Biosensors and Environmental Health

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Editors

Victor R. Preedy PhD DSc

Professor of Nutritional Biochemistry
School of Medicine
King's College London
and
Professor of Clinical Biochemistry
King's College Hospital
UK

Vinood B. Patel

Department of Biomedical Science
School of Life Sciences
University of Westminster
London
UK

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Preface

Biosensors have a simplistic concept but a great deal of sophistication in design, manufacture and application. They essentially have a biological component within them and are used to detect, monitor or quantify substances. They use a variety of physical platforms and technologies. The biological components may include enzymes, membranes and cells or any other naturally occurring biological product. Some have artificial biological components such as modified molecules or polymers. Biosensors may be used to detect single or groups of molecules and have wide applicability to the life sciences. In this book we aim to disseminate the information on biosensors in a readable way by having unique sections for the novice and expert alike. This enables the reader to transfer their knowledge base from one discipline to another or from one academic level to another. In this book we focus on environmental issues. Chapters in **Biosensors and Environmental Health** have an abstract, key facts, applications to other areas of health and disease and a "mini-dictionary" of key terms and summary points. The book describes new methods, prototypes and applications. For example coverage includes: personal toxicity testing, soil and risk assessment, pesticide, insecticides, parasites, nitrate, endocrine disruptors, heavy metals, food contamination, whole cell bioreporters, bacterial biosensors, antibody-based biosensors, enzymatic, amperometric and electrochemical aspects, quorum sensing, DNA-biosensors, cantilever biosensors, bioluminescence and other methods and applications.

Contributors to **Biosensors and Environmental Health** are all either international or national experts, leading authorities or are carrying out ground breaking and innovative work on their subject. The book is essential reading for environmental scientists, toxicologists, medical doctors, health care professionals, pathologists, biologists, biochemists, chemists and physicists, general practitioners as well as those interested in disease and sciences in general.

The Editors

Contents

List of Contributors

Vojtech Adam
Mendel University in Brno, Department of Chemistry and Biochemistry, Zemedelska 1, 61300 Brno, Czech Republic.
E-mail: vojtech.adam@mendelu.cz

Erol Akyilmaz
Ege University Faculty of Science Biochemistry Department 35100 Bornova/ Izmir, Turkey.
E-mail: erol.akyilmaz@ege.edu.tr

Silvana Andreescu
206 Science Center, Clarkson University, PO Box 5810, Potsdam, NY 13699-5810 USA.
E-mail: eandrees@clarkson.edu

Maria Daniela Angione
Department of Chemistry, University of Bari, Via Orabona 4, I-70126 Bari, Italy.
E-mail: angione@chimica.uniba.it

Mariarita Arenella
Dipartimento di Scienze delle Produzioni Vegetali, del Suolo e dell'Ambiente Agroforestale, Università di Firenze, Piazza delle Cascine 18, 50144 Firenze, Italy.
E-mail: mariarita.arenella@unifi.it

Umberto Bencivenga
Istituto of Genetica and Biofisica del CNR, Napoli, Italia.
E-mail: benciven@igb.cnr.it

S. Buchinger
German Federal Institute of Hydrology (BfG), Division of Qualitative Hydrology, Am Mainzer Tor 1, 56068 Koblenz, 56068 Germany.
E-mail: Buchinger@bafg.de

Xinxia Cai
State Key Laboratory of Transducer Technology, Institute of Electronics, Chinese Academy of Sciences, Beijing 100190, P.R. China.
E-mail: xxcai@mail.ie.ac.cn

Swati Choudhary
Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, 140 Gortner Laboratory, St. Paul, MN 55198, USA.
E-mail: swati@umn.edu

Nicola Cioffi
Department of Chemistry, University of Bari, Via Orabona 4, I-70126 Bari, Italy.
E-mail: cioffi@chimica.uniba.it

Serafina Cotrone
Department of Chemistry, University of Bari, Via Orabona 4, I-70126 Bari, Italy.
E-mail: cotrone@chimica.uniba.it

Liu Deng
State Key Laboratory of Electroanalytical Chemistry, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun, Jilin, 130022, P. R. China.
E-mail: dengliu@ciac.jl.cn

Shaojun Dong
State Key Laboratory of Electroanalytical Chemistry, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun, Jilin, 130022, P. R. China.
E-mail: dongsj@ciac.jl.cn

Gabriele Favero
Department of Chemistry and Drug Technologies, Sapienza University of Rome, P.le Aldo Moro, 5 – 00185 Roma, Italy.
E-mail: gabriele.favero@uniroma1.it

Zhixian Gao
Tianjin Key Laboratory of Risk Assessment and Control Technology for Environment and Food Safety, Tianjin Institute of Health and Environmental Medicine, No. 1 Dali Road, Heping District, Tianjin, China.
E-mail: Gaozhx@163.com

Maria Hepel
Department of Chemistry, State University of New York at Potsdam, Potsdam, New York 13676, USA.
E-mail: hepelmr@potsdam.edu

Wei E. Huang
Kroto Research Institute, Department of Civil and Structural Engineering, North Campus, University of Sheffield, Broad Lane, Sheffield S3 7HQ, UK.
E-mail: w.huang@shef.ac.uk

Jaromir Hubalek
University of Technology in Brno, Department of Microelectronics.
E-mail: hubalek@feec.vutbr.cz

Georges Istamboulie
IMAGES EA 4218, Biosensors group, Building S, Université de Perpignan Via Domitia, 52 Av Paul Alduy 66860 Perpignan cedex – France.
E-mail: georges.istamboulie@univ-perp.fr

Stephen L. Kaattari
Virginia Institute of Marine Science, Department of Environmental and Aquatic Animal Health, College of William & Mary, PO Box 1346, Gloucestor Point, Virginia 23062-1346 USA.
E-mail: kaattari@vims.edu

Rene Kizek
Mendel University in Brno, Department of Chemistry and Biochemistry, Zemedelska 1, 61300 Brno, Czech Republic.
E-mail: kizek@sci.muni.cz

Maria Lepore
Dipartimento di Medicina Sperimentale, Seconda Università di Napoli, Napoli, Italia.
E-mail: maria.lepore@unina2.it

Guanghe Li
Department of Environmental Science & Technology, Tsinghua University, Beijing 100084, P.R. China.
E-mail: ligh@mail.tsinghua.edu.cn

Nan Liu
Tianjin Key Laboratory of Risk Assessment and Control Technology for Environment and Food Safety, Tianjin Institute of Health and Environmental Medicine, No. 1 Dali Road, Heping District, Tianjin, China.
E-mail: LNQ555@126.com

Xiaohong Liu
Graduate School of Chinese Academy of Sciences, Beijing 100080, P.R. China.
E-mail: xinqi_0120@163.com

Jinping Luo
State Key Laboratory of Transducer Technology, Institute of Electronics, Chinese Academy of Sciences, Bejing 100190, P.R. China.
E-mail: jpluo@mail.ie.ac.cn

Maria Magliulo
Department of Chemistry, University of Bari, Via Orabona 4, I-70126 Bari, Italy.
E-mail: magliulo@chimica.uniba.it

Antonia Mallardi
CNR-IPCF, Istituto per i Processi Chimico-Fisici, Via Orabona 4, I-70126 Bari, Italy.
E-mail: a.mallardi@ba.ipcf.cnr.it

Jean-Louis Marty
IMAGES EA 4218, Biosensors group, Building S, Université de Perpignan Via Domitia, 52 Av Paul Alduy 66860 Perpignan cedex – France.
E-mail: jlmarty@univ-perp.fr

Franco Mazzei
Department of Chemistry and Drug Technologies, Sapienza University of Rome, P.le Aldo Moro, 5 – 00185 Roma, Italy.
E-mail: franco.mazzei@uniroma1.it

Damiano Gustavo Mita
Dipartimento di Medicina Sperimentale, Seconda Università di Napoli, Napoli, Italia.
E-mail: mita@igb.cnr.it

Fumio Mizutani
Graduate School of Material Science, University of Hyogo, 3-2-1 Kouto, Kamigori-cho, Ako-gun, Hyogo 678-1297, Japan.
E-mail: mizutani@sci.u-hyogo.ac.jp

Raj Mutharasan
Department of Chemical and Biological Engineering, Drexel University, Philadelphia, PA 19104, USA.
E-mail: mutharasan@drexel.edu

Thierry Noguer
IMAGES EA 4218, Biosensors group, Building S, Université de Perpignan Via Domitia, 52 Av Paul Alduy 66860 Perpignan cedex – France.
E-mail: noguer@univ-perp.fr

Anna Nowicka
Department of Chemistry, State University of New York at Potsdam, Potsdam, New York 13676, USA.
Permanent address: Department of Chemistry, University of Warsaw, PL-02-093 Warsaw, Poland.
E-mail: anowicka@chem.uw.edu.pl

Gerardo Palazzo
Department of Chemistry, University of Bari, Via Orabona 4, I-70126 Bari, Italy.
E-mail: palazzo@chimica.uniba.it

Rosa Pilolli
Department of Chemistry, University of Bari, Via Orabona 4, I-70126 Bari, Italy.
E-mail: pilolli@chimica.uniba.it

Karen Polizzi
Division of Molecular Biosciences & Centre for Synthetic Biology and Innovation, Imperial College London, London SW7 2AZ, UK.
E-mail: k.polizzi@imperial.ac.uk

Marianna Portaccio
Dipartimento di Medicina Sperimentale, Seconda Università di Napoli, Napoli, Italia.
E-mail: marianna.portaccio@unina2.it

Victor R. Preedy
Diabetes and Nutritional Sciences, School of Medicine, Kings College London, Franklin Wilkins Buildings, 150 Stamford Street, London SE1 9NU, UK.
E-mail: victor.preedy@kcl.ac.uk

Edoardo Puglisi
Istituto di Microbiologia, Università Cattolica del Sacro Cuore, Via Emilia Parmense 84, 29122 Piacenza, Italy.
E-mail: edoardo.puglisi@unicatt.it

Rajkumar Rajendram
Locum Consultant Physician, Department of Medicine, John Radcliffe Hospital, Oxford OX3 9DU, UK.
E-mail: rajkumarrajendram@doctors.org.uk

Javier Ramón-Azcón
Graduate School of Material Science, University of Hyogo, 3-2-1 Kouto, Kamigori-cho, Ako-gun, Hyogo 678-1297, Japan.
E-mail: ramonazconjavier@gmail.com

G. Reifferscheid
German Federal Institute of Hydrology (BfG), Division of Qualitative Hydrology, Am Mainzer Tor 1, 56068 Koblenz, 56068 Germany.
E-mail: Reiffersceid@bafg.de

Giancarlo Renella
Dipartimento di Scienze delle Produzioni Vegetali, del Suolo e dell'Ambiente Agroforestale, Università di Firenze, Piazza delle Cascine 18, 50144 Firenze, Italy.
E-mail: giancarlo.renella@unifi.it

Claudia Schmidt-Dannert
Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, 140 Gortner Laboratory, St. Paul, MN 55198, USA.
E-mail: schmi232@umn.edu

Li Shang
Institute of Applied Physics and Center for Functional Nanostructures (CFN), Karlsruhe Institute of Technology (KIT), Wolfgang-Gaede-Strasse 1, 76131 Karlsruhe, Germany.
E-mail: lishang208@gmail.com

Pavlina Sobrova
Mendel University in Brno, Department of Chemistry and Biochemistry, Zemedelska 1, 61300 Brno, Czech Republic.
E-mail:pavlina.sobrova@seznam.cz

Yizhi Song
Department of Environmental Science & Technology, Tsinghua University, Beijing 100084, P.R. China.
E-mail: song.ezhi@gmail.com

Candace R. Spier
Virginia Institute of Marine Science, Department of Environmental and Aquatic Animal Health, College of William & Mary, PO Box 1346, Gloucestor Point, Virginia 23062-1346 USA.
E-mail: cspier@vims.edu

Magdalena Stobiecka
Department of Chemistry, State University of New York at Potsdam, Potsdam, New York 13676, USA.
E-mail: stobiemk@potsdam.edu

Qing Tian
State Key Laboratory of Transducer Technology, Institute of Electronics, Chinese Academy of Sciences, Beijing 100190, P.R. China.
E-mail: tianqing@ustc.edu

Luisa Torsi
Department of Chemistry, University of Bari, Via Orabona 4, I-70126 Bari, Italy.
E-mail: torsi@chimica.uniba.it

Marco Trevisan
Istituto di Chimica Agraria ed Ambientale, Università Cattolica del Sacro Cuore, Via Emilia Parmense 84, 29122 Piacenza, Italy.
E-mail: marco.trevisan@unicatt.it

Libuse Trnkova
Masaryk University, Brno, Department of Chemistry.
E-mail: libuse@chemi.muni.cz

Daniela Di Tuoro
Dipartimento di Medicina Sperimentale, Seconda Universita di Napoli, Napoli, Italia.
E-mail: daniela.dituoro@libero.it

Michael A. Unger
Virginia Institute of Marine Science, Department of Environmental and Aquatic Animal Health, College of William & Mary, PO Box 1346, Gloucestor Point, Virginia 23062-1346 USA.
E-mail: munger@vims.edu

Mona Wells
Helmholtz Centre for Environmental Research, Department of Environmental Microbiology, Permoserstraβe 15, Leipzig, 04318, Germany.
E-mail: mona.well@ufz.de

Sen Xu
Department of Chemical and Biological Engineering, Drexel University, Philadelphia, PA 19104, USA.
E-mail: xuzhonghou@gmail.com

Tomoyuki Yasukawa
Graduate School of Material Science, University of Hyogo, 3-2-1 Kouto, Kamigori-cho, Ako-gun, Hyogo 678-1297, Japan.
E-mail: yasu@sci.u-hyogo.ac.jp

Weiwei Yue
State Key Laboratory of Transducer Technology, Institute of Electronics, Chinese Academy of Sciences, Beijing 100190, P.R. China.
E-mail: physics_yue@163.com

Dayi Zhang
Kroto Research Institute, Department of Civil and Structural, Engineering, North Campus, University of Sheffield, Broad Lane, Sheffield S3 7HQ, UK.
E-mail: d.zhang@shef.ac.uk

Eko Ge Zhang
Fetal and Maternal Medicine, Imperial College NHS Trust, London SW7 2AZ, UK.
E-mail: GeEko.Zhang@Imperial.nhs.uk

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Immunochips for Personal Toxicity Testing

Zhixian Gao,[1,a] Nan Liu[1,b] and Rajkumar Rajendram[2]

ABSTRACT

People are exposed to many chemicals in the course of day-to-day life. Measurement of the exposure of the environment and its inhabitants to pollutants is a useful estimate of the toxic effects of environmental pollution on health. The microarray is a sensitive and precise device which can be used to obtain this information from complex biological samples. Microarrays can thus be used to assess the "health" of the environment or an individual person. The use of microarrays allows complex, automated, high-throughput processes to be performed in small devices. The "immunochip" is a one of the formats of protein microchip based on the molecular specific immunological recognition of antigens (Ags) by antibodies (Abs) immobilized on a certain surface that together respond in a concentration-dependent manner. Recent work from our laboratory demonstrated that immunochip technology can simultaneously detect at least five different chemicals. This chapter discusses the various types of immunochips available and their application in personal toxicity testing. The definition, main features,

[1]Tianjin Key Laboratory of Risk Assessment and Control Technology for Environment and Food Safety, Tianjin Institute of Health and Environmental Medicine, No. 1 Dali Road, Heping District, Tianjin, China.
[a]E-mail: Gaozhx@163.com
[b]E-mail: LNQ555@126.com
[2]Locum Consultant Physician, Department of Medicine, John Radcliffe Hospital, Oxford OX3 9DU, England.
E-mail: rajkumarrajendram@doctors.org.uk

List of abbreviations after the text.

and probes of "conventional" immunochips, "Lab-on a chip" and "suspension arrays" are included. "Lab-on a chip" integrates several laboratory processes including preparation, incubation, detection and analysis on a single microchip. This eliminates the need for several different pieces of laboratory equipment to prepare and analyze a biological sample. The "high-throughput suspension" array is a novel method for multi-analysis of veterinary drugs. It is easy to use, very sensitive and inexpensive. However, immunochips are difficult to use in the field. A high-quality Ab with good bioactivity and specificity is the key reagent in the production of immunochips. Although further investigation is required, the potential advantages of immunochip technology for the detection of chemicals for environmental assessments are of great interest.

INTRODUCTION

Millions of chemicals have been created as civilization has advanced and industry and agriculture have developed. Modernization has simultaneously facilitated human existence and wrought havoc on the environment. Pollution is a global phenomenon of major concern.

The air quality of large- and medium-sized cities is poor worldwide. Air pollution affects many countries but is the worst in the developing world. Soil is commonly contaminated with solvents, hydrocarbons derived from petrochemicals, heavy metals and pesticides. Organic pollutants are a major problem affecting water quality. Eutrophication occurs naturally as bodies of water age, but the process is accelerated by pollution. Many lakes in China are in the intermediate or advanced stages of eutrophication. Pollution from industry, agriculture and domestic life damages the ecosystem and is a major hazard to human health. Air pollution increases excess mortality rates especially in the developing countries. The incidence of gastrointestinal tract tumors is increased with consumption of contaminated water. Many environmental contaminants, pollutants and toxins interfere with immune defense, immune signal transduction, and induce hypoimmunity and do harm to population health.

The general population is exposed to many chemicals in the course of day-to-day life. Measurement of the exposure of the environment and its inhabitants to pollutants is a useful estimate of the toxic effects of environmental pollution on health. "Safe" levels of exposure for many chemicals have yet to be determined. Newly synthesized compounds are released regularly and data on their intermediate products and by-products are scanty. However, relationships between levels of exposure and adverse effects on health remain unclear. So, even when guidance is available, standards for safe or acceptable concentrations or levels of exposure often

vary between countries. Assessment of the toxicity of chemicals *in vivo* is urgently in need of further research.

Personal toxicity testing is used to detect environmental toxins/pollutants/contaminants and/or their metabolites and biomarkers. Chromatogram (Koblížek et al. 2002), liquid chromatography with mass spectrometry (LC-MS) technology (Ramos et al. 2003) and enzyme-linked immunoadsorbent assay (ELISA) (Estévez et al. 2006; Mart'ianov et al. 2005) are widely employed for the detection of chemicals. However, these techniques require meticulous sample preparation and are complex to perform, so are expensive and time consuming. These shortcomings cannot be easily overcome.

The use of recently developed scientific techniques such as proteomics, metabolomics and metallomics could enhance personal toxicity testing and environmental assessments. The research, development and use of these and other new techniques, could improve monitoring of environmental pollution and the effects on human health. The recent production of microchips which can perform these techniques has enabled production of small, automated analytical systems for this purpose.

Microarray or biochip technology allows the simultaneous monitoring of several biological processes in a single experiment. These sensitive and precise, automated techniques can obtain vast amounts of data from each biological sample. Small, high-throughput systems utilizing microarrays can be used to detect and analyze samples containing bacteria (Song and Dinh 2004), viruses (Zhou et al. 2005), veterinary drug residues (Du et al. 2005) and tumor biomarkers (Ghobrial et al. 2005; Huang et al. 2004; Miller et al. 2003; Ghobrial et al. 2005; Huang et al. 2004).

Biochips have several uses in the agricultural, veterinary, healthcare, and medical sectors. DNA microarrays, which contain single strand nucleotide probes bound to a solid substrate, are used to identify DNA or RNA. DNA arrays function on the principle of nucleic acid hybridization on a surface-immobilized template. Protein arrays (protein microchips) contain various proteins probes imprinted on solid surface and can detect interactions between different proteins (Chiem and Harrison 1998; Wildt et al. 2000).

The immunochip is a protein microchip based on the specific recognition of Ags by Abs immobilized on a solid planar surface (e.g. glass) that responds in a concentration-dependent manner to a chemical target. Although plastic, gold and silicon have been used, and several novel surfaces have been developed including porous polyacrylamide gel pad slides (Arenkov et al. 2000) and microwells (Zhu et al. 2000), the most common surface used for immobilization of bio-recognition agents is the glass microscope slide.

When complementary Ags or Abs react with probes on the array, the resulting array image or fluorescent intensity (FI) can be captured by laser

scanner and analyzed by software. Microarray images are necessarily of high resolution and are therefore large (typically at least 1500 × 3500 pixels). Multifunctional data analysis software such as GenePix Pro, ScanAlyze and BlueFuse can be used to analyze FI data.

Several modifications of the conventional immunochip platform have been described. These include the "lab-on a chip" (Du et al. 2005), a hydrogel-based immunochip (Rubin et al. 2005) and the suspension array (Connolly et al. 2010; Kalogianni et al. 2007) which immobilizes bio-active materials on a fluorescent coded bead/microsphere.

High-output, automated, sensitive, analysis of several chemicals can be performed simultaneously. The potential scope for use of the immunochip is therefore remarkable. Although immunochip platforms are fairly versatile, configurations are limited as chips must be designed to detect a specific group of compounds. Abs with good bioactivity and specificity are the best probes currently available. The use of high titres of Abs with high specificity; especially monoclonal Ab (mAb), can significantly increase the accuracy and sensitivity of the immunochip. Phage display technology and ribosome display technology are tools for the generation of high-quality Abs. However, acquiring the key reagent, i.e. the specific Ab required, is still a limiting factor in the production of immunochips.

The Luminex (xMAP®) suspension array is another type of immunochip. It is a microarray on a microsphere surface which enables greater efficiency and output than a glass slide (planar, solid microarray). This system is based on internally color-coded microspheres with surface linked Abs, receptors, or oligonucleotides. Beads containing one of 100 specific dye sets can be differentiated by flow-cytometry. Binding of the surface label indicates analyte binding and can also be detected using a second, shorter-wavelength dye and a dual laser detector (Dasso et al. 2002).

It is inexpensive, versatile and considered as an open platform that has been widely employed for multiplex analysis of ribosomal RNA (rRNA), microRNA, cytokines and single-nucleotide polymorphisms (SNP); selecting and screening of mAbs; detection and testing for Abs, bacterial toxins, polysaccharides and autoantigens in serum, cerebrospinal fluid, dried blood spot specimens, and stool samples (Liu et al. 2009).

Binding to the solid support of ELISA or other microarrays denatures or dries bioactive materials. However, due to robust multiplexing, the Luminex suspension array can obtain more data more quickly from samples in the aqueous phase. Analysis of samples in the aqueous phase maintains their bioactivity for probes. Suspension array technology also offers several other advantages over traditional methods. It is versatile, flexible, accurate, reproducible and high-throughput.

APPLICATION TO HEALTH AND DISEASE

The aim of personal toxicity testing and environmental assessments is to determine the effect of pollution on health. Traditionally the diagnosis of disease was based on a single diagnostic test. However, modern clinical practice, bases the diagnosis of disease on the synthesis of data from several sources. Similarly environmental medicine and environmental health assessments can now use biomarkers and cytokines as well as the detection of toxins to determine the effects of pollution. The accuracy and validity of environmental health assessments are improved simply by taking more factors into account.

Immunochip based analytical techniques have a wide range of potential uses for personal toxicity testing and environmental assessments. Immunochips are already used extensively in many similar fields. For example, clinical diagnostics, biomedicine and pharmaceutical analysis use immunochips to detect disease related protein changes. Immunochips have also been used for the assessment of food and drink. Potential uses for immunochips include analyses of nutrients, organic toxins (e.g. bacterial toxins, mycotoxins and hormones) and inorganic toxins (e.g. pesticides, and heavy metals). These chemicals pollute the environment and cause disease. In comparison to other diagnostic techniques, those which use immunochips can provide large amounts of data in simple, rapid, automated, and relatively inexpensive processes.

KEY FACTS ON THE USE OF IMMUNOCHIPS FOR PERSONAL TOXICITY TESTING

- Many environmental contaminants, including polychlorinated biphenyl compounds, formaldehyde, heavy metals, organ phosphorus pesticide, tobacco and smog affect white blood cell function and immune signal transduction. This impairs cellular and non-cellular immunity and reduces population health.
- Personal toxicity testing requires rapid, accurate detection of a diverse range of chemicals which are often present in small traces and analysis of the interactions of these toxins with health and disease. This remains a formidable challenge.
- Analytical techniques which use microarray or biochip technology allow sensitive and precise monitoring of multiple processes in biological samples in a single experiment.
- Immunochips have a wide range of potential uses and can provide large amounts of data in simple, rapid, automated, and relatively inexpensive processes.

- Abs with good bioactivity and specificity are the best probes currently available and are the key reagents in the production of immunochips.
- The use of high titres of Abs with high specificity; especially monoclonal Abs, significantly increases the accuracy and sensitivity of the immunochip.
- Phage display technology and ribosome display technology are other alternative ideal tools for the generation of high-quality Abs.

DEFINITIONS OF KEY TERMS, GENES, CHEMICALS AND PATHWAYS

- Endocrine disruptor chemical (EDC) is the external agent that interferes with hormonal function *in vivo*. Any stage of hormone production and activity can be affected. For example by preventing hormones synthesis, binding directly to hormone receptors, or interfering with the natural breakdown of hormones. These agents can impair endocrine function *in vivo* and are toxic to human health and the environment.
- Immunochip technology is based on the specific, concentration-dependent, immunological recognition of Ags by immobilized Abs. The most common surface used for immobilization of Abs is a glass slide.
- Suspension array is another kind of immunochip and is simply a transfer of the microarray format from a glass slide (planar and solid microarray) to a high-throughput and efficient microsphere format. This system is based on internally color-coded microspheres with surface linking chemistry to accommodate Abs, receptors, or oligonucleotides. Beads containing one of 100 specific dye sets can be differentiated using flow-cytometry. Binding of the surface label indicates analyte binding. This can be detected using a second, shorter-wavelength dye and a dual laser detector (Dasso et al. 2002).
- "Lab-on-chips" integrate several laboratory processes on a single microchip. These procedures include preparation, incubation, detection and analysis.
- Phage display technology is widely used to display Ab libraries on the surface of filamentous bacterio-phages. The libraries allow the selection of Abs with high specificity and affinity for any Ags. A phagemid-based Ab display library is made by cloning Ab genes into a phagemid vector at the 5′end of the g III within the

phagemid vector. This is followed by transformation of *Escherichia coli* (Hoogenboom et al. 1991).

- Ribosome display is a powerful *in vitro* display technology. It exploits cell-free translation to generate a selection unit comprising a "stalled" ribosome linking a protein to its encoding mRNA (McCafferty et al. 1994). This pro-karyotic cell-free translation system can develop high affinity Abs.

THE USE OF CONVENTIONAL IMMUNOCHIPS FOR PERSONAL TOXICITY TESTING

Based on the molecular weight (MW) and the structure of the target molecules, chemicals or toxins can be divided into macromolecules and "small molecules". The immunochips use patterned Abs/Ags and sandwich immunoassays for the detection of their complimentary Ags/Abs. Most toxins are small molecules. As there is generally only one binding site in the structure of small molecules, direct or indirect competitive immunoassays are used to detect toxins in the immunochip.

Sapsford et al. (2006) employed the Naval Research Laboratory (NRL) array biosensor, an indirect competitive immunochip, to detect aflatoxin B_1 (AFB_1) in corn and nut products. The NeutrAvidin slides patterned with biotinylated AFB_1 were designed so that the orientation of the flow channels of the Poly(dimethyl)siloxane (PDMS) flow cells was perpendicular to the strips of immobilized biotinylated molecules (Fig. 1.1; Sapsford et al. 2006).

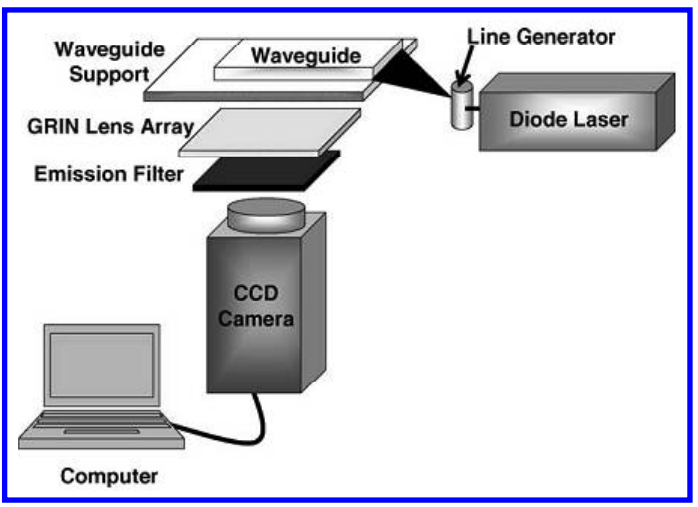

Figure 1.1. Schematic of the NRL array biosensor used by Sapsford et al. 2006.

After washing the PDMS channels, spiked food sample, containing "free" AFB_1 and the Cy5-labeled monoclonal mouse anti-AFB_1, was applied to each channel. AFB_1-spiked foods were extracted with methanol and Cy5-anti-AFB_1 then added. The mixture of the extracted sample and Ab was passed over a waveguide surface patterned with immobilized AFB_1. The resulting fluorescence signal decreased as the concentration of AFB_1 in the sample increased. The limit of detection for AFB_1 in buffer (0.3 ng/mL) increased to between 1.5 and 5.1 ng/g and 0.6 and 1.4 ng/g when measured in various corn and nut products, respectively.

In a recent work, we explored the feasibility of using immunochips to detect five different chemicals (Gao et al. 2009) simultaneously. We used atrazine (Ar), nonylphenol (NP) and 17-beta estradiol (E_2), paraverine and chloramphenicol. Atrazine (Ar), nonylphenol (NP) and 17-beta estradiol (E_2) are endocrine disruptor chemicals (EDCs) which are harmful to human health and the ecological environment (Cooper et al. 2000; Han et al. 2004; Spearow et al. 1999). Effects include heteroplasia (Kavlock et al. 1996), metabolic disorders (Friedmann, 2002), changes in sexual characteristics (Hayes et al. 2002) and development of some tumors (Choi et al. 2004). Papaverine (Pap) is an isoquinoline alkaloid, derived from poppies which can cause addiction and chronic poisoning. Chloramphenicol (CAP) may cause aplastic anemia.

Different concentrations of Ar, NP, E_2, Pap and CAP were used and standard curves were produced for each of the five chemicals (Fig. 1.2). The equations for these standard curves are shown in Table 1.1.

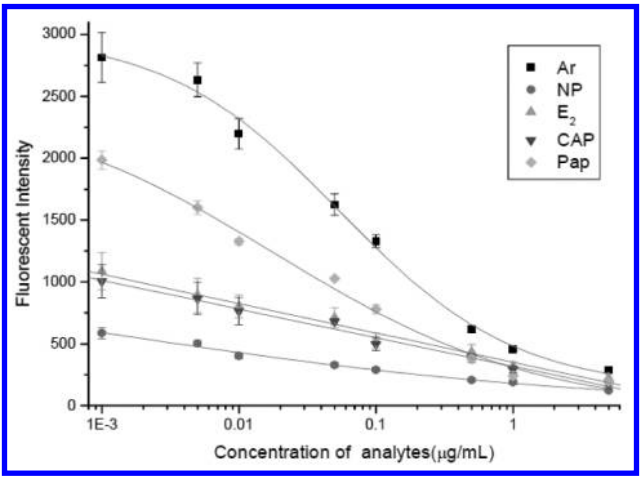

Figure 1.2. Standard curves of fluorescent intensity produced from immunochip analysis of five different chemicals (Liu et al. 2009).

Table 1.1. Equations for standard curves. Comparison of real values and those found by immunochip detection.

Item	Standard curve	R^2	Real (ng/mL)	Found (n=3, $x \pm s$, ng/mL)	RSD(%)
CAP	y_{CAP}=317.6746–231.6716x	0.9933	10.00	10.39 ± 0.84	3.9
NP	y_{NP}=–9.15721+1171.6080/ $(1+(x/0.00123)^{0.2455})$	0.9917	10.00	9.41 ± 0.31	5.9
Pap	y_{Pap}=–49.5254+2557.6292/ $(1+(x/0.0183)^{0.4544})$	0.9928	10.00	10.98 ± 0.53	9.8
E_2	y_{E_2}=351.0196–236.9911x	0.9914	10.00	9.42 ± 0.40	5.8
Ar	y_{Ar}=131.3843+2873.1845/ $(1+(x/0.0549)^{0.6803})$	0.9956	10.00	10.89 ± 0.66	8.9

Our observations demonstrated that simultaneous quantitative assessment of these five chemicals can be performed by the immunochip. Fluorescent intensity decreased as concentrations of the added standard chemicals increased. The detection ranges were 0.001–5 μg/mL with logistic and linear correlation. The determination coefficients-R^2 were all greater than 0.99 implying good correlation between the target chemicals and the FIs. Concentrations of any of the five chemicals within the detection range could quantify the standard curves. To simplify the experimental procedure the concentrations were increased in eight steps. Concentrations under 0.001 μg/mL and over 5 μg/mL, may deviate from the standard curve. Due to inter-chip variation and human error, validity, reliability, and stability are still major problems when immunochip technology is used for multi-analysis. For this reason, it is necessary to plot standard curves with simultaneous assessment of real samples when new multi-analyses are performed with immunochips.

To simplify and accelerate multianalytical procedures, each aldehyde glass slide was divided into 10 relatively small units. Each small unit was made up of a 6 × 4 array. From left to right, 3% BSA (negative control), mAbs of CAP, Pap, E_2, NP and Ar were spotted as probes in turn and repeated four times. The five corresponding Ag conjugates were successively added homogeneously to each of the 10 units on the slide. After spotting, the slide was deposited and incubated in an enclosed box for 2 hr at 37°C. The slide was then washed with PBST and water before being blocked with 2% BSA. After incubation in an enclosed box for another 2 hr the slide was washed again with PBST and water. The addition sequences of Ag conjugates are shown in Fig. 1.3. The slide was then incubated in an enclosed box for another 2 hr at 37°C for specific Ag-Ab competitive reactions. The GenePix™ 4000B scanner and GenePix™ Pro 4.0 software were used to analyze the results.

The specificity of the immunochip was assessed by expose to a mixture of Ags. Fig. 1.3A, demonstrates four mAbs of CAP, NP, E_2 and Ar with their complementary Ag conjugates spotting on a slide showing strong

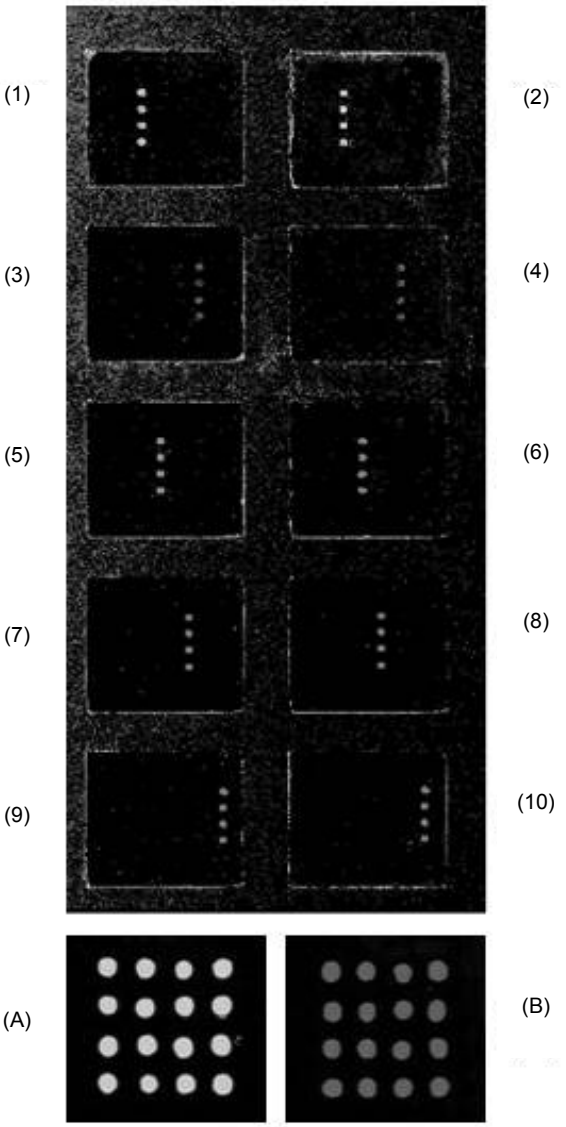

Figure 1.3. Multi-analysis of five different chemicals. Abs addition: In one unit, from left to right: 3% BSA (as the negative control), monoclonal antibodies of CAP, Pap, E_2, NP and Ar were spotted as probes; addition of the 10 units was homogeneous. Antigen conjugates addition: (1) and (2) units: CAP-BSA-Cy3; (3) and (4) units: NP-OVA-Cy3; (5) and (6) units: Pap-OVA-Cy3; (7) and (8) units: E_2-OVA-Cy3; (9) and (10) units: Ar-OVA-Cy3. **(A)** Four mAbs of CAP, NP, E_2 and Ar with their complementary Ag conjugates spotting on slide. **(B)** CAP, NP, E_2 and Ar with the same concentration of 0.01μg/mL mixture were added on the chip (Gao et al. 2009).

Color image of this figure appears in the color plate section at the end of the book.

FIs. After addition of the mixture of Ags including CAP, NP, E_2 and Ar at the same concentration (0.01µg/mL), the FIs significantly decreased (Fig. 1.3B). However, FIs were not reduced significantly after addition of erythromycin, gentamicin, BPA, diethylstilbestrol and 17-α-ethinylestradiol onto the immunochip. This observation suggested that these chemicals did not significantly cross-react on the immunochip. Phage display technology and ribosome display technology are other tools for the generation of high-quality Abs. Antibodies with higher titres and specificity, especially mAb, can significantly increase the accuracy and sensitivity of the immunochip.

Our observations indicate that immunochips can simultaneously detect five different chemicals. This offers exciting opportunities for detection of small molecules in online environmental and food hygiene assessments. However, the sensitivity is too low to detect traces of chemicals and further studies are required to determine whether more target chemicals can be integrated onto a single microchip. Improvements in the production of immunochip technology and access to high-quality Abs, are important for the rapid, sensitive, and high-throughput detection of chemicals, toxins and pollutants in food, water and the environment.

Kloth et al. (2009) developed a regenerable (i.e. reusable) immunochip for the rapid determination of 13 different antibiotics in raw milk. The newly developed hapten microarrays which use an indirect competitive chemiluminescence microarray immunoassay (CL-MIA) are designed to analyze 13 different antibiotics in milk within 6 min. Antigen immobilization was performed by the contact spotter system BioOdyssey Calligrapher miniarrayer (Bio-Rad, München, Germany) using a TeleChem Stealth SNS 9 microspotting solid pin. Printing conditions were set via the Bio-Rad Calligrapher Software. The relative humidity was 35% and the air temperature inside the spotting chamber was 21ºC. Two 15 × 5 clusters were set on one microarray (grid spacing 1100 mm; cluster distance 11.75 mm).

To regenerate the immunchip for reuse the high affinity Abs that bound Ag must be removed from the chip surface. This was based on epoxy-activated PEG chip surfaces, onto which microspotted antibiotic derivatives like sulfonamides, β-lactams, aminoglycosides, fluorquinolones and polyketides are coupled directly without further use of linking agents. Using the chip reader platform MCR 3, this Ag solid phase is stable for at least 50 consecutive analyses. Fig. 1.4A shows the scheme of the direct covalent coupling of different types of antibiotics and the assessment of samples of milk.

Figure 1.4B shows the two different CCD exposures of microarrays detecting 13 different antibiotics and the reference substance DNT with five replicates in each row. Normal milk and a sample of milk containing cloxacillin which tested positive were measured on the same biochip after one regeneration cycle. The concentration of cloxacillin was high enough

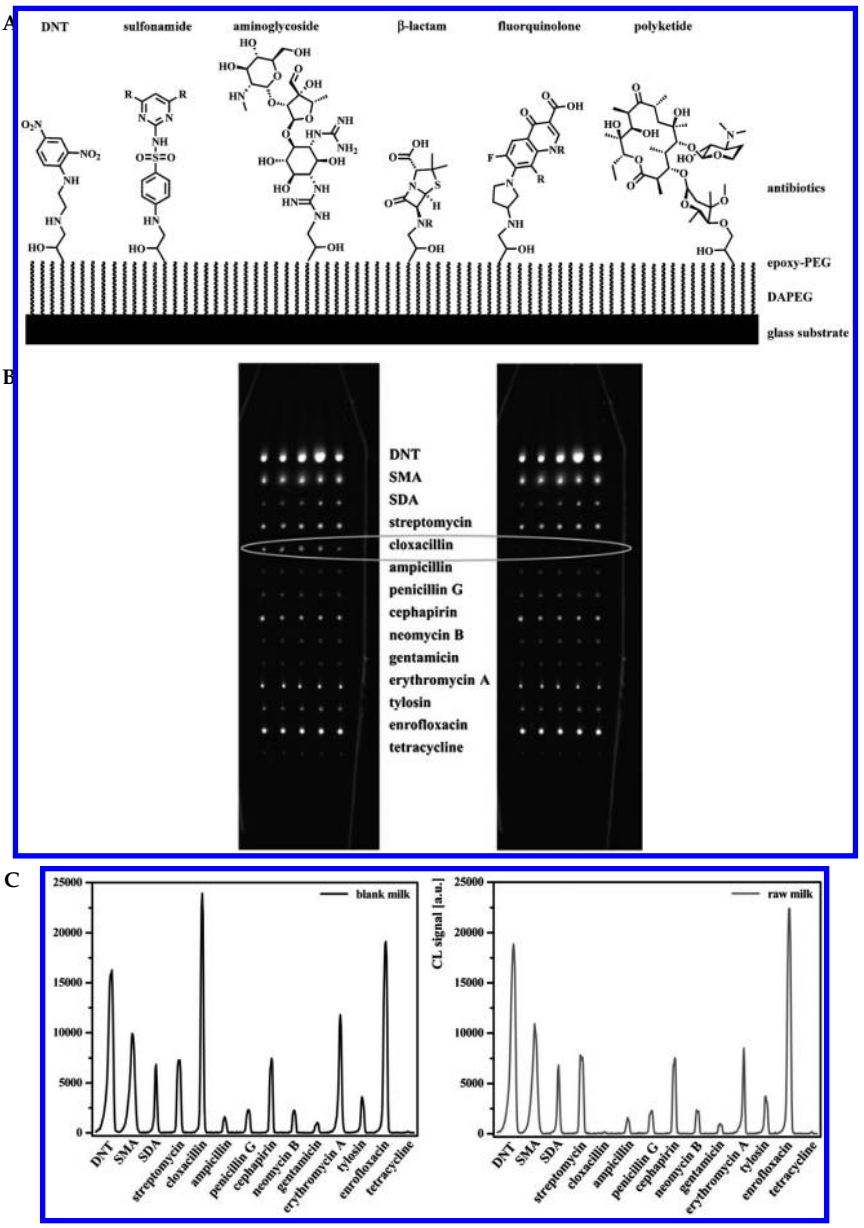

Figure 1.4. Direct covalent coupling of different antibiotics without further use of linking agents and the assessment of samples of milk. **(A)** Direct covalent coupling of different types of antibiotics without use of linking agents; **(B)** Measurements of milk samples: CCD exposures of blank milk without any added antibiotics (left) and of a raw milk sample contaminated with cloxacillin (right); **(C)** CL signal variation profiles of both analyses: blank milk (black) and raw milk (gray) (Kloth et al. 2009).

to reduce the signal intensity to background level. The signal intensities of all other analytes were reproducible (Fig. 1.4B; average CL signal deviation: 5.3%). The concentration of cloxacillin in this sample was estimated to be about 370 mg/L which is significantly greater than the MRL (30 mg/L). This is similar to the semi-quantitative result obtained by a microbial inhibitor test (cloxacillin: >220 mg/L). With this test, Kloth et al. 2009 also found all samples of milk containing one analyte and an inhibitor could be identified and quantified by the MCR 3. Similar residue levels were found in contaminated samples analyzed with both methods. Furthermore, milk samples which tested negative were identified correctly. The new microarray system offers a means for rapid, routine, identification and quantification of antibiotics in milk and will therefore aid the food industry to maintain quality and safety levels.

LAB-ON-CHIP FOR PERSONAL TOXICITY TESTING

The micrototal analysis system (μ-TAS) or "lab-on-a-chip" has been popular since it was introduced around 10 yr ago (Lee and Lee 2004). The ability to tailor-make an integrated system for a specific immunoassay is very useful. The lab-on-a-chip integrates several laboratory processes including all preparation procedures, incubation, detection and analysis on a single chip. It eliminates the need for several different pieces of laboratory equipment to prepare and perform a single assay. Technology from semi-conductor industries was adapted to design and fabricate a myriad of interconnecting micro-sized channels, chambers and reactors, required to produce the micrototal analysis system (Lim and Zhang 2007). Like microfluidic circuits, the micrototal analysis system offers the possibility of developing small, easy-to-use, fully integrated, automated devices for analysis of chemicals. The lab-on-a-chip should include fluidic systems, like channels, pumps, and valves, able to perform tasks of separation, transfer of liquids, purification, amplification etc., as well as bioarrays and the array-readers.

Lab-on-chip technology greatly exceeds the ability of conventional bioanalytical techniques to detection of environmental toxins. Other advantages include automated analysis, miniaturization, multiplex analysis, use of minute samples, reduced reagent consumption. However, perhaps most importantly, lab-on-chip technology facilitates integration of systems which share characteristics in respect to their production, bio-recognition interfaces, and signal enhancement and transition processes.

One approach to the production of a whole-cell lab-on-chip integrated system was described by Rabner et al. 2006. This disposable plastic biochip is prepared with a 4 × 4 micro-lab (mLab) chamber array of bioluminescent *E. coli* reporter cells that responds to a predetermined class of chemical agents and microfluidic channels for liquids translocation. The device includes

electro-optics for signal acquisition with motorized read out calibration accessories, hydropneumatic modules for water sample translocation into biochip mLabs and electronics for control and communication with the host computer. This prototype is sensitive to broad classes of water-borne toxins including naladixic acid (a model genotoxic agent), botulinum toxin, and acetylcholine esterase inhibitors.

Many immunochips incorporate nanotechnology. This does not mean that the devices are manufactured on nanoscale dimensions, but refers to the use of, for example, molecular monolayers of a material inside the structure, or the immobilization of individual molecules (proteins, DNA) inside the channels. However, some microfabricated fluidic devices are smaller than 1μm, for example, the carbon nanotube shown in Fig. 1.5.

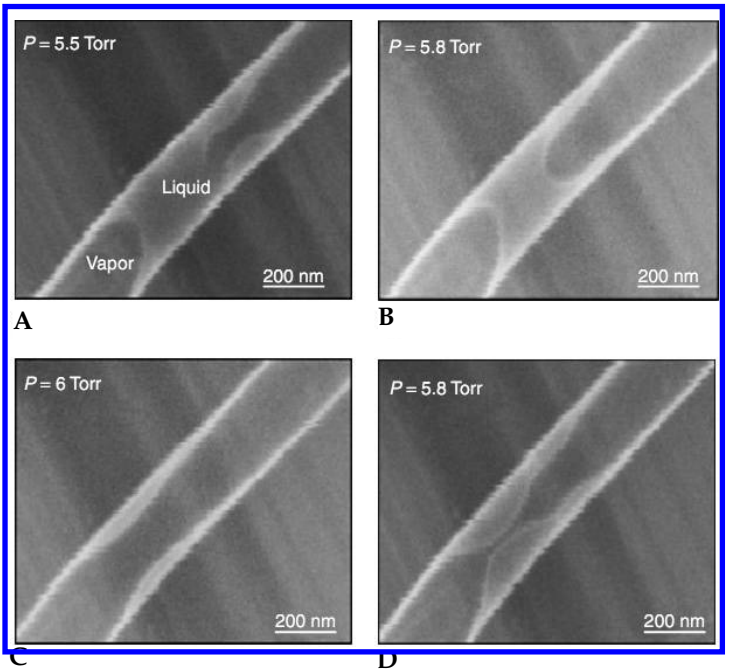

Figure 1.5. Sequence of environmental scanning electron microscopy (ESEM) images. These were obtained when partial pressure of water in the ESEM chamber was gradually raised in a controlled manner, while observing a single open carbon nanotube filled with water **(A–C)**. Note the liquid-volume recovery during subsequent pressure decrease **(C–D)** (Rabner et al. 2006).

SUSPENSION ARRAY FOR PERSONAL TOXICITY TESTING

We assessed the ability of suspension array technology to simultaneously assess three different veterinary drugs, chloramphenicol (CAP), clenbuterol

(CL) and 17-beta-estradiol (E_2) (Liu et al. 2009). The high-throughput suspension array is a novel method for multi-analysis of veterinary drugs. It is easy to use, inexpensive and very sensitive. Three different conjugate-coupled beads were mixed in the same proportions. The optimized mAbs were then added into the 96-well plate. Meanwhile, CAP, CL and E_2 were diluted into eight concentration gradients as 5×, 5× and 2×, respectively. Mixed 6000 beads were added to each well and the concentrations of CAP, CL and E_2 were adjusted to 0, 50, 250, 1250, 6250, 31250, 156250, 781250 ng L^{-1}; 0, 56, 280, 1400, 7000, 35000, 175000, 875000 ng L^{-1} and 0, 1, 3, 9, 27, 81, 243, 729 µg L^{-1}, respectively in the total volume of 50 µL per well in a plate. The plate was then spun for 1 hr at medium speed at 37°C for Ag-Ab competitive reaction. Then, SA-PE was added and the plate was spun for 15 min at medium speed at 37°C. 100 microspheres were read out well by well by Bio-Plex™ suspension array to obtain the MFIs. Based on the MFIs for each target, standard curves could be plotted.

There are negative logistic correlations between MFIs and the concentrations of the veterinary drugs, and all determination coefficients-R^2 were greater than 0.989 implying good correlation. The detection ranges are 40–6.25 × 10^5 ng L^{-1}, 50–7.81 × 10^5 ng L^{-1} and 1 × 10^3–7.29 × 10^5 ng L^{-1} for CAP, CL and E_2, respectively (Fig. 1.6).

Within these ranges, any concentration of the three veterinary drugs can be quantified using the standard curves. The sensitivity means the minimum

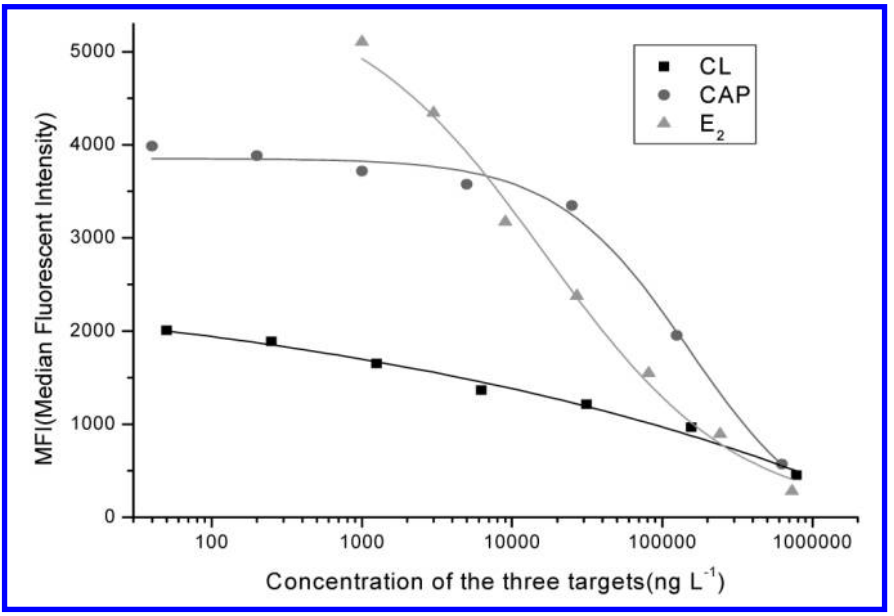

Figure 1.6. Standard curves produced by simultaneous detection of three veterinary drugs by suspension array technology (Liu et al. 2009).

detectable concentrations (Min DC) in suspension array detection, and MFIs of the blank controls or background for the three veterinary drugs are very close to that of the Min DC. On statistical analysis (Table 1.2) there were no significant differences between the MFIs of the blank controls and the groups of the Min DC. Consequently, the Min DC are the lowest detection limits (LDLs) for CAP, CL and E_2, i.e. 40, 50 and 1000 ng L^{-1}.

Table 1.3 shows that the resulting MFIs had no significant differences from blank control groups after addition of different concentrations of salbutamol, ractopamine, gentamicin, erythromycin or 17-alpha-estradiol, $P > 0.05$ indicating no significant alteration by or cross-reaction with other chemicals. Salbutamol, ractopamine and 17-alpha-estradiol are structural analogues of CL and E_2. The specificity of the suspension array is dependent on the specificity of the mAbs.

As competitive ELISA is an established and widely used method of quantifying small molecules, it was employed to assess real samples to confirm the feasibility in comparison with suspension array technology. The results from the two methods are shown in Table 1.3 and the relative standard deviations (RSDs) were between 8.09–17.03% and 9.19–17.74% from the real values. These are relatively small detection ranges. However,

Table 1.2 Standard curves for CAP, CL and E_2 detection and MFI for blank control and Min DC.

Item	Standard curve	R^2	MFI ($\bar{x}$ ± s, n=3)	
			Blank Control	MinDC
CAP	Y_{CAP}=–250.323 + 4103.517/ [1+(x/30121.243)$^{0.980}$]	0.994	2247.167 ± 90.423[a]	2071.667 ± 85.0431
CL	Y_{CL}=–263012.682 + 265751.765/ [1 + (x/6.063)$^{0.115}$]	0.989	4516.833 ± 177.340[b]	4195.833±185.581
E_2	Y_{E_2}=8.368 + 5651.421/ [1+(x/16409.422)$^{0.679}$]	0.995	5249.000 ± 201.765[c]	5110.833 ± 134.612

Compared with blank control, a, b and c in CAP, CL and E_2 group respectively: $P > 0.05$

Table 1.3 MFIs for different groups of chemicals.

Item	MFI ($\bar{x}$ ± s, n=3)				
	Concentration (ng L^{-1})	0ng L^{-1} (blank control)	50 ng L^{-1}	1250 ng L^{-1}	31.25 µg L^{-1}
Salbutamol		1750.17 ± 92.59[a]	1691.82 ± 25.59	1727.20 ± 42.06	1529.48 ± 7.26
Ractopamine		1750.17 ± 92.59[a]	1749.90 ± 51.65	1618.16 ± 76.21	1609.67 ± 61.94
Gentamicin		3662.58 ± 75.47[b]	3719.23 ± 74.68	3849.20 ± 56.60	3970.16 ± 82.41
Erythromycin		3662.58 ± 75.47[b]	3527.83 ± 52.53	3673.25 ± 63.37	3485.62 ± 47.75
17 α-estradiol		5520.50 ± 127.08[c]	5460.80 ± 118.95	5510.83 ± 339.50	5497.94 ± 219.81

Compared with 0 ng L^{-1} group (Negative control), a, b and c: $P > 0.05$.

the detection ranges of the suspension array are broader and more sensitive than ELISA, especially for the detection of the three veterinary drugs. Moreover, as multiplex analysis is possible with the suspension array, it will be a very useful application.

As a booming technology, suspension array has revealed great developing prospects and potentials not only for the research and detection of macromolecules (protein and nucleic acid) detection, but also for providing a novel pathway for analysis and assessment of small molecules such as pesticides, veterinary drugs and toxins. With the increasing availability of commercial kits multiplex analysis of these small molecules by suspension array could be applied extensively.

SUMMARY POINTS

- Immunochips provide large amount of information with rapid, timely, simple, low cost, wide range of usage and advantages of automatization.
- High-quality Ab with good bioactivity and specificity is the key reagent in the production of immunochips.
- Suspension array has great potential for use in personal toxicity testing not only for the research and detection of macromolecules (protein and nucleic acid), but also for providing a novel method of analysis and assessment of small molecules such as pesticides, veterinary drugs and toxins.
- All forms of immunochips are difficult to use for field detection and require further development and improvement.
- The online environmental and health inspection by suspension array for a large numbers of chemicals, pollutants and toxins requires further investigation and development.

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ABBREVIATIONS

AFB_1	:	aflatoxin B_1
Ags	:	antigens

Abs	:	antibodies
Ar	:	atrazine
CAP	:	chloramphenicol
CL-MIA	:	competitive chemiluminescence microarray immunoassay
E_2	:	17-beta estradiol
EDC	:	endocrine disruptor chemical
ELISA	:	enzyme-linked immunoadsorbent assay
FI	:	fluorescent intensity
LC-MS	:	liquid chromatographic method with mass spectrometry
LDLs	:	lowest detection limits
MFI	:	median fluorescent intensity
Min DC	:	minimum detectable concentrations
MW	:	molecular weight
mAbs	:	monoclonal antibodies
GalNAc	:	N-acetyl galactosamine
Neu5Ac	:	N-acetyl neuraminic acid
NP	:	nonylphenol
Pap	:	papaverine
PDMS	:	Poly (dimethyl) siloxane
PBS	:	phosphate-buffered saline
RSDs	:	relative standard deviation
RT	:	room temperature
SEM	:	scanning electron microscopy
SEB	:	staphylococcal enterotoxin B
SNR	:	signal-to-noise ratio
SA-PE	:	streptavidin-R-phycoerythrin

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Detection of Pesticide Residues Using Biosensors

Javier Ramón-Azcón,[1,a] Tomoyuki Yasukawa[1,b] and Fumio Mizutani[1,c]

ABSTRACT

Pesticides are substances used in food production in order to minimize or prevent damage caused by pests. Thus, unlike other groups of chemicals, pesticides are intentionally released into the environment, and there is a high risk of these chemicals appearing in the food chain. Unfortunately, the exponential increase in the demand for food in the world today makes it impossible to eliminate them from food production. Therefore, consumers and governments consider pesticide regulation and control a very relevant issue for the economy as well as human health. Often expensive and instrumental single-analyte methods are applied by regulatory and industrial laboratories. There is an urgent need for validated screening tools that are not only simple, inexpensive, and rapid but also show multiplex capabilities by detecting simultaneously as many contaminants as possible. In recent years, many efforts have been made to develop new analytical techniques integrating biorecognition elements and detection components in order to obtain small devices with the ability to carry out direct, selective, and continuous measures for one or several analytes present in samples. In

[1]Graduate School of Material Science, University of Hyogo, 3-2-1 Kouto, Kamigori-cho, Ako-gun, Hyogo 678-1297, Japan.
[a]E-mail: ramonazconjavier@gmail.com
[b]E-mail: yasu@sci.u-hyogo.ac.jp
[c]E-mail: mizutani@sci.u-hyogo.ac.jp

List of abbreviations after the text.

this context, biosensors can fulfill these requirements. Biosensors offer a good alternative to conventional methodologies in pesticide analysis due to their high sensitivity and selectivity.

INTRODUCTION

Today, environmental contamination is a problem recognized worldwide. A significant portion of environmental pollution is caused by the application of pesticides in agriculture, horticulture, and forestry. Pesticide is a term used in a broad sense for a chemical, synthetic or natural, which is used for the control of insects, fungi, bacteria, weeds, nematodes, rodents, and other pests. A large number of these compounds and/or their degradation products are highly toxic, and they have negative effects not only on the ecosystem but also on human health. Surveillance of the environment and food for pesticide residues has become essential in recent years for the prevention of risks to the population. It is necessary to control the presence of pesticides in the environment and at the same time to assess the risk to human health due to the presence of these chemicals in workplaces and in food in order to prevent adverse effects.

To control environmental contamination and to protect the population from it, governmental agencies have established several directives. In 1976, European Union (EU) set a "black list" of 132 dangerous substances (based on their toxicity, stability, and bioaccumulation) that should be monitored in water (Directive 76/464/CE). With the aim to protect the health of the general population, the EU has established a value of 0.1 μg/L as the maximum individual concentration and 0.5 μg/L as the total concentration of pesticides and related products in drinking water (Directive 80/778/CEE). In the United States of America (USA), the Environmental Protection Agency (EPA) has established a maximum level for each pesticide or its transformation products according to their toxicity. Similarly, for the protection of the public against the toxic effects of pesticides, regulatory agencies in many countries have established standards specifying the residue levels of each pesticide in various foodstuffs. Thus, the World Health Organization (WHO) has evaluated and reviewed the acceptable daily intakes (ADI) of pesticides (Lu 1995). An example of the degree of exposure to the European population suffers from pollution is reflected in the detection of more than 41 toxic chemicals in the blood tests to 39 Member of the European Parliament (MEP) in 2004.

Control of the environment and food must be assessed by reliable, fast, and sensitive analytical techniques. Chromatographical systems are conventional analytical techniques characterized by high precision and

sensitivity. Nevertheless, these sophisticated techniques need experienced personnel and costly instruments and are not easily adoptable for field analysis. For all these reasons, there is considerable delay between sample collection and data display, thus resulting in loss of money for the food industry and a possible risk to the population. A simple and advantageous alternative is the use of biosensors.

In the early 1950s, potentiometric detection was adopted for pesticide detection, and in the middle of the 1980s, it was used for the construction of the first integrated biosensor for the detection of pesticides based on inhibition of acetylcholinesterase (AChE). In the following decades, important advances have been achieved in the field of biosensors with new elements of recognition and new systems of transduction. The advances in nanotechnology and microelectronics in recent years have been particularly important for this field. However, the commercialization of biosensor technology in the environmental and food industries has significantly lagged behind the research output. In clinical diagnostics, commercial biosensors are well established, and an important number of companies produce them. It is not easy to explain the slow transfer of technology within research and industry, but it could be attributed to cost considerations and some key technical barriers such as stability, detection sensitivity, and reliability. Furthermore, the level of acceptation for governmental agencies of standard analytical techniques is low because of the lack of well-established methodologies of validation. Table 2.1 lists EPA requirements for biosensors that may be used in field assay applications (Rogers and Lin 1992). Medical applications overshadow the other applications, but there are some companies that work in environmental and food control. Table 2.2 summarizes companies with biosensors specially designed for the environmental or agro-alimentary industries.

Table 2.1. General requirements for biosensors in environmental field applications.

Requirement	Specific range
Cost	US$1–15 per analysis
Portable	Can be carried by one person; no external power requirements
Fieldable	Easily transported in a van or truck; limited external power required
Assay time	l–60 min
Personnel training	Can be operated by minimally trained personnel after l–2 hr training period
Matrix	Minimal preparation for ground water, soil extract, blood, urine, or saliva
Sensitivity	Parts per million/parts per billion
Dynamic range	At least two orders of magnitude
Specificity	Enzymes/receptors: specific to one or more groups of related compounds Ab: specific to one compound or one group of closely related compounds

Adapted from Rogers and Lin (Rogers and Lin 1992) with permission from Elsevier.

Table 2.2. List of companies commercializing biosensors for the agricultural and food industries.

Company name	Country	URL address	Biosensor name
Abtech Scientific	USA	www.abtechsci.com	ToxSen™
Affinity Sensors	United Kingdom	www.affinity-sensors.com	IASys plus™ e IASys, Auto+Advantage™
Ambri Limited	Australia	www.ambri.com	AMBRI Biosensor
Applied Biosystems	USA	www.appliedbiosystems.com	8500 Affinity Chip Analyzer
Biacore AB	Sweden	www.biacore.com	Biacore®Q
BioFutura Srl	Italy	www.biofutura.com	PerBacco 2000 y 2002
Biomerieux	France	www.biomerieux.com	Vitek™ Bactometer™
Biosensor Systems Desing	USA	www.biosd.com	OptiSense Technology™
Biosensores S.L.	Spain	www.biosensores.com	Politox
Biotrace	USA	www.biotrace.com	Uni-lite® Bev-Trace™
Chemel AB	Sweden	www.chemel.com	SIRE®
Innovative Biosensors, Inc	USA	www.innovativebiosensors.com	CANARY™
Molecular Devices	Switzerland	www.moleculardevices.com	Threshold® System
MicrooVacuum	Hungary	www.microvacuum.com	OWLS 210
Nippon Laser Electronics	Japan	www.nle-lab.co.jp	SPR-670M, SPR-MACSNANOSENSOR
Reichert Analytical Instruments	USA	www.reichertai.com	Reichert SR 7000™
Research International	USA	www.resrchintl.com	Analyte 2000™ FAST 6000™ Raptor™
Texas Instruments Inc.	USA	www.ti.com	Spreeta™
Universal Sensors	USA	intel.ucc.ie/sensors/universal	ABD 3000 Biosensor Assay System
Yellow Springs Instruments Co	USA	www.ysi.com	YSI 2700 SELECT™ Biochemical Analyzer

Unpublished table.

PESTICIDES BIOSENSOR CLASSIFICATION

Biosensors can be classified according to the type of recognition element (catalytic or affinity-based biosensor) used or the transduction system (optical, electrochemical, piezoelectric and nanomechanical) (see Fig. 2.2).

Catalytic Biosensors: Enzymatic Biosensors

Enzymatic biosensors for the detection of pesticides in food and environmental samples have been extensively described in several reviews (Cock et al. 2009; Manco et al. 2009). Enzymatic biosensors, which are based

on the use of enzymes, use either of the two principles: enzyme inhibition or hydrolysis of the pesticide.

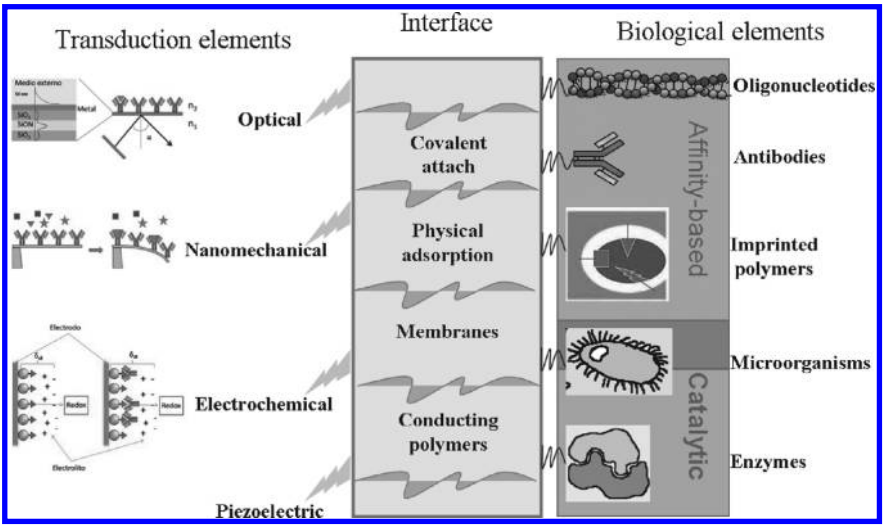

Figure 2.1. Biosensors classification. Classification of the biosensors depends on the bioactive element of recognition or the transduction system. The immobilization of the biological element onto a transducer is a key step in optimizing the analytical performance of a biosensor in terms of response, sensitivity, stability, and reusability. The immobilization strategies most generally employed are physical or chemical methods. Figure unpublished.

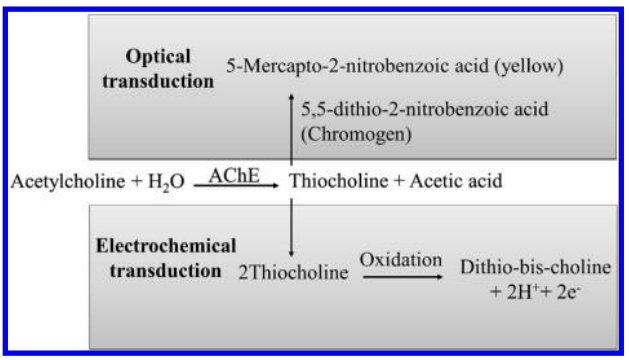

Figure 2.2. Enzymatic formation of thiocholine and transduction systems. Unpublished scheme.

Enzyme inhibition-based biosensors

In the case of inhibition, enzymes used for the detection of pesticides are inhibited by the pesticide, and the extent of inhibition is correlated to the concentration of the analyte. Other enzymatic methods such as the organophosphorus hydrolase assay use the analyte as a substrate, thus

giving the result that a positive signal is generated through the production of hydrolysis products rather than merely the inhibition of the enzyme. Although the most widely used transduction system by enzymatic biosensors has mainly been electrochemical, it is possible to find some examples of enzymatic biosensors using piezoelectric transduction (Abad et al. 1998) or optical transduction (Vamvakaki and Chaniotakis 2007).

Inhibition-based methods have some disadvantages and can be prone to false positives because handling and storage can cause the loss of enzyme activity (Shimomura et al. 2009). Furthermore, many pesticides irreversibly inhibit enzymes, and therefore regeneration of the sensor is required after each sample; this further extends the testing time. Finally, the main problem found in the use of enzymatic biosensors is the lack of specificity and selectivity in the detection of pesticides (Luque de Castro and Herrera 2003). Despite these drawbacks enzyme-based biosensors are effective tools that can be used in general screening methods and further investigation in chemometrics has been conducted for the differentiation of mixtures of pesticides (Ni et al. 2004).

Most pesticide biosensors are designed based on the inhibitory property of enzymes. AChE and butyrylcholinesterase (BChE) are widely used in the development of pesticide biosensors. Inhibition leads to a decrease in activity, which is indirectly proportional to the amount of inhibitors or pesticides in the sample. The other often-employed enzymes in pesticide biosensors are acetolactate synthase, acid phosphatase, alkaline phosphatase (AP), and tyrosinase. Pesticides can also inhibit the activity of luciferase, which is a major enzyme in bioluminescence reactions. By employing fireflies, luciferase pesticide concentrations have been determined on the basis of the fact that the pesticide concentration is indirectly proportional to the bioluminescence (Trajkovska et al. 2005).

Organophosphorus pesticides (OPP) and carbamate pesticides are potent inhibitors of the enzyme cholinesterase. The thiocholine produced during the catalytic reaction can be monitored using spectrometric, amperometric (see Fig. 2.2), or potentiometric methods, but as it has been previously stated, electrochemical methods are more common. As an example of the applications, Arduini et al. (Arduini et al. 2006) analyzed different pesticides with AChE and BChE enzymes. AChE-based biosensors have higher sensitivities toward aldicarb (50% inhibition with 50 μg/L) and carbaryl (50% inhibition with 85 μg/L) while BChE biosensors have higher affinities for diethyl-p-nitrophenyl phosphate (50% inhibition with 4 μg/L) and chlorpyrifos-methyl oxon (50% inhibition with 1 μg/L). The limits of detection (LOD) were 12 and 25 ppb (μg/L) for aldicarb and carbaryl, respectively, using the AChE enzyme and 2 and 0.5 ppb (μg/L) for paraoxon and chlorpyrifos-methyl oxon, respectively. BChE and AChE biosensors were then tested using both wastewater and river water samples,

and no inhibition of the signals was observed, thus obtaining good recovery percentages with spiked samples.

Catalysis-based biosensors

Inhibition-based biosensors are poor in selectivity and are rather slow and tedious since the analysis involves multiple reaction steps such as measurement of initial enzyme activity, incubation with an inhibitor, measurement of residual activity, and regeneration and washing. Biosensors based on direct pesticide hydrolysis are more straightforward. The enzyme organophosphorus hydrolase (OPH) hydrolyzes esters in a number of OPP and insecticides (e.g. paraoxon, parathion, coumaphos, diazinon) (Jeffrey et al. 1987). According to one example found in the literature, Mulchandani et al. (Mulchandani et al. 1999) purified OPH from recombinant *E. coli* and immobilized it on a pH electrode to develop a potentiometric biosensor by catalyzing the hydrolysis of OPP (parathion, paraoxon, and methyl parathion) to release protons, the concentrations of which were proportional to the amounts of hydrolyzed substrates.

Affinity Based Biosensors: Immunosensors for Pesticides

Immunosensors are based on the immunological reaction derived from the binding of the antibody (Ab) to the corresponding antigen (Ag). This reaction is reversible and is stabilized by electrostatic forces, hydrogen bonds, and Vander Waals interactions. The formed complex has an affinity constant (k_a) that can achieve values on the order of 10^{10} M^{-1}. In immunosensors procedures the quantification of pesticides molecules is performed under competitive conditions. The general strategy of competitive assays is based on the competition between the free Ag (analyte) and a fixed amount of labeled Ag for a limited amount (low concentration) of Ab. At the end of the reaction the amount of labeled Ag and subsequently the free Ag is determined. The labels used to quantify the immunoreaction can be of a different nature. A wide variety of antibody biosensors reported for different pesticides in food and environmental applications exists and are summarized and discussed in several reviews (González-Martínez et al. 2007; Bojorge Ramírez et al. 2009).

Electrochemical immunosensors

Because of its simplicity, electrochemical transduction is the oldest and most common method used in biosensors (for recent review see (Rivas et al. 2007)). Researchers can determine the level of pesticides by measuring

the change in potential, current, conductance, or impedance caused by the immunoreaction.

Amperometric biosensors are based on the measurement of the current generated by oxidation/reduction of redox species at the electrode surface, which is maintained at an appropriate electric potential. The current observed has a linear relationship with the concentration of the electroactive species. For the simultaneous analysis of several samples using only one device, Skládal and Kaláb (Skládal and Kaláb 1995) developed a multichannel immunosensor. The 2,4-D molecule conjugated to horseradish peroxidase was used as a tracer, which was determined amperometrically using hydrogen peroxide and hydroquinone as substrates. 2,4-Dichlorophenoxyacetic acid (2,4-D) is widely used in amounts of 10^5 tons per year as a herbicide for the control of broadleaf weeds. "Agent Orange," which was used extensively during the Vietnam war for defoliation, is composed of a 50:50 mixture of n-butyl esters of 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) and can still be detected in human tissues, soil as well as in the biosphere of Vietnam. A similar immunosensor coupled with an enzyme-linked immunosorbent assay (ELISA) microtiter plate has been also reported (Deng and Yang 2007), and a detection limit of 0.072 µg/L was achieved. The advantages of the presented electrochemical detector were high stability and sample throughput, a low detection limit, the ability to be repeatedly used without the need for regeneration.

Recently, several papers have been published combining microparticles with electrochemical amperometric detection. The use of microbeads greatly improves the performance of the immunological reaction, minimizing the matrix effect due to improved washing and separation steps. This strategy has been used for the detection of atrazine herbicide in orange juice (Zacco et al. 2006). The Ab is immobilized on the surface of magnetic beads. The immunological reaction for the detection of atrazine is based on a direct competitive assay using a peroxidise tracer as the enzymatic label (see Fig. 2.3). The LOD obtained in orange juices was 0.025 µg/L, which is below the maximum residue level (MRL) established by actual European legislation (0.1 µg/L) in oranges. Furthermore, in the case of orange juice, preliminary experiments performed with the magneto-ELISA demonstrated that nonspecific interferences from a matrix can be easily eliminated by a very simple sample pre-treatment, which consists of simply adjusting the pH to 7.5 (the original pH of the sample was 3.5), filtering the sample through a 0.2 µm filter, and diluting the filtrate five times with buffer.

Electrochemical impedance spectroscopy (EIS) is being rapidly developed because of the possibility to directly record information on biorecognition events occurring at the electrode surfaces and inducing capacitance and resistance changes (Katz and Willner 2003), allowing

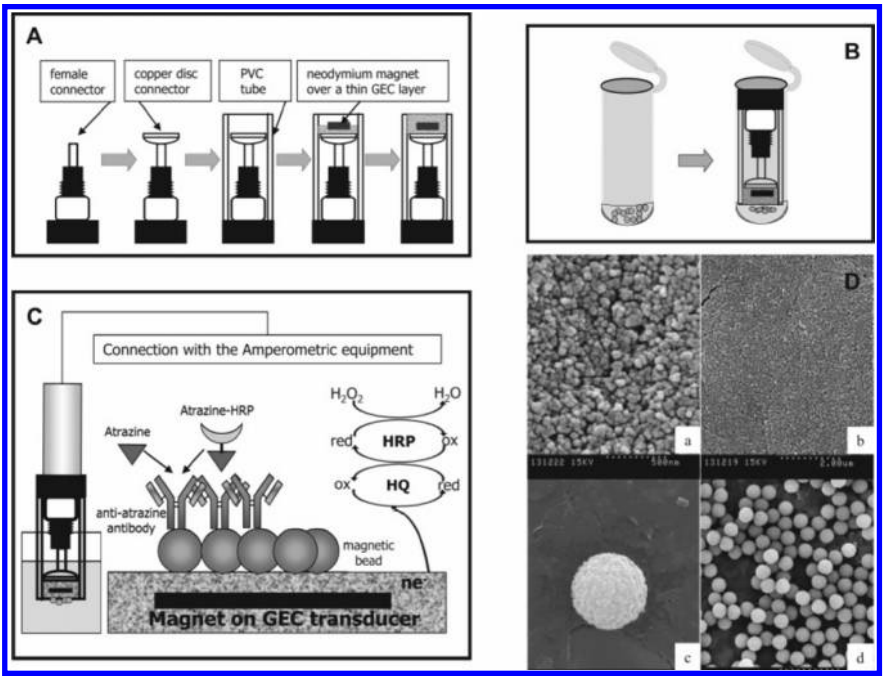

Figure 2.3. Schematic representation of the electrochemical magnetoimmunosensing strategy for the detection of atrazine. **(A)** Preparation of the magneto graphite-epoxy composite (m-GEC) electrode. **(B)** After the immunoreaction, the antibody (Ab) modified magnetic beads were captured for the m-GEC electrode. **(C)** Chemical reactions occurring at the m-GEC surface polarized at −0.150 V (vs Ag/AgCl) upon the addition of H_2O_2 in the presence of a mediator (hydroquinone). **(D)** Scanning electron microphotographs of carboxylated magnetic particles (MP-COOH) **(A, B)** on the surface of sensors taken at 0.5 and 2 μm of resolution, respectively and tosylated magnetic beads (MB-tosyl) **(C, D)** taken at 2 and 10 μm of resolution, respectively. An identical acceleration voltage (15 kV) was used in all cases. This figure is from Zacco et al. (Zacco et al. 2006) with permission from ACS.

the development of label-free biosensing devices. EIS in connection with immunochemical methods was tested for the direct determination of the herbicide 2,4-D (Navratilova and Skladal 2004). The changes in the impedance parameters (ϕ_{max} and Z_{min}) due to immunocomplex formation, which served as a parameter characterizing changes on the sensing surfaces, were evaluated. It was possible to measure the response to 2,4-D in a concentration range from 45 nM to 450 μM. In this context, interdigitated microelectrodes (IDμE) have recently received enormous attention because their sensitivity is higher than that of conventional electrodes (Berggren et al. 2001; Navratilova and Skladal 2004). Using thin Au/Cr (~200-nm thickness) IDμEs (3.85-μm thick and with electrode gaps of 6.8 μm) on a Pyrex 7740 glass substrate, researchers have recently reported the detection of atrazine without the use of any label with a limit of detection of 0.04 μg/L

(Ramón-Azcón et al. 2008) (see Fig. 2.4). The sensor has been evaluated to assess its potential to analyze pesticide residues in a complex sample matrix, such as red wine. An atrazine hapten-bovine serum albumin (BSA) conjugate was covalently immobilized within the microelectrodes on the glass substrate.

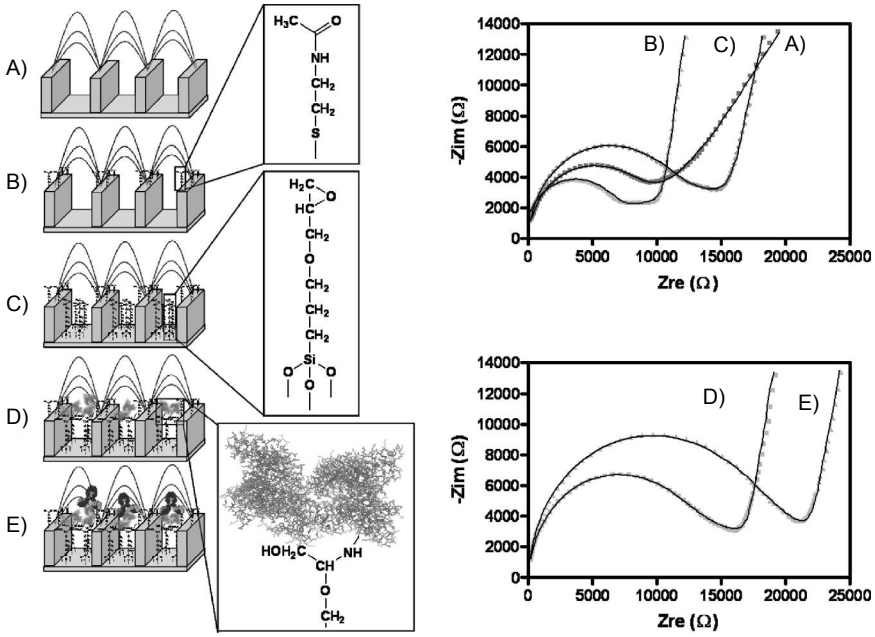

Figure 2.4. Scheme showing steps used to prepare the immunosensor surfaces and antibody (Ab) binding. Nyquist plots of impedance corresponding to **(A)** ID m E; **(B)** Step I: N-acetylcysteamine, gold protection; **(C)** Step II: functionalization of the Pyrex substrate with (3-glycidoxypropyl)trimethoxysilane; **(D)** Step III: coating atrazine hapten-bovine serum albumin (BSA) conjugate, covalent immobilization (1 µg/mL); and **(E)** Step IV: specific Ab produced versus atrazine herbicide, incubation step (1 µg/mL). Symbols represent the experimental data. Solid curves represent the computer fitting data with the parameters calculated by the commercially available software Zplot/Zview (Scibner Associates). Parts of this figure are reprinted from Ramón-Azcón et al. (Ramón-Azcón et al. 2008) with permission from Elsevier.

Optical biosensors

Optical transducers are based on various technologies involving optical phenomena, which are the result of an interaction between the analyte and receptor. This group may be further subdivided according to the optical properties applied in sensing (i.e. absorbance, reflectance, luminescence, fluorescence, refractive index and SPR, and light scattering).

An evanescent wave (EW) is a near field standing wave with an intensity that decays exponentially with increase in distance from the boundary at which the wave was formed. When biomolecules are located in the evanescent field, they absorb energy, leading to attenuation in the reflected guide of the waveguide. In attempts to improve detectability, many researchers reported that immunosensors combine this principle with the use of labeled molecules that are able to re-emit the absorbed evanescent photons at a longer wavelength as fluorescence. This phenomenon is known as total internal reflection fluorescence (TIRF). As an example, a TIRF immunosensor was shown to allow the detection of a multitude of analytes in one single test cycle (Klotz et al. 1998). Calibration curves obtained for 2,4-D and simazine had detection limits of 0.035 and 0.026 µg/L respectively. One limiting factor on the ability to simultaneously perform more than one assay on the same transducer was the availability of low cross-reactant Ab combined with a high affinity between the antibody and the analyte. Similarly, the River Analyzer (RIANA) is also a highly sensitive, fully automated biosensor able to rapidly and simultaneously detect multiple organic targets (Mallat et al. 2001; Rodriguez-Mozaz et al. 2006). Thus, this system was used to measure two herbicides, atrazine and isoproturon, in raw river water and in water obtained after each treatment step in the waterworks. The analysis of these compounds could be performed in one unique run and in a very short period (one measurement cycle, including the regeneration step, took 15 min), and the LOD reached the legislation requirements (0.1 µg/L, as set in the EU drinking water directive 2000/60/EC as the maximum admissible concentration for individual pesticides). The performance of the immunosensor method developed was evaluated against a method based on solid phase extraction, followed by liquid chromatography-mass spectrometry (LC-MS). In conclusion, the chromatographic method was superior in terms of linearity, sensitivity, and accuracy, and the biosensor method in terms of repeatability, speed, cost, and automation.

Other EW immunosensor approaches such as grafting couplers (Grego et al. 2008) and Mach–Zehnder Interferometers (MZI) (Prieto et al. 2003) have been investigated in order to obtain possible measurements of pesticides without the use of fluorescent labels. An optical waveguide lightmode spectroscopy (OWLS)-based biosensor is a recently developed device in the field of integrated optics, and exploits the science of light guided in structures that are smaller than the wavelength of light. This technique can be applied for the detection of the herbicide trifluralin (Székács et al. 2003). Within the immobilized Ab-based immunosensors, this method allowed the detection of trifluralin only above 100 µg/L due to the small molecular size of Ag, while the immobilized Ag-based OWLS system allowed the detection of trifluralin in the concentration range of 2×10^{-7} to 3×10^{-5}