optical or piezoelectric, which are used for pesticides determinations, are based on the detection of the catalytic activity of several enzymes in the presence of pesticides. The enzymes which have been most extensively employed for the realization of pesticide-sensitive biosensors are the cholinesterases, especially acetylcholinesterase (primarily among them butyrylcholinesterase and acetyl-cholinesterase) owing to the strong and mostly irreversible inhibition of their catalytic activity operated by different classes of pesticides. The major drawback of these biosensors is due to the fact that the irreversible inhibition of the enzyme activity leads to a rapid decrease in the sensitivity and performance of most of the cholinesterase based biosensors, so that each biocatalytic membrane can be used only for few assays. To overcome this limitation of cholinesterase-based biosensors, and on the basis of the same approach that has already been followed in the past for the realization of organophosphorous pesticide biosensor, we are proposing an improvement of this pesticide-selective sensor, based on the reversible inhibition of acid phosphatase (AcP) coupling with the screen printing technology. The method here proposed is based on the following catalyzed reaction:

\[ \text{A2P} \rightarrow \text{Ascorbic Acid} + \text{H}_2\text{PO}_4^- \]

The determination of Malathion was carried out by measurements of its inhibition values of the catalytic activity of AcP by means of the A2P-selective sensor.

EXPERIMENTAL SECTION

Materials:

- L-Ascorbic Acid 2-phosphate, Acid Phosphatase (Orthophosphoric-monooester phosphohydrolase, EC 3.1.3.2 from potato). Malathion was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The reference solution was obtained from BAS (Biotechnical Systems Inc. IN, USA). Carbon paste, silver conductive ink and dielectric polymer ink were purchased from Anchor Products.

Apparatus:

- Screen-printing was performed with an manual printing machine (Flexo). All electrochemical measurements were performed using an AUTOLAB PGST 10 (Ecochemie) potentiostat interfaced to a computer and controlled with Autolab-GPES version 4.7 for Windows 98.

Procedure to obtain screen-printed electrodes: Screen-printed graphite (SPG) electrodes (A, B, C) were designed for use in batch and flow cell measuring systems. Graphite electrodes were supported on a PyC layer. Silver ink acting as conductive medium was printed and cured at 70°C for 15 minutes. Carbon paste ink was printed and cured at the same temperature. An insulator layer was finally applied to cover the body of the electrode as reported in the figure 1.

Electrode pretreated: Electrodes were cut from the printed sheet and placed in 10 mL of 0.1 M NaOH. Voltammetric cycles were carried out between -500 and +500 mV vs. Ag/AgCl at a scan rate of 100 mV/s for 40 minutes.

APPEROMETRIC MEASUREMENTS

Calibration of the sensors

Amperometric measurements were carried out by connecting the previously described electrodes to a potentiostat at a constant potential of +400 mV vs. Ag/AgCl. Experiments were carried out in a glass cell, by using an uniformity of solution by a constant magnetic stirring, in 2.5 mL of citrate buffer 0.1 M, pH 5.5 and KCl 0.01 M. The electrode was employed both in the absence of the enzyme, to the direct determination of ascorbic acid concentration, and in the presence of AcP in the buffer solution, for the determination of A2P concentration.

Pesticide determinations

All the assays were performed under the same experimental conditions as described for the calibration experiments. The measurements were performed by dropping the sensor in citrate buffer 0.1 M, pH 5.5 and KCl 0.01 M containing A2P and AcP at concentrations of respectively 0.125 U/mL and 25 mM. After stabilization of the current signal, increasing quantities of the pesticide solution were added under constant stirring to calibrate the system. The current decrease, proportional to the lower ascorbic acid production due to the A2P inhibition, was recorded for 30 minutes. It represents the best compromise between the optimal response of the electrode and short time of analysis. In this way the calibration curve gives the decrease of current as a function of the concentration of added pesticide in an easy and reliable way.

RESULTS

The calibration graph, obtained with the standard solution of ascorbic acid without AcP, is reported in figure 2.

Figure 3 refers to a calibration curve performed in the presence of AcP and carried out on standard solution of A2P.

Figure 4 shows measurements carried out in the presence of several concentrations of pesticide. Table summarizes the main electroanalytical features of the screen-printed electrode referred to the inhibition assays.

CONCLUSIONS AND FUTURE STUDIES

The performance of the AcP based screen printed electrode proves to be a close correlation with previously obtained analogous bionzymatic biosensor, with the advantage of the requirement of only a single enzyme.

The another advantage of the AcP based sensor with respect to the cholinesterase based sensor is that the inhibition of AcP by organophosphorous pesticides is almost completely reversible, so that no reactivating treatment is required. The reversibility of the inhibition of AcP, in the case of the immobilized enzyme biosensor, could be also responsible for the relatively long shelf life of the sensor, leading to a drastic reduction of the overall costs of operation.

Additional experiments carried out to study the appropriate enzyme immobilization procedure for the optimization of the electroanalytical features of the screen-printed biosensor, are currently in progress.

REFERENCES:


An original procedure suitable for chemical immobilisation of engineered proteins was previously reported. This procedure involves the use of Au or graphite substrates, oriented and highly specific immobilisation of two engineered proteins, with specific binding properties of proteins to sensor surfaces. The ability of protein pre-concentration from crude extracts was demonstrated in these experiments. In this paper, the chemical synthesis of the chelator on Au or Au-graphite electrodes is reported with respect to chemical synthesis of antibodies. The higher Ni-NTA surface density (increased 30-fold), lower treatment time (1-12 with respect to 16h), ability to address the chelator on one electrode in a sensor matrix, and deposition of CYS-SAM on Au or Pt particles in a carbon matrix as a lower density of other molecules was deposited on Au electrode for faster diffusion of inhibitors and mediators (OCT) or for mediatorless direct electron transfer (PAN) from PSII to Au electrode.

**EXPERIMENTAL**: a) Chemical Au plating on Cu paths. Au electrodes were obtained from a commercial Cu sheet deposited on fiberglass. Real surface (A) of Au electrode was chronocoulometrically obtained and a roughness factor of 1.13 was determined.

b) Deposition of CYS-SAM and synthesis of Ni-NTA. CYS 20 mM in PB for 16h was used for chemical deposition. Screen printed Au-graphite or Pt-graphite composites allowed deposition of CYS only on the metal particles dispersed in the carbon pastes at 0.85 V versus SCE. Ni-NTA chelator was prepared as reported above. The surface density of Ni-NTA (513 pmol/m²) was achieved electrochemically for 20' greater (15 fold) than that obtained with a chemical treatment of 16h) and 30 fold after 20'

d) Synthesis of PAN molecular wire

e) Purification and immobilisation of PSII Thermophilic cyanobacterial Synecococcus elongatus 43H cells expressing psbC with an His tag were used. Immobilisation of PSII was obtained by incubation of electrodes in MES buffer containing PSII equivalent of 300 µg Chl/mL at 4°C in complete darkness for 20'.

**RESULTS AND DISCUSSION**: i) Comparison of PSII monolayer vs crosslinked PSII in BSA-GA-MATRIX. Fig 2 reports the velocity of reoxidation of reduced DQ in the case of BSA-GA-PSII electrode and BSA-GA-SAM-NTA-PSII. In the case of PSII monolayer, a rapid inhibition of PSII electrode is observable directly after the addition of herbicide. On the contrary, for BSA-GA-SAM-NTA-PSII gel matrix a stable signal of inhibited PSII electrode is observed after 15' of herbicide exposition. 

**CONCLUSIONS**: Electrochemical deposition of CYS-SAM on Au surfaces results a good method to lower treatment time (1-12' with respect to 16h of the chemical treatment), and address the synthesis on only one Au electrode in a sensor matrix. With screen printed Au-graphite or Pt-graphite composites, selective deposition of CYS-SAM on Au or Pt particles can be achieved. The number of Ni(II) heads available for histag mediated immobilisation can be decreased and different molecules deposited (i.e. PAN conducting films or mixed hydrophobic SAMs) on the Au surface, hopefully obtaining better performances of the deposition in terms of substrate diffusion or direct electron transfer onto the electrode.

**REFERENCES**

**ACKNOWLEDGEMENTS**: This work was supported partly by the ROGERS/MILK Project (Q102079) of the Biobolus Project, the 5th WP, Quality of Life Program, partly by COSMIC Project (INCA Target Project on Biosensors and Bioimmobilizations) and partly by INTEC Project (SMA 'Project on Biosensors and Bioimmobilizations').
SCREEN PRINTED ELECTROCHEMICAL BIOSENSORS: FANCY OR VALID ALTERNATIVE FOR ENVIRONMENTAL ANALYSIS?

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Introduction
Pesticides and herbicides are largely diffused in agriculture because of their high efficiency but their recycling processes result in water and soil pollution, with dangerous and acute effects on the living organisms, i.e., inhibiting either the active site of enzymes, or the photosynthetic activity. HPLC, GC-MS, ELISA methods were shown to be expensive and time consuming in the sample treatment, do not provide any information about toxicity on living organisms. AcChE and OP inhibitors biosensors were already developed to detect paraxon, methyl-paraxon and diazinon, with LOD of 0.5-1.8 x 10^{-7} M, 0.6-9.1 x 10^{-7} M and 0.46-8.8 x 10^{-7} M. Two specific environmental applications are reported: an engineered yeast, which express acetyl cholinesterase activity from rat, used for detection of OP compounds and an engineered PSII for herbicides detection were both immobilized on SPEs.

SCREEN PRINTED ELECTRODES PREPARATION
SPGEs on PVC substrate according to different layouts and configurations were electrochemically tested, in order to improve TPT performance in detector production.
1) ink doping (Pt or Rh adsorbed on graphite particles) improve the sensitivity up to a maximum value of doping (0.5%). carbon ink can be doped with several electrochemical mediators as well as ferrocene derivatives or pyrrole-SPEs; photochemically RE: reference electrode (Ag/AgCl); RSD: relative standard deviation; SAm: self-assembled monolayer; SEM: scanning electron microscopy; SPE: screen printed electrode; SPGE: screen printed graphite electrode; TPT: thin film technology; WE: working electrode

GOLD PLATING ON Cu PATHS

CYS-SAM and synthesis of NiNTA
Au-graphite or graphite SPEs allowed chemical deposition of CYS (20 mM in PB for 16h) on the metal particles dispersed in the carbon pastes at 0.85V vs RE. Synthesis of NiNTA arms was also made electrochemically with or without spacers. The surface density of NiNTA (513 pmol/mm²) resulted greater (15-fold) than that one obtained with chemical treatment for 16h.

Electrochemical CYS-SAM synthesis

Screen printed electrodes by thick film technology

CONCLUSIONS:
Stable biosensors for triazine herbicides were developed, using an original procedure for oriented and highly specific immobilization of (His)₆-tag-PSII (via NiNTA chelator) on gold and graphite surfaces. Different Au-air electrodes or Au-graphite/SPGEs were made on RSDS, resulting in: i) specific binding, ii) sensitive detection of analytes, iii) "chip" protein pre-concentration starting from crude unpurified bacterial extracts, iv) lower treatment times and consistently biosensors working on microwells.
1st announcement

International Association of Environmental Analytical Chemistry

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Biosensors and BioAnalytical μ-Techniques in Environmental and Clinical Analysis

October 8-12, 2004
ENEA, S. Maria di Galeria, Rome, Italy
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Keywords:
Optrode, electrode, acrode based biosensors, Oriented and/or Reversible Immobilization, Molecular recognition, Immunossay, Immunosensors, Electronic/Bioelectronic Noses, Enzyme-biosensors, Metabolic Biosensors, Photosynthetic biosensors, DNA chips, GM(O) based sensors, Protein chips, Amplification strategies, FIA, Biochips, μ-arrays, μ-fluidics, Lab on a chip, Environmental Bioanalysis, Clinical Analysis, Food Analysis.

You can find additional details and news about the workshop on www.biosensing.net/iaeac.html
please send a message to rome2004@biosensing.net to be added to the mailing list of the 2nd circular

Recently, the word “ENVIRONMENT” has been acquiring a broader meaning. Prevention, monitoring, and depuration are now focused not only on the chemical detection of air, water and soil pollutants but also on the health of the ecosystem, quality of life, including not only man but all living beings clinical diagnostics and food safety, industrial activities and products, effects from chemical, biological and physical agents. In this sense the need for controls of such a complex ENVIRONMENT reflects the request for an increased measurement capability, mainly in terms of number of analyses and costs, but also in terms of knowledge of the relationship between causes and effects. For instance, effects of radiations from electromagnetic fields on animals, cell metabolism, genes and proteins represent a relevant topic which is still to be understood and studied in many aspects. Genetically modified organisms (GMO) and microorganisms (GMMO), for new processes and products in the field of agriculture, food and therapy (new drugs and vaccines), represent new challenges for the sustainable progress of mankind. However, they have to be well known and controlled, to rule out their possible negative effect on health and biodiversity. For these reasons, the use of sensor-based analytical method, originally focused on chemical and biochemical tests, is gaining increasing interest in the fields of environmental toxicity testing, for ecosystem monitoring as well as testing of crops and foods of animal origin, clinical diagnosis and therapy.

The increased interest in sensor-based techniques is proven by the significant number of both scientific papers and registered patents on this subject. Multidisciplinarity between chemistry, material sciences, biochemistry, molecular biology, physics, microelectronic technologies, and engineering has created important new ideas in several research fields, including biosensing, and remarkable results for improving quality of life on our planet can be expected. For these reasons,

the workshop chairs, the scientific and the local organizing committees are certain that the workshop will be a successful occasion for researchers to meet and generate new ideas and relevant results. Young researchers are encouraged to attend in order to contribute their enthusiasm and new ideas to the biosensing field. For this purpose, travel awards are being made available from sponsors.

Scientific papers will be published in a special issue of Intern. J. Environ. Anal. Chem (http://www.tandf.co.uk/journals/titles/03067319.htm)

Chairs:
Roberto Pilgtoin, ENEA, Rome, Italy
Ursula Spichiger, CCS, ETH Technopark, Zurich, Switzerland

Scientific Committee
Antje J. Beurmer, Cornell University, Ithaca, USA
Luigi Campagnella, Rome University, La Sapienza, Italy
Carlo Cremisini, Enea, Rome, Italy
Elena Dominguez, University of Alcala, Spain
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Bertold Hock, Technical University of Munich, Germany
Marco Mascini, Florence University, Italy
Giuseppe Palmacci, Rome University, Tor Vergata, Italy
Aldo Roda, Bologna University, Italy

Local Organizing Committee
Dario Compagnone, University of Teramo, Italy
Franco Macge, University of Rome, La Sapienza, Italy
Lucia Moscillo, ENEA, Rome, Italy
The 6th Workshop on
Biosensors and BioAnalytical μ-Techniques in Environmental and Clinical Analysis (BioμTECA)
October 8-12, 2004, ENEA S. Maria di Galeria, Rome, Italy
www.biosensing.net/iaceucl.html

Registration form

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Participate with a presentation:

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*Visit www.biosensing.net/iaeucl.html for a list of suggested key-words

Date, __________________________ Sign __________________________

Send this form immediately to: rome2004@biosensing.net or by fax to +390630484096
or by ordinary mail to:
The sixth BioμTECA Workshop
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