Electrosensing applications by using titania as a support for bio(inspired) molecules

Promoter: Prof. Dr. K. De Wael
Co-promoter: Prof. Dr. V. Meynen

Antwerpen
2018
Members of the jury:

Chair of the jury:
Prof. Dr. Pegie Cool, University of Antwerp, Belgium

Promoter:
Prof. Dr. Karolien De Wael, University of Antwerp, Belgium

Co-promoter:
Prof. Dr. Vera Meynen, University of Antwerp, Belgium

Members:
Prof. Dr. Sylvia De Wilde, University of Antwerp, Belgium
Prof. Dr. Cecilia Cristea, Luliu Hatieganu University of Medicine and Pharmacy, Romania
Prof. Dr. Koodlur Lokesh, Vijayanagara Sri Krishnadevaraya University, India
Prof. Dr. Jorge Garrido, School of Engineering (ISEP), Polytechnic of Porto, Portugal
Acknowledgment

This thesis is the end of my journey in obtaining a PhD. It would have been impossible to write this thesis without the help and support of so many talented people around me, to only some of whom it is possible to address my gratitude here:

First and foremost, I would like to express my sincere appreciations to my promoter, Prof. Karolien De Wael and my co-promoter, Prof. Vera Meynen, for giving me the opportunity to pursue a PhD research under their guidance at the University of Antwerp and to explore the interesting field of electrochemical (bio)sensors combined with material science allowing me to expand my horizons.

I would like to thank my doctoral committee members: Prof. Pegie Cool, Prof. Sylvia De Wilde and my jury members: Prof. Cecilia Cristea, Prof. Koodlur Lokesh and Prof. Jorge Garrido for their insightful comments and encouragements.

Prof. Sabine Van Doorslaer, whose expert knowledge about EPR and characterization techniques were of great value for my work. Thank you so much for your valuable comments and advices. I have to acknowledge Zainab for the EPR measurements. I would like to thank Prof. Bart Partoen, Prof. Dirk Lamoen, Prof. Koen Janssens and Dr. Nasrin Sarmadian for the nice collaboration and wonderful DFT calculations, as well as many helpful discussions. I am also grateful to Prof. Lo Gorton from University of Lund (Sweden), It was fantastic to have the opportunity to work with the flow injection system in your lab. In addition, I would like to thank Prof. Ana Cabal and Prof. Piet Van Espen. I appreciate your kindness and support.

My sincere thanks also for Dr. Stanislav Trashin, I am deeply grateful for your valuable advice and many helpful discussions throughout the entire period of my PhD. I never imagined so much help and support from you. Next, I would like to express special thanks to all my colleagues in the AXES and LADCA research group: Balwinder, Cheryl, Willemien, Alba, Andrea, Liselotte, Fabio, Jeroen, Mats, Nick, Oli, Gert, Frederik, Stijn, Steven, Sanne, Stefano, Elena, Ward and Bert. I would like to thank my friends Ezat, Maryam and Amin for their constant support during the initial years of my PhD. Anca and Saranya, you are amazing people that I will never forget. Thank you for your spiritual support and helping me, it is really my pleasure to have friends like you. Thank you, for being my home away from home. Ehab and Ermanno, it was my good fortune to work with you during the weekends in the last few months 😊. Thank you for all your support.

Special thanks to my uncle (Babak), I really appreciated your support. Last, but by no means least, I would like to thank to my wonderful dad, my adorable mom and my lovely sisters (Solmaz and Diyardokht). I love you! Thank you so much for your unconditional support and encouragement, and without which I would not have come this far.
Table of Contents

Abbreviations

Chapter 1 Introduction .................................................................................................................. 1
  1.1. Porous materials .................................................................................................................. 2
    1.1.1. Microporous materials ................................................................................................. 2
    1.1.2. Mesoporous materials ................................................................................................. 2
    1.1.3. Macroporous materials ............................................................................................... 3
  1.2. Titanium dioxide ................................................................................................................. 4
  1.3. Typical TiO$_2$ based sensors ............................................................................................. 5
    1.3.1. TiO$_2$-based photocatalytic sensors ......................................................................... 6
    1.3.2. TiO$_2$-based biosensors ............................................................................................ 7
      1.3.2.1. Optical TiO$_2$-based biosensors ......................................................................... 7
      1.3.2.2. Potentiometric TiO$_2$-based biosensors ......................................................... 7
      1.3.2.3. Amperometric TiO$_2$-based biosensors ......................................................... 8
  1.4. Enzymes ............................................................................................................................ 11
  1.5. Enzyme-based amperometric biosensors .......................................................................... 12
  1.6. Enzyme immobilization techniques .................................................................................. 16
    1.6.1. Physical methods for enzyme immobilization ....................................................... 16
    1.6.2. Chemical methods for enzyme immobilization ..................................................... 18
  1.7. Common enzymes used in amperometric biosensors ...................................................... 22
    1.7.1. Laccase .................................................................................................................... 22
    1.7.2. Tyrosinase ............................................................................................................... 22
    1.7.3. Glucose oxidase ....................................................................................................... 23
    1.7.4. Horseradish peroxidase ......................................................................................... 24
  1.8. Bio-inspired strategies to tackle enzymes instability ....................................................... 27
  1.9. References ......................................................................................................................... 30

Rationale and Objectives ............................................................................................................. 37

Chapter 2 Instrumentation and techniques ............................................................................. 39
  2.1. Electrochemical techniques ............................................................................................... 40
    2.1.1. Cyclic voltammetry .................................................................................................. 40
    2.1.2. Linear sweep voltammetry ...................................................................................... 41
    2.1.3. Chronoamperometry ............................................................................................... 42
  2.2. Electrochemical setup ....................................................................................................... 43
2.2.1. Electrodes

2.2.1.1. Reference electrodes

2.2.1.2. Counter electrodes

2.2.1.3. Working electrodes

2.2.2. Potentiostat

2.3. Flow injection analysis

2.4. Photoelectrochemical setup

2.5. UV-vis spectroscopy

2.6. Diffuse reflectance UV-vis spectroscopy

2.7. N₂ sorption

2.8. Electron paramagnetic resonance

2.9. Thermogravimetric analysis

2.10. References

Chapter 3 Incorporation of horseradish peroxidase in mesoporous TiO₂

3.1. Introduction

3.2. Experimental section

3.2.1. Reagents

3.2.2. Apparatus

3.2.3. Horseradish peroxidase immobilization in TiO₂

3.2.4. Preparation of the enzyme electrode

3.3. Results and discussion

3.3.1. Optimization of the experimental parameters

3.3.1.1. Matrix composition

3.3.1.2. Incubation time

3.3.1.3. Working potential

3.3.1.4. Concentration of the mediator

3.3.2. Amperometry detection of H₂O₂

3.3.3. Conformational studies: UV-vis, UV-DR and N₂ sorption

3.3.3.1. UV-vis

3.3.3.2. UV-DR

3.3.3.3. N₂ sorption

3.3.3.4. TGA
Chapter 6 Bio-inspired molecular photosensitizers for the photo-electrochemical detection of phenolic compounds .................................................................103

6.1. Introduction ...........................................................................................................104

6.2. Experimental section ..........................................................................................105
  6.2.1. Reagents ......................................................................................................105
  6.2.2. Apparatus ..................................................................................................106
  6.2.3. SPE modifications ......................................................................................106
  6.2.4. Electrochemical measurements ................................................................106

6.3. Results and discussion .......................................................................................107
  6.3.1. Enzymatic and photosensitizer electrode mechanisms ...............................107
  6.3.2. Structural and spectroscopic features of the photosensitizer .....................108
  6.3.3. Oxygen- and electron-based photocatalysis ...............................................109
  6.3.4. Dependency on electrodes and applied potentials ......................................110
  6.3.5. How selective is the proposed detection for phenols? ...............................113

6.4. Conclusion ..........................................................................................................120

6.5. References ..........................................................................................................121

Summary and future perspectives ...............................................................................123

Abstract ......................................................................................................................128

Samenvatting ..............................................................................................................129

List of scientific contributions ..................................................................................130
**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Adenine</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>AG</td>
<td>Antigen</td>
</tr>
<tr>
<td>AMP</td>
<td>Amperometry</td>
</tr>
<tr>
<td>AP</td>
<td>Aminophenol</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BQ</td>
<td>Benzoquinone</td>
</tr>
<tr>
<td>C</td>
<td>Cytosine</td>
</tr>
<tr>
<td>CE</td>
<td>Counter electrode</td>
</tr>
<tr>
<td>CMC</td>
<td>Carboxymethyl cellulose</td>
</tr>
<tr>
<td>CV</td>
<td>Cyclic voltammetry</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECL</td>
<td>Electrochemiluminescence</td>
</tr>
<tr>
<td>EMR</td>
<td>Electron magnetic resonance</td>
</tr>
<tr>
<td>Epa</td>
<td>Anodic peak potential</td>
</tr>
<tr>
<td>EpC</td>
<td>Cathodic peak potential</td>
</tr>
<tr>
<td>EPR</td>
<td>Electron paramagnetic resonance</td>
</tr>
<tr>
<td>ESR</td>
<td>Electron spin resonance</td>
</tr>
<tr>
<td>ET</td>
<td>Electron transfer</td>
</tr>
<tr>
<td>FIA</td>
<td>Flow injection analysis</td>
</tr>
<tr>
<td>FET</td>
<td>Field-effect transistor</td>
</tr>
<tr>
<td>G</td>
<td>Guanine</td>
</tr>
<tr>
<td>GCE</td>
<td>Glassy carbon electrode</td>
</tr>
<tr>
<td>GOx</td>
<td>Glucose oxidase</td>
</tr>
<tr>
<td>HQ</td>
<td>Hydroquinone</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>Ip</td>
<td>Peak current</td>
</tr>
<tr>
<td>IpA</td>
<td>Anodic peak current</td>
</tr>
<tr>
<td>IpC</td>
<td>Cathodic peak current</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>ISC</td>
<td>Intersystem crossing</td>
</tr>
<tr>
<td>ITO</td>
<td>Indium tin oxide</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>LED</td>
<td>Light-emitting diode</td>
</tr>
<tr>
<td>LSV</td>
<td>Linear sweep voltammetry</td>
</tr>
<tr>
<td>MPc</td>
<td>Phthalocyanine metal complexes</td>
</tr>
<tr>
<td>MQ</td>
<td>Milli-Q water</td>
</tr>
<tr>
<td>NP</td>
<td>Nano particles</td>
</tr>
<tr>
<td>NW</td>
<td>Nano wire</td>
</tr>
<tr>
<td>ODN</td>
<td>Oligodeoxynucleotide</td>
</tr>
<tr>
<td>PPO</td>
<td>Tyrosinase, poly-phenol oxidase</td>
</tr>
<tr>
<td>PS</td>
<td>Photosensitizer</td>
</tr>
<tr>
<td>PTFE</td>
<td>Polytetrafluoroethylene</td>
</tr>
<tr>
<td>RE</td>
<td>Reference electrode</td>
</tr>
<tr>
<td>RGO</td>
<td>Reduced graphene oxide</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SCE</td>
<td>Saturated calomel electrode</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
</tr>
<tr>
<td>SHE</td>
<td>Standard hydrogen electrode</td>
</tr>
<tr>
<td>SPE</td>
<td>Screen printed electrodes</td>
</tr>
<tr>
<td>T</td>
<td>Thymine</td>
</tr>
<tr>
<td>TCNQ</td>
<td>Tetracyanoquinodimethane</td>
</tr>
<tr>
<td>TGA</td>
<td>Thermogravimetric analysis</td>
</tr>
<tr>
<td>TTF</td>
<td>Tetrathialfulvalene</td>
</tr>
<tr>
<td>UV-DR</td>
<td>Ultraviolet diffuse reflectance</td>
</tr>
<tr>
<td>UV-vis</td>
<td>Ultraviolet-visible</td>
</tr>
<tr>
<td>WE</td>
<td>Working electrode</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction

This chapter aims to provide an overview of mesoporous titania (TiO$_2$) materials bearing enzymes applied in biosensing strategies. Electrochemical biosensors offer an attractive tool for analysing the content of an analyte due to the direct conversion of a biological event into an electronic signal.

It also includes an overview of different transduction principles in TiO$_2$-based biosensors, is provided in this chapter. In addition, common enzymes used for TiO$_2$-based electrochemical biosensor constructions will be reviewed. Furthermore, a bio-inspired strategy will be introduced and the advantages of this system over the common enzymatic system will be addressed.
1.1. Porous materials

In recent years, the number and variety of materials of particular interest to an engineer or a scientist in general have increased tremendously. Each type of material with a given composition possesses particular properties for a specific application. Porous materials are ubiquitous due to their many advantages such as a large surface area and the ability to anchor different chemical functionalities on their surface. They have attracted the attention of chemists and materials scientists due to their employability in chemical separation and heterogeneous catalysis as well as the scientific interest in the challenges posed by their synthesis, processing, and characterisation.

The International Union of Pure and Applied Chemistry (IUPAC) classifies porous materials according to their pore diameter into three categories: those with pore diameters less than 2 nm are microporous; materials with pore sizes between 2 and 50 nm are mesoporous; and materials with pore diameters greater than 50 nm are called macroporous materials. Nanoporous materials are defined as those materials with pore diameters less than 100 nm; therefore, all three of the above porous materials can be designated as nanoporous materials. However, in most of the literature, nanoporous materials refer to microporous (pore size up to 2 nm) or/and mesoporous (2-50 nm) materials.

1.1.1. Microporous materials

Microporous materials (with pores of less than 2 nm in diameter) have attracted considerable attention due to the variety of applications in which they can be deployed, including heterogeneous catalysis, gas purification, and separation, adsorbents and carriers (catalyst supports). Traditional microporous materials, such as zeolites Y, ZSM-5 and beta, have excellent properties related to their uniform pores, large surface area and controlled acidity, with numerous applications in catalysis and separation processes. The structural capabilities on the scale of a few nanometers can however not meet the demands of the growing applications emerging in processes involving large molecules, for example, biomolecules. Therefore, materials with larger dimensions in the mesoporous range (2-50 nm) should be an appropriate answer to this problem.

1.1.2. Mesoporous materials

The term ‘mesoporous materials’ refers to solids based on either ordered or disordered networks with a broad or narrow distribution of pores in the range between 2 and 50 nm. Initially, the aim of developing these materials was to overcome the ‘1 nm restriction’ imposed by the use of zeolites. Theoretically, using materials with similar features to zeolites (regular pore size, high surface area) but with larger pores could open the door to processing large molecules that are unable to enter into a zeolite porous framework.
Mesoporous materials applied to electrochemical biosensors can be classified in three main categories: silica-based materials\(^\text{11-13}\), carbon-based materials\(^\text{14-16}\) and metal oxides other than silica\(^\text{17-22}\). These materials have a wide range of framework compositions, morphologies, and porous structures. Mesoporous materials possess attractive properties, such as high surface areas, tunable pore sizes and shapes, various structures, and a multitude of compositions, which endow them with potential applications in catalysis, adsorption, sensors, lithium-ion batteries, drug delivery, and nanodevices.\(^\text{5, 23-26}\) Table 1.1 summarises some examples of different types of mesoporous materials and a selection of their applications.

Table 1.1 Some examples of mesoporous materials with a selection of their applications.

<table>
<thead>
<tr>
<th>Material</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>TiO(_2)</td>
<td>solar cells(^\text{27}), pollutants removal(^\text{28}), photocatalysis(^\text{29}) and catalysis(^\text{30})</td>
</tr>
<tr>
<td>SiO(_2)</td>
<td>drug delivery(^\text{31}), catalysis(^\text{32}), gas separation(^\text{33}) and pollutants removal(^\text{34})</td>
</tr>
<tr>
<td>MnO(_2)</td>
<td>batteries(^\text{35}), catalyst(^\text{36}), oxidation of organic pollutants(^\text{37}) and ozone decomposition(^\text{38})</td>
</tr>
<tr>
<td>WO(_3)</td>
<td>light-emitting-diodes(^\text{39}), solar cells(^\text{40}) and photocatalysis(^\text{41})</td>
</tr>
<tr>
<td>Microgels/nanogels</td>
<td>tissue engineering(^\text{42}), bionanotechnology(^\text{43}) and drug delivery(^\text{44})</td>
</tr>
<tr>
<td>Mesoporous carbon</td>
<td>batteries(^\text{44}), fuel cells(^\text{46}), supercapacitors(^\text{47}), gas storage(^\text{48}) and catalysis(^\text{49})</td>
</tr>
</tbody>
</table>

1.1.3. Macroporous materials

Macroporous materials are porous materials with pore diameters larger than 50 nm. There are numerous examples of macroporous materials, including polymers\(^\text{50}\), glasses\(^\text{51}\), wood replicas\(^\text{52}\), gels\(^\text{53}\) and others.\(^\text{54}\) These materials can be used in a wide range of chemical applications such as catalysis and catalyst support, chemical filtration and separation, biomaterials and chromatography.\(^\text{55, 56}\)

From the viewpoint of applications, for instance in catalysis, the macropores favour mass transfer and reduce transport limitations. This becomes particularly important for large molecules (e.g. polymers) or in viscous systems, where diffusion rates are low. Traditionally, macroporous materials with pore diameters >50 nm were believed to be the most suitable support material, ensuring no spatial restrictions when enzyme molecules enter such large pores.\(^\text{57}\) In recent years, however, there has been
a growing emphasis on the use of mesoporous supports with pore diameters ranging between 2 and 50 nm. It is thought that this smaller pore range may offer better retention and enhanced conformational stability to immobilize enzymes, while not being too small for adsorption of the enzymes and access of chemical substrates to be processed in enzymatic reactions.\textsuperscript{58}

1.2. Titanium dioxide

Among the mesoporous non-siliceous materials, mesoporous metal oxides, an important family including most of the metals in the periodic table, are of great interest because of their different functional properties and their industrial application potential.\textsuperscript{59} They hold promise in applications involving electron transfers such as photocatalysis and catalyst support.\textsuperscript{60-62} Mesoporous titanium dioxide is one of the metal-oxide mesoporous materials of particular interest and has been undergoing the most explosive growth due to its outstanding features such as low cost, environmental friendliness, good chemical and mechanical stability, and optical properties.\textsuperscript{63}

Titanium dioxide (TiO\textsubscript{2}), also known as titania, has been the most intensively investigated transition metal oxide in the past two decades.\textsuperscript{64} Titania is commonly seen in three crystalline polymorphs: rutile, anatase, and brookite.\textsuperscript{65} These three types of TiO\textsubscript{2} consist of TiO\textsubscript{6} octahedra, but differ in the distortion of the octahedron units and share edges and corners in different manners\textsuperscript{66} (Figure 1.1).

The octahedron in anatase and rutile is slightly distorted from the perfect octahedron.\textsuperscript{67} The bond length in the axial directions is slightly larger than that in the equatorial directions.\textsuperscript{68} The bond lengths also differ between these two phases. The Ti–O bond lengths are 1.937 and 1.966 Å for anatase and 1.946 and 1.983 Å for rutile in the equatorial and axial directions, respectively.\textsuperscript{68} Anatase phase is more commonly seen for particles synthesised at room temperature and rutile at high temperatures.\textsuperscript{69} Brookite is the least studied of the three natural phases of TiO\textsubscript{2}, largely because of the extreme
difficulty in synthesising it. Brookite always appears as a minor by-product of anatase or rutile in most synthesis methods. In comparison with anatase and rutile, brookite has a low structural symmetry. Moreover, anatase and rutile have wider applications because they are more stable than brookite. Bulk anatase and rutile have band gaps of 3.2 (390 nm) and 3.0 eV (413 nm), respectively. In this thesis mesoporous TiO\textsubscript{2} PC500 (anatase) is used with an anatase content of \~ 85 wt%.

From the beginning of the 20\textsuperscript{th} century, titanium dioxide steadily replaced toxic oxides used as pigments for white paint (e.g. antimony(III) oxide Sb\textsubscript{2}O\textsubscript{3}). At present the annual production of TiO\textsubscript{2} exceeds 5,480,000 million tons, which accounts for 70\% of the total production volume of pigments. It is widely used to provide whiteness in products such as paints, plastic and paper and is also a permitted colour in foodstuffs. Additionally, titania is used as an opacifier in textiles, leather, glass and pharmaceuticals, cosmetics and skin care products. Moreover, TiO\textsubscript{2} has excellent photocatalytic properties and effectively transforms light energy into chemical energy. It is no surprise that titanium dioxide was found to be useful for application in solar cells, environmental remediation, sensing, and coatings.

1.3. Typical TiO\textsubscript{2} based sensors

TiO\textsubscript{2} materials have been used for various sensors such as photocatalytic sensors (1.3.1) and biosensors (1.3.2).

![Figure 1.2 TiO\textsubscript{2}-based photocatalytic applications.](image-url)
1.3.1. TiO$_2$-based photocatalytic sensor

Titania has emerged as an excellent photocatalyst material for environmental and renewable energy fields among others (Figure 1.2).\textsuperscript{81} Most famous are the environmental purification and the decomposition of organic materials.\textsuperscript{82-87}

Titania is a semiconductor material with a band gap of $\sim$3.2 eV (anatase), corresponding to a wavelength of $\sim$390 nm.\textsuperscript{88} When excited with light of a wavelength equal to or greater than the band gap energy (corresponds to UV irradiation), electron-hole ($e^-/h^+$) pairs are generated in the conduction and valence bands, respectively. The photogenerated electron-hole pairs can then follow several pathways: (i) recombination of electrons and holes on the surface or in the bulk of the semiconductor; (ii) reduction of electron acceptors by photogenerated electrons; and (iii) oxidation of electron donors by photogenerated holes. Electrons and holes transported to the particle surface can therefore initiate redox chemistry.\textsuperscript{82} The basic principles involved in the photocatalytic mechanism are shown in Figure 1.3.

![Figure 1.3. Schematic overview of TiO$_2$ photoexcitation to initiate electron–hole pairs ($e^-/h^+$) and redox chemistry at the TiO$_2$ surface. Separated $e^-/h^+$ pairs at the TiO$_2$ surface can result in the reduction of electron acceptors (A) by photogenerated electrons and the oxidation of electron donors (D) by photogenerated holes.](image)

Photogenerated electrons and holes, for example, can react with H$_2$O and O$_2$ molecules, leading to the formation of reactive oxygen species (ROS).\textsuperscript{88} The reaction mechanism can be depicted as follows (equations 1.1-1.3):

\begin{align*}
\text{TiO}_2 + h\nu (\lambda < 390 \text{ nm}) &\rightarrow e^- + h^+ & \text{(eq. 1.1)} \\
 h^+ + \text{H}_2\text{O} &\rightarrow \text{OH}^- + \text{H}^+ & \text{(eq. 1.2)} \\
e^- + \text{O}_2 &\rightarrow \text{O}_2^- & \text{(eq. 1.3)}
\end{align*}
Photocatalytic reactions can be initiated by the resulting ROS. The hole, \( h^+ \), can also react directly with an adsorbed organic donor (D). A is considered an adsorbed molecule that can accept electrons. However, reductive titanium dioxide photocatalysis is less studied due to the lower reducing power of \( e^- \) compared to the high oxidizing power of \( h^+ \).

1.3.2. TiO\(_2\)-based biosensor

Biosensors are sensing devices comprising a biological entity and a transduction element converting the biomolecule-analyte event into a readable signal. Biosensors can be classified on the basis of (1) the type of bio-recognition elements (i.e. enzyme-, antibody-, DNA-, or RNA-based) and (2) the signal transduction method (optical, electrochemical, gravimetric, etc.) used for detection. The biosensors specificity towards a target analyte is mainly determined by the bio-recognition element (1.4), while a careful design of the transducer (e.g. modification of the electrode) can improve the sensitivity (1.3.2). TiO\(_2\) is an excellent candidate for the immobilization of biological components due to its low cost, high stability, and biocompatibility.

The detection principle of a TiO\(_2\)-based biosensor varies according to its sensing mechanism. Three typical TiO\(_2\)-based biosensors are described: optical (1.3.2.1), potentiometric (1.3.2.2), and amperometric (1.3.2.3) sensors.

1.3.2.1 Optical TiO\(_2\)-based biosensors

The binding of biomolecules immobilized on titania with targets changes the optical properties of titania so that the variation of reflected light is in proportion to the target concentration. The unique photocatalytic properties of TiO\(_2\) make it an attractive metal oxide for the fabrication of optical based biosensors. Several types of optical models are generally used for sensing signals based on a light transducer. The most common optical model is based on electrogenerated chemiluminescence, or electrochemiluminescence (ECL). ECL is a kind of luminescence produced during electrochemical reactions in solutions where one/all reactants are electrochemically produced at the electrodes. The ECL signal intensity is positively related to the concentration of luminescent species, so a specific analyte can be accurately determined according to the wavelength and intensity of the emitted photon of light.

1.3.2.2. Potentiometric TiO\(_2\)-based biosensors

Potentiometric sensors work by measuring the potential difference while drawing a negligible current. Many studies have illustrated the fabrication of nanostructure sensors using
semiconducting titanium dioxide materials. An important benefit of nanostructure TiO$_2$ materials is their wide energy band gap (between 1.8 and 4.1 eV), which may provide a better sensitivity range than those of other available matrices for field-effect transistor (FET) devices.$^{101}$

Various field-effect transistor (FET) devices were designed as potentiometric devices$^{96}$. FET devices are three terminal semiconductor devices, with source, drain and gate terminals (Figure 1.4). The semiconductor titania is located between source and drain terminal. The general working mechanism of a FET device is using an electric field to control the conductivity of a semiconductor film such as titania between two electrodes (i.e., the source and drain). The charge carries are electrons or holes, which flow from the source to drain though an active channel. This flow of electrons from source to drain is controlled by a voltage applied across the gate and source terminals. The conductivity control is achieved by changing the electric field potential, relative to the third electrode, known as the gate. FET devices can detect weak signals based on high impedance semiconductors; hence they are widely used in the growing field of electrochemical biosensing.$^{102,103}$ The change in the gate potential resulting from the sensing events provides a label-free detecting method, which is ideal for the electronic transduction of (bio)chemical recognition.

![Figure 1.4. Metal oxide semiconductor field effect transistor.](image)

1.3.2.3. Amperometric TiO$_2$-based biosensor

Amperometric detection is usually adopted in a three-electrode system in which an electrode modified by TiO$_2$ serves as a working electrode, combined with a reference electrode and a counter electrode.$^{104}$ Porous TiO$_2$ is a good candidate for biosensing materials due to its high surface area and tunable pore size.$^{105-109}$ In this type of biosensor the target chemicals are dispersed in the electrolyte solution, and a redox reaction takes place at the electrode surface at a given potential. The current signal generated from the redox reaction of the electroactive species can be continuously collected using an electrochemical workstation.$^{103}$
In the amperometric experiment, changes in current generated by the electrochemical oxidation or reduction are monitored directly with time, while a constant potential is maintained at the working electrode with respect to a reference electrode. In amperometry the current is directly proportional to the bulk concentration of the analyte. In addition, hydrodynamic amperometric techniques can provide significantly enhanced mass transport to the electrode surface, for example when the working electrode moves with respect to the solution by rotation, or in flow conditions where the sample solution passes over the stationary electrodes. TiO$_2$ material has been previously studied and emerged as a matrix well suited for the immobilization of biomolecules (e.g. enzymes, protein molecules, and antibodies) and the construction of biosensors. Among them, amperometric biosensors based on immobilized enzymes in the vicinity of the electrode material are most commonly used, combining the advantages of enzyme selectivity and direct transduction of the interaction with the target in an electric current. Since reactions are usually only detected in the vicinity of the electrode surface, the electrodes themselves play a crucial role in the performance of electrochemical biosensors. The function of electrode material, surface modifications or dimensions, greatly affect their ability to detect analytes.

Over the past decade, studies have been conducted to improve the material’s performance, particularly on lowering the rate of photogenerated charge recombination and increasing the diffusion rate of reactants. To tackle these issues, TiO$_2$ can be integrated with other nanoparticles/nanomaterials. Nanoparticles/nanomaterials can provide a more efficient electron transport and show an excellent capability to immobilize enzymes as well. For example, a TiO$_2$-graphene nanocomposite has a larger surface area, higher conductivity, and chemical stability with DNA. On the other hand, nanostructured TiO$_2$ with different dimensionalities has been proven to be interesting due to their properties. Reducing the dimensions of electrode materials from the micron to the nanoscale can boost the performances of electrochemical devices.

Nanostructured TiO$_2$ including TiO$_2$ nanowires, TiO$_2$ nanoneedles, TiO$_2$ nanospheres, and TiO$_2$ nanotubes can be used in the context of sensor fabrication. For example, vertically aligned TiO$_2$ nanotubes are widely employed to facilitate the electron transfer as well as to serve as a prospectively ideal “vessel” for immobilizing various biological components, such as enzymes, haemoglobins, antibodies, antigens, cells, DNA, and bacteria.

The presence of polymer structures, such as nafion, chitosan, and cellulose, mechanically stabilizes TiO$_2$ matrix. Table 1.2 summarises some amperometric TiO$_2$-based biosensors reported in recent years.
Table 1.2: Amperometric TiO$_2$-based biosensors reported in recent years.

<table>
<thead>
<tr>
<th>TiO$_2$ material</th>
<th>Electrode</th>
<th>Biomolecule</th>
<th>Analyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>TiO$_2$ microspheres</td>
<td>GCE</td>
<td>HRP</td>
<td>H$_2$O$<em>2$$</em>{127}$</td>
</tr>
<tr>
<td>TiO$_2$NPs</td>
<td>Ti foil</td>
<td>HRP</td>
<td>H$_2$O$<em>2$$</em>{128}$</td>
</tr>
<tr>
<td>TiO$_2$ NWs</td>
<td>GCE</td>
<td>HRP</td>
<td>H$_2$O$<em>2$$</em>{122}$</td>
</tr>
<tr>
<td>Au/ TiO$_2$ hollow microsphere</td>
<td>CPE</td>
<td>probe DNA</td>
<td>DNA sequence$_{129}$</td>
</tr>
<tr>
<td>Mesoporous TiO$_2$</td>
<td>ITO</td>
<td>alcohol dehydrogenase</td>
<td>H$_2$O$<em>2$$</em>{108}$</td>
</tr>
<tr>
<td>TiO$_2$ nanofibers</td>
<td>Pt electrodes</td>
<td>glucose oxidase</td>
<td>glucose$_{119}$</td>
</tr>
<tr>
<td>TiO$_2$ NPs</td>
<td>GCE</td>
<td>tyrosinase</td>
<td>phenol$_{130}$</td>
</tr>
<tr>
<td>TiO$_2$ NPs</td>
<td>Ti wires</td>
<td>urease</td>
<td>urea$_{131}$</td>
</tr>
<tr>
<td>TiO$_2$ NPs/LDH/silica</td>
<td>gold electrode</td>
<td>lactate dehydrogenase</td>
<td>lactic acid$_{132}$</td>
</tr>
<tr>
<td>TiO$_2$ nanoneedles</td>
<td>ITO</td>
<td>cytochrome c</td>
<td>H$_2$O$<em>2$$</em>{120}$</td>
</tr>
<tr>
<td>PbO$_2$/TiO$_2$/Ti</td>
<td>Ti foil</td>
<td>acetylcholinesterase enzyme</td>
<td>organophosphorus pesticides$_{133}$</td>
</tr>
<tr>
<td>Ag/TiO$_2$ NPs</td>
<td>gold electrode</td>
<td>protein kinase A</td>
<td>ATP$_{134}$</td>
</tr>
<tr>
<td>chitosan/CdSe$<em>x$Te$</em>{1-x}$/TiO$_2$ NTs</td>
<td>Ti foil</td>
<td>bovine serum albumin</td>
<td>pentachlorophenol$_{135}$</td>
</tr>
<tr>
<td>CdSe/TiO$_2$-RGO</td>
<td>ITO</td>
<td>antibodies</td>
<td>carcinoembryonic antigen$_{136}$</td>
</tr>
</tbody>
</table>

Abbreviations: GCE - glassy carbon electrode; HRP - horseradish peroxidase; NP - nano particles; NW - nano wire; CPE - carbon paste electrode; ITO - indium tin oxide; LDH - lactate dehydrogenase; ATP - adenosine triphosphate; RGO - reduced graphene oxide.
1.4. Enzymes

Enzymes are composed of α-amino acids (amino acids containing an amino group bounded to the alpha carbon), connected together by amide linkages. Almost all enzymes are proteins with the exception of a few ribonucleoprotein enzymes, in which the catalytic activity is in the RNA rather than the protein part. Enzymes operate by specific binding capability to a substrate, lowering the activation energy necessary for a reaction to take place (Figure 1.5). The specific binding of enzymes with substrates occurs at the catalytic (active) site of the enzyme. Using enzymes as bio-receptors not only provides biosensors with a high degree of specificity, but their catalytic activity leads to sensitive analyses.

![Figure 1.5. Induced-fit model: the enzyme active site forms a complementary shape to the substrate.](image)

The significant efficiency of these biocatalysts at rather low temperatures (below 50°C) and at physiological pH (5.0 to 8.0) represents one of the most important reasons for the wide use of enzymes in biosensor applications. However, the instability and reproducibility of the reactivity of enzymes, combined with the need for chemical reagents for sensing remain challenging for the construction of biosensors.

The enzymes are divided into six main classes according to their function, which are oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases.

1. **Oxidoreductases** involve redox reactions in which hydrogen or oxygen atoms or electrons are transferred between molecules.

2. **Transferases** catalyse the transfer of an atom or group of atoms (e.g. acyl-, alkyl-, and glycosyl) between two molecules, but exclude transfers that are classified in the other groups (e.g. oxidoreductases and hydrolases).
(3) **Hydrolases** involve hydrolytic reactions and use water to cleave chemical bonds, usually dividing a large molecule into two smaller molecules.

(4) **Lyases** involve the elimination of a functional group from the substrate with the formation of double bonds.

(5) **Isomerases** which transfer groups from one position to another position in the same molecule.

(6) **Ligases**, also known as synthetases, form a relatively small group of enzymes that involve the formation of a covalent bond joining two molecules together, coupled with the hydrolysis of a nucleoside triphosphate (ATP).

The major classes are further subdivided into subclasses. Of the commercially available enzymes, the oxidoreductases are used most frequently. These enzymes are divided into four groups: dehydrogenases (hydride transfer), oxidases (electron transfer to molecular oxygen), oxygenases (oxygen transfer from molecular oxygen), and peroxidases (electron transfer to peroxide).  

1.5. **Enzyme-based amperometric biosensors**

Enzyme-based amperometric sensors hold a leading position among biosensor systems presently available by reaching a large commercial market for example for glucose sensing. The concept includes the placement or immobilization of an enzyme in close proximity to or on an electrode surface. The enzyme is the most critical component of such devices: combining the specificity of the enzyme for a given target analyte and catalysing the formation of an electroactive product allowing the electrochemical detection. The electroactive product can be monitored directly using amperometry, in which the current is measured in response to an applied, constant voltage.

Enzyme-based biosensors can be historically divided into three generations. First- and second-generation biosensors are oxygen- and mediator-based respectively. Third-generation biosensors are so-called directly coupled enzyme electrodes (Figure 1.6).
Figure 1.6. Three generations of enzyme-based amperometric sensors (a) based on the use of natural secondary substrate, (b) redox mediators, or (c) the direct electron transfer between the enzyme and the electrode (c).

(a) First-generation biosensors

The first-generation biosensors are based on the use of oxygen as a substrate or on the detection of hydrogen peroxide as a product (Figure 1.7). In the enzymatic reaction, molecular oxygen acts as the oxidising agent to produce hydrogen peroxide. Detection of oxygen consumption at a negative potential (-0.6 V vs Ag/AgCl) is the simplest way to monitor the reaction. Alternatively, the oxidation of H$_2$O$_2$ to O$_2$ at a platinum electrode or its reduction to H$_2$O at a prussian blue modified electrode can be used as the current response of the enzyme electrode.

Figure 1.7. Oxygen-dependent first-generation biosensor with amperometric detection.
The first-generation biosensors may suffer from interference due to a high applied overpotential that might lead to a background signal from the other electroactive species. Moreover, the response of the first-generation biosensors depends on the oxygen concentration.

(b) Second-generation biosensors

Second-generation biosensors address many of the first generation biosensor issues with the incorporation of a mediator. Mediators, small redox active molecules diffuse in and react with the active site of the enzyme and diffuse out and react with the electrode surface. They are capable of shuttling the electrons between the enzyme and the electrode. These mediated enzyme electrodes are called second-generation biosensors. Mediators that can be used to assist the electron transfer include: ferrocene, ferricyanide, methylene blue, hydroquinone, etc. (Figure 1.8). As a result of using artificial electron acceptors (mediators), measurements become insensitive to oxygen fluctuations and can be performed at lower potentials that do not provoke interfering reactions. In order to function effectively, the mediator should react rapidly with the enzyme, must be nontoxic and chemically stable (in both reduced and oxidized forms), and must have a practically suitable redox potential.

![Chemical structures of some common redox mediators](image)

*Figure 1.8. Chemical structures of some common redox mediators: (a) ferrocene; (b) ferricyanide; (c) methylene blue; (d) hydroquinone.*

Optimization of the mediator as a replacement of oxygen in glucose sensors resulted in existence of fast and accurate personal glucose sensors that capable to measure glucose in a volume of 1 µL of blood in five seconds.
(c) Third-generation biosensors

Intensive efforts are being made to improve the electronic transfer (ET) between the redox centre of the enzymes and electrode surfaces in the development of third-generation biosensors. In this generation, the redox enzyme (oxidoreductase) exchanges electrons directly with an electrode facilitating electrochemical oxidation/reduction of a corresponding substrate molecule with no mediator involved in the process. Direct electron transfer should occur at the redox potential of the prosthetic group itself.

An optimally designed a third-generation biosensors’ configuration has to ensure the proximity of the enzyme to the electron conducting transducer surface to make possible the ET. Unfortunately, proteins directly adsorbed on carbon, platinum or gold surfaces tend to denature at least partially, leading to electrode fouling and unfavourable conditions for direct ET. To avoid this, the electrodes are often chemically pre-modified before immobilization of the enzymes. This modification can also promote the direct ET between the enzyme and the electrode due to orienting the enzyme on the surface. In the optimal orientation, most of the enzyme molecules in the first monolayer can contribute to the sensor signal (Figure 1.9, right), instead of only a part of them in the case of random adsorption (Figure 1.9, left).

![Figure 1.9. Enzymes immobilized by non-specific absorption (left) and in an oriented way on the surface of a monolayer (right).](image)

Only a limited number of enzymes have shown direct electron transfer reactions. The most intensively studied and best characterised enzymes showing direct ET properties belong to the group of peroxidases, such as cytochrome c peroxidase, horseradish peroxidase, fungal peroxidase, immobilized mainly on carbonaceous materials and in some cases on noble metals.
1.6. Enzyme immobilization techniques

The main reason for enzyme immobilization is to allow the re-use of immobilized enzymes over an extended period of time, hence generating cost savings. Furthermore, it allows easier biosensor manipulation and operation. Immobilized enzymes are enzymes that are retained or localised in or on a material, whilst retaining their catalytic activity, so that they can be used repeatedly and continuously.¹⁵⁸ Basically, all enzyme immobilization techniques can be classified in two methods (Figure 1.10): physical and chemical methods.¹⁵⁹

![Diagram of enzyme immobilization techniques]

*Figure 1.10. Overview of the techniques being used for enzyme immobilization.*

1.6.1. Physical methods for enzyme immobilization

Enzyme attachment can be achieved via physical forces involving van der Waals forces, hydrophobic interactions and hydrogen bonding.¹⁶⁰ One discriminates between entrapment, adsorption, and microencapsulation (Figure 1.11).
Figure 1.1. There are three physical methods available for immobilising enzymes: (a) adsorption, (b) entrapment, and (c) microencapsulation.

(a) Adsorption

A first easy method of physical immobilization is adsorption. The enzyme is attached to the support material by non-covalent linkages including hydrogen bonding, and van der Waals forces without any pre-activation of the support. The nature of the forces involved in noncovalent immobilization results in a process that can be reversed by changing the conditions that influence the strength of the interaction (e.g. pH, ionic strength, temperature, or polarity of the solvent). This method is mild and causes generally little or no enzyme inactivation. Such methods are therefore economically attractive, but may suffer from problems such as enzyme leakage when the interactions are relatively weak.\(^{161}\)

(b) Entrapment

This method does not involve chemical bonds between the supporting matrix and the protein, which is in fact simply included in the three-dimensional (3D) network of the supporting matrix.\(^{162}\) Enzymes can be immobilized in a wide diversity of 3D matrices such as an electropolymerised film, an amphiphilic network composed of polydimethylsiloxane (PDMS), a silica gel, a polysaccharide or a carbon paste.\(^{163}\) This method has several advantages: simplicity, no change in intrinsic enzyme properties, no chemical modification, minimal enzyme requirement and matrices available in various shapes. Disadvantages of this method are as follows: enzyme leakage, only small sized substrate/products can be used, requires delicate balance between mechanical properties of the matrix and its effect on enzyme activity and presence of diffusional constraints.
(c) Microencapsulation

Enzymes are immobilized by enclosing them within spherical semi-permeable polymer membranes with controlled porosity (1-100 μm). The semi-permeable membrane allows the free passage of the substrate and product but confines the enzyme in a fixed cavity between the membrane and transducer. The selectivity can be maintained by controlling the porosity and chemical properties of the membrane. Semi-permeable membranes can either be permanent or non-permanent membranes based on the constituents. Permanent membranes are made of cellulose nitrate and polystyrene while non-permanent membranes are made of liquid surfactant.

1.6.2. Chemical methods for enzyme immobilization

Chemical methods for enzyme immobilization involve the attachment of enzymes onto different matrices using covalent or ionic bonds (Figure 1.12). In general, chemical immobilization can provide a longer shelf-life and stable biosensors; however, the enzymatic activity is often not as good as that in immobilized enzymes prepared by physical immobilization.

![Figure 1.12. Chemical methods for enzyme immobilization (a) covalent attachment, (b) cross-linking, (c) ionic binding and (d) conjugation by affinity ligands.](image-url)
(a) Covalent attachment

The most popular chemical immobilization for enzyme-based biosensors is covalent bonding. Covalent bonding involves bonding between a functional group present in the biomaterial to the supporting matrices. Enzyme molecules are attached either directly to the reactive groups (e.g. hydroxyl, amide, amino, carboxyl groups) present on the matrix or by a spacer arm, which is artificially attached to the matrix through various chemical reactions. The binding of the enzymes to the solid support is achieved by activating the surface of the support, followed by coupling to the activated surface. An advantage of these methods is that, because of the stable nature of the bonds formed between enzyme and matrix, the enzyme is not released into the solution upon use. However, this method sometimes leads to drastic changes in the conformation and catalytic properties of the enzyme, due to harsh immobilization conditions and concurrence of similar amino-groups at the active site being involved during interaction of the enzyme with the matrix.

(b) Cross-linking

Cross-linking involves the formation of a number of covalent bonds between the enzyme molecule and the matrix consisting of crosslinking agents, to increase the attachment significantly (e.g. glutaric acid dialdehyde, glyoxal, diisocyanates, hexamethylene diisocyanate, toluene diisocyanate). Generally, amino groups of lysine, sulfhydryl groups of cysteine, phenolic OH groups of tyrosine, or imidazol groups of histidine are used for enzyme binding under mild conditions. The main advantage of this method is that the covalent nature of the interaction is reflected in a minimal leakage boosting the operational stability of the enzymes. However, the negative side is the possible chemical modification of the protein surface induced by crosslinking.

(c) Ionic interaction

The ionic interaction is based on the interactions between the charged enzyme molecules and a charged matrix. Here, the higher the surface charge density on the matrix, the greater the amount of enzyme being bound to the matrix. Sometimes, in addition to ionic interactions, enzyme molecules are also physically adsorbed on the matrix. The method is straightforward and reversible but, in general, it is difficult to find conditions under which the enzyme remains both strongly bound and fully active. Enzyme binding via ionic interactions during immobilization depends on the pH of the solution, the concentration of the enzyme, the charges in the enzyme, and the temperature. This method of immobilization leads to minimal changes in enzyme conformation. However, problems may arise from the use of a highly charged supports when the substrates or products themselves are charged; the kinetics are distorted as a result of partition, competitive adsorption or diffusion phenomena.
(d) Conjugation by affinity ligands

Attachment of the enzyme to the matrix using specific ligands is a fourth strategy to chemically bind enzymes to a support. This method of immobilization leads to minimal changes in the conformation of the enzyme, with high stability and catalytic efficiency of the immobilized enzyme due to non-involvement of active site residues and higher immobilization efficiency due to the presence of high densities of ligands on the matrix. The major advantage of using affinity interactions for enzyme immobilization lies in the selectivity of the method. An important benefit of this type of binding is the possibility to control the orientation of the immobilized enzyme and minimal conformational changes, which results in high retention of the immobilized molecule activity.169

The advantages and drawbacks of the five common immobilization methods (physical and chemical) are summarised in Table 1.3.163
Table 1.3. Advantages and drawbacks of the five most common immobilization methods (physical and chemical).

<table>
<thead>
<tr>
<th>Method</th>
<th>Binding nature</th>
<th>Advantages</th>
<th>Drawbacks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adsorption (Physical)</td>
<td>Weak bonds</td>
<td>○ Simplicity&lt;br&gt; ○ Generally no loss of enzyme activity</td>
<td>○ Desorption/Leaking&lt;br&gt; ○ Non-specific adsorption</td>
</tr>
<tr>
<td>Covalent coupling (Chemical)</td>
<td>Chemical binding between functional groups of the enzyme and those on the support</td>
<td>○ Stable&lt;br&gt; ○ Fast response</td>
<td>○ Matrix not regenerable&lt;br&gt; ○ Coupling sometimes with toxic reagent</td>
</tr>
<tr>
<td>Entrapment (Physical)</td>
<td>Incorporation of the enzyme within a gel, polymer or a solid</td>
<td>○ No chemical reaction between the monomer and the enzyme that could affect the activity&lt;br&gt; ○ Several types of enzymes can be immobilized within the same polymer</td>
<td>○ Diffusion barrier&lt;br&gt; ○ Enzyme leakage&lt;br&gt; ○ High concentrations of monomer and enzyme needed for the specific case of electropolymerisation</td>
</tr>
<tr>
<td>Cross-linking (Chemical)</td>
<td>Bond between enzyme/cross-linker</td>
<td>○ Simple&lt;br&gt; ○ No leakage/desorption</td>
<td>○ Risk of severe loss of enzyme activity</td>
</tr>
<tr>
<td>Affinity (Chemical)</td>
<td>Affinity bonds between a functional group on a support and an affinity tag on a protein sequence</td>
<td>○ Controlled and oriented immobilization</td>
<td>○ Need for the presence of specific groups on the enzyme</td>
</tr>
</tbody>
</table>
1.7. Common enzymes used in amperometric biosensors

Commonly, amperometric biosensors use the following enzymes: laccase\textsuperscript{170}, tyrosinase\textsuperscript{171,172}, glucose oxidase, and peroxidase.\textsuperscript{142,173}

1.7.1. Laccase

Laccases are oxidoreductases containing copper, have been reported in many plants, and are secreted by numerous fungi.\textsuperscript{174-176} Laccases are able to oxidise various substrates: mono-, di-, phenols, and polyphenols, aromatic amines, anilines, with concomitant reduction of oxygen to water according to the reaction (equation 1.4):

\[ 4 \text{AH} + \text{O}_2 \rightarrow 4\text{A} + 2\text{H}_2\text{O} \quad \text{(eq. 1.4)} \]

where AH and A are the reduced and oxidized states of the substrate, respectively.\textsuperscript{177-179} In the last decade, the use of laccases has been explored for a wide variety of applications, including detoxification of industrial effluents - mostly in paper/pulp, textile and petrochemical industry; laccase is also employed as a medical diagnostic tool and an agent for bioremediation of herbicides and pesticides. It has also been used as a catalyst for the manufacture of anti-cancer drugs and even as an ingredient in cosmetics.\textsuperscript{180}

1.7.2. Tyrosinase

Tyrosinase, also known as poly-phenol oxidase (PPO), is widely distributed in plants, fungi and animals has the ability to oxidise several kinds of phenolic compounds to homologous o-quinones.\textsuperscript{181} The catalytic process comprises two successive reactions known as cresolase and catecholase, which are illustrated in Figure 1.13.\textsuperscript{182,183} The first step is hydroxylation of monophenols to o-diphenols. The second step is to oxidise o-diphenols to homologous o-quinones in the presence of molecular oxygen.\textsuperscript{182}
1.7.3. Glucose oxidase

In the basic concept of the glucose biosensor, immobilized glucose oxidase (GOx) catalyses the oxidation of β-D-glucose by molecular oxygen producing gluconic acid and hydrogen peroxide. Hydrogen peroxide is further oxidized at a platinum (Pt) anode thus indicating about the reaction and the glucose concentration. The electron flow at the working electrode (measured current) is proportional to the number of glucose molecules present in a sample.

\[
\text{glucose} + O_2 \rightarrow \text{gluconic acid} + H_2O_2 \quad (\text{eq. 1.5})
\]

\[
H_2O_2 \rightarrow 2H^+ + O_2 + 2e^- \quad (\text{eq. 1.6})
\]

In general, three strategies are used for the electrochemical sensing of glucose: measuring oxygen consumption, measuring the amount of hydrogen peroxide produced by the enzyme reaction, or using a diffusible or immobilized mediator to transfer the electrons from GOx to the electrode. The usage of the mediator improves the analytical performance of the sensors in application to personal glucose blood test.

The first electrochemical blood glucose monitor for self-monitoring of diabetic patients was pen-sized and was launched in 1987 as ExacTech by Medisense Inc. Various strategies to facilitate electron transfer between the GOx redox centre and the electrode surface have been employed since then.

\[
\text{glucose} + \text{GOx}_{(\text{ox})} \rightarrow \text{gluconic acid} + \text{GOx}_{(\text{red})} \quad (\text{eq. 1.7})
\]

\[
\text{GOx}_{(\text{red})} + 2\text{M}_{(\text{ox})} \rightarrow \text{GOx}_{(\text{ox})} + 2\text{M}_{(\text{red})} + 2H^+ \quad (\text{eq. 1.8})
\]

\[
2\text{M}_{(\text{red})} \rightarrow 2\text{M}_{(\text{ox})} + 2e^- \quad (\text{eq. 1.9})
\]
1.7.4. Horseradish peroxidase

Peroxidases are widely distributed in nature and can be extracted from most plant cells, some animal organs and tissues. Peroxidases are a group of redox enzymes (oxidoreductases) catalysing the oxidation of various electron donors with hydrogen peroxide or alkyl hydroperoxidase. Horseradish peroxidase (HRP) is the most intensively studied peroxidase. The horseradish (Armoracia rusticana) is a herb cultivated in temperate regions of the world.

The enzyme HRP belongs to the group of plant peroxidases and it contains two different types of metal centre, iron(III) protoporphyrin IX (usually referred to as the 'heme group') and two calcium atoms (Figure 1.14). Both are essential for the structural and functional integrity of the enzyme. The heme group is attached to the enzyme at His170 (the proximal histidine residue). The second axial coordination site (on the so-called distal side of the heme plane) is unoccupied in the resting state of the enzyme but available to hydrogen peroxide during enzyme turnover (Figure 1.14-right).

Figure 1.14. Three-dimensional representation of the X-ray crystal structure of HRP. The heme group (coloured in red) is located between the distal and proximal domains, which each contain one calcium atom (shown as green spheres) - left. HRP heme active site - right.

Peroxidases occur as a large family of isoenzymes. Isoenzymes (or isozymes) are different molecular forms of the same enzyme, which catalyse the same biochemical reaction but have distinct physical, chemical and kinetic properties arising from small differences in their amino acid sequence. In 1958, Paul isolated five different forms of peroxidases from horseradish roots. These were labelled with
the capital letters A, B, C, D and E. In 1966, Shannon and co-workers\textsuperscript{192} confirmed these results and further reported that fraction A could be resolved into three additional fractions, designated as A1, A2, and A3. These authors performed an extensive study, reported in a series of papers, on the characterisation of HRP isoenzymes in terms of their physical, catalytic, and structural properties.\textsuperscript{193-195} The research group of Morita isolated and characterised five neutral (B1, B2, B3, C1 and C2)\textsuperscript{196} and six basic isoenzymes (E1, E2, E3, E4, E5, and E6).\textsuperscript{197} Neutral isoenzymes contain a high carbohydrate content and have similar physico-chemical and kinetic properties.\textsuperscript{198} Basic isoenzymes contain a lower content of carbohydrates than the neutral isoenzymes.\textsuperscript{187}

HRP catalyses the oxidation of a wide variety of electron donor substrates, such as phenols, aromatic amines, and iodide, by H\textsubscript{2}O\textsubscript{2}. The reaction is a three-step cyclic process (A-C), in which the enzyme is first oxidized by H\textsubscript{2}O\textsubscript{2} (A) and then reduced back to the native form in two sequential steps (B-C) involving the formation of two enzyme intermediates, Compounds I and II (Figure 1.1).\textsuperscript{187, 190, 199}
Figure 1.15. Reaction cycle of HRP, showing the enzyme intermediates, Compounds I, II and III.
The first step (A) consists of the cleavage of the H₂O₂ molecule, with the concomitant production of water and incorporation of one of the oxygen atoms of H₂O₂ into Compound I. It is now known that Compound I contains an oxoferryl group (Fe⁴⁺=O), in which the iron is in +4 oxidation state, and a porphyrin π-cation radical. Compound I is then capable of oxidising a wide range of substrate molecules (AH) by a mechanism involving a single-electron transfer, in which the π-cation radical is first discharged, leading to the formation of the second enzyme intermediate called Compound II (B). Compound II, which also contains an oxoferryl group (Fe⁴⁺=O), is then reduced by a second substrate molecule (AH) to the native ferric enzyme (Fe³⁺). During this one-electron reduction, the ferryl iron returns to its native state, whereas the oxygen accepts two protons to form a water molecule and is released from the heme (C).

Aside from the three described oxidation states of HRP, two more oxidation states have been identified: a ferrous species and compound III. The structures of these five intermediate states were solved in 2002. In the absence of reducing substrates, excess peroxides react as reductants with Compound I, giving rise to several spectroscopically distinct species. In the case of H₂O₂, three pathways have to be considered subsequent to the formation of a Compound I-H₂O₂ complex: (1) a catalase-like (i.e. pseudocatalase) two-electron reduction reaction that restores the enzyme to the resting state (D), (2) another catalytic pathway that leads to the formation of Compound III which decays back to the resting state (E), and (3) a competing pathway that leads to the irreversible inactivation of the enzyme (F). The presence of a reducing substrate, as well as the two catalytic pathways, plays an important role in preventing enzyme inactivation by H₂O₂. The enzymatic production of molecular oxygen via the pseudocatalase activity of HRP was described as a major protective mechanism against H₂O₂ inactivation.

1.8. Bio-inspired strategies to tackle enzymes instability

The utilisation of enzymes as catalysts has two main drawbacks. The first one is related to the complexity of the catalytic system, which makes it fragile and active only under special reaction conditions. The second drawback is in the extreme selectivity of enzymes. This seems to be a rather provocative statement but, indeed, this selectivity restricts applications of enzymes-based sensors to only a limited number of compounds which is for some applications not always to strive for. In the bioinspired chemistry approach, the active site of an enzyme in the form of a small synthetic chemical material will be imitated. This reduces the complexity of the reaction mechanism and provides a way of broadening the scope of possible substrates since the enzymatic active sites’ analogues are more substrate-accessible and flexible.
Biomimicry or bioinspiration involves learning nature’s design principles in building highly complex and sophisticated engineering models at various length scales and utilising the wealth of knowledge to solve the critical challenges faced by humanity.\textsuperscript{208-210} Considering that natural materials and systems have evolved to perform various functions, including structural support, signal transduction, sensing, catalysis, light harvesting, charge transfer, molecular recognition, self-assembly, and self-organisation, or combinations of two or more of these functions, the structures, and properties of these materials and systems are extremely diverse. Learning from nature and applying nature’s engineering principles is a promising approach not only to find sustainable solutions for various critical global challenges such as food, water, public health, and clean energy but also to achieve revolutionary advances in the design and fabrication of novel functional materials and systems.\textsuperscript{211}

Bioinspired materials are synthetic materials whose structure, properties or function mimic those of natural materials or living matter.\textsuperscript{212} One of the common examples of bioinspired materials is light-harvesting photonic materials that mimic photosynthesis.\textsuperscript{212}

Phthalocyanine metal complexes (MPc’s)\textsuperscript{213} are structurally related to porphyrin complexes (Figure 1.16), consisting of four isoindole units presenting an 18 π-electron aromatic cloud delocalised over an arrangement of alternated carbon and nitrogen atoms\textsuperscript{214}, which are widely used by nature in the active sites of enzymes responsible for catalytic aerobic oxidations, reduction, and transport of dioxygen, and destruction of peroxides.\textsuperscript{215} Although phthalocyanines are purely synthetic ligands they can, therefore, be regarded as related to bioinspired chemistry usually associated with porphyrin complexes. Among a large variety of porphyrinoid macrocyclic complexes such as porphyrins, porphyrazines\textsuperscript{213}, corroles\textsuperscript{216}, and corrolazines\textsuperscript{217}, MPc’s are probably the most accessible from a preparation point of view. Synthetic metalloporphyrin complexes have generally been used for a variety of catalytic transformations.\textsuperscript{218} In turn, MPc’s are very attractive as catalysts not only because of their structural analogy with porphyrin complexes but also due to their accessibility in terms of the cost and straightforward preparation on a large scale as well as their chemical and thermal stability. These complexes are widely used in different fields of material science, including semiconductor, and optical devices.\textsuperscript{214}
Photochemical properties of phthalocyanine complexes have been the subject of several investigations, due in part to the prospect of their use in photovoltaic and photocatalytic processes.\textsuperscript{219} Phthalocyanines are efficient at forming the singlet oxygen (\(^{1}O_{2}\)) after excitation by light.\textsuperscript{220} Excitation of a photosensitiser (PS) yields the excited singlet state (1PS*) and by intersystem crossing (ISC) the excited triplet state (3PS*). Then spin-allowed triplet-singlet energy transfer from 3PS* to triplet oxygen (\(^{3}O_{2}\)) results in the highly reactive singlet oxygen (\(^{1}O_{2}\)) followed by oxidation of a substrate (Sub).\textsuperscript{221}

\[
\begin{align*}
\text{PS} & \rightarrow 1\text{PS}^* \rightarrow 3\text{PS}^* & \text{(eq. 1.10)} \\
3\text{PS}^* + ^{3}O_{2} & \rightarrow \text{PS} + ^{1}O_{2} & \text{(eq. 1.11)} \\
^{1}O_{2} + \text{Sub} & \rightarrow \text{oxidized Sub} & \text{(eq. 1.12)}
\end{align*}
\]

The efficient production of singlet oxygen from this triplet state has found applications in photocatalytic degradation of pollutants.\textsuperscript{214} The photosensitisers that are commonly used for the generation of singlet oxygen are zinc or aluminium phthalocyanines.\textsuperscript{222}
1.9. References

121. M. A. Fox and M. T. Dulay, Chemical reviews, 1993, 93, 341-357.
129. Z.-D. Gao, Y. Qu, T. Li, N. K. Shrestha and Y.-Y. Song, Scientific Reports, 2014, 4, 6891.
173. S. Yang, Y. Li, X. Jiang, Z. Chen and X. Lin, *Sensors and Actuators B: Chemical*, 2006, **114**, 774-780.
Rationale and Objectives

The overall goal of this thesis is to develop new robust strategies for the detection of environmentally important compounds such as phenolic compounds and persistent organic pollutants by using titania (TiO₂) and horseradish peroxidase (HRP). In order to accomplish this goal, different innovative strategies to overcome drawbacks of current bioanalysis are developed. Strategies avoiding the addition of hydrogen peroxide (H₂O₂) and the use of bio-inspired materials will be applied for the detection of a selection of targets.

The pathway to reach the main goal is passing through the following objectives: (1) mesoporous TiO₂ impregnated with HRP will be immobilized onto a gold substrate and evaluated for the electrochemical detection of H₂O₂, then (2) innovative approaches to avoid the presence of H₂O₂ during sensing will be developed by pre-activation of the electrode in H₂O₂. Next (3) TiO₂ impregnated with HRP will be used in a flow injection system to detect the target, and finally (4) bio-inspired materials will be used to outcompete current bioanalysis by proposing a sensitive and selective photo-electrochemical sensor for phenolic compounds.

- To accomplish objective 1, commercial mesoporous TiO₂ will be used. The incorporation of HRP in titania will be achieved at room temperature for 18 h. Several spectroscopic methods will be used to characterize impregnated TiO₂ with HRP. The later will be used for the detection of H₂O₂ by amperometry in the presence of a mediator (Chapter 3).

- In chapter 4, a new strategy to avoid the presence of H₂O₂ in HRP-TiO₂ based biosensing will be developed. This strategy is based on the pre-activation of a titania electrode in H₂O₂ solution. The influence of the pre-activation on the properties of the HRP-TiO₂ electrode will be evaluated by the detection of 4-aminophenol (4-AP). In addition, the presence of reactive oxygen species (ROS) upon the pre-activation of the surface of HRP-TiO₂ will be proven via electron paramagnetic resonance (EPR) (Chapter 4).

- In order to improve the recyclability of the electrode, a flow injection analytical setup will be employed (Chapter 5). The short contact time between the sensor and the sample ensures the recovery of the sensor. The HRP-TiO₂ sensor will be applied for the detection of 4-AP, without any pre-activation of the surface by H₂O₂. The performances of this strategy will be compared with the system in the presence of H₂O₂.

- In the last chapter of the thesis, bio-inspired molecules will be used for the detection of phenolic compounds (Chapter 6). In enzymatic based electrochemical biosensing, the
utilization of enzymes as catalysts has one main drawback related to the stability of enzymes. Enzymatic systems, however, are ideal systems to mimic given the catalytic property which is taking place and responsible for a low limit of detection. A novel photo-electrochemical sensor for the detection of 4-AP will be achieved by immobilizing photosensitizers on the electrode.

Chapter 6

**Goal:** To overcome the instability of enzymes.

**Solution:** Bio-inspired molecules as a replacement for enzymes.

Chapter 5

**Goal:** Mechanization of the analytical procedure for the detection of phenolic compounds.

**Solution:** Using flow injection analysis to detect phenolic compounds.

Chapter 4

**Goal:** Avoiding the addition of H$_2$O$_2$ during the sensing of HRP.

**Solution:** Incubation of HRP-TiO$_2$ in H$_2$O$_2$ solution to allow a hydroperoxyl functionalization.

Chapter 3

**Goal:** Providing a protective environment for enzymes.

**Solution:** Incorporation of HRP into mesoporous TiO$_2$.

Robust strategies for the electro-sensing of phenolic compounds

Chapter 1

Introduction-Inspiration

Chapter 2

Techniques
Chapter 2

Instrumentation and techniques

The focus of this chapter is to briefly describe the instruments and analytical techniques (UV-vis, diffuse reflectance UV-vis, N\textsubscript{2} sorption, electron paramagnetic resonance, and thermogravimetric analysis) used to perform the electrochemical measurements and the characterization of the materials. The fundamental principles of all techniques will be touched, references are included to gain an in-depth understanding.
2.1. Electrochemical techniques

There are several major electrochemical methods which record various electrochemical signals such as current, potential, charge, and impedance that are further related to the concentration of the analytes. Voltammetric methods (controlled-potential methods) are based on monitoring the current as a function of the potential applied to a working electrode (against the potential of a reference electrode). The most commonly used voltammetric methods are cyclic voltammetry (CV) and linear sweep voltammetry (LSV). When the measurements are conducted at one given potential, the current is plotted as a function of time and the method is called chronoamperometry or just amperometry (AMP).

2.1.1. Cyclic voltammetry

Cyclic voltammetry (CV) is the most widely used electroanalytical method that acquires information on electroactive species. In CV, a triangular potential waveform (Figure 2.1 a) is applied at the working electrode and the resulting current is plotted against the applied potential. This plot is called a cyclic voltammogram (Figure 2.1 b). The current passing through the electrode changes with the potential mainly due to oxidation or reduction processes. An electrochemically reversible process with fast electron transfer shows a small peak to peak separation ($\Delta E_p = E_{p^{ox}} - E_{p^{red}}$) with symmetric oxidation ($I^{ox}_{p}$) and reduction ($I^{red}_{p}$) current. In reversible system, the current is directly proportional to concentration and increases with the square root of the scan rate, which is indicative of an electrode reaction controlled by mass transport (diffusion controlled). On the other hand, if the peak to peak separation slightly increases, the reaction is quasi-reversible. When $\Delta E_p$ is larger than that observed for the reversible and quasi-reversible reaction, a cyclic voltammogram corresponds to an irreversible electrochemical process, the electron transfer rates are slow and noticeable overpotentials are required to drive the reaction (Figure 2.1 c).
2.1.2. Linear sweep voltammetry

Linear Sweep Voltammetry (LSV) is a voltammetric method in which the current at a working electrode is measured while the potential is linearly at a constant sweep (or scan) rate. The slope of this ramp has units of volts per unit time and is referred to as the scan rate of the experiment (Figure 2.2 a). Oxidation or reduction of species is registered as a peak at the potential at which the species begins to be oxidized or reduced (Figure 2.2 b). The principle advantage of this method is its rapidity of response. This principle enables performance of a single determination of concentration in less than a second. Thus many samples can be evaluated for kinetic purposes and the changes in concentration can be readily monitored.4
2.1.3. Chronoamperometry

Chronoamperometry involves stepping the potential of the working electrode from an initial value $E_1$, at which no electrode reaction occurs, to a potential $E_2$, at which the electrode reaction is complete and the surface concentration of the electroactive species is effectively zero (Figure 2.3 a). When a potential step is large enough to cause an electrochemical reaction, the current changes with time. The resulting current-time dependence is monitored. As the mass transport under these conditions is solely by diffusion, the current-time curve reflects the change in the concentration gradient in the vicinity of the surface (Figure 2.3 b). This involves a gradual expansion of the diffusion layer associated with the depletion of the reactant, and hence a decreased slope of the concentration profile as time progresses.\(^1\) \(^2\) Thus this method usually is performed in nonquiescent solution by using stirrer or rotating electrode. Such an approach, where convection is used to enhance the rate of mass transport to the electrode surface, is known as hydrodynamic electrochemistry and offers improvements in the analytical sensitivity in comparison to measurements performed in stagnant solution. In chronoamperometry, the observed current at a potential step decays with time (Figure 2.3 b), as given by the Cottrell equation (equation 2.1):

$$I(t) = nFAD^{1/2} C (\pi t)^{-1/2}$$

(eq. 2.1)

where $n$ is stoichiometric number of electrons involved in the reaction, $F$ is the Faraday’s constant, $A$ is the electrode area, $C$ is the concentration of electroactive species, and $D$ is the diffusion coefficient.

---

Figure 2.3. Chronoamperometric experiment: (a) potential–time waveform and (b) the current-time response.
2.2. Electrochemical setup

Electrochemical experiments usually employ a “three-electrode” cell containing a working electrode, a counter electrode (also called auxiliary electrode), and a reference electrode, connected to a potentiostat (Figure 2.4). While the working electrode (WE) is the electrode at which the reaction of interest occurs, the reference electrode (RE) provides a stable and reproducible potential, against which the potential of the working electrode is compared. The counter electrode (CE) is to provide a pathway for current to flow in the electrochemical cell without passing significant current through the reference electrode. These electrodes will be described further.

![General scheme of a three-electrode electrochemical cell (left) and screen printed electrode 3.4 x 1.0 x 0.05 cm (SPE) (right) with counter electrode (CE), working electrode (WE) and reference electrode (RE).](image)

2.2.1. Electrodes

2.2.1.1. Reference electrode

As previously mentioned the role of the reference electrode is to provide a known and stable potential against which the potential of the working electrode is compared. It is made from a highly reversible redox system so that small passages of current are ineffective to its potential. Commonly used reference electrodes are the silver/silver chloride electrode (Ag/AgCl), the saturated calomel electrode (SCE), standard hydrogen electrode (SHE). For practical reason SHE is rarely used in practice in comparison to the first two reference electrodes mentioned.

(a) Saturated calomel electrode (SCE)

The calomel electrode is widely used as a reference electrode and provides a fixed and reproducible potential. The SCE is composed of mercurous chloride (Hg₂Cl₂, calomel) in contact with a mercury pool
immersed in a saturated potassium chloride (KCl) solution (Figure 2.5). The electrode net reaction can be formulated in the following way:

\[
\frac{1}{2} Hg_2Cl_2 + e^- \leftrightarrow Hg + Cl_{aq}^-
\]  

(eq. 2.2)

Thus, the potential of this electrode against the standard hydrogen electrode (SHE) is given by the equation 2.3:

\[
E = E^0_{Hg,Hg_2Cl_2} - \frac{RT}{F} \ln a_{Cl^-}
\]  

(eq. 2.3)

SCE has a potential of 0.241 V with respect to the SHE at 25°C.

(b) Silver-silver chloride electrode (Ag/AgCl)

The Ag/AgCl electrode is commonly used in electrochemical measurements. It generally consists of a cylindrical glass tube containing a silver wire coated with a layer of solid silver chloride, immersed in a solution that is saturated with potassium chloride and silver chloride. The Nernst equation below describes the dependence of the potential of the Ag/AgCl electrode on the activity of chloride ions:

\[
E = E^0_{Ag,AgCl} - \frac{RT}{F} \ln a_{Cl^-}
\]  

(eq. 2.4)
For the reaction $\text{AgCl(s)} + e^- \rightarrow \text{Ag(s)} + \text{Cl}^-$, the standard electrode potential for this half-cell is +0.2046 V relative to the Standard Hydrogen Electrode at 25°C. A schematic representation of the Ag/AgCl reference electrode is shown at Figure 2.6.

![Figure 2.6. Ag/AgCl reference electrode.](image)

2.2.1.2. Counter electrode

The role of the counter electrode (CE), also called auxiliary electrode, is to provide a pathway for the current to flow in the electrochemical cell without passing significant current through the reference electrode. It is necessary for the CE to be placed at a sufficient distance from the working electrode since electroactive species can undergo reaction at the CE surface and the chemicals produced at CE surface could interfere with the reactions that occurring at WE surface. The CE should be designed to prevent any reaction occurring at its surface that would affect the working solution. To this end, the CE should be fabricated out of inert conductive materials and should have much larger area than that of the WE. The larger area leads to a low current density at the CE. The most commonly used auxiliary electrodes are platinum, glassy carbon, or graphite electrodes.

2.2.1.3. Working electrode

The working electrode (WE) is the surface where the reaction of interest occurs. The performance of the electrochemical procedure is strongly influenced by the working electrode material. The WE may be composed of any conducting material and it should provide high signal to noise characteristics, as
well as a reproducible response. It must be recognized that the shape, and area of this electrode affects the resulting current. A range of materials have found application as working electrodes for electroanalysis. The most popular are those involving mercury, carbon, or noble metals (particularly platinum and gold). In this thesis, gold electrodes (Chapter 3), screen printed graphite electrodes (Chapter 4, 6), and rod graphite electrodes (Chapter 5) are used as working electrodes.

2.2.2. Potentiostat

All the electrodes are connected to a potentiostat controlled by a computer. A potentiostat accurately controls the current passing between the counter and the working electrodes in a way that it result in the potential difference between the working and the reference electrodes exactly as specified by the operator or the software. In this thesis, the electrochemical measurements were performed with a AUTOLAB PGSTAT302N potentiostat controlled by Nova 1.9 software (ECO Chemie, Utrecht, The Netherlands) and PalmSens (Utrecht, The Netherlands) with PStrace software (version 5.3).

2.3. Flow injection analysis

Flow injection analysis (FIA) belongs to a family of analytical methods which is based on the injection of a liquid sample (containing the analyte or its reaction products) into a moving, non-segmented continuous carrier stream of a suitable liquid. Figure 2.7 a shows the components of the simplest flow injection analyzer consisting of (1) a pump, which is used to propel a constant flow of carrier solution (buffer) through a narrow tube, (2) an injection loop, which is used as an introduction port for a well-defined volume of sample solution to merge into the carrier stream, and (3) a flow cell, in which the sample zone is progressively dispersed into the carrier stream and is later sensed by a flow through detector. The output has a peak-shaped signal with height H, width W, and area A which is related to the concentration of the analyte. The period of time the analyte spends in the flow cell is defined by \( t_b \), while the time interval between the sample injection S and the peak maximum H, during which the chemical reaction takes place, is defined as the residence time T (Figure 2.7 b).
FIA is based on a combination of three important principles: sample injection, controlled dispersion of the injected sample zone and reproducible timing of processes in the flowing stream. Therefore, there is no need for the reaction to reach equilibrium before the detection is performed, the chemical reaction takes place while the sample is dispersing within the reagent without undergoing homogeneous mixing with the carrier solution (Figure 2.8).
Figure 2.8. Four phases of FIA (a) sample injection, (b) dispersion process, (c) yielding an analytical readout, and (d) washout of the sample.

Usually the injected sample volumes are in the range of 20–200 μL, which in turn requires about 0.5 mL of reagent per sampling cycle, but they can be much smaller in miniaturized flow injection systems. In a well-designed FIA system the whole sampling cycle can be accomplished in a few seconds to 10’s of seconds.\(^{14}\) This makes FIA an easy-to-use, automated technique capable of having a high sampling rate by using minimum amounts of sample and reagents, with a high degree of precision.

2.4. Photoelectrochemical setup

A photoelectrochemical setup is usually a combination of the common electrochemical setup and a light source (Figure 2.9). It has been over a decade since scientists use light-emitting diodes (LEDs) to initiate photochemical reactions on lab scale.\(^{15-17}\) The light source which is used in this thesis is a diode red laser 655 nm from Roithner Lasertechnik, Austria.
2.5. UV-vis spectroscopy

In UV-vis (ultraviolet-visible light) spectroscopy, light absorption as a function of wavelength provides information about electronic transitions occurring in the material. It has been well documented that changes in the conjugation pathway and symmetry of a molecule can be identified by UV/Vis absorption spectroscopy. Quantifying the interaction of visible light with a chemical sample allows for the determination of an unknown solution concentration, the monitoring of reaction progress as a function of time, and many other quantitative uses. One of the basics is the use of the Beer-Lambert law to determine the concentration of a sample. According to the Beer-Lambert law, the fraction of light transmitted is defined as the fraction of the light measured after interaction with the sample ($I/I_0$, usually measured as transmittance (T) or reflectance) versus the incident intensity ($I_0$) which is dependent on the path length of light through the sample ($l$).\textsuperscript{18}

$$A = εcl = -\log_{10}(\frac{I}{I_0}) = -\log_{10} T$$  \hspace{1cm} (eq. 2.5)

where $A$ is the absorbance, $ε$ is the molar absorptivity coefficient of the material, $c$ is the concentration of the absorbing species, and $l$ is the path length of light through the sample (usually 1 cm for liquid samples). Figure 2.10 depicts the various components in a transmission measurement.
2.6. Diffuse reflectance UV-vis spectroscopy

Diffuse reflectance UV-vis spectroscopy is based on measuring the diffusely reflected light, rather than the transmitted light, from a sample. Samples are in form of powders or films. This method is frequently used to characterize materials and offers a convenient method of estimating the optical band gap for semiconductors, since it probes electronic transitions between the valence band and the conduction band. The optical band gap is not necessarily equal to the electronic band gap, which is defined as the energy difference between the valence band maximum (VBM) and the conduction band minimum (CBM). A typical configuration for a diffuse reflectance spectrometer comprises of an integrating sphere to capture all photons that are reflected (in all directions) from the sample (Figure 2.11). The integrating sphere has an input port connected to the light source, an output port connected to a detector that collects the diffusely reflected light, and an aperture against which the working or reference samples can be placed for measurement.
The inner part of the integrating sphere is covered with a highly reflective material such as polytetrafluoroethylene (PTFE) or BaSO\(_4\), which distributes the light uniformly throughout the entire surface of the integrating sphere and is effective over a large wavelength region. There are two types of reflection: specular and diffuse. Specular reflection occurs when the angle of the incident beam is equal to the angle of reflected light. In diffuse reflectance, the incident light penetrates the sample surface, is partially absorbed, and a fraction of its photons is reflected at various angles. For powders, dilution in a non-absorbing material increases diffuse reflectance while minimizing specular reflectance. Common non-absorbing materials include KBr, KCl, and BaSO\(_4\).

2.7. \(\text{N}_2\) sorption

Gas adsorption is a common method for the characterisation of a wide range of porous materials. Surface area and porosity are two important physical properties that can be measured by this type of analysis. There are many gases available as adsorptive among them nitrogen has remained universally pre- eminent. It is possible to use nitrogen adsorption at 77 K for both routine quality control and the investigation of new materials. In sorption techniques, solid materials will be exposed to nitrogen at 77 K with a gradual increasing pressure from high vacuum to vapour pressure (Figure 2.12) and will be evaluated by either the weight uptake or relative vapour pressure. In this thesis, volumetric sorption is used (vapour pressure). Analysis of these data provides information regarding the physical characteristics of the solid including: skeletal density, porosity, total pore volume, surface area, pore morphology and pore size distribution.
Figure 2.12. The sample surface begins to adsorb N\textsubscript{2} molecules upon gradual increasing of N\textsubscript{2} pressure.

2.8. Electron paramagnetic resonance

Electron paramagnetic resonance (EPR) spectroscopy (also known as electron spin resonance, ESR, or electron magnetic resonance, EMR, spectroscopy) is a technique to investigate directly free radicals and paramagnetic compounds, transition metal ions, triplet states, etc.\textsuperscript{28} It is often described as the “gold standard” for the detection and characterisation of radicals in chemical, biological, and medical systems.\textsuperscript{29} This technique is based on the absorption of electromagnetic radiation by a paramagnetic sample (i.e. a species with an unpaired electrons) when placed in a suitable strength magnetic field.\textsuperscript{30,31}

In principle, EPR is similar to NMR spectroscopy where the transition of protons between two energy levels is observed.\textsuperscript{32} However, in EPR spectroscopy, it is the spins of electrons that are excited (Figure 2.13). The magnetic moment associated with the electron spin \( S \) is randomly oriented and, for \( S = \frac{1}{2} \), the two energy levels are degenerated in the absence of an external magnetic field. The interaction between a paramagnetic centre with an electron spin \( S = \frac{1}{2} \) and the external magnetic field \( B_0 \) results in a splitting of the two energy levels, indicated as b and a in Figure 2.13. The splitting between the two energy states is called the Electron Zeeman interaction and is proportional to the magnitude of \( B_0 \).\textsuperscript{33}
Contrary to NMR studies, in which continuous wave (CW) experiments have been replaced with pulsed methods, CW EPR is the most frequently used spectroscopic technique. In a typical CW-EPR experiment, the substance under investigation interacts with a homogeneous magnetic field which is allowed to vary in a selected range and is irradiated by a continuous flow of microwaves at fixed frequency which, when resonance conditions are fulfilled, entails a transition between two spin states. The resonance condition is described by the following equation:

$$\Delta E = h\nu = g_e \beta_e B_0$$

(eq. 2.6)

where $h$ is Planck’s constant, $\nu$ is the microwave frequency, where $g_e$ is a dimensionless factor that depends on the environment in which the unpaired electron resides. It is a proportionality factor that can considered to be similar to a chemical shift parameter in nuclear magnetic resonance (NMR), $\beta_e$ is the electron Bohr magneton ($9.27 \times 10^{-24} \text{ J.T}^{-1}$), and $B_0$ is the strength of the static magnetic field. For a free electron, the $g$ factor, $g_e$, is found to be 2.0023. The $g$ factor is the most easily measurable EPR parameter, dictating at what field strength an EPR signal occurs during a CW experiment. In addition to the field ramp, a sinusoidal magnetic field modulation is introduced that frequency labels the EPR signal for detection, while background noise and electrical interference are not responsive to this modulating field and thereby can be suppressed by a high-pass filter. This mode of detection, gives rise to the well-known CW EPR line-shape that appears as the first derivative of a microwave absorption signal as a function of the magnetic field $B$.

EPR spectroscopy applied to enzymes can provide a clear picture of the local geometry and electronic structure of the heme-iron (the paramagnetic center). The EPR spectrum of HRP has been interpreted
from a thermal or chemical mixture of high- and low-spin forms of HRP.\textsuperscript{35} For horseradish peroxidase (HRP) two oxidizing equivalents are stored in the heme protein molecule and there is a ferric iron having an S = 3/2 ground state with Ms = ± 1/2 higher in energy than Ms = ± 3/2. A signal is expected only from the Ms = ± 1/2 state.\textsuperscript{36} Samples can be measured at room temperature by EPR spectroscopy, but in most cases lower temperatures are needed. The reason why we need to do EPR measurements at lower temperatures than room temperature is the fact that, in biological samples, the solvent is commonly an aqueous buffer. Water will absorb the microwaves, just like in a regular microwave oven, and the sample will heat up.\textsuperscript{37} To maintain lower temperatures the sample has to be cooled from the outside with a stream of cold gas (nitrogen or helium) which also helps battling the heating up of the sample.\textsuperscript{38} The underlying physics behind this process is described in detail in a number of text books.\textsuperscript{39, 40, 41}

\textbf{2.9. Thermogravimetric analysis}

Thermogravimetric analysis (TGA) or thermogravimetry (TG) is a technique in which the weight or, strictly speaking, the mass of a sample is measured as a function of temperature as it is heated or cooled under a defined and controlled environment with respect to heating rate, gas atmosphere, flow rate, etc.\textsuperscript{42} Breaking of physical and chemical bonds at high temperature leads to the evolution of volatile products or formation of reaction products which causes the changes in the mass of the sample.\textsuperscript{43} A plot of mass as a function of temperature is known as a thermogram. In this method, the sample is typically heated at a constant heating rate (dynamic measurement) or held at a constant temperature (isothermal measurement). The type of required information about the sample will alter the choice of the temperature program and the atmosphere applied. The atmosphere used in the TGA experiment plays an important role and can be oxidising (e.g. air or oxygen), inert (e.g. nitrogen, argon, or helium) or reducing (e.g. forming gas, 8-10% hydrogen in nitrogen).\textsuperscript{44} The instrument is usually equipped with a sensitive recording analytical microbalance, a furnace, a temperature controller and mass flow controllers for the flowing gas atmosphere, and a programmer that provides a plot of the mass as a function of temperature.
2.10. References

Chapter 3

Incorporation of horseradish peroxidase in mesoporous TiO$_2$

In this chapter, the development of a sensitive electrochemical mesoporous TiO$_2$ enzyme based biosensor for the detection of H$_2$O$_2$ will be described. Enzymes are commonly used for the development of biosensors because of their high specificity. However, considering their moderate stability, in many instances it is necessary to stabilize them or protect them from denaturation. Immobilization of enzymes in porous materials provides important advantages, such as enzyme reutilization, enhancing the thermal stability of enzymes and may provide a better environment for the enzymes. For this purpose, first, in this chapter the immobilization of horseradish peroxidase in mesoporous titania will be achieved. Finally, the titania based enzyme biosensor will be used for the detection of H$_2$O$_2$. The detection of H$_2$O$_2$ is important as it plays a crucial role in the life phenomena of nature as well as in the environment and industry.

This chapter was adapted from:

vanoushe Rahemi, Stanislav Trashin, Vera Meynen, Karolien De Wael
Talanta 146 (2016) 689–693.
3.1. Introduction

Mesoporous materials are finding many applications in the fields of science and technology as adsorbents, supports for catalysis and sensing elements\textsuperscript{1-3}. Owing to large surface area and uniform pore-size distribution\textsuperscript{4} that can be tuned to fit dimensions of biomolecules, mesoporous materials have also been applied in biosensors as advanced immobilization matrix impregnated with biomolecules.\textsuperscript{4-7}

Titania (TiO\textsubscript{2}) is a typical inorganic mesoporous material with good biocompatibility, stability, and environmental friendliness.\textsuperscript{8} Particularly, Y. Wang et al. stabilized gold nano-seeds by TiO\textsubscript{2} colloid using commercial non-porous TiO\textsubscript{2} P25.\textsuperscript{9} The resulted conductive composite was well compatible with HRP and could provide direct electron transfer between the adsorbed enzyme and an electrode. Jiang et al. encapsulated HRP in TiO\textsubscript{2} through phospholipid-templated synthesis.\textsuperscript{10} The immobilized HRP showed improved thermal stability and tolerance against extreme pH and inactivating agents. Therefore, there is interest in the use of (commercial) mesoporous TiO\textsubscript{2} with uniform pore size distribution as a support for enzymes for applications in biosensing. Such solid material impregnated with enzyme can be used as a stable ready-to-use component for producing carbon paste or screen-printed electrodes.\textsuperscript{11}

Horseradish peroxidase (HRP) is a well-characterized enzyme widely used in bioanalytical applications, for example, as a label in immunoassays or redox enzyme in electrochemical biosensors. The last ones were particularly designed for detection of H\textsubscript{2}O\textsubscript{2}, phenols and its derivatives.\textsuperscript{12-14} Recently, it has been shown that HRP encapsulated in TiO\textsubscript{2} shows improved stability and can be applied for phenolic compounds removal.\textsuperscript{10} Electrochemical detection of H\textsubscript{2}O\textsubscript{2} was previously demonstrated for HRP immobilized on home-made TiO\textsubscript{2}.\textsuperscript{15, 16} The drawback of these approaches is the time consuming preparation step of the sensing material.

In the present work, for the first time, we suggest using (commercial) mesoporous TiO\textsubscript{2} (Millennium PC500) as an efficient matrix impregnated with a model redox enzyme HRP for applying it as an active component in conductive adhesive electrode material for the detection of H\textsubscript{2}O\textsubscript{2}.
3.2. Experimental section

3.2.1. Reagents

Graphite, H₂O₂, nafion 117 (5% in a mixture of lower aliphatic alcohols and water) and sodium hydroxide were purchased from Sigma-Aldrich. 2-[4-(2-hydroxyethyl)-piperazinyl] ethane sulfonic acid (HEPES) was obtained from VWR, hydroquinone (HQ) from Acros, Aeroxide TiO₂ P25 from Evonik. The N₂ adsorption isotherm and the pore diameter distribution of TiO₂ (Millennium PC500) are shown in Figure 3.1. Horseradish peroxidase (HRP) (EC 1.11.1.7) with the activity of 293.0 U/mg was purchased from Calbiochem. The HEPES buffer solution of 10 mM was set to pH 7.0 using NaOH solution. All reagents were used without further purification and all solutions were prepared with deionized water.

![Figure 3.1](image1.png)

*Figure 3.1. (a) N₂ adsorption isotherm for pure TiO₂ (Millennium PC500) (b) the pore diameter distribution of TiO₂ sample without degassing, N₂-sorption was carried out at 77 K.*
3.2.2. Apparatus

Electrochemical measurements were carried out with the AUTOLAB PGSTAT302N equipped with a Metrohm 628-10 electrode rotator (Metrohm, The Netherlands). A conventional three-electrode electrochemical cell was used with a saturated calomel reference electrode (SCE), a glassy carbon rod electrode as an auxiliary electrode, and the working electrodes were gold rotating disk electrodes (Au) with a diameter of 3 mm. The rotating speed of gold electrode was 2000 rpm in all experiments. All solutions were purged by pure nitrogen for 30 min prior to the experiment and maintained under nitrogen atmosphere during measurements. Electrochemical measurements were performed at room temperature. UV-vis spectroscopic characterization in liquid state was performed in the range of 190–800 nm using a SYNERGYTM MX (Biotek, USA). UV-vis Diffuse Reflectance measurements on solid samples were done on an Thermo Electron Evolution spectrometer equipped with an integrating sphere. All of the materials were characterized by nitrogen adsorption-desorption isotherms measured in liquid nitrogen at 77 K on a QUADRASORB SI (Quantachrome Instruments, USA). Samples were measured without degassing as sample preparation in order not to denature the enzyme. As a consequence some of the solvent might remain in the pores, resulting in a slight decrease of the volume of adsorbed nitrogen.

3.2.3. Horseradish peroxidase immobilization in TiO₂

Prior to use, the TiO₂ was calcined to 450 °C to enlarge its pore size. A surface area of 96.6 m²/g was found for this material from N₂ sorption experiments (Table 3.1). When pure TiO₂ is mentioned in the text, this means that no HRP is present. Horseradish peroxidase entrapped in TiO₂ was prepared according to the following procedure: Firstly, 250 mg of mesoporous TiO₂ was added to 3 mL 0.66 mg/mL HRP solution in pH 7.0 buffer. The mixture was stirred for 18 h at room temperature. Then, the suspension was washed with HEPES buffer on a membrane filter (0.45 µm) to remove non-immobilized enzyme and loosely held HRP-TiO₂. Finally, the HRP-TiO₂ paste was dried in air.
Table 3.1. Surface areas of different types of titania, calculated by nitrogen sorption experiments.

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Surface area (m²/g)</th>
<th>Pore Volume (cm³/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TiO₂ (PC500)</td>
<td>96.6</td>
<td>0.272</td>
</tr>
<tr>
<td>TiO₂ incubated in MQ water (MQ: Milli-Q water)</td>
<td>91.0</td>
<td>0.265</td>
</tr>
<tr>
<td>TiO₂ incubated in HRP+MQ</td>
<td>32.4</td>
<td>0.144</td>
</tr>
<tr>
<td>TiO₂ incubated in HEPES</td>
<td>15.1</td>
<td>0.080</td>
</tr>
<tr>
<td>TiO₂ incubated in HRP+HEPES</td>
<td>14.0</td>
<td>0.052</td>
</tr>
<tr>
<td>TiO₂ incubated in HEPES and then washed with MQ</td>
<td>94.8</td>
<td>0.281</td>
</tr>
<tr>
<td>TiO₂ incubated in HRP+HEPES and then washed with MQ</td>
<td>94.9</td>
<td>0.270</td>
</tr>
</tbody>
</table>

3.2.4. Preparation of the enzyme electrode

The gold electrodes were used as a support for the HRP-TiO₂ containing matrix. Prior to coating, the electrodes were polished on a polishing cloth (Buehler, Germany) with alumina powder of 1 and 0.05 µm particle size and rinsed thoroughly with distilled water in an ultrasound bath. Then suspension of a composite mixture (7 µL) consisting of graphite, nafion, and TiO₂ impregnated with enzyme was dropped on the surface of the electrode and dried at +4 °C. The obtained electrode was marked as Au/Gr/HRP-TiO₂/Nafion electrode. To reveal the role of the porous TiO₂ (Millennium PC500), non-porous commercial TiO₂ P25 powder was mixed with HRP in the same way and the obtained electrode was denoted as Au/Gr/HRP-TiO₂(P25)/Nafion. For comparative studies, Au/HRP-TiO₂/Nafion and Au/Gr/HRP-Gr/Nafion were fabricated with the similar procedures leaving out one of the ingredients.

3.3. Results and discussion

3.3.1. Optimization of the experimental parameters

In general, direct oxidation/reduction of HRP at an electrode is prevented due to deep embedding of its redox active center. The long distance between the active site and the electrode surface decelerates the electron transfer rate. As a consequence, HRP-based enzyme-electrodes require a mediator such as hydroquinone (HQ) to shuttle electrons between HRP and an electrode. In our work we used HQ mediated bioelectrocatalysis for the detection of H₂O₂. First, the experimental parameters such as matrix composition, incubation time, working potential and concentration of the mediator were optimized.
3.3.1.1. Matrix composition

Titania is an inorganic mesoporous material that is aimed at immobilizing biomolecules. To improve the electrical characteristics of the matrix, TiO$_2$ was applied in a mixture with graphite powder as a well conductive supplement. To deposit the graphite-TiO$_2$ mixture on the electrode we also included ion exchange polymer nafion as an adhesive binder. First, the percentage of nafion was optimized via graphite-nafion mixtures with various percentage ratios. The cyclic voltammograms of those mixtures were recorded between -0.6 and 0.6 V in pure HEPES buffer solution (pH 7.0). In the recorded voltammograms (Figure 3.2) an unwanted ohmic drop and increase in capacitance of the electrode appeared when the amount of ion exchange polymer was 2% or less. In contrast, 5% weight or more of nafion gave satisfying background and stability of the modified electrodes, similar to results found in literature.$^{19}$ To avoid effects of the polymer on the accessibility of biomolecules, a minimal amount of 5% was taken as an optimum, which also provide excellent characteristics of the three component mixture graphite-TiO$_2$-nafion.

![Figure 3.2. Cyclic voltammograms in 10 mM HEPES (pH 7.0) at Au/Gr/Nafion electrodes with different ratio of nafion. Scan rate, 50 mV s$^{-1}$. Applied potential, -0.30 V vs. SCE.](image)

Next, the effect of the graphite to TiO$_2$ ratio on HQ electrochemistry was investigated. Figure 3.3 shows the electrochemical behavior of the gold electrodes modified with matrices consisting of different percentages of TiO$_2$ in the presence of 1 mM HQ. A redox system related to the presence of HQ is observed. Up to 50% of TiO$_2$, no noticeable effect is observed on the electrochemical behavior of HQ giving an oxidation peak current of ca. 30 µA.
Figure 3.3. Cyclic voltammamograms of 1 mM HQ in 10 mM HEPES (pH 7.0) at Au/Gr/TiO\textsubscript{2}/Nafion electrodes with different content of TiO\textsubscript{2}. Nafion content, 5%. Scan rate, 50 mV s\textsuperscript{-1}. An increase of TiO\textsubscript{2} content up to 70% resulted in about a 30% decrease in HQ currents. It was also clear that TiO\textsubscript{2} without graphite could not provide good electrical conductivity through the layer which resulted in more than one order decrease of oxidation/reduction currents. The mixture 1:1 of graphite to TiO\textsubscript{2} was taken as an optimum since it provides electrical characteristics similar to graphite and contained plenty of mesoporous TiO\textsubscript{2} impregnated with the enzyme. In all further measurements, this ratio was used unless stated otherwise.

3.3.1.2. Incubation time

To optimize the incubation time, TiO\textsubscript{2} was incubated in a 15 µM HRP solution for different times (1-24 h). The recorded amperometric curves at an applied potential of -0.3 V suggest that incubation up to 7 hours resulted in only a minor activity of the enzyme immobilized electrodes. A maximum current is obtained often overnight incubation for 18 hours, the reduction current reached 28.6 ± 4.0 µA, which was more than one order higher than after 1 h incubation. This indicates a kinetic (diffusion) delay for enzyme immobilization in the mesoporous TiO\textsubscript{2}. Longer incubation (24 h) did not increase the activity of the electrodes. Therefore, the incubation time of 18 h was taken in the subsequent. The supernatant (after adsorption for 18 h) was clear and colorless (section 3.3.3.1) suggesting an adsorption of all proteins corresponding to 0.8 wt% or 1.8·10\textsuperscript{-7} mol per g of TiO\textsubscript{2}.
3.3.1.3. Working potential

The sensitivity of the electrodes to H$_2$O$_2$ is mainly influenced by the working potential of the amperometric measurements and the concentration of the mediator. The influence of the operating potential on the amperometric response of the Au/Gr/HRP-TiO$_2$/Nafion electrode was investigated over a potential range from 0 to -0.8 V in solution containing 0.1 mM HQ and 1 mM H$_2$O$_2$ (Figure 3.4). The response of the biosensor proportionally increased as the applied potential shifted towards more negative values reaching a plateau at -0.4 V. To keep low background current, the potential of -0.3 V was taken for further measurements.

![Figure 3.4. Effect of the applied potential on the performance of Au/Gr/HRP-TiO2/Nafion electrodes. (A) Chronoamperometry in the presence of 1 mM H2O2 and 0.1 mM HQ. Numbers denote potentials applied; the asterisk marks the effect of HQ injection. (B) Current-potential profile constructed for a series of the electrodes, each point was measured at least three times. The error bars show the standard deviations.]

3.3.1.4. Concentration of the mediator

To optimize the concentration of the mediator, the effect of the HQ concentration on the HRP electrode response was studied in the presence of 1 mM H$_2$O$_2$. As shown in Figure 3.5, the current response increased with mediator concentration in the range from 0.001 to 0.1 mM and then leveled off similarly to the Michaelis-Menten behavior.$^{20}$ The maximal current was 61.0 ± 11.9 µA and a concentration of HQ at half-maximum was around 0.02 mM. To minimize the background current, the concentration of HQ was fixed at 0.1 mM for the following experiments. Though HQ plays a role of the mediator in the present work while H$_2$O$_2$ is the analyte of the interest, the same detection principle can be applied for detection of phenolic compounds such as phenol or pentachlorophenol.$^{21}$ In that case the analogue of graph in Figure 3.5 would be considered as a calibration curve.
Figure 3.5. Effect of HQ concentration on the current of Au/Gr/HRP-TiO\textsubscript{2}/Nafion electrode in the presence of 1 mM \(\text{H}_2\text{O}_2\). The error bars give the standard deviation for series of three different electrodes. The inset shows an amperometry curve recorded for successive addition of HQ, the numbers denote the concentrations of HQ in \(\mu\text{M}\) injected in the cell. Applied potential, -0.30 V vs. SCE.

3.3.2. Amperometry detection of \(\text{H}_2\text{O}_2\)

The amperometric response to \(\text{H}_2\text{O}_2\) at Au/Gr/HRP-TiO\textsubscript{2}/Nafion electrodes was studied and compared with other HRP electrodes prepared as described above (see 3.2.4) to reveal the role of TiO\textsubscript{2}. As it is shown in Figure 3.6, in all cases the calibration plots show that the current response of the electrodes increases with \(\text{H}_2\text{O}_2\) concentration. In the same time, the amperometric response of Au/Gr/HRP-TiO\textsubscript{2}/Nafion electrodes was much higher compared to the other modified electrodes. This evidence confirms that the concept of using a (commercial) mesoporous TiO\textsubscript{2} as host for HRP is a feasible approach. As a comparison, an electrode modified by non-porous TiO\textsubscript{2} (p25) gave only minor current, which was even lower than that for the electrode modified by graphite with adsorbed HRP. This blank experiment with P25 indicates that the enzyme attached to the outside of the TiO\textsubscript{2} particles (61 m\textsuperscript{2}/g surface area) did not contribute to the catalytic current or was simply removed during washing steps. Assuming the same enzyme activity in all systems prepared, one can estimate from the maximal currents that the enzyme loading was at least one order higher in the coating with porous TiO\textsubscript{2} compared to the systems based on non-porous TiO\textsubscript{2} or only graphite.
Figure 3.6. Chronoamperometry curves (A) and calibration plots (B) for different matrices obtained in the presence of 0.1 mM HQ in 10 mM HEPES (pH 7.0). Numbers denote the concentration of H$_2$O$_2$ injected into the cell. Applied potential, -0.30 V vs. SCE.

The concentration-response profile (Figure 3.6) for Au/Gr/HRP-TiO$_2$/Nafion behaved closely to the Michaelis-Menten model giving saturation kinetics at concentration higher than 1 mM. The dependence was linear up to 0.4 mM of H$_2$O$_2$ with an average sensitivity $\pm$ SD (three different electrodes) of 1.09 $\pm$ 0.16 A M$^{-1}$ cm$^{-2}$ and the limit of detection around 1 µM. The long-term stability of the impregnated TiO$_2$ with HRP was checked to confirm the activity of HRP. The HRP-TiO$_2$ powder kept its activity after more than two years storage at +4 °C. The freshly prepared powder resulted in the average response to 1 mM H$_2$O$_2$ of 28.6 µA with SD of 4.0 µA (n=3) and after two and half years the same material gave 27.6 µA with SD of 1.7 µA (n=3).

3.3.3. Conformational studies: UV–vis, UV-DR and N$_2$ sorption

3.3.3.1. UV-vis

UV-vis spectroscopy was applied to monitor the enzyme concentration before and after immobilization of HRP on TiO$_2$. Figure 3.7 displays the spectra of HEPES buffer, HRP in HEPES buffer of pH 7.0 and the supernatant after adsorption of the enzyme into TiO$_2$. The UV–Vis spectrum of HRP solution gave a typical heme band at 402 nm$^9$, while there is no absorbance in the supernatant collected after incubation of mesoporous TiO$_2$ in HRP solution. The data suggest an efficient adsorption of HRP onto TiO$_2$. 
3.3.3.2. UV-DR

In an additional experiment, UV-DR measurements were performed on the powder with incorporated HRP (HRP-TiO₂). As it can be seen in Figure 3.8, both HRP and HRP-TiO₂ show the absorption band originating from HRP around 402 nm (heme band). The disappearance of this heme band from the liquid phase and its presence in the solid material confirms the presence of HRP on or in the porous TiO₂ material.

Figure 3.7. UV-vis spectra of 0.015 mM HRP solution in 10 mM HEPES buffer (pH 7.0) before (in blue) and after (in red) incubation with TiO₂ for 18 h (250 mg of TiO₂ were added to 3 mL of 0.015 mM HRP) in comparison with pure buffer (in green).

Figure 3.8. UV-DR spectra of HRP powder (in blue), TiO₂ impregnated with HRP (in red), blank TiO₂ incubated 18 h in pure buffer (in green). The arrow shows the absorption band of HRP.
3.3.3.3. N\textsubscript{2} Sorption

To clarify whether HRP actually resided within the TiO\textsubscript{2} pores, pore volume and pore size distribution in the mesoporous TiO\textsubscript{2} were characterized by the nitrogen sorption method \textsuperscript{22} before and after HRP loading (Figure 3.9). The pore volume of TiO\textsubscript{2} decreased sharply upon adsorption of HRP. This indicates that the mesopore network of TiO\textsubscript{2} is (1) occupied by enzyme molecules and some possible remainder of solvent and (2) the available sites for nitrogen adsorption have been reduced.\textsuperscript{23}

The comparison of isotherms of HEPES-TiO\textsubscript{2} and HRP-TiO\textsubscript{2} both show the loss of nitrogen uptake indicating that the pores were totally blocked (Figure 3.9 a). As the size of the HEPES molecule is small compared to the pore size (5-10 nm) of TiO\textsubscript{2}, this was quite surprising. This strong decrease in porosity can be related to HEPES that strongly adsorbs in the pores together with the remainder of the solvent (water) as no degassing was done as sample preparation. Nevertheless, even though there was no degassing, pure TiO\textsubscript{2} mixed with water doesn’t show the strong decrease in nitrogen sorption uptake. Therefore, it gives a strong indication that a large amount of HEPES is blocking the pores. This blocking can be caused in several ways: it is possible that a higher amount of water could remain in the pores as HEPES is more hydrophilic than TiO\textsubscript{2} and keeps water stronger than TiO\textsubscript{2} inside of the pores resulting in a lower removal of water during drying and short degassing at the start of the measurement. Another suggestion is aggregation of HEPES molecules. HEPES aggregates might block the entrance of the pores, so that water can maintain in the pores. To make this clear, HEPES-TiO\textsubscript{2} powder was mixed with MQ (Milli-Q water) for 90 min, and then centrifuged (three times). The isotherm demonstrated that the pores were totally opened and no more HEPES blocked the pores; all HEPES molecules were washed off by the MQ and the pores were opened again (Figure 3.9 b). To verify that HEPES salt did not contribute to the N\textsubscript{2} adsorption results, the experiments were performed on samples of MQ-TiO\textsubscript{2} and MQ-HRP-TiO\textsubscript{2} which was a mixture of MQ water, HRP and TiO\textsubscript{2} (Figure 3.9 c).
Figure 3.9. Changes of N$_2$ adsorption isotherms in (a) pure TiO$_2$ without degas, HEPES-TiO$_2$ and HRP-TiO$_2$ (b) pure TiO$_2$ without degas, HEPES-TiO$_2$ powder was mixed with MQ water and HRP-TiO$_2$ powder was mixed with MQ water (c) pure TiO$_2$ degas, TiO$_2$-MQ water and MQ-HRP-TiO$_2$ in the absence of HEPES.
3.3.3.4. TGA

Thermogravimetric analysis was conducted using a Mettler Toledo TGA/SDTA851e Thermogravimetric Analyzer. 30 mg of TiO$_2$, HRP-TiO$_2$ and HEPES-TiO$_2$ are pyrolysed under approximately 50 mL/min O$_2$ gas flow at a heating rate of 0.5 °C/min from 30 to 300 °C. As it can be seen from TGA measurements (Figure 3.10) water is more retained in case of HRP and HEPES presence than in pure TiO$_2$.

![Figure 3.10. TGA graphs of TiO$_2$, HEPES-TiO$_2$ and HRP-TiO$_2$ 0.5 °C/min heating rate.]

3.4. Conclusion

We suggest using (commercial) mesoporous TiO$_2$ (Millennium PC 500) as a matrix for the impregnation of biomolecules such as HRP as a model redox enzyme. Amperometric, UV-vis and N$_2$ sorption studies revealed that the chosen matrix (Gr-HRP-TiO$_2$-Nafion) provide an excellent platform for the immobilization of HRP. The good biocompatibility of TiO$_2$ allows that HRP retains its active state within the matrix by showing good electrocatalytic responses to the reduction of hydrogen peroxide. In addition, our study demonstrates the importance of a mixed matrix electrode where each component adds to the beneficial performance of the electrode. The presence of graphite in the matrix was shown to greatly increase the conductivity of the sensor, while the HRP impregnated TiO$_2$ causes high affinity to the substrate. This study is expected to provide a great potential for straightforward design of environmental friendly sensing materials that can be used in the construction of other enzyme based biosensors.
3.5. References


Chapter 4

Impregnated TiO$_2$ with HRP incubated in H$_2$O$_2$ for the detection of phenolic compounds

This chapter reports on the development of a new strategy which can be applied to avoid the presence of hydrogen peroxide (H$_2$O$_2$) during horseradish peroxidase (HRP) sensing. The catalytic cycle of horseradish peroxidase starts by the binding of H$_2$O$_2$ to the heme in the Fe(III) state, which causes the heterolytic cleavage of the oxygen oxygen bond of H$_2$O$_2$. A water molecule is released during this reaction with the concomitant two-electron oxidation of the heme to form an intermediate compound I. Compound I is then converted back to the resting enzyme via two successive single-electron transfers from reducing substrate molecules. So the presence of H$_2$O$_2$ is very crucial for the HRP mechanism reaction. However, adding H$_2$O$_2$ during the sensing has some disadvantages. These disadvantages will be circumvented in this chapter. Finally, the novel hydrogen peroxideless titania based HRP biosensor developed in this chapter will be used for the detection of some phenolic compounds.

This chapter was adapted from:

- A new direction for enzymatic sensors for phenols based on redox cycling with peroxidase and a surface confined sacrificial electron acceptor. 
  
  Vanoushe Rahemi, Stanislav Trashin, Zainab Hafideddine, Sabine Van Doorslaer, Vera Meynen, Karollen De Wael
  Submitted
4.1. Introduction

Numerous bioassays and -analyses are based on the ability of HRP to catalyze the oxidation of a wide range of substrates upon adding hydrogen peroxide\(^1\text{--}^3\) (Figure 4.1 A). The addition of \(\text{H}_2\text{O}_2\) typically causes a large drop in background current for electrochemical based systems, consequently, additional time is needed to stabilize the background. Even more, in a flow injection analysis (FIA) setup both the \(\text{H}_2\text{O}_2\) solution and the substrate solution should be mixed before detection. Due to the ability of \(\text{H}_2\text{O}_2\) to oxidize agents in the substrate solution, this phenomenon might influence the actual detection. In other words, the question raised whether there is any way to avoid the addition of \(\text{H}_2\text{O}_2\) in HRP based electrosensing.

In general, titania is an environmental friendly mesoporous material with good biocompatibility and stability.\(^4\text{,}^5\) It has been reported that it may successfully encapsulate HRP for the removal of phenolics\(^6\text{,}^7\) motivating our focus on the detection of 4-aminophenol (4-AP) and its model compound hydroquinone (HQ). Phenolic compounds belong to the large group of organic pollutants in medical, food, and environmental matrices.\(^8\text{,}^9\) Among these phenolic compounds, 4-AP has industrial relevance due to its occurrence as intermediate in the synthesis of pharmaceuticals\(^10\) or as a dyeing agent for fur and feathers.\(^11\) 4-AP was also identified as a degradation product of paracetamol, therefore frequently present in wastewater.\(^12\text{,}^13\)

The activity of the HRP based electrodes towards the selected phenolics is measured by the back reduction of the oxidized HQ (benzoquinone, BQ) or oxidized 4-AP. The suggested approach solves practical difficulties of using HRP in biosensors, including suppression and stabilization of the background signal, greatly simplifying the biosensing strategy.

4.2. Experimental section

4.2.1. Reagents

Hydrogen peroxide (\(\text{H}_2\text{O}_2\)), nafion 117 (5% in a mixture of lower aliphatic alcohols and water), potassium chloride (KCl), and potassium phosphate monobasic (\(\text{KH}_2\text{PO}_4\)) were purchased from Sigma-Aldrich. Hydroquinone (HQ) was obtained from Acros, TiO\(_2\) (Millennium PC500, mesoporous) from Crystal Global (Prior to use, the TiO\(_2\) was calcined to 450 °C to enlarge its pore size).\(^14\) Horseradish peroxidase (HRP) (EC 1.11.1.7) with the activity of 293.0 U/mg was purchased from Calbiochem. 10 mM phosphate buffer (\(\text{KH}_2\text{PO}_4\)) and 0.1 M KCl solution was used as supporting electrolyte. The phosphate buffer solution was set to pH 7.0 using NaOH solution. All reagents were used without further purification, and all solutions were prepared with deionized water.
4.2.2. Apparatus

Electrochemical measurements were carried out using PalmSens (Utrecht, The Netherlands) with PSTrace software (version 3.0). A conventional three-electrode electrochemical cell was used with a saturated calomel reference electrode (SCE), a glassy carbon rod electrode as an auxiliary electrode, and the working electrodes were ItalSens graphite screen printed electrodes (SPE) with a diameter of 3 mm. HQ was detected in a 10 mL cell by using SCE as an external reference electrode and 4-aminophenol was detected in droplet by using an internal Ag reference electrode. To evaluate the electrochemical response obtained with an external reference electrode (SCE) compared to an internal reference electrode, a more negative potential (−0.14 V) is applied onto the SPE. Electrochemical measurements were performed at room temperature. For EPR measurement, X-band (~9.44 GHz) continuous-wave (CW) EPR spectra were performed on a Bruker ESP300E spectrometer equipped with a liquid Helium cryostat (Oxford Inc.). The EPR spectra were recorded at 2.5 K, with a modulation amplitude of 5 G, microwave power of 1 mW and a modulation frequency of 100 kHz. A vacuum pump was used during the experiments to remove paramagnetic oxygen from the sample. All EPR spectra are normalized and simulated using the MATLAB toolbox Easyspin.15

4.2.3. Modification of the electrode

The procedure of immobilization of HRP in TiO₂ is reported in chapter 3.16 The screen printed graphite electrode were used as a support for HRP-TiO₂. Prior to coating, the working electrodes were soaked first in 2 µL 80% aqueous ethanol and left till nearly dry, then a suspension of a composite mixture (5 µL) consisting of TiO₂ impregnated with enzyme and naftion was dropped on the surface of the electrode and dried at room temperature for approximately 1 h. Herein we report an original strategy to avoid H₂O₂ spiking in HRP based bioanalysis illustrated by focusing on the detection of phenolics. Our strategy includes the pre-activation of a screen printed electrode (SPE) modified with titania and impregnated with HRP, by incubation in a 1 mM H₂O₂ solution to functionalize the titania with -OOH functionalities (Figure 4.1 B). To avoid the presence of excess H₂O₂ and to remove non-attached H₂O₂, the electrode was rinsed with buffer solution after the pre-activation step. These rinsed electrodes are further marked as SPE/HRP-TiOOH, their non-activated versions (no prior incubation in 1 mM H₂O₂) are indicated as SPE/HRP-TiO₂. To reveal the role of TiO₂, SiO₂ impregnated with HRP was prepared following similar procedures which is reported chapter 3.16
4.2.4. Estimation the oxidized amount of HQ on the surface of TiO$_2$

To estimate the oxidized of HQ on the surface of TiO$_2$, UV-vis measurements were performed. First a mixture of HRP-TiO$_2$ was incubated in 1 mL of 1 mM H$_2$O$_2$ for 5 min allowing the formation of •OOH functionalities. This suspension was centrifuged for 1 min and washed with 1 mL phosphate buffer (HRP-TiOOH), repeated three times. Then, 1 mL of 25 μM hydroquinone (HQ) was added to HRP-TiOOH and incubated for 1 h before the UV-vis measurements. Buffer, HQ solution and supernatant HRP-TiOOH incubated with HQ were measured by UV-vis. By taking into account the difference in the absorbance of HQ before and after incubation with HRP-TiOOH, the oxidized amount of HQ can be calculated.

4.3. Results and discussion

4.3.1. The feasibility of avoiding H$_2$O$_2$

The amperometric response of a non-activated and a pre-activated electrode towards HQ is shown in Figure 4.1. The enzymatic activity of SPE/HRP-TiO$_2$ in the presence of 1 mM H$_2$O$_2$ upon addition of HQ is demonstrated by the amperometric reduction current generated by the produced BQ (pH 7.0) (Figure 4.1 C). As shown in Figure 4.1 C, the disadvantage of using H$_2$O$_2$ in a cell solution is clearly illustrated being a considerably long background stabilization time (ca. 600 s) after adding H$_2$O$_2$ in the cell solution. Additionally, a suppression of the HQ/BQ current response is observed for low concentrations of HQ as seen in the corresponding calibration curve due to the oxidative properties of H$_2$O$_2$ in any solution (inset of Figure 4.2). To solve these issues, a SPE/HRP-TiOOH is prepared by pre-activation of a SPE/HRP-TiO$_2$ in a 1 mM H$_2$O$_2$ solution followed by a rinsing step (buffer solution). After the pre-activation and from the onset of the amperometric experiment, the background current is stable (Figure 4.1 D). Also, at low concentrations the sensitivity of the pre-activated electrode to HQ is improved in comparison with the electrodes tested in the presence of H$_2$O$_2$ in the cell solution (Figure 4.2).
Figure 4.1. HRP reaction mechanism A) in the presence of H$_2$O$_2$ in solution and B) at a pre-activated Ti-OOH electrode. (C) Baseline fluctuation due to the addition of 1 mM H$_2$O$_2$ and amperometric detection (after 600 s of stabilization time) of different concentrations (µM) of HQ at SPE/HRP-TiO$_2$. (D) Detection of HQ at the surface of a pre-activated electrode SPE/HRP-TiOOH (incubation in 1 mM H$_2$O$_2$ for 2 min before the amperometric detection) Inset: Amperometric responses at a pre-activated SPE/HRP-SiO$_2$ for different concentrations of HQ (µM) in 10 mM phosphate buffer (pH 7.0) containing 0.1 M of KCl. Applied potential, -0.10 V vs. SCE.
Figure 4.2. Calibration curve of SPE/HRP-TiOOH incubated for 2 min in 1 mM H₂O₂ (red) and SPE/HRP-TiO₂ in the presence of 1 mM H₂O₂ (black) for different HQ concentrations (µM), inset: lower HQ concentration range in 10 mM phosphate buffer (pH 7.0) containing 0.1 M of KCl. Applied potential: -0.10 V vs. SCE.

The sensitivity of a pre-activated and non-activated electrode in the presence of HQ was calculated to be respectively 2.6 ± 0.8 and 0.54 ± 0.05 A M⁻¹ cm². Based on those observations, the higher sensitivity of the pre-activated electrode towards HQ sensing might be explained by the presence of the hydroperoxyl functionalities (-OOH) created on the surface of the activated electrode, delivering e.g. •OOH as intermediate species to the adsorbed HRP. Furthermore, no H₂O₂ is present in solution to initiate the (background) oxidation reactions.

To further emphasize the role of pre-activated titania, a SPE/HRP-SiO₂ was prepared and immersed in a H₂O₂ solution. Upon adding HQ no electrochemical response (due to the back reduction of BQ) could be observed (Figure 4.1 D inset) meaning that no functionalization with reactive species or modification took place. In contrast, after adding 1 mM H₂O₂ in the cell solution, SPE/HRP-SiO₂ gave a similar response for HQ as was observed at non-activated SPE/HRP-TiO₂ with H₂O₂ present in the cell solution (Figure 4.3). This indicates that the pre-activation step in 1 mM H₂O₂ is only applicable when TiO₂ is employed as support. The activated titania electrodes keep their ability to oxidize phenolic compounds during a period of about twenty minutes, even incubated in a large volume of pure buffer solution.
4.3.2. Continues wave-EPR (CW-EPR) and UV-vis characterization

To study the functionalities obtained at the TiO₂ surface in detail, EPR analysis is performed. Initially, blank TiO₂ (in HEPES buffer and without HRP) is measured and subsequently 10 mM H₂O₂ is added. By subtracting the EPR spectrum of blank TiO₂ with 10 mM H₂O₂ from the EPR spectrum of blank TiO₂, the resulting anisotropic EPR spectrum shown in Figure 4.4 A is obtained. Simulations (Figure 4.4 B) reveal g-values of \( g_z = 2.0288 \), \( g_y = 2.009 \) and \( g_x = 2.0037 \). These values correspond to the superoxide anion \( O_2^- \) bound on the Ti⁴⁺-centers.¹⁸-²¹ Another weak signal is observed for \( O_2^- \) with \( g_z = 2.0365 \). The two \( O_2^- \) signals show the same \( g_y \) and \( g_x \) values. The difference in \( g_z \) component originates from the sensitivity in the crystal field.¹⁹,²¹-²³ It is suggested that through homolysis of H₂O₂ to HO• radicals the side reaction occurs:¹⁸,²⁰

\[
H_2O_2 \rightarrow 2HO• \rightarrow TiOOH
\]  
(eq. 4.1)
This further leads to the formation of $\text{O}_2^-$ due to the -OOH functionalization of TiO$_2$. No such effect was found for silica treated with $\text{H}_2\text{O}_2$ (Figure 4.5). The full EPR spectra of non-activated and $\text{H}_2\text{O}_2$-activated HRP-TiO$_2$ are shown in Figure 4.6. In the 100-150 mT region of Figure 4.6 A, the low-field component of the high-spin ferric forms of HRP are observed. A multitude of components is seen, in line with earlier observations for peroxidases that show that the heme pocket is very heterogeneous.\textsuperscript{24} By adding $\text{H}_2\text{O}_2$, many components of this signal disappear, in line with the expected two-electron oxidation to compound I, which is an oxoferryl iron (Fe$^{4+}$=O) and a porphyrin π cation radical.\textsuperscript{25,26} The amount of HRP compound I depends on the amount of $\text{H}_2\text{O}_2$ added.\textsuperscript{25} In this case a high amount of $\text{H}_2\text{O}_2$ (10 mM) is used to investigate the influence of $\text{H}_2\text{O}_2$ on both the enzyme and TiO$_2$. 

---

*Figure 4.4. CW-EPR spectrum of (A) the subtraction of the EPR spectrum of TiO$_2$ + $\text{H}_2\text{O}_2$ from the EPR spectrum of TiO$_2$ and (B) the corresponding simulation.*
Figure 4.5. CW-EPR spectra of (A) SiO$_2$ in HEPES buffer and (B) SiO$_2$ with 10 mM H$_2$O$_2$.

Figure 4.7 zooms into the region where radical contributions are expected for HRP-TiO$_2$ in HEPES buffer (Figure 4.7 A) and activated HRP-TiO$_2$ (Figure 4.7 B). The difference between spectrum 4.7 B and 4.7 A is shown in Figure 4.7 C. Besides contributions of O$_2^-$ radicals as observed for TiO$_2$ treated with H$_2$O$_2$ (Figure 4.4), an extra radical signal stemming from the porphyrin radical of compound I is observed (indicated with red dashed line). By raising the temperature above 20 K, the radical signal could not be observed anymore.$^{25}$

Figure 4.6. CW-EPR spectra of (A) HRP-TiO$_2$ in HEPES buffer and (B) HRP-TiO$_2$ with 10 mM H$_2$O$_2$. * indicates a non-heme iron signal and ** the Cu$^{2+}$ background signal.
The calculated amount of oxidized HQ at HRP-TiOOH by UV-vis measurements was 0.013 µmol ± 0.0014 (n=4). As a comparison, similar experiments were performed with silica and titania without HRP and H₂O₂ (Table 4.1).

Table 4.1. The oxidized amount of HQ at the surface of different matrices which are activated by pre-incubation in 1 mM H₂O₂ in comparison with non-activated.

<table>
<thead>
<tr>
<th>Matrix at the electrode</th>
<th>The oxidized amount of HQ (µmol)</th>
<th>±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>TiO₂+HRP (pre-activated)</td>
<td>0.013</td>
<td>0.0014</td>
</tr>
<tr>
<td>TiO₂+HRP (non-activated)</td>
<td>0.0013</td>
<td>0.0003</td>
</tr>
<tr>
<td>TiO₂ (pre-activated)</td>
<td>0.0012</td>
<td>0.0002</td>
</tr>
<tr>
<td>SiO₂ (pre-activated)</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

4.3.3. Sensitivity and operational stability

To study the effect of the enzyme loading, titania was impregnated with different amounts of HRP (Table 4.2). A maximal loading of titania with HRP was calculated experimentally from UV-vis absorption being 1.06 µmol HRP per g TiO₂. The sensitivity of the electrodes increased linearly with the loading of HRP, for both SPE/HRP-TiO₂ with H₂O₂ in the cell solution and SPE/HRP-TiOOH. Clearly, for all loadings the sensitivity was higher in the case of the pre-activated electrodes. However, the operational stability of the pre-activated electrode with the highest loading was the worst, the
electrode lost 44% of its activity in 30 min (Figure 4.8). The electrode with a loading of 0.77 µmol/g kept 80% activity after 30 min in 0.5 µM HQ and was used in further experiments.

**Table 4.2.** Dependence of the amperometric response to the amount of adsorbed HRP at SPE/HRP-TiOOH (after incubation for 2 min in 1 mM H₂O₂), compared to SPE/HRP-TiO₂ in the presence of 1 mM H₂O₂ in the cell solution.

<table>
<thead>
<tr>
<th>Spec ads (µmol/g)</th>
<th>SPE/HRP-TiO₂ with 1 mM H₂O₂ in the cell (non-activated electrode)</th>
<th>SPE/HRP-TiOOH (pre-activated electrode)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Slope (nA/µM)</td>
<td>SD (nA/µM)</td>
</tr>
<tr>
<td>0.18</td>
<td>33.7</td>
<td>1.4 (n=6)</td>
</tr>
<tr>
<td>0.77</td>
<td>108</td>
<td>15 (n=9)</td>
</tr>
<tr>
<td>1.06</td>
<td>206</td>
<td>15 (n=3)</td>
</tr>
</tbody>
</table>

**Figure 4.8** Stability of SPE/HRP-TiOOH with different concentrations of HRP incubated for two min in 1 mM H₂O₂ in the presence of 0.5 µM HQ during 2 hours, the currents are recorded after 10 min for each concentration of HRP in 10 mM phosphate buffer (pH 7.0) containing 0.1 M of KCl. Applied potential: -0.10 V vs. SCE.

4.3.4. Effect of incubation time on pre-activation step

Variation of the time of the pre-activation step revealed that even a short incubation of two minutes is enough to activate the electrode surface, while the sensitivity gradually decreased with the treatment time, probably due to bleaching and inactivation of the enzyme (Figure 4.9). The result indicates that a short treatment time in the activation step is sufficient and, thus, compatible with the
strategy to prepare a rapid on-site biosensor. A pretreatment time of two minutes was selected for all measurements. Interestingly, SPE/HRP-TiO$_2$ gives a minor response (order of 70 nA) even without pre-activation in H$_2$O$_2$ (black square in Figure 4.9). The most probable explanation for the observed current in case of non-activated SPE/HRP-TiO$_2$ electrodes is that TiO$_2$ could accumulate small amounts of reactive groups without treatments by H$_2$O$_2$ due to unintended photocatalytic water decomposition.\textsuperscript{27}

![Figure 4.9](image)

*Figure 4.9. Effect of pre-incubation time on the reduction current of different concentrations of HQ in 10 mM phosphate buffer (pH 7.0) containing 0.1 M of KCl. Applied potential: -0.10 V vs. SCE.*

4.3.5. Effect of the working potential

The sensitivity of the electrodes is influenced by the working potential of the amperometric detections. Its influence was investigated over a potential range from 0.1 to -0.4 V in a solution containing 1 µM HQ (Figure 4.10). The response of the pre-activated electrodes proportionally increased as the applied potential shifted towards more negative values reaching a plateau at -0.3 V. At more negative potential values, the electrochemical reduction of molecular oxygen contributes considerably to the response of the phenolic compound and it may cause a slow irreversible deactivation of adsorbed HRP.\textsuperscript{29} A low background current and no influence of oxygen reduction motivates the selection of a working potential of -0.10 V.
4.3.6. Applicability of the electrode

The amperometric response of SPE/HRP-TiOOH was monitored for different phenolic compounds and referred to the current response obtained by SPE/HRP-TiOOH in the presence of HQ (Table 4.3). The trend of the reactivity can be explained by the nature of the phenolic structures, the position of the functionalities and the ability of the substituents to form electron-donor conjugation.\textsuperscript{30, 31} Among the phenolic substrates tested, hydroquinone shows the highest sensitivity and reactivity followed by 2-aminophenol (2-AP), quercetin and 4-aminophenol (4-AP). In the case of quercetin, 2-AP, caffeic acid, and 4-AP, the corresponding conjugation structure could be easily formed due to the strong electron-donor conjugation of the substituents meaning the free electron on the resulting quinone radical is stabilized by the charge distribution through the conjugated system.\textsuperscript{32, 33}
Table 4.3. Reactivity of SPE/HRP-TiOOH towards different phenolic compounds.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Reduction current (nA) ± SE at -0.1 V</th>
<th>Relative electrochemical activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hydroquinone</td>
<td>238 ± 11.9</td>
<td>100</td>
</tr>
<tr>
<td>2-aminophenol</td>
<td>233</td>
<td>98</td>
</tr>
<tr>
<td>quercetin</td>
<td>170</td>
<td>71</td>
</tr>
<tr>
<td>caffeic acid</td>
<td>167</td>
<td>70</td>
</tr>
<tr>
<td>4-aminophenol</td>
<td>166.8 ± 15.9</td>
<td>70</td>
</tr>
<tr>
<td>catechol</td>
<td>115</td>
<td>48</td>
</tr>
<tr>
<td>phenol</td>
<td>62.7 ± 6.9</td>
<td>26</td>
</tr>
</tbody>
</table>

To explore the applicability of electrodes such as SPE/HRP-TiOOH for on-site applications, the amperometric response to 4-AP was measured at \( E = -0.14 \text{ V} \) (with internal reference electrode) in a droplet of 100 µL. As shown in Figure 4.11, the calibration plots exhibit a typical current response of the electrode with increasing 4-AP concentration. The concentration-response profile (Figure 4.11 B) for SPE/HRP-TiOOH was near linear in the concentration range 0.025-1 µM and saturated at 2-5 µM. The average sensitivity ± SD (three different electrodes) was \( 2.73 ± 0.99 \text{ A M}^{-1} \text{ cm}^{2} \) and the limit of detection was 24 nM.

![Figure 4.11. Chronoamperometry curves (A) and calibration plot (B) obtained for 4-AP at SPE/HRP-TiOOH in 10 mM phosphate buffer (pH 7.0). Applied potential, -0.14 V vs. internal reference electrode.](image-url)
4.4. Conclusion

We offer a sensitive and robust sensing strategy for phenolics by activation of a titania modified electrode by prior incubation in a H$_2$O$_2$ solution which circumvents the drawback of H$_2$O$_2$ injection in typical HRP based electrosensing. Our results indicate that a short treatment time in the activation step is sufficient and, thus, compatible with the strategy to prepare a rapid on-site biosensor. The amount of oxidized HQ at HRP-TiOOH was calculated by UV-vis measurements. This work indicates that the pre-activation step in 1 mM H$_2$O$_2$ was only applicable when TiO$_2$ was employed as support. EPR spectroscopy revealed the presence of •OOH at the surface of TiO$_2$ after the pre-activation step. The proposed enzyme electrode is expected to provide new possibilities for a straightforward design of sensors to detect a wide range of substrates.
4.5. References

Chapter 5

Monitoring of phenolic compounds by flow injection analysis

This chapter describes a system based on flow injection analysis (FIA) for the enzymatic detection of phenolic compounds. A particularly successful concept of mechanization of analytical procedures is carrying out part or whole of an analytical procedure in flow conditions, resulting in an enhancement of the efficiency of analytical determinations. FIA is an accurate, reliable, and reproducible method because of automation and sample throughput. The measurement of an analytical signal takes place during the flow of a sample segment through an electrochemical cell connected to a potentiostat. This allows a larger sampling rate than in similar determinations carried out manually. The moving flow enhances the mass transport of the analyte towards the electrode and improves the sensitivity and the limit of detection.

In this chapter a wall-jet cell with a carbon electrode coupled to a flow-injection analysis system will be applied for the detection of phenolic compounds in the absence and presence of $\text{H}_2\text{O}_2$.

This chapter was adapted from:

- Hydrogen peroxide-less impregnated titania with horseradish peroxidase for bioelectrochemical monitoring of phenolic compounds in flow system. 
  *In preparation*
5.1. Introduction

Lately, applications of titania as catalyst support\textsuperscript{1-3} and in photocatalysis\textsuperscript{4-6} have arisen increasing interest. Solar daylight provides 3 to 5\% of its radiation intensity within the absorption spectral range of titanium dioxide.\textsuperscript{7} The electron-hole pair that is generated upon irradiation with sunlight may separate and result in charge carriers. These excited-state electrons and holes can (i) recombine and dissipate the input energy as heat, (ii) get trapped in metastable surface states, or (iii) react with electron donors and electron acceptors adsorbed on the semiconductor surface.\textsuperscript{4, 8} The excited-state electrons and holes after reaction with water and oxygen can produce radical species.\textsuperscript{9} Hydroxyl radical \textsuperscript{*}OH, hydroperoxyl radical \textsuperscript{*}OOH and superoxide ion radical \textsuperscript{*}O\textsubscript{2} are commonly produced reactive species, which participate in the reduction and oxidation reactions. They are all strong oxidants able to oxidize organic compounds directly to CO\textsubscript{2}, H\textsubscript{2}O or other oxidation products.\textsuperscript{10}

Titanium dioxide (TiO\textsubscript{2}) is a typical inorganic (mesoporous) material with good biocompatibility, stability and environmental friendliness.\textsuperscript{11, 12} These properties of TiO\textsubscript{2} make it a suitable electrode material to immobilize biomolecules.\textsuperscript{13, 14} TiO\textsubscript{2}-based enzyme biosensors for phenolic compounds determination usually comprise tyrosinase\textsuperscript{15-17}, peroxidase\textsuperscript{18-21}, laccase\textsuperscript{22-25} enzymes. These enzymes exploit different mechanisms in their corresponding electrochemical biosensing strategies.\textsuperscript{26} The general working principle is the enzyme molecules will be oxidized by oxygen (for tyrosinase and laccase) or hydrogen peroxide (for peroxidase) on the surface of the electrode. Afterwards, the oxidized form will be reduced back to the native state by phenolic compounds.\textsuperscript{27} The tyrosinase biosensors are applicable for monitoring phenolic compounds with at least one free ortho-position\textsuperscript{28, 29}, the laccase biosensors can detect phenolic compounds with free para- and meta-position.\textsuperscript{30} Numerous bioassays and analyses are based on the ability of horseradish peroxidase (HRP) to catalyse the oxidation of a wide range of substrates upon adding hydrogen peroxide.\textsuperscript{31-33} The addition of hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) typically causes a large drop in background current for electrochemical based systems, consequently, additional time is needed to stabilize the background (as illustrated in chapter 3).

Herein we exploit the use of reactive oxygen species generated under artificial or natural light on the surface of titania in a biosensing strategy that avoids H\textsubscript{2}O\textsubscript{2} spiking in titania impregnated with HRP. The strategy is illustrated by focusing on the detection of phenolic compounds in flow injection (FI) system. The major advantage of flow electroanalysis is an enhancement of mass transport in voltammetric techniques.\textsuperscript{34} Due to the reproducible use of a transient signal from the detector without the need of obtaining a steady-state equilibrium signal, flow injection techniques provide shortening of time of a single analytical determination. In addition, the short time of exposure of the electrode surface to the
reaction products formed in the enzymatic and electrochemical reactions partly prevents the inactivation of the enzymes.²⁹

5.2. Experiment section

5.2.1. Reagents

Hydrogen peroxide (H₂O₂), nafion 117 (5% in a mixture of lower aliphatic alcohols and water), potassium chloride (KCl), 4-Aminophenol (4-AP), and potassium phosphate monobasic (KH₂PO₄) were purchased from Sigma-Aldrich. Hydroquinone (HQ) was obtained from Acros, TiO₂ (Millennium PC500, mesoporous) from Crystal Global (Prior to use, the TiO₂ was calcined to 450 °C to enlarge its pore size).³⁵ Horseradish peroxidase (HRP) (EC 1.11.1.7) with the activity of 293.0 U/mg was purchased from Calbiochem. 10 mM KH₂PO₄ phosphate buffer and 0.1 M KCl solution (pH 7.0) was used as supporting electrolyte. The phosphate buffer solution was set to pH 7.0 using NaOH solution. All reagents were used without further purification and all solutions were prepared with deionized water.

5.2.2. Apparatus

A double line flow injection system with a three-electrode wall-jet flow-through cell³⁶ (Figure 5.1) was used for the amperometric measurements of phenols. A peristaltic pump (Perkin-Elmer, France) propelled the phosphate buffer (pH 7.0) as the carrier into the flow line using Tygon tubing (1 mL/min flow rate). A 50 µL sample solution containing substrate (phenolic compounds) was injected into the carrier stream via a omnifit labware (Diba) manual sample injection valve (USA). The flow line was made from Teflon tubing (1.58 mm o.d.). A HRP-TiO₂ modified graphite electrode, an Ag/AgCl (0.1 M KCl) electrode, and a platinum wire were used as the working, reference, and auxiliary electrodes, respectively. Electrochemical measurements were carried out using PalmSens (Utrecht, The Netherlands) with PSTrace software (version 5.3). Electrochemical measurements were performed at room temperature. For EPR measurement, X-band (~9.44 GHz) continuous-wave (CW) EPR spectra were performed on a Bruker ESP300E spectrometer equipped with a liquid Helium cryostat (Oxford Inc.). The EPR spectra were recorded at 2.5 K, with a modulation amplitude of 5 G, microwave power of 1 mW and a modulation frequency of 100 kHz. A vacuum pump was used during the experiments to remove paramagnetic oxygen from the sample. All EPR spectra are normalized and simulated using the MATLAB toolbox Easyspin.³⁷
5.2.3. Modification of the electrode

The procedure of the immobilization of HRP in TiO$_2$ is reported in our previous work (Chapter 3). The pure rod graphite electrode was used as a support for HRP-TiO$_2$. A suspension of a composite mixture (5 µl) consisting of TiO$_2$ impregnated with enzyme and nafion was dropped on the surface of the electrode and dried at room temperature for approximately 1 h.

5.3. Results and discussion

5.3.1. HRP-TiO$_2$ electrode with and without H$_2$O$_2$ in the flow toward monitoring HQ

HRP catalyzes the oxidation of organic substrates such as phenolic compounds by H$_2$O$_2$. However, the addition of H$_2$O$_2$ complicates the analysis due to background drifting and fluctuations, and increased noise. The problem can be partially solved by generation of H$_2$O$_2$ in flow during flow injection analysis as proposed by Munteanu et al. In their work, a reactor with glucose oxidase was connected next to the injector loop and glucose was added into all solutions as a stable molecule that along with oxygen leads to the formation of hydrogen peroxide in the flow when passing the reactor with glucose oxidase. In our first model we used the simplest configuration to introduce H$_2$O$_2$ into the flow-injection system: 0.5 mM H$_2$O$_2$ was added directly in the carrier buffer (Figure 5.2). However, the solutions injected in the injection loop (blank, standard solutions, or samples) contained no H$_2$O$_2$, which leads to background fluctuations expressed as peaks or pre-peaks of opposite direction. The pre-peak height for blank injection is +0.013 µA ± 0.0014 (n=4). The latter can be explained by the fact that the background current caused by the electrochemical reduction of H$_2$O$_2$ at the electrode, and the H$_2$O$_2$ concentration drop during the injection since the injection solution does not contain H$_2$O$_2$. 
The experiments were done in the presence and absence of H$_2$O$_2$ (blank phosphate buffer). As observed in Figure 5.3 the current response for HRP-TiO$_2$ with H$_2$O$_2$ in the flow (4.28 µA ± 0.05 (n=4)) at 100 µM HQ is 33 times bigger than the response at HRP-TiO$_2$ without H$_2$O$_2$ in the flow (0.11 µA ± 0.017 (n=4)). The sensitivity of the electrode in the presence of H$_2$O$_2$ in the flow and in the absence of H$_2$O$_2$ for different concentration of HQ was calculated to be 0.51 A M$^{-1}$ cm$^{-2}$ and 0.33 A M$^{-1}$ cm$^{-2}$ respectively. The higher sensitivity obtained in presence of H$_2$O$_2$ can be explained by the fact that H$_2$O$_2$ reaches the cell earlier than HQ and could create hydroperoxyl functionalities (-OOH) on the surface electrode which is further oxidize HRP and the oxidize HRP will oxidize HQ to BQ (the presence of hydroperoxyl functionalities has proven in Chapter 4). Another explanation can be related to the small amount of H$_2$O$_2$ that will remain in the cell and can’t be washed and therefore will participate in the oxidation reaction of HQ.

![Figure 5.2. Schematic flow injection system.](image)
To improve the blank fluctuation due to the presence of H$_2$O$_2$ in the carrier buffer, a mixing coil was added to the system. The mixing coil was placed after the injection loop, with the role to mix the sample with H$_2$O$_2$ before reaching the surface of the electrode (Figure 5.4). As it can be seen in Figure 5.5 the background current improved and there is no fluctuation and pre-peak upon injection of blank sample (buffer) for the new system. In this setup, because H$_2$O$_2$ is mixed with HQ after injection, H$_2$O$_2$ reduces the concentration of the injected HQ to half of the main concentration that is injected into the loop. In this model, the measurements were done with (Figure 5.4 a) and without H$_2$O$_2$ (Figure 5.4 b) in the flow. To be able to compare the results of the system with H$_2$O$_2$ in the flow with the ones without H$_2$O$_2$ in the flow, both channels of the pump were filled with buffer solution (Figure 5.4 b).
Figure 5.4. Schematic flow injection system with the mixing coil that is placed after loop before cell (a) in the presence of H$_2$O$_2$ in the carrier stream and (b) in the absence of H$_2$O$_2$ in the carrier stream.

Figure 5.5. Comparison of background fluctuation upon injection of buffer for two different models.

The sensitivity of the electrode without H$_2$O$_2$ and with H$_2$O$_2$ in the flow to HQ was calculated to be 0.31 A M$^{-1}$ cm$^{-2}$ and 0.18 A M$^{-1}$ cm$^{-2}$, respectively. Also, at low concentrations, the sensitivity of the HRP-


TiO$_2$ without H$_2$O$_2$ to HQ is improved (around two times) in comparison with the electrodes tested in the presence of H$_2$O$_2$ in the flow solution (Figure 5.6 A). However, as it can be seen in Figure 5.6 B the current response for HRP-TiO$_2$ with H$_2$O$_2$ in the flow (0.81 µA ± 0.015 (n=4)) at 50 µM HQ is 3.5 times higher than the response at HRP-TiO$_2$ without H$_2$O$_2$ in the flow (0.23 µA ± 0.013 (n=4)).

Based on these observations, the higher sensitivity of the electrode towards HQ in the absence of H$_2$O$_2$ might be explained by the presence of reactive oxygen species (ROS) created on the surface of the electrode upon artificial or natural light, delivering ROS as intermediate species to the adsorbed HRP.

\[ \text{Figure 5.6. Calibration curve of Gr/HRP-TiO}_2 \text{ with H}_2\text{O}_2 \text{ in the flow (black) and Gr/HRP-TiO}_2 \text{ without 1mM H}_2\text{O}_2 \text{ in the flow (red) for (A) low HQ concentrations (0.15 µM- 5 µM) and (B) high HQ concentration (µA). Applied potential, -0.1 V vs. Ag/AgCl. Flow rate: 1 mL/min.} \]
5.3.2. CW-EPR characterization

To study the functionalities obtained at the TiO$_2$ surface in detail, EPR analysis was performed. TiO$_2$ and HRP-TiO$_2$ are both measured. Figure 5.7 shows the EPR spectrum of blank TiO$_2$ in powder form. Oxygen-radical related (g>2) and Ti$^{3+}$ (g<2) paramagnetic species can be seen. HRP-TiO$_2$ was measured by EPR (Figure 5.8). In the 100-150 mT region, the low-field component of the high-spin ferric forms of HRP is observed. A multitude of components is seen, in line with earlier observations for peroxidases that show that the heme pocket is very heterogeneous.\textsuperscript{39} In other words the incorporation keeps the heme pocket intact, which is the most important part during the incorporation process. However, probably the wide and broad spectrum of HRP will cover the existing peak related to reactive oxygen species which can be seen for TiO$_2$ without HRP.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure5_7.png}
\caption{CW-EPR spectra of TiO$_2$.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure5_8.png}
\caption{CW-EPR spectra of HRP-TiO$_2$.}
\end{figure}
5.3.3. Effect of working potential

The sensitivity of the electrodes is influenced by the working potential of the amperometric detections. Its influence was investigated over a potential range from +0.2 to -0.4 V in a solution containing 10 µM HQ (Figure 5.9). The response of HRP-TiO₂ electrode proportionally increased as the applied potential shifted towards more negative and reaching a plateau at -0.2 V. At more negative potential values, the electrochemical reduction of molecular oxygen contributes considerably to the response of the phenolic compound and it may cause a slow irreversible deactivation of adsorbed HRP. A low background current and no influence of oxygen reduction motivates the selection of a working potential of -0.10 V.

![Figure 5.9. Influence of the applied potential on the amperometric response of 20 µM HQ at Gr/HRP-TiO₂ in 10 mM phosphate buffer (pH 7.0) containing 0.1 M of KCl. Flow rate: 1 mL/min. inset: changes in the background current upon applying different potentials.](image)

5.3.4. Applicability of electrode

To explore the applicability of the electrode for on-site applications, the amperometric response to 4-AP was measured at E= -0.1 V. As shown in Figure 5.10, the calibration plots exhibit a typical current response of the electrode with increasing 4-AP concentration. The concentration-response profile for HRP-TiO₂ was near linear in the concentration range 0.025-0.25 µM and get saturated from 20-50 µM. The limit of detection was 0.026 µM.
The results obtained for electrodes modified with horseradish peroxidase in comparison with the results from other methods, the data of these methods are listed in Table 5.1.

Table 5.1. Comparison of the detection limit for 4-aminophenol using different methods.

<table>
<thead>
<tr>
<th>Method</th>
<th>Limit of detection (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>This method</td>
<td>0.026</td>
</tr>
<tr>
<td>Flow system combined with a glucose oxidase-mutarotase reactor$^{38}$</td>
<td>0.1 ± 0.01</td>
</tr>
<tr>
<td>Flow system combined graphite electrodes modified with laccases$^{40}$</td>
<td>0.39</td>
</tr>
<tr>
<td>Flow system combined graphite electrodes modified with laccases$^{41}$</td>
<td>0.37</td>
</tr>
<tr>
<td>Flow system with spectrophotometric detection$^{42}$</td>
<td>10</td>
</tr>
<tr>
<td>Flow system using chemiluminescence detection$^{43}$</td>
<td>17.6</td>
</tr>
<tr>
<td>HPLC$^{44}$</td>
<td>3</td>
</tr>
</tbody>
</table>
5.4. Conclusion

The TiO$_2$-HRP electrode was used and optimized for hydroquinone (HQ) and 4-aminophenol (4-AP) determination in flow injection mode. At the optimum conditions for HQ, the electroanalytical behaviour of the HRP-TiO$_2$ for 4-AP was studied and compared with reported data related to 4-AP in literature by using different methods. It is well known that HRP needs H$_2$O$_2$ as a oxidant in the reaction mechanism. Two strategies were employed and compared in this work, in the presence and absence of H$_2$O$_2$ in the flow. It was found that the electrode modified with HRP in the absence of H$_2$O$_2$ is more sensitive to the one in the presence of H$_2$O$_2$ in the flow. This difference might be caused by the presence of reactive oxygen species (ROS) at the surface of TiO$_2$ upon artificial and natural light. This work supports the idea of exploiting HRP without H$_2$O$_2$ or any oxidant for the development of HRP based biosensors for the detection of phenolic compounds, e.g. in wastewater, by flow injection analysis.
5.5. References

27. S. Yang, Y. Li, X. Jiang, Z. Chen and X. Lin, *Sensors and Actuators B: Chemical*, 2006, **114**, 774-780.
Chapter 6

Bio-inspired molecular photosensitizers for the photo-electrochemical detection of phenolic compounds

Enzyme-based electrochemical biosensors are an inspiration for the development of (bio)analytical techniques. However, the instability and reproducibility of the reactivity of enzymes, combined with the need for chemical reagents for sensing remain challenges for the construction of useful devices. In this chapter, we present a sensing strategy inspired by the advantages of enzymes and photoelectrochemical sensing, namely the integration of aerobic photocatalysis and electrochemical analysis. The photosensitizer, a bioinspired perfluorinated Zn phthalocyanine, generates singlet-oxygen from air under visible light illumination and oxidizes analytes, yielding electrochemically-detectable products. Compared with enzymatic detection methods, the proposed strategy uses air instead of internally added reactive reagents, features intrinsic baseline correction via on/off light switching. It also affords selectivity imparted by the catalytic process and nano-level detection in µL sample volumes.

This chapter was adapted from:

- Singlet oxygen-based electrosensing by molecular photosensitizers. Stanislav Trashin, Vanoushe Rahemi, Karpagavalli Ramji, Liselotte Neven, Sergiu M. Gorun, Karolien De Wael
  Nature communications, 2017, 8, 16108.
6.1. Introduction

The use of enzymes for chemical analysis is well documented. Horseradish peroxidase (HRP), a typical example, has been employed either as a selective catalyst to transform an analyte into an easily detectable product\textsuperscript{1, 2} or as an enzymatic label in immunosorbent assays and related techniques.\textsuperscript{3-5} The advantage of enzymes is their catalytic signal amplification, which translates into high sensitivity and low limits of detection (LOD). Enzyme based reagents, in combination with a cost efficient and straightforward electrochemical detection method could lead to portable, selective and sensitive sensors\textsuperscript{6, 7} not unlike the widely used personal glucose meters.\textsuperscript{8}

Several research groups have advanced over last three decades the electrochemical detection of phenols at HRP modified electrodes\textsuperscript{9-11} as well as the use of HRP and alkaline phosphatase (ALP) as enzyme labels in electrochemical immunoassays.\textsuperscript{12-14} However, despite these efforts and commercial interests, progress in commercialization of electrochemical biosensors remains slow.\textsuperscript{15-17} The reasons include the complexity of fabrication of sensing elements, the thermal and chemical instability of enzymes during fabrication, sterilization and storage\textsuperscript{18}, the reproducibility of enzyme activity and immobilization procedures, as well as challenges related to field-use of reagents, for example unstable hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}). Thus, sensitive yet robust, renewable reagents and simple, reliable detection approaches are needed.

The elegant idea of using light to activate the chemical conversion of an analyte has been recently introduced in the field of electrochemical analysis.\textsuperscript{19, 20} Chronoamperometry using disposable, screen-printed electrodes (SPE) yields a detection platform similar to that of glucose meters. In the case of so-called photo-electrochemical sensors an analytical signal (photocurrent) is triggered only by light and thus it can be cleanly distinguished from background by simply switching the light off. Advantageously, relatively stable reagents could be photoactivated to start a measurement. For example, an illuminated semiconductor can become a strong electron acceptor, capable to oxidize an organic compound and exchange electrons with an electrode.\textsuperscript{21}

Here, we describe a detection paradigm based upon the reliability of photoelectrochemical detection and the principles of enzyme detection, i.e. catalytic signal amplification, but translated into a bio-inspired catalyst that needs only air and light to function. The active, bioinspired enzyme replacement is a Type II molecular photosensitizer which, under visible light illumination generates reactive singlet oxygen, $^1\text{O}_2$, which, in turn triggers the appearance of a photocurrent due to its, or its daughter species (Reactive Oxygen Species, ROS) reactions with analytes such as 4-aminophenol (4-AP) and amoxicillin.
6.2. Experimental section

6.2.1. Reagents

Perfluorophthalocyanine Zn, $F_{64}\text{PcZn}$ was synthesized and characterized as described earlier. Briefly, perfluoro-(4,5-bis-isopropyl)phthalonitrile was prepared from perfluorophthalonitrile and perfluoropropene and reacted with Zn acetate. The obtained product was purified by chromatographically and recrystallized twice from acetone. The $^{19}\text{F}$ NMR and UV-vis spectra were identical with the literature data.

Titanium dioxide, TiO$_2$ (Aeroxide® P25) and silicon dioxide, SiO$_2$ (AEROSIL® OX 50) were obtained from Evonik Inorganic Materials (USA). Horseradish peroxidase (HRP) (EC 1.11.1.7) with the activity of 293.0 U/mg was purchased from Calbiochem and 2-[4-(2-hydroxyethyl)-piperazinyl] ethane sulfonic acid (HEPES) was obtained from VWR.

Prior to impregnation with $F_{64}\text{PcZn}$, SiO$_2$ and TiO$_2$ were dried at 100 °C for 2 hours. After dissolving $F_{64}\text{PcZn}$ in 10 mL of absolute ethanol, either SiO$_2$ or TiO$_2$ were added to this solution. The ethanol was evaporated under vacuum and the impregnated materials were dried at 100 °C for 6 hours. Loadings of 0.5-3.0 % wt $F_{64}\text{PcZn}$ were confirmed spectrophotometrically by back extracting the phthalocyanine from the impregnated materials with acetone (Soxhlet) until the oxide appeared white and no phthalocyanine was observed via reflectance UV-vis spectroscopy. TiO$_2$ containing 3 wt % $F_{64}\text{PcZn}$ was used in all experiments, if not mentioned otherwise.

For the impregnation of TiO$_2$ with HRP, TiO$_2$ was suspended in a solution of 0.125 mM HRP in 10 mM HEPES (pH 7.0) buffer and the mixture was agitated overnight on a rotatory shaker. The suspension was centrifuged and the pellet washed three times with 10 mM HEPES (pH 7.0) buffer and dried at room temperature for 8 hours. The resulting powder was refrigerated prior to use. A loading of 1.06 µmol g$^{-1}$ (4.6 wt %) was calculated from the concentration of HRP in the supernatant collected after adsorption. To calculate the loaded amount of HRP, a solution of 0.125 mM HRP was prepared and the absorption of the solution was measured by UV-vis. Then 0.02 g TiO$_2$ was incubated in 0.125 mM HRP solution for 18 h. Afterwards the suspension was centrifuged and the supernatant was collected and its absorption of the supernatant solution was measured. By taking into account the difference between the absorption of HRP solution before and after mixing with TiO$_2$ (supernatant), we could calculate the loaded amount of HRP, adsorbed on TiO$_2$. The supernatants of washing solutions did not contain any noticeable amount of HRP.
Amoxicillin of 99.4% purity was obtained from TCI Europe (Belgium), HQ of 99.9% purity was purchased from Acros Organics (Belgium). 4-Aminophenol (4-AP) of ≥99% purity was purchased from Sigma-Aldrich. Other phenolic compounds were 98% purity or better and obtained from different suppliers. Ultrapure water was used for all experiments.

6.2.2. Apparatus

The electrochemical measurements were conducted using a µAutolab III (Metrohm-Autolab BV) instrument. Data for calibration curves were obtained using PalmSens3 (PalmSens BV) instrument. UV-vis-diffuse reflectance (UV-vis-DR) spectra were measured using an Evolution 500 double-beam spectrophotometer equipped with RSA-UC-40 DR-UV integrated sphere (Thermo Electron Corporation) or a Cary 5000 instrument. A diode laser pointer operating at 655 nm (Roithner Lasertechnik, Austria) was adjusted to 30 mW power using a light power meter. A power supply was programmed to switch on and off the light beam at given time intervals.

6.2.3. SPE modifications

Screen-printed carbon electrodes (SPE) were purchased from DropSens (Asturias, Spain). The modification of electrodes by F64PcZn to give SPE|F64PcZn was performed by depositing a 5 μL drop of 0.3 mg mL⁻¹ of F64PcZn solution in ethanol on SPEs and letting the solvent evaporate. The SPE|TiO2-F64PcZn were manufactured by adding a 5 μL drop of an aqueous suspension containing 10 mg mL⁻¹ TiO2-F64PcZn on the working electrode surface of SPEs and allowing the water to evaporate completely at room temperature.

6.2.4. Electrochemical measurements

Measurements with SPE were performed in a drop of 80 μL. Measuring buffer consisted of 0.1 M KCl and 20 mM KH2PO4 (pH 7.0) dissolved in ultrapure water. A saturated calomel electrode (SCE, Radiometer, Denmark) was used as an external reference electrode whenever necessary. The quasi-reference electrode of SPEs had the potential of +0.04 V versus SCE in the measuring buffer. All potentials in the text are given versus SCE. To study the effect of oxygen, a tightly closed three electrode cell was used with SPE, SCE, and a glassy carbon rod as the working, reference and counter electrodes, respectively. The beam of the diode laser was directed to the working electrode surface through the glass wall of the cell. Some, constant power loss was noted due to the glass wall absorption.
6.3. Results and discussion

6.3.1. Enzymatic and photosensitizer electrode mechanisms

Figure 6.1 illustrates the similarity between the mechanisms of action for the enzymes and the photosensitizers at an electrode. Specifically, H$_2$O$_2$ reacts with the metal site of HRP leading to a highly reactive ferryl heme iron intermediate which, in turn rapidly oxidizes an appropriate electron donor, for example hydroquinone (HQ) to benzoquinone (BQ). The formed BQ can be electrochemically reduced at an electrode back to HQ leading to electrocatalytic loop (Figure 6.1 a,b). In case of ALP, the enzyme catalyzes hydrolysis $p$-aminophenyl phosphate to form a redox active product, which is further detected at an electrode (Figure 6.1 c). Evidently, the enzymatic reactions are only possible by the consumption of the oxidant (H$_2$O$_2$) in case of HRP or the phosphate in case of ALP. The photosensitizer mimics these mechanisms but only needs dissolved air oxygen turning into singlet oxygen under light illumination (Figure 6.1 d,e).

![Figure 6.1. Electrode mechanisms. The white arrows indicate the flow of electrons to/from electrodes. Schematic illustrations of enzymatic reactions mechanisms (a-c). Mechanisms (a) and (c): the electrochemical detection of enzyme-labelled reagents. Mechanism (b): the detection of phenolic compounds. Schematic illustrations of photosensitized reactions mechanisms (d-f). Mechanisms (d) and (e): photosensitized analogues mechanisms of (a) and (b) respectively. Mechanism (f): schematic representation of the generation of singlet-oxygen, its reduction and the final production of water as the result of activity of the photocatalyst. HRP, horseradish peroxidase; ALP, alkaline phosphatase; HQ, hydroquinone; BQ, benzoquinone.](image-url)
6.3.2. Structural and spectroscopic features of the photosensitizer

The photosensitizer used is chemically robust perfluorinated phthalocyanine complex $F_{64}^{15}PcZn$ that produces singlet oxygen. Structural details of the photocatalyst, $F_{64}^{15}PcZn$ are shown in Figure 6.2. The bulky $i$-$C_3F_7$ groups effectively protect the molecules from aggregation and facilitate dissolution in organic solvents such as ethanol. The photosensitizer $F_{64}^{15}PcZn$ was deposited on TiO$_2$ (Aeroxide $^\circ$ P25), a carrier matrix with a high specific surface area, producing a TiO$_2$-$F_{64}^{15}PcZn$ material containing from 0.5 to 3 wt% of $F_{64}^{15}PcZn$ according to the experimental procedure.

Diffuse reflectance UV-vis spectrum for TiO$_2$-$F_{64}^{15}PcZn$, Figure 6.2, exhibited the characteristic Q-band$^{25,26}$ of phthalocyanines in the range of wavelengths of 600-700 nm and continuously increasing absorbance at wavelengths below 400 nm, attributed to the intrinsic absorbance of TiO$_2$. The Q-band for TiO$_2$-$F_{64}^{15}PcZn$ appeared to be relatively broad, but it remains located at the same position as that for $F_{64}^{15}PcZn$ in solutions. The wavelength of a common red diode laser pointer (655 nm) essentially matched the absorbance band of TiO$_2$-$F_{64}^{15}PcZn$. Thus, a clear photocurrent could be measured under illumination by the laser. The photocurrent depended on measuring conditions and composition of measuring solution as discussed below. It is important that the reaction that results in photocurrent is driven by light and, thus, every time when light is off, measurements automatically reveal the actual baseline position. The light sensing leads to an efficient and straightforward way for baseline correction at any desirable moment of measurements even in the presence of a sample.

Figure 6.2. Structural and optical features of $F_{64}^{15}PcZn$. (a) Structural formula and X-ray structure depiction. Color code and representation: C, gray, ball-and-stick; N, blue, ball-and-stick; F, green, space-filling model, van der Waals radii; Zn, orange, space-filling model, van der Waals radii. (b) Diffuse reflectance UV-vis spectrum of TiO$_2$-$F_{64}^{15}PcZn$ dry powder in comparison with the UV-vis spectrum of 10 μg mL$^{-1}$ $F_{64}^{15}PcZn$ in ethanol.
6.3.3. Oxygen- and electron-based Photocatalysis

The essential role of O$_2$ for substrate detection was confirmed by comparing photocurrents obtained in an air-saturated buffer with those obtained under an inert N$_2$ atmosphere (Figure 6.3). The photocurrents were completely suppressed upon N$_2$ purges, whether HQ was present or not, but recovered completely upon admission of air. Intriguingly, for SPE|F$_{64}$PcZn the photocurrent (under N$_2$) exhibits a sign reversal in the presence of HQ, suggesting a direct electron transfer from HQ to F$_{64}$PcZn and, eventually, to the electrode, in agreement with a recent report on the direct photoreduction of F$_{64}$PcZn by an electron donor in strictly oxygen-free conditions.$^{25}$ In this case the electrode plays a role of the electron acceptor that continuously oxidizes the reduced form of F$_{64}$PcZn, thus, recovering its initial form. In case of SPE|TiO$_2$-F$_{64}$PcZn, there is no direct connection between F$_{64}$PcZn and the electrode surface and, thus, photocurrent in the absence of O$_2$ is not possible.

Figure 6.3. The effect of O$_2$ on the photocurrent. The amperometric response was recorded for SPE|TiO$_2$-F$_{64}$PcZn (a) and directly adsorbed F$_{64}$PcZn, SPE|F$_{64}$PcZn (b). The measurements were performed under 655 nm red laser illumination, in the blank buffer (0.1 M KCl, 20 mM KH$_2$PO$_4$, pH 7.0) and in the presence of 10 μM HQ. Amperometry voltage vs SCE: −0.1 V.
6.3.4. Dependency on electrodes and applied potentials

Variations in photocurrents function of the applied potential were investigated by linear sweep voltammetry under light-chopped illumination. The SPEs were subjected to alternating illumination and dark periods of 50 s each, with and without the photosensitizer (Figure 6.4).

![Figure 6.4](image_url)

*Figure 6.4. The effect of the electrode modification and the applied potential. Linear sweep voltammetry (LSV) traces recorded for the bare electrode, SPE (a); the electrodes modified by TiO₂, i.e. SPE|TiO₂ (b); SPE|F₆₄PcZn, (c); and SPE|TiO₂-F₆₄PcZn, (d) in the absence (solid, black trace) and presence of 10 μM HQ (dashed, red trace). Scans range from 0.24 to −0.21 V with a scan rate of 0.25 mV s⁻¹.*

The bare SPE and the SPE|TiO₂ showed no photocurrent response either in the absence or presence of HQ (Figure 6.4 a,b). In contrast, a photocurrent response was observed for SPE|F₆₄PcZn and SPE|TiO₂-F₆₄PcZn (Figure 6.4 c,d) due to the formation of ¹O₂ and its subsequent reduction at the electrode surface (Figure 6.1 f). Higher values of the photocurrent are noted in the presence of HQ due to its redox cycling (Figure 6.1 d). Noteworthy, at similar F₆₄PcZn loadings and in the absence of HQ the response of SPE|F₆₄PcZn is higher relative to that of SPE|TiO₂-F₆₄PcZn. Moreover, when 10 μM HQ is added, the SPE|TiO₂-F₆₄PcZn photocurrent increases 20-fold, while the response of SPE|F₆₄PcZn is unchanged. More detailed amperometric measurements at higher HQ concentrations revealed that SPE|F₆₄PcZn is 50-fold less sensitive to HQ relative to SPE|TiO₂-F₆₄PcZn (Figure 6.5). These observations could be rationalized considering that ¹O₂, the photocurrent trigger has a limited lifetime in water, namely 3.5 μs.³⁷ Taking into account the 2·10⁻⁵ cm² s⁻¹ diffusion coefficient of O₂ in water ¹O₂ can diffuse...
only about 200 nm, commensurate with the thin F₆₄PcZn layer of SPE|F₆₄PcZn. In contrast, since the TiO₂-F₆₄PcZn layer of SPE|TiO₂-F₆₄PcZn is obviously more voluminous, a portion of the F₆₄PcZn is outside the O₂ diffusion radius and thus the O₂ it produces decays before reaching the electrode, leading to a lower value of the photocurrent. However, once HQ is present the more stable but redox active BQ occupies a much larger diffusion volume and thus all F₆₄PcZn of SPE|TiO₂-F₆₄PcZn (same loading as SPE|F₆₄PcZn) contributes now to the photocurrent. The significantly higher activity of SPE|TiO₂-F₆₄PcZn vs. SPE|F₆₄PcZn is due to the highly dispersed state of F₆₄PcZn supported on nano-size TiO₂, as opposed to it being present as a thin film.

\[
\text{Photocurrent response (μA)} \quad \text{Concentration of HQ (μM)}
\]

\[
\begin{align*}
0.00 & \quad 0.01 & \quad 0.02 & \quad 0.03 & \quad 0.04 & \quad 0.05 & \quad 0.06 & \quad 0.07 \\
0 & \quad 20 & \quad 40 & \quad 60 & \quad 80 & \quad 100
\end{align*}
\]

Figure 6.5. Dependence of the photocurrent on the HQ concentration for SPE|F₆₄PcZn. Deposited F₆₄PcZn on SPE: 5 μL of 0.3 mg mL⁻¹ solution in ethanol. Potential applied: -0.1 V; background, 0.1 M KCl, 20 mM KH₂PO₄, pH 7.0; the photocurrent of the blank buffer was subtracted; Red diode laser, 655 nm, 30 mW. The error bars represent SD of four consecutive measurements.

The expected photoinactivity of semiconducting TiO₂ under low-energy, 655 nm illumination was verified by testing the similar SPE|SiO₂-F₆₄PcZn electrode prepared from SBA-15 SiO₂ (Figure 6.6). The test device exhibited roughly the same activity as SPE|TiO₂-F₆₄PcZn, thus verifying TiO₂ exclusive role as a supporting matrix²⁸. Coatings of SiO₂-F₆₄PcZn, however are non-uniform and mechanically unstable and thus have not been used further.
Figure 6.6. Silica as carrier for the photosensitizer. (a) Linear sweep voltammetry recorded for SPE|SiO$_2$-F$_{64}$PcZn. (b) Amperometry measurements at a constant potential of -0.1 V and different concentrations of HQ. Background, 0.1 M KCl, 20 mM KH$_2$PO$_4$, pH 7.0. Sample volume, 80 µL. Diode laser, 655 nm, 30 mW.
6.3.5. How selective is the proposed detection for phenols?

Ampicillin, which lacks the aromatic hydroxyl group of amoxicillin, as well as benzylpenicillin, nafcillin, and 6-aminopenicillanic acid produced no noticeable photocurrents even in concentrations as high as 100 μM (Table 6.1 and Figure 6.7). Several phenols, were evaluated next (Table 6.2). Setting hydroquinone to 100, the relative sensitivities are: 4-aminophenol, 113; 2-chlorophenol, 44; bisphenol A, 39; amoxicillin, 32. Surprisingly, only a minor response was observed for 4-methylphenol, phenol, bisphenol A, 4-nitrophenol, and 2-chlorophenol have longer response times, 50-100 s, in comparison to HQ, 10 s, likely due to their slow reactions with \( ^1\text{O}_2 \).

*Table 6.1. Selectivity to the phenolic moiety. The amplitude of the photocurrent responses to 100 μM concentrations of three different penicillins and the 6-APA penicillin precursor in comparison to amoxicillin and 4-AP. Data were presented as mean (± SD) of four consecutive measurements.*

<table>
<thead>
<tr>
<th>Compound</th>
<th>Photocurrent response ± SD (nA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>amoxicillin</td>
<td>1387 ± 23</td>
</tr>
<tr>
<td>ampicillin</td>
<td>1.2 ± 0.8</td>
</tr>
<tr>
<td>benzylpenicillin</td>
<td>-1.0 ± 0.8</td>
</tr>
<tr>
<td>nafcillin</td>
<td>6.8 ± 3.7</td>
</tr>
<tr>
<td>6-aminopenicillanic acid (6-APA)</td>
<td>7.6 ± 2.9</td>
</tr>
<tr>
<td>4-aminophenol (4-AP)</td>
<td>2152 ± 24</td>
</tr>
</tbody>
</table>

*Table 6.2. Photoelectrochemical responses towards phenolic compounds.*

| Phenolic compounds          | SPE|TiO\(_2\)|F\(_{64}\)PcZn | SPE|HRP-TiO\(_2\) |
|----------------------------|----------------|---------------|----------------|----------------|
| Hydroquinone               | 100            | 100           |
| Catechol                   | 12             | 38            |
| Phenol                     | 16             | 35            |
| Bisphenol A                | 39             | 0.9           |
| 2-Chlorophenol             | 44             | 10            |
| 3-Nitrophenol              | 1.3            | 0.3           |
| 2-Aminophenol              | 10             | 26            |
| 3-Aminophenol              | 17             | 7.5           |
| 4-Aminophenol              | 113            | 70            |
| 2-Amino-4-chlorophenol     | 19             | 96            |
| 3-Cyanophenol              | 5.3            | No response   |
| 4-Cyanophenol              | 6.5            | No response   |
| 4-Methylphenol             | 1.2            | 0.7           |
| Amoxicillin                | 32             | No response   |
Figure 6.7. Selectivity study. Amperometry measurements at TiO$_2$-F$_{64}$PcZn modified SPE at a potential of $-0.1$ V in pure buffer and in the presence of 100 μM amoxicillin and 10 μM 4-AP in comparison to three different penicillins and 6-aminopenicillanic acid (6-APA). Potential applied: $-0.1$ V; background, 20 mM pH 7.0 phosphate buffer containing 0.1 M KCl; red diode laser, 655 nm, 30 mW.

Plots of the photocurrent for amoxicillin as a function of its concentration reveal that the sensitivity was 0.14 A M$^{-1}$ cm$^{-2}$ in the low concentration range, corresponding to the limit of detection (LOD) 22 nM, calculated from the 3 SD value of the background signal (SD = 0.13 nA, n = 16). The sensitivity for 4-AP was 0.62 A M$^{-1}$ cm$^{-2}$ in the low concentration range, corresponding to the limit of detection (LOD) 1 nM, calculated from the 3 SD value of the background signal (SD = 0.03 nA, n = 5). The photocurrents for HQ were about three times higher compared to amoxicillin possibly because of the slower photo-oxidation kinetics for amoxicillin compared to HQ (Figure 6.8). The sensitivity for HQ was 0.41 A M$^{-1}$ cm$^{-2}$ with a LOD of 12 nM.
Figure 6.8. Photocurrent response of HQ. Amperometry measurements (a) and the calibration curve (b) obtained for HQ at −0.1 V in 0.1 M KCl, 20 mM KH₂PO₄, pH 7.0. Sample volume, 80 µL. Diode laser, 655 nm, 30 mW. Data for the calibration curve were presented as mean (±SD) of four consecutive measurements.

Notably, the LOD values for both amoxicillin and HQ were about two orders of magnitude lower compared to a recently reported system using iron phthalocyanine designed for dopamine detection⁴⁰, and one-two orders of magnitude lower compared to HRP-modified electrodes used for the detection of phenolic compounds⁹, ¹⁰, ³¹, ³². Similar, favourable results are noted following a comparison with previously published phenolic analytes (Table 6.3).
Table 6.3. Performance of HRP-based electrodes designed for detection of phenols.

<table>
<thead>
<tr>
<th>Method</th>
<th>Electrode/Modification</th>
<th>Sensitivity[a] (nA/µM/cm²)</th>
<th>Linear range (µM)</th>
<th>Limit of detection (µM)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amperometry at -0.1 V &quot;in drop&quot; combined with photocatalytic oxidation.</td>
<td>Screen-printed electrode; commercial TiO₂ was impregnated with the photocatalyst and drop casted on the electrode surface.</td>
<td>410 (HQ) 49<a href="catechol">b</a> 66<a href="phenol">b</a> 180<a href="2-CP">b</a> 41[b] (2-AP)</td>
<td>0.1-100 (HQ) 0.012 (HQ)</td>
<td>This work</td>
<td></td>
</tr>
<tr>
<td>Amperometry at -0.05 V and continuous stirring at 600 rpm; three-electrode cell.</td>
<td>Modified gold disk electrode; HRP was incorporated in a CNT/polypyrrole nanocomposite matrix.</td>
<td>255 (HQ) 64 (catechol) 32 (phenol) 255 (2-CP) 1273 (2-AP)</td>
<td>16-240 (HQ) 1.8-8 (catechol) 16-44 (phenol) 1.6-8 (2-CP) 8-60.8 (2-AP)</td>
<td>6.4 (HQ) 0.93 (catechol) 3.5 (phenol) 0.26 (2-CP) 1.53 (2-AP)</td>
<td></td>
</tr>
<tr>
<td>Amperometry at 0 V; flow-injection analysis at flow rate of 0.8 mL/min</td>
<td>Modified carbon paste electrode; HRP was immobilized onto SiO₂/Nb₂O₅ sol/gel matrix by adsorption and cross-linking with glutaraldehyde.</td>
<td>235.7[b] (HQ) 107.5[b] (catechol) 45.3 (phenol)</td>
<td>5-25 (phenol) 0.5 (phenol)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amperometry at -0.05 V in a stirred electrochemical cell.</td>
<td>Modified carbon paste electrode; HRP was immobilized onto silica–titanium containing DNA additive and cross-linked with glutaraldehyde.</td>
<td>38.0[b] (catechol)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amperometry at -0.05 V; flow-injection analysis at flow rate of 0.25 mL/min.</td>
<td>HRP adsorbed on a graphite disk electrode.</td>
<td>110.9 (catechol) 27.4 (phenol) 488 (2-AP)</td>
<td>1.3 (catechol) 3.6 (phenol) 0.1 (2-AP)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amperometry at -0.05 V in a three-electrode cell.</td>
<td>Modified carbon paste electrode. MWCNT were chemically modified with methylene blue. HRP was immobilized on the modified MWCNT by cross-linking with glutaraldehyde in the presence of BSA.</td>
<td>50 (catechol) 40.3[b] (HQ) 18.2[b] (phenol)</td>
<td>1-150 (catechol) 0.5 (catechol)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amperometry at 0 V; flow-injection analysis at flow rate of 0.225 mL/min.</td>
<td>HRP adsorbed on SWCNT modified screen-printed carbon electrodes.</td>
<td>83.6 (catechol)</td>
<td>0.11 (catechol)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amperometry at 0 V in a three electrodes cell.</td>
<td>Modified carbon paste electrode; HRP was immobilized on silica gel modified with titanium dioxide.</td>
<td>16.3 (phenol) 39[b] (HQ) 80[b] (catechol)</td>
<td>10-50 (phenol) 1 (phenol)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

[a]If a sensitivity value was reported in nA/µM, the sensitivity in nA/µM/cm² was recalculated from a corresponding electrode surface area. [b]Recalculated values from relative response obtained for different phenolic compounds at a single concentration. Abbreviations: 2-AP – 2-aminophenol; 2-CP – 2-chlorophenol; HQ – hydroquinone; CNT – carbon nanotubes; MWCNT – multi-walled carbon nanotubes SWCNT – single-walled carbon nanotubes.
A direct comparison between F$_64$PcZn and HRP modified electrodes provides more insights into the proposed method. To our knowledge, the detection of amoxicillin using an enzymatic biosensor has not been reported. Thus, a SPE|HRP-TiO$_2$ electrode, analogous with SPE|TiO$_2$-F$_64$PcZn was constructed. The HRP-based electrode detected HQ with sensitivity similar to that of TiO$_2$-F$_64$PcZn (Figure 6.9), but it did not detect amoxicillin, substantiating the advantages of catalytic, O$_2$-mediated detection strategy.

![Figure 6.9. Amperometry measurements at a HRP-TiO$_2$ modified electrode. The amperometric response curve obtained using a HRP-TiO$_2$ modified electrode operated under the same conditions as the TiO$_2$-F$_64$PcZn electrodes. The baseline was stabilized in the measuring buffer (0.1 M KCl, 20 mM KH$_2$PO$_4$, pH 7.0) containing 1 mM H$_2$O$_2$. The total volume of the measuring solution was 100 µL. Before the introduction a compound into the drop, amoxicillin and HQ were mixed with H$_2$O$_2$ to keep its concentration unchanged during the measurements. The numbers denote the final concentration of amoxicillin or HQ. Blanks contained only H$_2$O$_2$. The favourable sensitivity reported above is dependent on the signal-to-noise ratio of the detection method. The photocurrent of the SPE|HRP-TiO$_2$ was observed to fluctuate upon the addition of H$_2$O$_2$ when low concentrations of HQ were measured (Figure 6.10), consistent with known H$_2$O$_2$ problems. Mitigation attempts by disconnecting and reconnecting the electrode exacerbated the baseline instability. A second remedy was tried by maintaining the reaction volume strictly constant. Hence, HQ was introduced by removing 20 µL from the total volume (100 µL) and returning the same volume of HQ solution also containing H$_2$O$_2$. Thus, measurements were performed continuously without switching off the cell or affecting noticeably the concentration of H$_2$O$_2$. The addition of 0.1 µM HQ after 20 min of the baseline stabilization elicited no clear response (Figure 6.10). The lowest concentration detected was 0.2 µM although the sensitivity of the electrode, 0.39 A M$^{-1}$ cm$^{-2}$ was similar to that of
SPE|TiO$_2$-F$_{64}$PcZn. The extrapolation of the baseline beyond the time the sample is introduced, however, is problematic rendering the LOD values uncertain.

In this respect, the strategy we suggest presents a distinct LOD advantage since switching off the light reveals the baseline current under the experimental measuring conditions, thereby affording a simple, straightforward correction if necessary. Moreover, while the photoelectrochemical and HRP-based approaches reveal similar selectivity patterns (Table 6.2), the phthalocyanine, unlike HRP is stable at elevated pH values. As mentioned above, a higher pH favours the reaction of phenols with $^1$O$_2$\textsuperscript{29}, and might be encountered when measuring analytes in basic wastewaters. In general, enzymes function only within narrow pH ranges, a condition which is less restrictive in the case of chemically robust photosensitizers.

Discussion

The use of enzymes for chemical analysis is reaching maturity. Photosensitizers generating $^1$O$_2$ have been explored and exploited in the fields of organic synthesis, medical photodynamic therapy and others, but not yet in the field of chemical sensors.\textsuperscript{40, 41} The proposed analogy between the photocatalytic and enzymatic detection schemes, Figure 6.1, might be the basis for new directions of exploration of $^1$O$_2$-generating photo-catalytic materials as chemical sensors.

The high reactivity of $^1$O$_2$ and its ROS daughters, however, limits the use of photocatalytic materials due to photosensitizers degradation.\textsuperscript{41} This is not surprising considering that C-H bonds containing photosensitizers are being attacked by the $^1$O$_2$ and ROS they produce. Moreover, the oxidation of
analytes may also generate reactive species, including radicals, an additional source of sensor degradation.

The photosensitizer photo-degradation is mitigated by using a fully fluorinated Zn phthalocyanine complex, F₆₄PcZn, an efficient yet stable ¹O₂ generator⁴²,⁴³ in solution and in the solid state. The manufacture of electrodes containing F₆₄PcZn revealed that robust, ROS and redox processes inactivation-resistant sensors can be produced. The bulky F₆₄Pc organic scaffold, complexed by a closed-shell metal ion like Zn²⁺ precludes deactivating aggregation, exhibits reversible electron addition and resistance to radical, electrophilic and nucleophilic attacks.⁴²

Electronic transfers to/from the sensor are thus feasible while the interactions of electrodes with analyte species occur without baseline interference. The latter process is possible since only light switching triggers the appearance of analyte products, the dark photocurrents being obtained from a chemical composition-invariable environment. Compared with enzymatic detection, significantly higher signal-to-noise ratios are generally observed, while selectivity based on the type of chemical oxidation, represents an additional bonus.

The proposed strategy compares favourably with HRP-based detection for a series of phenols, including pharmaceuticals bearing the phenol functionality. The analyte chosen as an example, amoxicillin, is the most used antibiotic⁴⁴, but also contaminates hospital trash and urban wastewaters and is therefore a marker in environmental management and pollution control.⁴⁵ 4-AP has industrial relevance due to its occurrence as intermediate in the synthesis of pharmaceuticals⁴⁶ or as a dyeing agent for fur and feathers.⁴⁷ 4-AP was also identified as a degradation product of paracetamol, therefore frequently present in wastewater. Amoxicillin, 4-AP, and other phenols’ selective detection suggests that the direct, reactivity of ¹O₂ operates, as noted previously for phenol⁴⁸ and dominates the selective sensing process. The shuttle of electrons between a site of ¹O₂ production/reactivity and an electrode occurs for both SPE|TiO₂-F₆₄PcZn and SPE|F₆₄PcZn electrodes that detect redox-active products, but the nano-dispersed TiO₂-F₆₄PcZn is more effective due to its high surface area and distribution of F₆₄PcZn in the large bulk volume of the support. The sensing efficiency can be understood and tuned using classical catalysis principles.

The kinetics of the ¹O₂ mediated oxidation of an analyte could be further tuned by modifying the reaction conditions such as temperature and pH, as shown for phenols. The large variation of pH, an important parameter for optimizing sensors’ sensitivity and selectivity, is unavailable in the enzymatic detection scheme.
6.4. Conclusion

In conclusion, robust, perfluorinated molecular photosensitizers, resistant chemically yet reactive, have been shown as proof-of-principle efficient enzymes mimics for electrochemical (bio)sensing applications, while favourably enhancing the useful feature of the enzymatic detection mechanism, namely the catalytic formation of an easily detectable product and redox cycling. The photosensitizer generates photocurrents using air oxygen without the need to add any supplementary reagents. The use of catalytic photosensitizers instead of enzymes for analytical sensing offers several advantages, including: chemical and thermal stability; on-off control of sensing by on-off light switching; facile dark baseline monitoring and adjustments; invariable structural and reactivity properties of photocatalytic, well-defined metal complexes; comparative simplicity and low preparation price; facile chemical modification and functionalization, attachment to biomolecules and surfaces. The bioinspired molecules, subject to chemical modifications, coupled to biomolecules may allow conjugates to function in the same way as fluorescent dye- and enzyme-labelled (e.g. HRP-labelled) reagents, but affording an additional, on-demand reactivity controlled by simply switching the light. An enhanced degree of flexibility in the design of biorecognition elements and the functionalization of sensor surfaces is envisioned. The present example of a bioinspired strategy of replacing an enzyme with synthetic components for analytical purposes could be useful for developing applications ranging from chemistry to biology and environmental monitoring.
6.5. References

Summary and future perspectives

1. Summary

In my thesis, new strategies were developed for the detection of phenolic compounds by using titania (TiO$_2$) and horseradish peroxidase (HRP). With HRP which exhibits affinity for phenolic compounds, the detection can only occur in the presence of hydrogen peroxide (H$_2$O$_2$) which creates a reactive HRP-H$_2$O$_2$ complex (strong oxidant initiating the further reaction). However, the addition of H$_2$O$_2$ complicates the detection strategy as time is needed for background stabilization and additionally, the stabilisation of enzymes is another issue. Enzymes which are dissolved in the electrolyte are more susceptible to change by the variations in the pH of a solution and temperature alternations. Therefore, different strategies were developed to tackle this drawback, i.e. the presence of H$_2$O$_2$ during HRP sensing.

First, the incorporation of HRP in (commercial) mesoporous titania (PC500) was performed. An adhesive conducting electrode material containing graphite, biocompatible ion exchange polymer nafion and (commercial) mesoporous TiO$_2$ impregnated with horseradish peroxidase (HRP) was prepared and characterized by amperometric, UV-vis and N$_2$ sorption methods. The role of mesoporous titania is to protect the incorporated enzyme from denaturation. The factors influencing the performance of the resulting biosensor towards detection H$_2$O$_2$ were studied in detail. The optimal electrode material consists of 45% graphite, 50% impregnated HRP-TiO$_2$ and 5% nafion. The optimum conditions for H$_2$O$_2$ reduction were an applied potential of -0.3 V and 0.1 mM hydroquinone. The sensitivity and limit of detection in the optimum conditions were 1 A M$^{-1}$ cm$^{-2}$ and 1 µM correspondingly. The N$_2$ sorption results showed that the pore volume of TiO$_2$ decreased sharply upon adsorption of HRP. The preparation process of the proposed enzyme electrode was straightforward and potentially useful for the preparation of carbon paste electrodes for bioelectrochemical detections.

Based on those observations, hydrogen peroxide addition is an essential step in electrochemical detection strategies in which horseradish peroxidase participates as an oxidant (e.g. the detection of phenolic compounds). The injection of H$_2$O$_2$ in solution is known to cause background instability during bioanalysis with an electrochemical readout. It was described how to overcome this drawback whilst focusing on the detection of phenolics by using HRP based systems. The innovative concept involved a pre-activation of a TiO$_2$ modified electrode by incubation in a H$_2$O$_2$ solution resulting in a reactive Ti-OOH functionalized surface, acting as an in-situ formed oxidant. EPR spectroscopy was used to
characterize the surface of the pre-activated electrode. Both hydroquinone and 4-aminophenol were selected as model target compounds to demonstrate the activity of such pre-activated electrodes. The proposed modified electrode was expected to provide new possibilities for a straightforward design of sensors to detect wide range of chromogenic substrates, azo dyes, aniline and phenolic compounds.

In addition, the special features of titania were used to construct a highly efficient electrochemical enzyme based biosensor in order to detect phenolic compounds in flow systems. These features are related to the presence of reactive oxygen species (ROS) at the surface of titania upon irradiation in day light. This ROS worked as the necessary oxidant for phenolic detection. For this purpose, HRP-TiO$_2$ was immobilized on the surface of rod graphite electrode through drop casting. Flow analysis is a method based on the injection of a liquid sample into a moving unsegmented continuous stream of a suitable liquid. Flow cells make it possible to achieve high sensitivity using very small amounts of sample with distinctive properties: (i) aqueous flow is generally laminar, not turbulent; (ii) diffusion is an efficient process for mixing the dissolved content of two or more fluids. The fabricated TiO$_2$-based biosensor showed excellent sensitivity, good stability and electrocatalytic activity toward detection of hydroquinone and 4-aminophenol as examples of phenolic compounds. The sensitivity of the developed sensing platform is 0.31 A M$^{-1}$ cm$^{-2}$ which was around two times higher compared to a system with hydrogen peroxide in flow with sensitivity 0.18 A M$^{-1}$ cm$^{-2}$.

Enzyme-based electrochemical biosensors were an inspiration for the development of (bio)analytical techniques. However, the instability and reproducibility of the reactivity of enzymes, combined with the need for chemical reagents for sensing remain challenging for the construction of devices. We presented a sensing strategy inspired by the advantages of enzymes and photoelectrochemical sensing, namely the integration of aerobic photocatalysis and electrochemical analysis. The photosensitizer, a bioinspired perfluorinated Zn phthalocyanine, generates singlet-oxygen from air under visible light illumination and oxidizes analytes, yielding electrochemically-detectable products. Compared with enzymatic detection methods, the proposed strategy used air instead of internally added reactive reagents, features intrinsic baseline correction via on/off light switching. It also affords selectivity imparted by the catalytic process and nano-level detection, such as 12 nM HQ in µL sample volumes. This example of a bioinspired strategy of replacing an enzyme with inorganic components for analytical purposes may find applications in other (bio)analytical areas. Scheme 1 summarizes the advantages and disadvantages of the (bio)sensing strategies described in each chapter of this thesis.
| Scheme 1. Summary of the advantages and disadvantages of the bio(inspired)sensor in each chapter. |
|---|---|
| **Incorporation of horseradish peroxidase in mesoporous TiO<sub>2</sub> (chapter 3)** | **Advantages** | **Disadvantages** |
|  | • Very stable HRP matrix (two years)  
• Saving time in the electrode preparation step due to use of the commercial mesoporous titania as a support  
• LOD for H<sub>2</sub>O<sub>2</sub> is 1 µM |  | • The addition of H<sub>2</sub>O<sub>2</sub> is necessary according to the general mechanism of HRP  
• Long background stabilization time (about 600 s) |
| **Impregnated TiO<sub>2</sub> with HRP incubated in H<sub>2</sub>O<sub>2</sub> for the detection of phenolic compounds (chapter 4)** |  | **Advantages** |
|  | • Titania can be activated by prior incubation (2 min) in 1 mM H<sub>2</sub>O<sub>2</sub> solution  
• No need for the addition of H<sub>2</sub>O<sub>2</sub> during the measurements  
• Short background stabilization time (about 30 s)  
• LOD for 4-AP is 24 nM |  | • Pre-activated electrode is only stable for 20 minutes  
• Higher concentration of H<sub>2</sub>O<sub>2</sub> in pre-activation step can cause the bleaching and denaturation of the enzyme  
• Pre-activation is not applicable for all types of materials |
| **Monitoring of phenolic compounds by flow injection analysis (chapter 5)** |  | **Advantages** |
|  | • Flow injection analysis (FIA) has higher sensitivity compared to the stationary setup (common electrochemical setup)  
• No need for the addition of H<sub>2</sub>O<sub>2</sub> or the pre-activation step  
• Using advantages of produced ROS at titania upon artificial and natural light illumination  
• LOD for 4-AP is 26 nM |  | • The setup contains a lot of components such as a pump, an injection loop, etc which causes challenges for in-situ measurements  
• The presence of air bubbles, moving in the fluidic setup can cause some problems during the measurements |
| **Bio-inspired molecular photosensitizers for the photo-electrochemical detection of phenolic compounds (chapter 6)** |  | **Advantages** |
|  | • Generating singlet oxygen under red light illumination (655 nm)  
• Allows detection in the presence of other redox active molecules  
• Higher signal to noise ratio in comparison with enzyme based biosensors (previous chapters)  
• High chemical (pH) and thermal stability  
• LOD for 4-AP is 1 nM |  | • Phthalocyanines should be mixed with a support (e.g. TiO<sub>2</sub>) |
2. Future perspectives

During this Ph.D. dissertation, it was shown that titanium dioxide possesses a great potential in the field of biosensors. However, titania has a large electronic bandgap of 3.0-3.2 eV, limiting its optical absorption to the ultraviolet (UV) region of the solar spectrum, which is a major drawback. In a typical photo-catalytic process, the semiconductor catalyst should harvest light with energy larger than its bandgap to produce excited electrons and holes. These excited charges should separate from each other and migrate to the surface to perform photo-catalytic reactions, before they are annihilated in the recombination process. The more light the photo-catalyst absorbs, the more excited charges are likely to be present on the surface. Therefore, in the case of using TiO$_2$, it is intriguing to explore the possibility of enhancing and/or extending the optical absorption properties of TiO$_2$ to improve its overall activity. Enormous efforts have been devoted to extend the light absorption range of TiO$_2$ into the visible region, such as nonmetal or metal elements doped TiO$_2$ and dye or narrow band-gap semiconductor composited TiO$_2$. However, the stability of these dyes and semiconductors is far from satisfactory and sometimes the doped impurity atoms can serve as carrier recombination centers and decrease the overall photo-conversion efficiency.

Recent advances in extending the light absorption range of titania (TiO$_2$) into the visible region has resulted in a new material for scientists, i.e. black TiO$_2$ with a bandgap around 1.5 eV. Black (reduced) TiO$_2$ is a promising candidate for photo-(electro)catalysis under near infrared light owing to its narrow band gap and its improved electronic conductivity, however, only limited attention was paid to using it as an advanced photo-electrochemical (bio) sensor material. Using photo-electrocatalysts in stationary electrochemical systems commonly face poisoning phenomena due to the generated products seriously affecting the electrochemical detection. In order to improve the recyclability of the photo-electrocatalyst, a photo-electrochemical flow cell is the best choice due to the continues movement of a carrier solution to the electrode surface. The combination of a flow cell and a photo-electrochemical setup integrates the benefit of different systems such as high mass diffusion and low amount of sample requirements, while warranting strong signals and a high detection sensitivity.

Enzyme based electrochemical sensors for polyphenols detection have been developed on the basis of enzymes such as tyrosinase, laccase and horseradish peroxidase. The most known one is horseradish peroxidase (HRP) which exhibits affinity for phenolic compounds, nevertheless, the detection can only occur in the presence of hydrogen peroxide which creates a reactive HRP-H$_2$O$_2$ (strong oxidant initiating the further reaction). Polyphenols are oxidized to phenoxy radicals by HRP-H$_2$O$_2$, followed by their back reduction to polyphenols at the electrode surface and generating a measurable signal (catalytic loop) which is proportional to the concentration of produced phenoxy radicals (Figure 1-left).
Similar events take place at tyrosinase biosensors (restricted to monitoring phenolic compounds with at least one free ortho phenol group) and laccase biosensors (to detect free para and meta positioned phenols). Upon the existence of such enzymatic based electrochemical biosensors, the utilization of enzymes as catalysts has one major drawback which is related to the stability of enzymes. Enzymes are fragile and active only under specific reaction conditions, however, enzymatic systems are ideal systems to mimic given the catalytic loop which is taking place and responsible for a low limit of detection and thus my source of inspiration.

When developing bio-inspired strategies, this electrocatalysis (catalytic loop) is definitely the property to retain. However, the addition of hydrogen peroxide complicates the detection strategy as time is needed for background stabilization and additionally, enzymes mostly have a restricted life time.

So, the idea is to replace the HRP-H$_2$O$_2$ system by a more robust system which can in-situ generate a strong oxidant (ROS from illuminated black TiO$_2$), to create a more simple, robust and rapid method retaining the main advantage of enzymatic detection (i.e. catalytic signal amplification) but overcoming the drawbacks of current enzymatic sensors being poor stability of biomolecules, additions of reagents and fluctuations in the readout signal over time. Therefore, the innovation of this project combines three aspects, i.e. (1) the idea of light to initiate the detection of polyphenols, (2) robust black TiO$_2$ which can be activated under red light illumination and (3) a flow system to have high mass diffusion, high sensitivity while protecting the photo-catalyst from poisoning during the measurements.
Abstract

In my thesis, new strategies were developed for the detection of phenolic compounds by using titania and horseradish peroxidase (HRP). HRP is a heme-containing enzyme that utilises hydrogen peroxide to oxidise a wide variety of organic and inorganic compounds. However, the application of free enzymes is often hampered by a lack of long-term operational stability and difficult recovery and re-use of the enzyme. These drawbacks can generally be overcome by immobilisation/incorporation of the enzyme in a support. As a first step, HRP was incorporated in titania. An adhesive conducting electrode material containing graphite, biocompatible ion exchange polymer nafion and (commercial) mesoporous TiO$_2$ impregnated with horseradish peroxidase (HRP) was prepared. The role of mesoporous titania is to protect the incorporated enzyme from denaturation and increases the operational stability of the enzyme. HRP exhibits affinity for phenolic compounds, the detection can only occur in the presence of hydrogen peroxide (H$_2$O$_2$) which creates a reactive HRP-H$_2$O$_2$ complex (strong oxidant initiating the further reaction).

However, the addition of H$_2$O$_2$ complicates the detection strategy as time is needed for background stabilization. Therefore, different strategies were developed to tackle this drawback. In the second step, a new strategy was described how to avoid the presence of hydrogen peroxide during HRP sensing. In the third step, the special features of titania were used to construct a highly efficient electrochemical enzyme based biosensor in order to detect phenolic compounds in flow systems without the need of the addition of supplementary reagent. These features are related to the presence of reactive oxygen species (ROS) at the surface of titania upon irradiation in day light. This ROS worked as the necessary oxidant for phenolic detection. Flow cells make it possible to achieve high sensitivity using very small amounts of sample with distinctive properties: (i) aqueous flow is generally laminar, not turbulent; (ii) diffusion is an efficient process for mixing the dissolved content of two or more fluids.

Enzyme-based electrochemical biosensors were an inspiration for the development of (bio)analytical techniques. However, the instability and reproducibility of the reactivity of enzymes, combined with the need for chemical reagents for sensing remain challenging for the construction of devices. In order to overcomes the drawbacks of current enzymatic sensors the photosensitizer, a bioinspired perfluorinated Zn phthalocyanine was explored that generates singlet-oxygen from air under visible light illumination and oxidizes analytes, yielding electrochemically-detectable products while resisting the oxidizing species it produces.
Samenvatting

In dit werk worden nieuwe strategieën voor de detectie van fenolische verbindingen ontwikkeld gebaseerd op het gebruik van titania en het enzyme peroxidase (HRP) of een bio-geïnspireerd alternatief (phthalocyanine). In essentie kan met HRP, dat affiniteit vertoont voor fenolverbindingen, de detectie van fenolen enkel plaatsgrijpen in aanwezigheid van waterstofperoxide ($H_2O_2$). In contact met $H_2O_2$ zal een reactief HRP-$H_2O_2$-complex ontstaan dewelke als sterk oxidatief middel de reactie met fenolen initieert. Echter, de toevoeging van $H_2O_2$ bemoeilijkt de detectiestrategie aanzienlijk aangezien extra tijd nodig is voor achtergrondstabilisatie. Bovendien vormt de stabilisatie van enzymen een bijkomend probleem; enzymen opgelost in een waterige elektrolyt oplossing zijn meer vatbaar voor variaties in pH en temperatuurwisselingen. In een eerste fase werd een adhesief geleidend elektrodemateriaal bereid dat bestaat uit grafiet, een biocompatibel nafion membraan en commercieel mesoporeus titaniumoxide ($TiO_2$) geïmpregneerd met HRP. Het $TiO_2$ beschermt het opgenomen enzym tegen denaturatie en verhoogt aldus de stabiliteit. Rekening houdend met het feit dat toevoeging van $H_2O_2$ een essentiële stap is bij op elektrochemie gebaseerde detectiestrategieën met HRP als oxidans, werd in een volgende fase een nieuwe strategie ontwikkeld om de nadelen van toevoeging van waterstofperoxide te overwinnen.

De bijzondere kenmerken van $TiO_2$ werden vervolgens gebruikt om een sensor te construeren om fenolische verbindingen in doortroomsystemen te detecteren. Deze kenmerken zijn gerelateerd aan de aanwezigheid van reactieve zuurstofgroepen (ROS) aan het oppervlak van $TiO_2$ bij bestraling in daglicht. Deze ROS werken als het noodzakelijke oxidatiemiddel voor fenolische detectie. Doorstroomcellen maken het mogelijk om met een hoge gevoeligheid kleine hoeveelheden fenolen te detecteren in een staal: (i) de waterige stroom is in het algemeen laminair, niet turbulent; en (ii) diffusie is een efficiënte werkwijze voor het mengen van twee of meer vloeistoffen.

Tot slot zijn enzymatische systemen vaak een inspiratie voor de ontwikkeling van (bio)analytische technieken. De instabiliteit en reproduceerbaarheid van de reactiviteit van enzymen, gecombineerd met de behoefte aan chemische reagentia voor detectie, blijven echter een uitdaging voor de ontwikkeling en het vermarkten van biosensoren. Om de nadelen van de huidige enzymatische sensoren te overwinnen, werd een fotogeoel materiaal, een geperfluoreerde Zn-ftalocyaine, bestudeerd. Dit materiaal kan, onder rood laserlicht, singlet-zuurstof (uit lucht) genereren en daaropvolgend analieten oxideren, i.e. elektrochemisch detecteerbare producten, met een hoge gevoeligheid.
List of scientific contributions during PhD

- Hydrogen peroxide less impregnated titania with horseradish peroxidase for bioelectrochemical monitoring of phenolic compounds in flow system. 
  *In preparation.*

- A new direction for enzymatic sensors for phenols based on redox cycling with peroxidase and a surface confined sacrificial electron acceptor. 
  *Vanoushe Rahemi,* Stanislav Trashin, Zainab Hafideddine, Sabine Van Doorslaer, Vera Meynen, Karolien De Wael 
  *In preparation.*

- Assessing the stability of arsenic sulfide pigments and influence of the binding media on their degradation by means of spectroscopic and electrochemical techniques. 
  Marc Vermeulen, Koen Janssens, Jana Sanyova, *Vanoushe Rahemi,* Chris McGlinchey, Karolien De Wael 

- Antarctic fish versus human cytoglobins–The same but yet so different. 
  Bert Cuypers, Stijn Vermeylen, Dietmar Hammerschmid, Stanislav Trashin, *Vanoushe Rahemi,* Albert Konijnenberg, Amy De Schutter, C-H Christina Cheng, Daniela Giordano, Cinzia Verde, Karolien De Wael, Frank Sobott, Sylvia Dewilde, Sabine Van Doorslaer 

- Singlet oxygen-based electrosensing by molecular photosensitizers. 
  Stanislav Trashin, *Vanoushe Rahemi,* Karpagavalli Ramji, Liselotte Neven, Sergiu M. Gorun, Karolien De Wael 
  *Nature communications 2017, 8, 16108.*

- Unique optoelectronic structure and photoreduction properties of sulfur-doped lead chromates explaining their instability in paintings. 
  *Vanoushe Rahemi,* Nasrin Sarmadian, Willemien Anaf, Koen Janssens, Dirk Lamoen, Bart Partoens, Karolien De Wael 
  *Analytical Chemistry 2017, 89, 3326-3334.*

- An adhesive conducting electrode material based on commercial mesoporous titanium dioxide as a support for horseradish peroxidase for bioelectrochemical applications. 
  *Vanoushe Rahemi,* Stanislav Trashin, Vera Meynen, Karolien De Wael 
  *Talanta 2016, 146, 689-693.*
List of scientific contributions before PhD

- Carbon nanotube β-cyclodextrin modified electrode as enhanced sensing platform for the determination of fungicide pyrimethanil. 
  Jorge MPJ Garrido, Vanoushe Rahemi, Fernanda Borges, Christopher MA Brett, E Manuela Garrido
  *Food Control* 2016, 60, 7-11.

- A new voltammetric sensor for hydrazine based on michael addition reaction using 1-amino-2-naphtol-4-sulfonic acid.
  Reza Ojani, Vanoushe Rahemi, Jahan-Bakhsh Raoof

- Electrochemical sensor for simultaneous determination of herbicide MCPA and its metabolite 4-chloro-2-methylphenol. Application to photodegradation environmental monitoring.
  Vanoushe Rahemi, Jorge MPJ Garrido, Fernanda Borges, Christopher MA Brett, E Manuela Garrido
  *Environmental Science and Pollution Research* 2014, 22, 4491-4499.

- Electrochemical determination of the herbicide bentazone using a carbon nanotube β-cyclodextrin modified electrode.
  Vanoushe Rahemi, Jorge MPJ Garrido, Fernanda Borges, Christopher MA Brett, E Manuela Garrido
  *Electroanalysis* 2013, 25, 2360-2366.

- Enhanced host–guest electrochemical recognition of herbicide MCPA using a β-cyclodextrin carbon nanotube sensor.
  Vanoushe Rahemi, Jeroen Vandamme, Jorge MPJ Garrido, Fernanda Borges, Christopher MA Brett, E Manuela Garrido

- Evaluation of sodium dodecyl sulfate effect on electrocatalytic properties of poly (4-aminoacetanilide)/nickel modified carbon paste electrode as an efficient electrode toward oxidation of ethylene glycol.
  Reza Ojani, Jahan-Bakhsh Raoof, Vanoushe Rahemi

- A simple and efficient electrochemical sensor for electrocatalytic reduction of nitrite based on poly (4-aminoacetanilide) film using carbon paste electrode.
  Reza Ojani, Jahan-Bakhsh Raoof, Vanoushe Rahemi
Conference contributions

- Oral presentation titled “How to avoid hydrogen peroxide in horseradish peroxidase based sensing?” at XXIV international symposium on bioelectrochemistry and bioenergetics, 3-7 July 2017, Lyon, France.


- Oral presentation titled “Impregnated commercial mesoporous titanium dioxide with horseradish peroxidase for bio-electrochemical applications” at SMOBE 2016, 17-19 August 2016, Antwerp, Belgium.

- Poster presentation titled “Unique optoelectronic structure and photoreduction properties of sulfur-doped lead chromates explaining their instability” at ChemCH, 6-8 July 2016, Brussels, Belgium.

- Poster presentation titled “An adhesive conducting electrode material based on commercial mesoporous titanium dioxide as a support for horseradish peroxidase for bioelectrochemical applications” at ESEAC 2016, 12-16 June 2016, Bath, UK.

- Oral presentation titled “An adhesive conducting electrode material based on commercial mesoporous titanium dioxide as a support for horseradish peroxidase for bioelectrochemical applications” at ChemCys 2016, 16-18 March 2016, Blankenberg, Belgium.

- Poster presentation titled “An adhesive conducting electrode material based on commercial mesoporous titanium dioxide as a support for horseradish peroxidase for bioelectrochemical applications” at SMCBS 2015, 6-10 November 2015, Pultusk, Poland.

- Oral presentation titled “An adhesive conducting electrode material based on commercial mesoporous titanium dioxide as a support for horseradish peroxidase for bioelectrochemical applications” at SMOBE-2015, 17-20 August 2015, Antwerp, Belgium.
• Poster presentation titled “An adhesive conducting electrode material based on commercial mesoporous titanium dioxide as a support for horseradish peroxidase for bioelectrochemical applications” at Electrochemistry 2014, 22-24 September 2014, Mainz, Germany.

• Poster presentation titled “Horseradish peroxidase entrapped on a mesoporous titania-coated gold electrode for electrochemical sensing of hydrogen peroxide” at ChemCYS 2014, 27-28 February 2014, Blankenberge, Belgium.

Scientific awards

• Best poster presentation for the poster titled “Unique optoelectronic structure and photoreduction properties of sulfur-doped lead chromates explaining their instability” at SMOBE, 17-19 August 2016, Antwerp, Belgium.

• Best poster presentation for the poster titled “Unique optoelectronic structure and photoreduction properties of sulfur-doped lead chromates explaining their instability” at ChemCH, 6-8 July 2016, Brussels, Belgium.