CRANFIELD UNIVERSITY

Biosensor stabilization using Hypersolutes

A thesis submitted

-part of the HotSolutes project-

Patent: PCT/PT2007/000013

"Utilização de solutos compatíveis para melhorar o desempenho de técnicas utilizando materiais biológicos imobilizados"

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And a thought for my son, Wolfram, who was born as I finished my thesis!

ABSTRACT

Introduction

A biosensor may be described as a sensor incorporating a biological element such as an enzyme, antibody, nucleic acid, microorganism or cell. A biosensor should exhibit both shelf-stability and operation stability. Compatible solutes from hyperthermophilic bacteria, called hypersolutes, are very efficient for the preservation of the performance of a wide variety of biomaterials; ranging from proteins to whole cells and artificial tissues. The overall objectives of this work have been to investigate the application of hypersolutes to enhance the performance of biosensors based on the stabilization properties offered by hypersolutes compounds, particularly with respect to storage and operational lifetime.

Materials *and* Methods

The stabilizing agents considered for this study were firoin, firoin A, ectoine®, hydroxyectoine, diglycerol phosphate (DGP) and potassium mannosyl-lactate (PML), provided either by Bitop AG (Witten, D) or StabVida (Oeiras, P).The following enzymes were selected due to their commercial importance: Glucose oxidase (GOx), alcohol oxidase (AOx), acetylcholinesterase (AchE) and lactate dehydrogenase (LDH).

On immunosensors, a model system was first designed using ELISA tests. The influence of hypersolutes was then studied using BIAcore. The antibody test system selected for examination of the effect of stabilizing agents on immunosensor performance was based on an anti-human immunoglobulin G (IgG) primary antibody, grown in goat, and an anti-goat secondary antibody conjugated to horseradish peroxidase enzyme.

A model DNA binding system was sought: The poly-A strand was tethered to the sensing surface within the BIAcore system via a biotin-streptavidin linkage whereas the complimentary poly-T strand contained a fluorescent Cy3 label, that offered the possibility to also use more conventional detection techniques to ensure that

hybridization between the two complimentary strands had been achieved, as well as add a significant weight to the strand, increasing its visibility on the BIAcore signal.

Results

The sensor range, operational stability, storage stability, pH tolerance and thermal stability have been investigated for enzyme based biosensors. Ectoine and firoin A presented a small stabilizing effect (+80%) against ionic strength variations. DGP gave the best stabilization against storage (+237%) with firoin A and hydroxyectoine (+218% and +176% respectively).

For immunosensors, ionic strength variation, over the range 0.1-1000mM PBS: ectoine, hydroxyectoine and DGP had a significant stabilizing effect with an antibody activity preservation of 161%, 145% and 125% respectively. After 2 months of storage at 920nM, with 7mM stabilizing agent, DGP gave the most promising result with a stabilization of 244%, followed by firoin A (178%) and hydroxyectoine (170%).

Careful optimization of the streptavidin-biotin binding complex was achieved and proved a time-consuming process.

Conclusion

The hypersolutes have proven to reduce enzyme deactivation at the screen-printed electrode surfaces. They do not influence the optimum operating pH for both enzyme sensors and immunosensors.

Enzyme sensors incorporating ectoine and firoin A, and immunosensors incorporating ectoine, hydroxyectoine and DGP had a stabilizing effect in the presence of varying ionic strength conditions.

The best enzyme activity preservation was observed with DGP and hydroxyectoine upon storage, DGP, firoin A and hydroxyectoine were the most efficient for immunosensors.

On DNA-based biosensors, an improved method of nucleic acid immobilization to the BIAcore chips was delivered, in comparison to those other methods reported in the literature.

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1. INTRODUCTION

Biosensor development over the past 20 years has occurred at an impressive rate. One major issue faced by biosensors is that due to biological stability. Whether for storage or prolonged uses, stability remains a challenge. The exploitation of bacteria, able to survive under extreme conditions (extremophiles), offers a potential answer to the challenge posed by stabilization. Deciphering the preservation mechanisms of these bacteria may be the key to the biosensor stability. Recently, new compounds from this source have been identified and isolated, called hypersolutes, which may prove particularly useful as stabilization factors for biosensors.

The HotSolutes project, funded by the E.U., has been developed in order to study those compounds and determine their properties and functions as well as find them applications. The study carried out is part of this project. The aim of this study is to determine whether these compounds can act as efficient stabilizers for biosensors. The project report presented here is a preliminary part to this study. Other studies are being carried out in order to characterize these solutes, by several industrial partners.

The contribution of Cranfield University to this project was in 4 deliverables:

- **HotSolutes-D26** – New formulations for existing biosensors, in which the application of hypersolutes were to be investigated as how to enhance the performance of enzyme based biosensors that are currently available
- **HotSolutes-D27** – The introduction of novel biosensors that have been previously difficult to produce as a result of the inherent instability of the biological components used. The overall objective of this workpackage was to develop a new range of biosensors based on the enhanced stability offered by the hypersolutes, in particular both storage and operational lifetime of the sensor.
- **HotSolutes-D28** – Assessment of hypersolutes for improving the stability of immunosensors, where the overall objective of this workpackage was to investigate the application of hypersolutes to improve the stability of

enzyme immunosensors and to exploit the enhanced stability offered by the hypersolutes to improve the performance of these devices.

- **HotSolutes-D29** – DNA based biosensors stability. The overall objective of this workpackage was to investigate the application of hypersolutes to improve the stability of DNA-based biosensors and to exploit the enhanced stability offered by the hypersolutes to improve the performance of these devices.

In the first part of this report, biosensors are presented and the different types studied are detailed. The systems chosen for this study are also explained, whether for the enzyme-based biosensors, immunosensors or DNA-based biosensors. A special attention was put into glucose biosensors, as they are the most widely studied biosensor and the first that was investigated in this study. The other enzymes sensors that were studied are then presented, namely: alcohol oxidase, lactate dehydrogenase an acetylcholine esterase. A model system was designed for both immunosensors and DNA-based biosensor, so that their presentation stays more general. A short background on the different detection techniques that were used is also given. Finally, the hypersolutes as well as the HotSolutes project are reviewed as they are the keystone to this thesis.

The second part of this report presents the experimental portion of the study that was realized. It begins with a short material and methods section. Most methods used in this study were optimized before any results pertaining to our study could be obtained and as such, these are detailed in the appropriate experiments report.

The Experimental part of this thesis then goes on to detail the work that was realized on biosensors, starting with the characterization of the system used with hydrogen peroxide. The system was then optimized for the enzymes studied, without then with hypersolutes before the stabilizing effect of these products was assessed. As 3 different types of biosensors were studied as part of our input in the Hotsolutes project, there are three distinct parts in this report as well, each concentrating on a

biosensor (enzyme-, antibody-or DNA- based), and each being concluded by the corresponding discussion on the results.

The immunosensor study is then detailed, starting with the choosing of a model system, tested first via ELISA tests and then translated to the BIAcore system and later on to the Akubio system. Again, as a system was chosen, it was first assessed before the hypersolutes were added. Only when the system thus created was comprehended, were stress factors introduced to determine the impact the hypersolutes have.

Finally, a short section is dedicated to DNA-based sensor. As a few difficulties in setting up this system were encountered, and as the summary of this entire experimental chapter is logical as well as chronological, there are few results on the actual hypersolutes influence on this type of sensor, and this would need to be investigated further.

This work was intended as a preliminary study, to determine the potential of hypersolutes on the stabilization of certain types of biosensors. As such, it isn't exhaustive. Also, the novelty of these compounds explains the lack of understanding about the stabilization mechanism of the hypersolutes that were made available to us in this study. It is also the reason for the shortage of PML, one of the hypersolutes at our disposition: the HotSolutes partner did not manage to find a satisfactory synthetic pathway for it, nor were they able to produce more of it via natural means.

This report ends with a summary of the studies realized and a conclusion on the hypersolutes used. As this study was done with potential commercialization in sight, it seems only natural that this part should have the same orientation.

2. LITERATURE REVIEW

2.1. Biosensors: A presentation

This study revolved around different types of biosensors and the optimization of their stability when facing various stress factors. This report will therefore start by presenting biosensors in general, and then concentrate on the biosensors that were chosen in particular. [Lowe (1984). Lowe (1985). Hall (1991). Tess and Cox (1999). Turner *et al.* (1987).]

2.1.1. Definition of a biosensor

A biosensor is an analytical device incorporating a biological or biologically-derived sensing element, either integrated within or intimately associated with a physicochemical transducer (cf. Fig.1). The biorecognition of the analyte by the receptor creates a signal that is converted into a measurable signal, thanks to the transducer. In essence, biosensor development encompasses a broad range of skills, as shown in Fig.2. Its usual am is to produce either a discrete or a continuous digital electronic signal that is proportional to a single analyte or a related group of analytes. [Setford *et al.* (2004).]

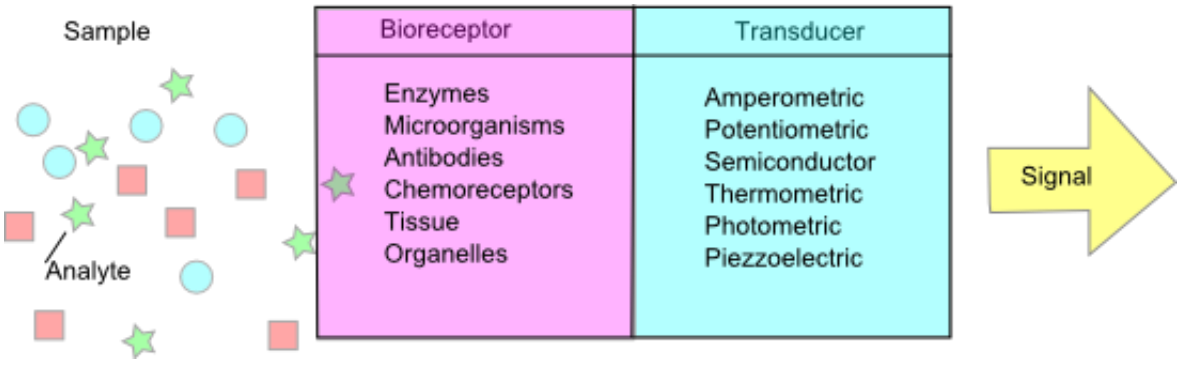


Figure 1: Biosensor schematic principle

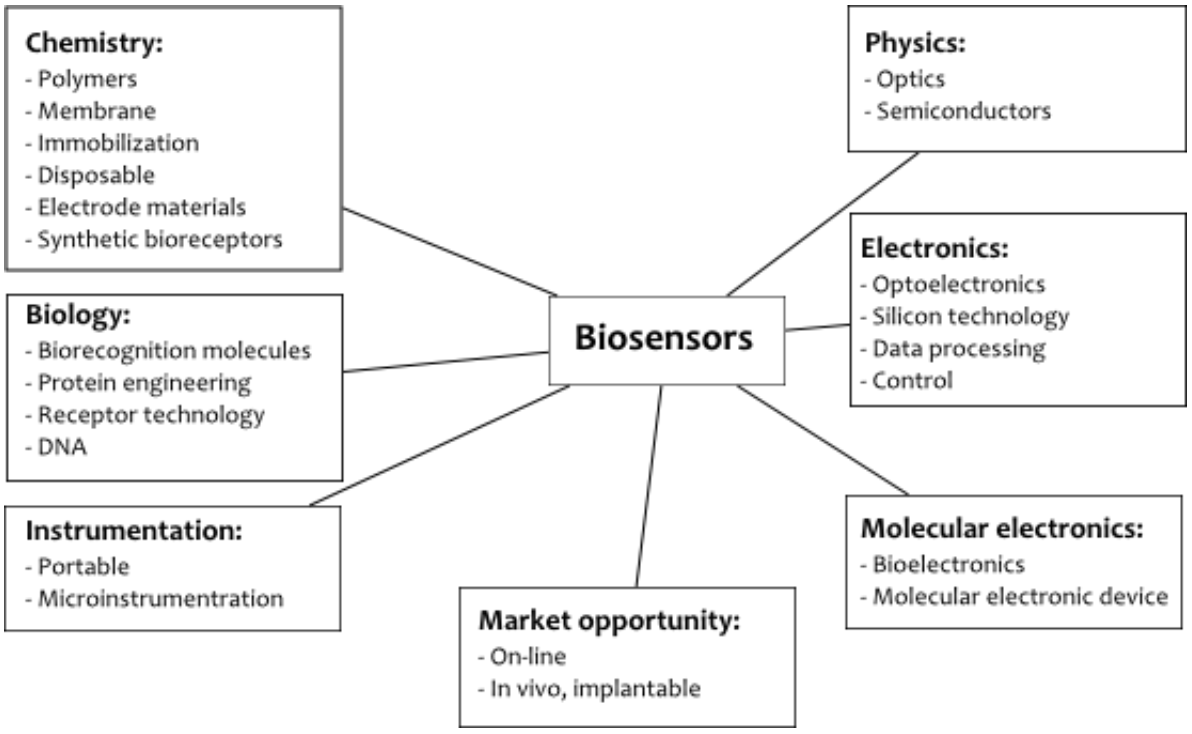


Figure 2: Multidisciplinary nature of biosensors

2.1.1.1. *Commercial importance*

Biosensors have many advantages, such as simple and low-cost instrumentation, fast response times, minimum sample pre-treatment, and high sample throughput. Increased research in this area demands the development of novel materials, new and better analytical techniques, and new and improved biosensors. [Andersen *et al.* (2004).]

It is estimated that the market size for worldwide biosensors at year end 2003 was about $7.3 billions. In the US alone, the market for biosensors in 2002 was estimated at $563 millions. Even with unfavorable geopolitical events occurring and a weak global economy, the market is projected to improve and grow to about $10.8 billions in 2007, with a growth rate of about 14% per annum. [www.fuji-keizai.com]

United States and Europe dominate the global market for medical biosensors, collectively capturing 69.73% share estimated in 2008. The market in Asia-Pacific is projected to reach $794 million by the year 2012. Sales in United States, the largest market for Glucose biosensors are expected to reach $1.28 billion by 2012. In Europe,

Germany, United Kingdom and France, together, collar 55.3% of the biosensors market estimated in 2008. Revenues in the German Environmental Biosensors market are projected to climb at the fastest rate to reach $32.7 million by the year 2015. [www.strategyr.com Karube (2003).]

This development is sustained by the collaboration from many areas of academia and industry; it finds application in 5 main fields:

- Bio/Pharmaceutical research,
- BioDefense,
- Food and beverage,
- Environment,
- Medical.

Some of the potential applications of biosensors are agricultural, horticultural and veterinary analysis; pollution, water and microbial contamination analysis; clinical diagnosis and biomedical applications; fermentation analysis and control; industrial gases and liquids; mining and toxic gases; explosives and military arena; and flavors, essences and pheromones. [www.fuji-keizai.com Marty *et al.* (1995). Radke (2003). Rogers (1995). Rodriguez-Mozaz *et al.* (2005). Scheller *et al.* (2001).]

2.1.1.2. *A brief history of biosensors*

The concept of a biosensor was developed from Prof. Leland C. Clark Jr., with his paper on the oxygen electrode in 1956. In 1962, he described how to make electrochemical sensors more "intelligent" by including an "enzyme transducer as membrane-enclosed sandwiches". Clark and Lyons followed the decrease in concentration of glucose oxidase (GOx) co-substrate, oxygen. [Setford *et al.* (2004). Lee (1998).]

The measurement of enzymatically generated hydrogen peroxide was first demonstrated in 1972 by Yellow Springs Instruments, U.S.A.. Subsequently, glucose biosensors were launched commercially in 1975, based on the amperometric detection of hydrogen peroxide, using glucose oxidase. In 1984, with the use of ferrocene and its derivatives as mediators for use with oxidoreductases, a way to produce inexpensive

enzyme electrodes, based on screen-printed electrodes (SPE), was developed. Since 1988, glucose oxidase has been studied using different solvents, for example in ethanol and with a range of transducer such as calorimetric and optoacoustic enzyme measurement. [Kröger (1997). Kissinger (2005).]

2.1.2. Main classes of biosensors

There are 3 main classes for biosensors: metabolic or catalytic, affinity and inhibition sensors. The type of interactions there are between the analyte and the biological component determines these classes. Two classes are using bio-recognition processes, namely bio-affinity recognition and bio-metabolic recognition. [Diamond (1998). Gauglitz *et al.* (1993). Kreuzer *et al.* (2001). Newman *et al.* (1995). Setford *et al.* (2002).]

2.1.2.1. Metabolic biosensors

Bio-recognition processes involve the binding of a chemical species with another, which has a complementary structure. This is referred to as shape-specific binding. In bio-metabolic recognition, the analyte and other co-reactants are chemically altered to form the product molecules.

In other terms, the metabolic biosensors rely on the ability of an organism or factors relating to an organism (for example, an enzyme) to use an analyte as a substrate. The biomaterials that can be recognized by the bio-recognition elements are as varied as the different reactions that occur in biological systems. The concept of shape-specific recognition is commonly used to explain the high sensitivity and selectivity of biological molecules.

2.1.2.2. Affinity biosensors

In bio-affinity recognition, the binding is very strong, and the transducer detects the presence of the bound receptor-analyte pair. The affinity sensors are based on receptors molecules specifically recognizing and binding an analyte.

The most common types of processes are receptor-ligand and antibody-antigen binding. The analyte molecule has a complementary structure to the antibody, and the bound pair is in a lower energy state than the two separate molecules. This binding is very difficult to break. [Young *et al.* (2001).]

2.1.2.3. *Inhibition biosensors*

The inhibition biosensors are biological elements ranging from whole cells to isolated enzymes, associated with a transducer able to detect a decrease in biological activity (for example, growth, photosynthesis, activity, respiration). Enzymes are very often used for this class, where their activity is inhibited by the analyte. [Wu *et al.* (2004).]

Inhibition biosensors find many applications in environmental applications. One common example is the detection of heavy metals or pesticides. [Turner (1989; 1991; 1992; 1993; 1995) Wang *et al.* (2005).]

2.1.3. Evolution of the biosensors

The evolution of biosensors can be broken down in three distinct stages, defining the corresponding so called biosensor "generations". [Dong and Chen (2002). Karube and Yokoyama (1993).] There are three generations of biosensors: First generation biosensors where the normal product of the reaction diffuses to the transducer and causes the electrical response, second generation biosensors which involve specific "mediators" between the reaction and the transducer in order to generate improved response, and third generation biosensors where the reaction itself causes the response and no product or mediator diffusion is directly involved. (Cf. Fig.3) [Wilkins and Atanasov (1996).]

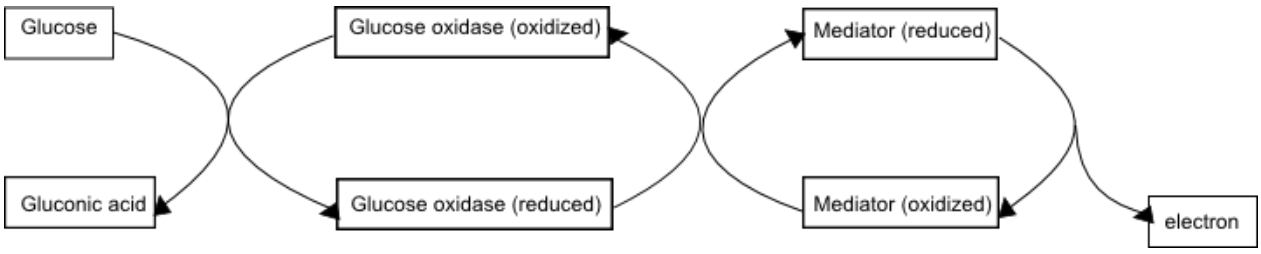


Figure 3: Mediator-based glucose biosensors mechanism

2.1.3.1. *First-generation biosensors*

One type of first-generation devices relied on the production and detection of hydrogen peroxide, as shown Equ.1. Hydrogen peroxide is a commonly detected analyte for biosensors, analytical methods for its detection including electrochemistry, chromatography and spectrophotometry. It is a substrate for peroxidases and the product of many enzymatic reactions.

$$H_2O_2 \rightarrow O_2 + 2H^+ + 2e^-$$

Equation 1: Detection of hydrogen peroxide on first-generation biosensors

The direct electrochemical detection of hydrogen peroxide is very attractive in its simplicity, but it requires a high over-potential for the oxidoreduction reaction to occur as to be prone to interferences; other coexisting species are electroactive at such potentials, like ascorbic acid.

The use of permselective coatings or membranes minimizes the access of such components to the transducer surface, restoring selectivity and enhancing stability by excluding surface-active macromolecules. The use of mediators, whether soluble or immobilized, allows the application of lower potential. Metalized carbon (especially rhodinized carbon) and metal-hexacyanoferrate based transducer effectively enhances the selectivity at glucose and allowing reactions to occur at significantly lower potentials. [Kröger (1997). Wang (2001).]

2.1.3.2. *Second-generation biosensors*

A major advance was achieved by replacing oxygen with a non physiological electron acceptor, in order to enhance the electron transfer between the oxidase enzyme and the electrode surface. Glucose oxidase, for example, allows no direct electron transfer, due to its thick protein and carbohydrate layer surrounding the flavin redox centre, this shell introducing a spatial separation of the electron donor-acceptor pair. It's important to minimize the electron transfer distance to ensure optimal performances.

The use of an artificial mediator is shown in the schematic below (Fig.4; where M stands for mediator), allows electrons to be shuttled between the flavine adenine dinucleotide (FAD) centre and the electrode surface, producing a current dependant of the glucose concentration. Diffusional electron mediators such as ferrocene derivatives, ferricyanide and conducting organic salts (for example tetrathiafulvalene-tetracyanoquinodimethane (TTF - TCNQ)) have been widely used. They also allow the experiment to be carried out at lower potentials reducing interference reaction. [Wang (2001).] An alternative approach may be the use of electrocatalysts, material reducing the redox potential of electroactive species of interest.

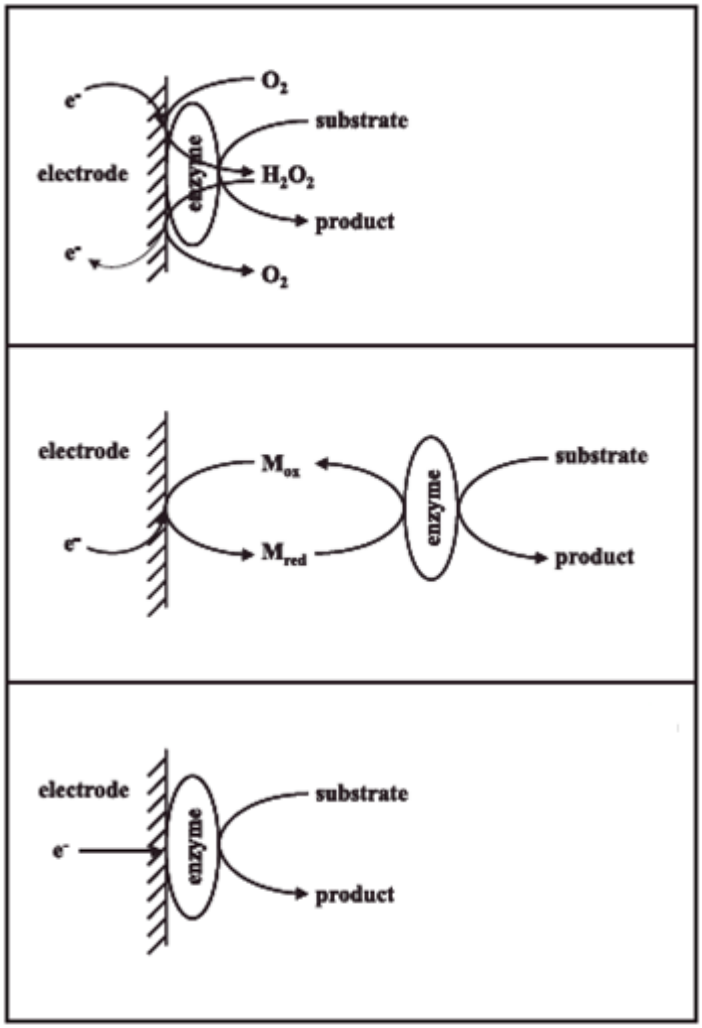


Figure 4: Different biosensor generations

However, the mediator itself may induce interferences: for example, ferrocene is reduced by ascorbic acid. The mediator prussian blue (PB), or ferric hexacyanoferrate, displays an effective electrochemical selectivity to hydrogen peroxide. [Ricci *et al.* (2003). Garcia Armada *et al.* (2003).]

A good mediator must fill in the following characteristics:

- React quickly with the enzyme,
- Exhibit reversible electrochemistry,
- Have good electrochemical properties,
- Have low solubility in aqueous solutions,
- Be non toxic (especially for in vivo systems),
- Be chemically stable.

Another method involves attaching electron transfer relays to the enzyme. It is based on chemical modification of the glucose oxidase with electron-relay group such as poly-pyridine polymer having a dense array of osmium-complex electron-relay, also known as molecular wires. It has lead to layer-by-layer (LbL) glucose oxidase / mediator network that also may be linked to functionalized gold electrode surface. [Wang (2001)]

2.1.3.3. *Third-generation biosensors*

In the third generation biosensors the electron transfer is associated with, or occurs during, the catalytic transformation of the substrate to the product. The redox enzyme acts as an electrocatalyst, facilitating the electron transfer between the electrode and the substrate molecule involving no mediator in this process. Thus, this kind of biosensor usually offers better selectivity, because they are able to operate in a potential range closer to the redox potential of the enzyme itself, becoming less exposed to interfering reactions. The higher integration between the biomolecule and the electrode surface can also improve the sensitivity of this kind of biosensor. Recently, a lot of studies have been carried out on the development of electron transferring interfaces between redox enzymes and electrodes to apply them as high-performance amperometric biosensors. Another attractive feature of the systems based

on direct electron transfer is the presumable simplicity of construction of the enzyme based amperometric devices. [Malhotra *et al.* (2005).]

One of the major obstacles to be overcome in the construction of third generation biosensors is how to optimize the electron transfer between the enzyme and the electrode. The best electron transfer mechanism in an amperometric biosensor is direct electrochemical recycling of the prosthetic group of the enzyme at the electrode surface involving an electron tunneling mechanism. Unfortunately, the distance between the prosthetic group and the electrode surface is often rather long for direct electron transfer, due to shielding by the protein shell, and electron transfer via a tunneling mechanism is therefore rarely encountered. Thus, the main aim in the design of optimized amperometric biosensors is to provide fast electron transfer processes based on electrode architectures with predefined electron transfer pathways interconnecting the redox site within the enzyme and the electrode surface. In this way, an optimally designed electrode configuration has to ensure that the electron transfer distance between an immobilized redox biomolecule and a suitable electrode surface is made as short as possible. Moreover, the immobilized biomolecule must have an appropriate orientation, which also should facilitate communication between the active center of the biomolecule and the electrode surface (cf. Fig.5). Thus, the performance of electron transfer depends strongly on the immobilization procedure. [Malhotra *et al.* (2005).]

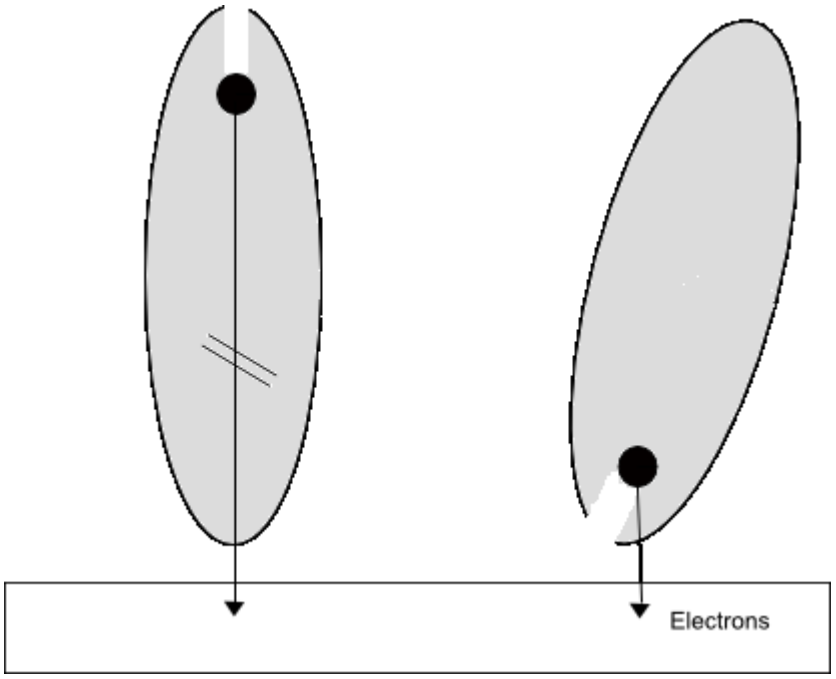


Figure 5: Effect of immobilized enzyme orientation on direct electron transfer

Although third generation biosensors present favorable characteristics, only a few groups of enzymes or proteins were found to be capable of interacting directly with an electrode while catalyzing the corresponding enzymatic reaction. Depending on the practical significance of the substrates of these enzymatic reactions, electroanalytical applications of bioelectrocatalysis began to appear in the late eighties. Later publications on this topic have reported use of heme containing peroxidases, for which the electrode works as an electron donor to oxidize peroxidase. Third generation biosensors are today still hardly reported, even though the number of examples is increasing each year, mostly focused on peroxidase, lactase, multi-cofactor enzyme and heme containing protein. [Gavrilescu *et al.* Willner and Willner (2001).]

2.2. Different biosensor configurations

Another way to characterize biosensors is through their various configurations, namely the association of a recognition system and a transduction system. These categories are shortly presented here.

2.2.1. Recognition systems

The biological components used for detection are usually selected from one of 6 main different types (cf.Fig.6):

- Enzyme (the most widely used),
- Antibodies (Ab),
- DNA,
- Entire cells or organisms,
- Cell fragments,
- Tissues (both animal and plant-based).

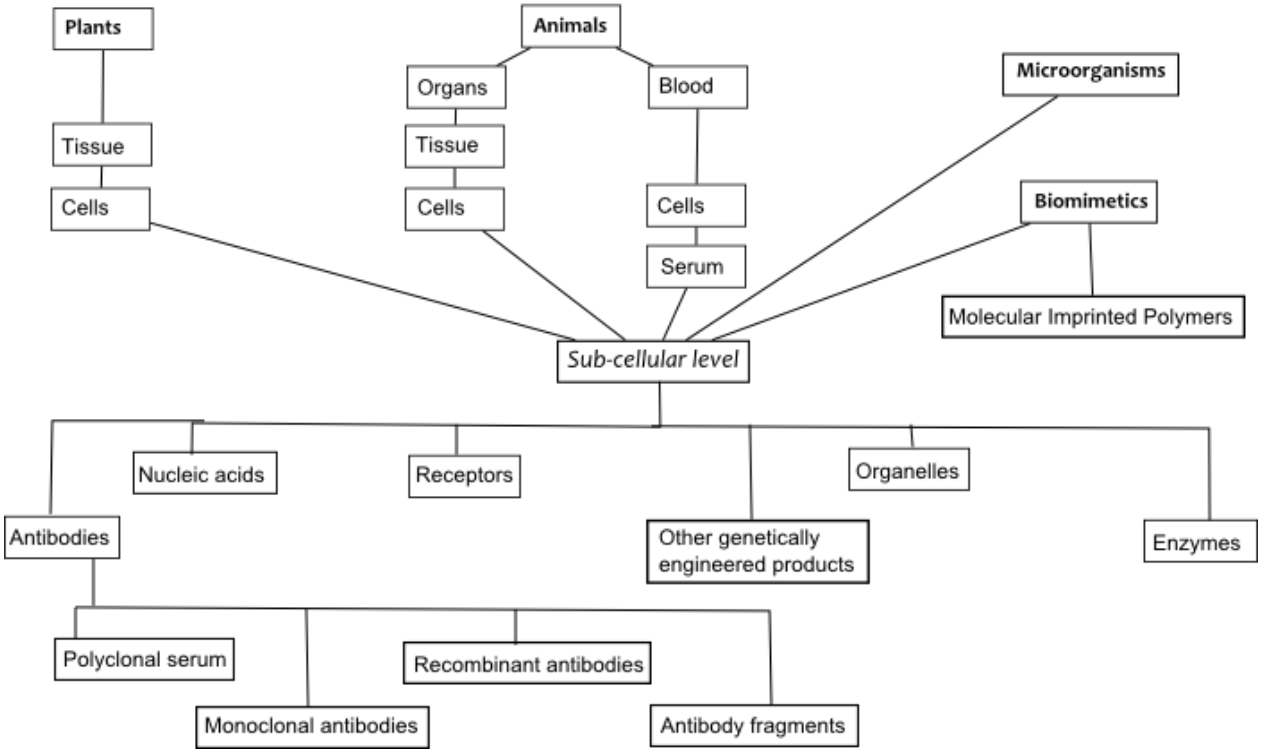


Figure 6: Detection systems for biosensors

The lifetime of these biological components is highly dependent on how they can be maintained and restored. Enzymes are the most commonly used biological elements; when retained in their natural environment, they show enhanced stability compared with the purified enzyme. The capacity of the biological component to maintain its ability to interact with the substrate (for example, the catalytic activity for

an enzyme), between manufacturing and use, is the storage stability or shelf life of the sensor. The maintenance of optimum activity during a process is its operational stability.

The first main difficulty in developing a biosensor is to find the right association of biological compound/substrate, for the required range of target analyte concentrations. Consideration must also be given to that its catalytic activity amplifies the signal in enzymatic systems, whereas DNA- or antibody-based systems have no catalytic effect, but form very strong bonds with the substrate, limiting the sensitivity while enhancing the selectivity of the biosensor. These systems generally require the addition of a tracer compound to visualize the extent of binding of the analyte. [Lee (1998). Guilminot *et al.*]

2.2.2. Transduction systems

The transducer is a physicochemical device and is usually based on one of 5 different principles:

- Electrochemical (AC impedance, Clark Electrode [Kwan *et al.* (2004).], Mediated Electrodes, Ion-Selective Electrodes, Field-Effect Transistor – ENFET – [Luo *et al.* (2004).]),
- Optical (Photodiodes, Waveguide systems, Integrated Optical devices, Evanescent Wave [McCormack *et al.* (1997]. Leatherbarrow and Edwards (1999).]),
- Piezoelectric (Quartz Crystals [Karousos *et al.* (2002).], Surface Acoustic Wave[Ho (1984). Jordan (1988).]),
- Calorimetric (Thermistor, Thermopile),
- Magnetic.

Its role is to convert the biochemical response into a signal that can be amplified and transformed in an electrical signal. Electrochemical biosensors use several measurement systems such as amperometry, conductimetry and potentiometry. Optical transducers, for their part, utilize fluorescence, chemiluminescence, colorimetry, evanescence waves. Other devices may use microbalances or semi-

conductor based systems (cf. Fig.7). [Lee (1998). Guilminot, E *et al.* Hahn (1988). Pickup *et al.* (2005). Ko (1994).]

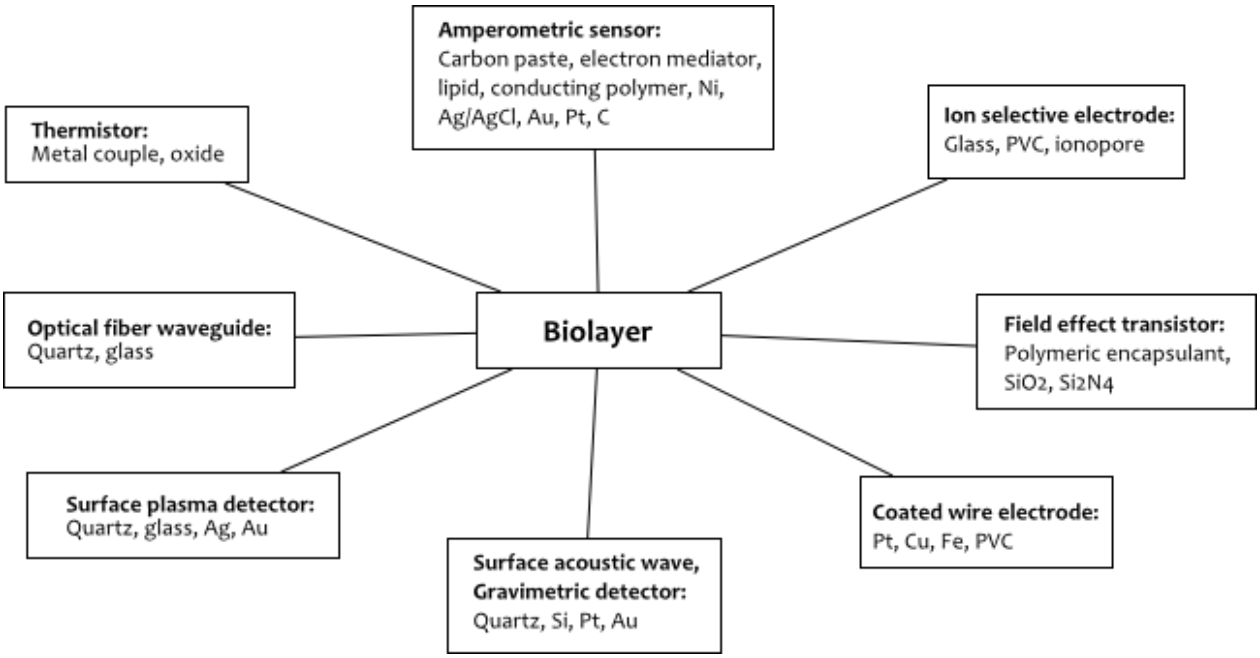


Figure 7: Various biosensor configurations

2.3. Enzyme-based biosensors

The first and main biosensor system that was studied was an enzyme-based biosensor. Here are presented the various components considered during this study. [Sokolov and Neykov (1995).]

2.3.1. Enzymes: a brief overview

There are many reasons for studying enzymes; their biological and chemical roles, medical, therapeutic and industrial uses, and their use as tools in molecular biology, to mention a few. Essentially, all life processes are controlled by enzymes and depend on them.

The first clear realization of the existence of enzymes came in 1879 by E. Buchner; as is so often the case it was a matter of serendipity. Until that time, it was believed that only living cells could be biochemically functional. Buchner realized living cells were not required for carrying out metabolic processes. Instead some smaller entities must be present. A major conceptual breakthrough came a couple of decades later when Emil Fisher proposed the "lock and key" model, where the substrate is analogous to the key and fits into the enzyme the same way a key fits into a lock. The first enzyme to be isolated in pure crystal form was urease from jack beans in 1926 by J.B. Summer.

2.3.1.1. Presentation

Enzymes are proteins, whose role is that of biological catalysts. They may be single polypeptide chains, or oligomers, of several subunits and often contain a prosthetic group, or cofactor. They speed the rate of attainment of equilibrium by lowering the energy barrier (ΔG, Energy of activation) between the substrate and the products. The enzyme is not used in the reaction but is regenerated. They are very efficient and also highly specific catalysts. A given enzyme will only catalyse one type of reaction for one type of compound. They also are stereospecific, and most enzyme reactions occur within a relatively narrow conditions range of temperature and pH.

Enzymes are subject to different regulatory controls.

- The rates of most enzymes are responsive to changes in substrate concentration because the intracellular level of many substrates is in the range of the enzyme K_m. Thus, an increase in substrate concentration increases the reaction rate, returning the substrate concentration towards normal.
- Another is product inhibition where, if the product accumulates, can inhibit some enzymes, limiting the rate of formation of the product when it is underused.
- The activity of enzymes catalyzing key regulatory reactions of metabolic pathways is often subject to allosteric regulation. The enzyme activity is modulated by the binding of allosteric effector (activators or inhibitors) to a site distinct from the active site. Feedback inhibition is here a negative modulation of the committed step of a metabolic pathway by its end product, preventing the production of an excess of end product by shutting down the pathway.
- Another important way is through covalent modification. For example, phosphorylation of a specific amino acid, usually serine (Ser), tyrosine (Tyr) or threonine (Thr) enhances or depresses activity. This modification is totally reversible, as is nuclotidylation, where the activity of the enzyme is regulated by the addition of a nucleotide to a specific amino acid.

The enzyme carries out its role, catalysis, in a specific region of the molecule, the active site. It usually is a pocket on the surface of the enzyme, involving only a small fraction of the molecule. As per the "lock and key" model, it is complementary to the substrate shape and polarity. It contains binding sites for the substrate as well as catalytic groups, the reactive side chains carrying out the reactions involved. The rest of the protein provides a structure to position substrate and catalytic groups, as well as a mean for regulatory control mostly. The enzyme localization is also a key factor in the tridimensional stabilization of its structure, although ions are sometimes necessary to help the enzyme maintain its conformation.

2.3.1.2. *Naming convention*

By common convention, an enzyme's name consists of a description of its function, with the word ending in "-ase". A nomenclature was developed for enzymes by the International Union of Biochemsitry and Molecular Biology, the EC numbers. The first number broadly classifies the enzyme based on its mechanism, thus defining the 6 main types of enzyme functions:

- EC 1 Oxidoreductases: They catalyze oxidation/reduction reactions
- EC 2 Transferases: They transfer a functional group (e.g., a methyl or phosphate group)
- EC 3 Hydrolases: They catalyze the hydrolysis of various bonds
- EC 4 Lyases: They cleave various bonds by means other than hydrolysis and oxidation
- EC 5 Isomerases: They catalyze isomerization changes within a single molecule
- EC 6 Ligases: They join two molecules with covalent bonds

For example, Glucose oxidase is also known as EC 1.1.3.4. EC 1 because it is an oxydoreductase, .1 because it is acting on the CH-OH group of donor, .3 because it is with oxygen as acceptor and finally .4 as it is fourth on the list in that specific category. [www.brenda-enzymes.org]

2.3.2. Choosing the systems studied

2.3.2.1. *Glucose biosensors*

a. Glucose

This small molecule (βD-Glucose: $C_6H_{12}O_6$, Molecular Weight: MW = 180.2) is of great importance within the living biosphere. It is the first of the carbohydrates to be oxidized, thereby producing energy, through glycolysis (Embden and Meyerhof way) and thereafter via the Krebs Cycle.

Glucose circulates within the human body through blood, its concentration being regulated by the hormone insulin. A lack of control on the release of insulin is at the origin of diabetes, one of the most widely spread diseases and a worldwide health problem. Diagnosis and management of diabetes requires a tight monitoring of blood glucose levels, glucose biosensors being thus the target of substantial research. [Vogel *and* Angermann (1994). Pickup *et al.* (2005).]

i. Interaction with enzymes

Several enzymes catalyze the conversion of glucose. These can be separated in 4 categories:

- Glucose dehydrogenase (GDH),
- Quinoprotein glucose dehydrogenase,
- Glucose 2-oxidase,
- Glucose 1-oxidase (GOx).

The latter is however the most widely used enzyme for biosensor application, because of its high specificity for glucose and of it being very close to an ideal enzyme for biosensors (e.g. high specificity, high turnover, high stability and low production costs). The first enzyme (glucose dehydrogenase, GDH) requires a soluble cofactor (a non peptide molecule capable of complexing the binding site for the transition state), whereas the second enzyme is very unstable and the third oxidizes other carbohydrate as well as glucose. [Wilson *and* Turner (1992). Florescu and Brett (2005).]

ii. Mutarotation

In aqueous solution, glucose is present in different forms at equilibrium. At 293K, if left a few hours to mutarotate, those different forms will be present at the percent of concentration shown in Fig.8.

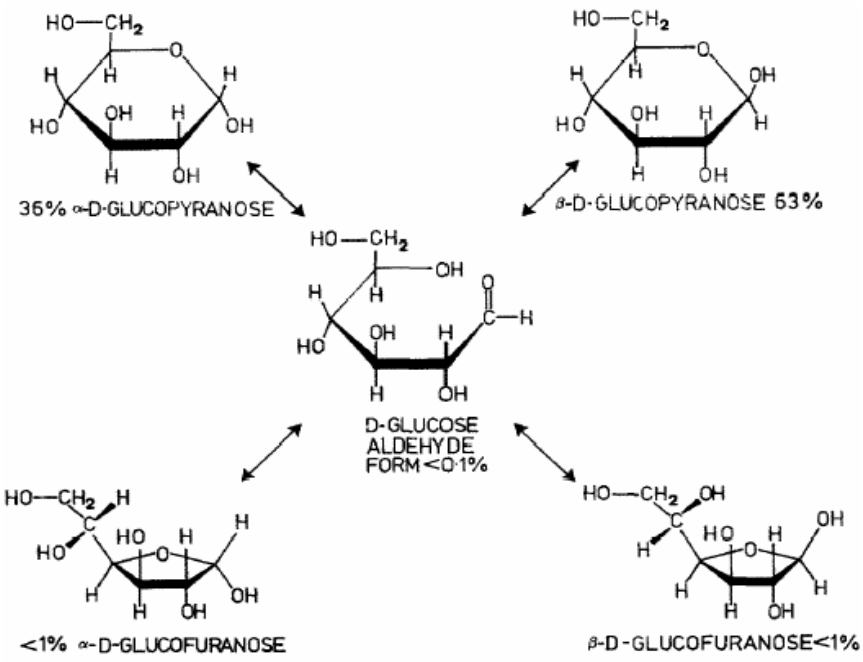


Figure 8: Different forms of glucose reaching equilibrium in water at 293K

It can be seen that αD-glucopyranose and ßD-glucopyranose are the predominant forms. As Glucose Oxidase is more active with the βD-glucopyranose, freshly prepared glucose solutions are left to mutarotate before use. The mutarotation of glucose is catalyzed by the enzyme mutarotatose and by phosphate anions. The equilibrium is reached within a few hours in buffer solutions instead of several days when phosphate anions and/or mutarotatose are absent. [Wilson *and* Turner (1992).]

b. Glucose 1-oxidase

i. Presentation of the oxidase enzyme

Oxidoreductases have widespread applications, especially in diagnosis, where their biochemical specificity is combined with either photometric or electrochemical transduction systems for sensitive and reliable assay procedures. They also find use as labels in immunoassay systems, and play an important role in the synthesis and stabilization of food supplements.

Oxidase enzyme conforms to the following reaction pathway, under classical enzyme kinetics, as shown Equ.2.

$$Substrate_{(ox)} + O_2 \xrightarrow{oxidase} Substrate_{(red)} + H_2O_2$$

Equation 2: Typical oxidase enzyme reaction pathway

Photometric transduction system is based on changes in the optical properties of the sample solution, due to the oxidation of an added dye, through peroxidase enzyme mediation. Electrochemically, the peroxide pre-product is also used, by monitoring the current generated by oxidation of peroxide at an electrode poised at a suitable potential. There are several ways for measuring the glucose oxidase activity: Measurement of oxygen consumption by an oxygen sensor, measurement of gluconic acid by a pH sensor and measurement of the production of hydrogen peroxide by a peroxide sensor. [Kröger *et al.* (1998).]

ii. Presentation of glucose oxidase

Glucose oxidase (βD-glucose oxygen 1-oxydoreductase) is classified under the denomination E.C.11.3.4. : Enzyme, oxidoreductases, acting on the CH-OH group of donors, with oxygen as acceptor, glucose oxidase. It was first discovered by Mueller in 1928 and can be purified from different sources, including red algae, citrus fruit, Penicillium amagakiense or Talaromyces flavus. Most commonly, it is obtained from Aspergillus Niger.

The enzyme is commercially used for the production of gluconic acid and food preservation. It is also the most commonly applied biocatalyst for enzyme based biosensors; the relevant literature is extensive and steadily increasing. [Kröger (1997). Jawaheer *et al.* (2002).]

iii. Structure and physical properties

Glucose oxidase is a slightly elongated globular protein of a known amino acid sequence of 583 residues. It has a diameter of 8nm, a specific volume of 75mL/g and its molecular weight average is 155±5kDa. 0.3g of water are associated with 1g of dry weight protein.

Glucose oxidase is a dimer composed of 2 identical subunits. The N-terminal regions are similar to the glutathione reductase, which bonds the adenine monophosphate (AMP) region of flavin adenine dinucleotide (FAD), to which electrons

from oxidoreductase activities are transferred. It also includes 2 disulphide bridges, which are not involved in maintaining the structure, as there is one in each monomer. It is also to be noted that each monomer contains a free cystein. The monomer folds into two structural domains, one of the domains binds flavin adenine dinucleotide and the other is involved with substrate binding. As with many oxidases, the active site of the enzyme (the flavin ring system) is located deep inside the bulk of the enzyme, near the bottom of a cavity, and is well protected by a glycoprotein shell. [www.biol.paisley.ac.uk]

As for the secondary structure of glucose oxidase , it is known that 28% is in the form of an α helix, and 18% as a sheet The tertiary structure of the enzyme is characterized by two separate and distinctly different β-sheet systems, one of them forming part of the flavin adenine dinucleotide binding domain. It is a branched glycoprotein, containing 10 to 17%carbohydrates, mainly D-mannose (14% of the enzyme by mass), D-glucosamine (3%), D-galactose (0.3%). The structure may also contain some glucose.

Glucose oxidase is an anionic enzyme at physiological pH. The carbohydrate components form a branched polysaccharide, partly surrounding a protein core. These components are not involved in catalysis, but can influence the enzyme stability: Increases in carbohydrate levels lead to an increase in stability. Carbohydrates are also responsible for many of the enzyme's physical properties, but they form a barrier to the transfer of electrons in amperometric biosensors, hence leading to the development of indirect detection techniques. [Wilson *and* Turner (1992).]

iv. Stability and inhibitors

When lyophilized, glucose oxidase is very stable (over 2 years at 0°C). In solution, its stability is directly dependant of the pH, with pH=5.6 having the highest stability. Its pI is 4.2. [www.sigmaaldrich.com] For pH < 2 or pH > 8, glucose oxidase catalytic activity decreases quickly. For example, at pH = 8.1, only 10% of its activity remains after 10 min. However, the rate of inactivation at higher pH is reduced if glucose is added to the solution. Glucose oxidase is also very unstable at temperature higher than 40°C, due to its low enthalpy of denaturation.

Glucose oxidase is inhibited by micromolar amounts of heavy metal. It is inhibited by millimolar amounts of hydrazine, phenylhydrazine, hydroxylamine, hydroxyquinoline, sodium nitrate, semicarbazide and aldohexoses, the latter behaving as competitive inhibitors. Halide ions at low pH also inhibate glucose oxidase, 0.1M KCl completely inhibiting glucose oxidase at pH = 3. [Wilson *and* Turner (1992)]

v. Reaction mechanism

Values for the Michaelis constant (Km) for glucose oxidase with glucose lie around 4mM and with oxygen around 0.25mM. The overall reaction, presented Equ.3, of glucose oxidase with glucose and oxygen is shown Fig.9 and involves the oxidation of the flavin adenine dinucleotide structure for the glucose oxidase (cf. Fig.10).

$$C_6H_{12}O_6 + O_2 \xrightarrow{GOx} C_6H_{10}O_6 + H_2O_2$$

Equation 3: Glucose oxidase reaction pathway against glucose

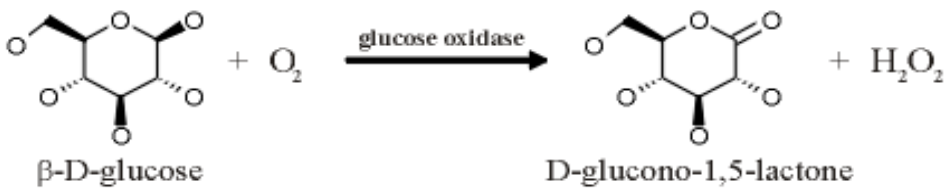


Figure 9: Glucose oxidation by glucose oxidase

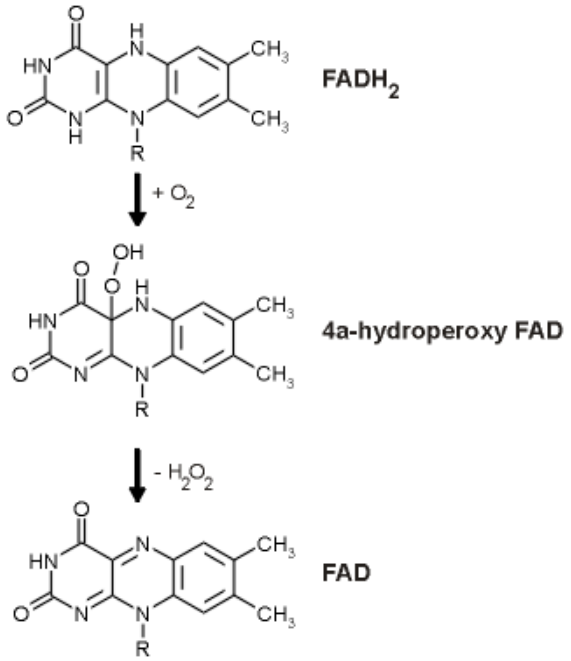


Figure 10: Mechanism of the flavin adenine dinucleotide oxidation by oxygen

The enthalpy variation associated with this reaction is sufficient to allow thermometrical detection. The use of catalase, that disproportionates hydrogen peroxide, enhances the sensitivity of thermometric glucose biosensors.

The initial product of the reaction is D-gluconolactone, a weak inhibitor of glucose oxidase, which hydrolyzes spontaneously to gluconic acid (cf. Fig.11). At pH = 8.0, this reaction occurs with a half-life of 10 min, whereas at pH = 3.0, it is significantly longer. The decrease of pH associated with the formation of gluconic acid is noticeable enough for its use in potentiometric and colorimetric biosensors. [Wilson *and* Turner (1992).]

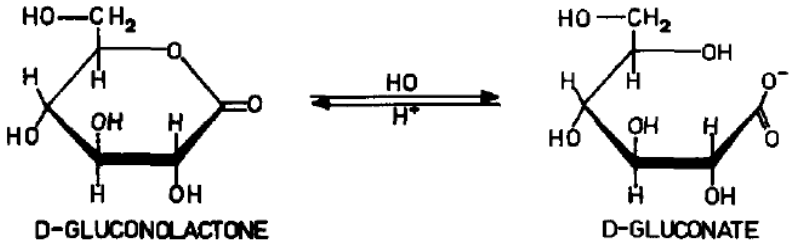


Figure 11: Hydrolysis of gluconolactone into gluconic acid

vi. Flavin adenine dinucleotide

Flavin adenine dinucleotide and riboflavin-5'-phosphate are perhaps the most versatile of all the redox coenzymes. Flavins are usually stronger oxidizing agents than nicotinamide adenine dinucleotide, a property that fits them for a function in the electron transport chain of mitochondria where a sequence of more and more powerful oxidizing agents is needed. Furthermore, flavin can be reduced by either one or two electron processes. This enables them to participate in oxidation reactions involving free radicals and in reactions involving metal ions. Finally, reduced flavins can be reoxidized by molecular oxygen (e.g. as in the case of glucose oxidase). This autooxidisability allows some enzymes to pass electrons directly to oxygen and also provides a basis for the functioning of flavins in hydroxylation reactions. Its structure is presented Fig.12.

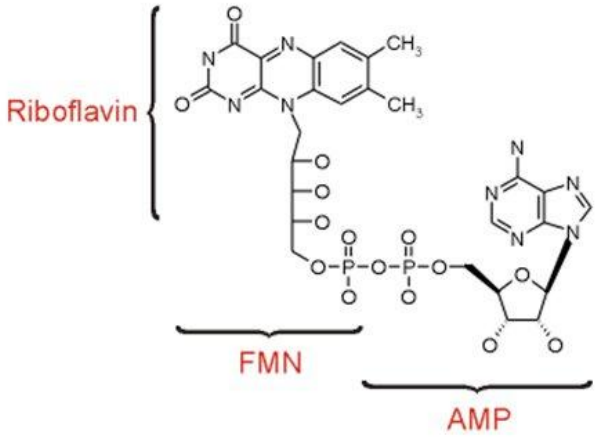


Figure 12: Chemical structure of the coenzyme flavin adenine dinucleotide. Riboflavin (vitamin B2) consists of the sugar alcohol D-ribitol attached to 7,8-dimethyl-isoalloxazine

Flavin coenzymes are usually tightly bound to proteins and cycle between the reduced and oxidized state while remaining attached to the same protein molecule. Depending upon the nature of the flavoprotein, the redox potential of flavin adenine dinucleotide will vary. The redox potentials of flavins spans the range (-0.49 to 0.19 volts). Thus, flavoproteins can take part in a diversity of biochemical reaction.

- Oxidation of hemiacetals to lactones (e.g. glucose oxidase)
- Oxidation of alcohols to aldehydes (e.g. glycolate oxidase)
- Oxidation of amines to imines (e.g. amino acid oxidase)

- Oxidation of NADH or NADPH to NAD^+ or $NADP^+$ (e.g. diaphorase)
- Oxidation of carboxylic acids to α,β-unsaturated carbonyl compounds (e.g. succinic dehydrogenase)

2.3.2.2. *Other enzyme sensors*

Glucose biosensors are widely studied and rather well known. However, the glucose oxidase based systems are also very stable and straightforward. Therefore it was interesting to look at other enzyme systems that would be either less stable or would require a co-factor to work.

a. Alcohol oxidase

i. Presentation

Alcohol oxidase (EC.1.1.3.13, AOx) belongs to oxidoreductases acting on the CH-OH group of donors and using oxygen as acceptor. Flavin adenine dinucleotide is a cofactor for this enzyme. Alcohol oxidase reacts with primary alcohols to form aldehydes, using the following pathway. (cf. Equ.4) [www.ebi.ac.uk]

$$R - CH_2OH + O_2 \rightarrow R - CHO + H_2O_2$$

Equation 4: Alcohol oxidase reaction pathway

Alcohol oxidase is an octamer of 600kDa. Each subunit weights 74kDa and is associated with a flavin adenine dinucleotide structure. It can be obtained from different sources, such as Candida sp., Hansenula sp. Poria sp. or Basidiomycete sp. .The alcohol oxidase used for this project was provided as vacuum-dried powder by Sigma-Aldrich and obtained from Hansenula specie, from its peroxisome, as alcohol oxidase from this origin has been found in literature to give better results in enzyme activity, sensibility and sensitivity. It is to be noted that enzymes commercially obtained from suppliers have been quite severely stressed in their preparation and are often delivered prestabilized, usually with dextran. Therefore, the enzyme studied is not quite as optimum, stability wise as a fresh enzyme would be. However, it is the fastest and most convenient way to obtain enzymes and the study was therefore done using enzymes obtained from suppliers, usually Sigma Aldrich. [www.brenba.uni-koeln.de. Patel *et al.* (2001).]

Alcohol oxidase belongs to one out of the 3 groups of enzymes oxidizing primary alcohols. Those groups are defined by the coenzyme employed:

- Flavin adenine dinucleotide -dependant enzymes (of which alcohol oxidase is a member),
- Nicotinamide adenine dinucleotide (NAD(P)) - dependant alcohol dehydrogenase,
- Nicotinamide adenine dinucleotide (NAD(P)) - independent alcohol dehydrogenase. [www.ub.rug.nl]

ii. Stability and inhibitors

Alcohol oxidase is irreversibly inhibited or completely inactivated by 1,4-butynediol and KBr + urea. Cyclopropanone acts as suicide substrate with alcohol oxidase. The enzyme is also inhibited by Ag^{+}, Cu^{2+}, Cl^{-}, hydroxylamine, KCN, propynal and cyclopropanol at various degrees. [www.brenba.uni-koeln.de. www.biocyc.org.1555]

The optimum pH for alcohol oxidase is 8.5; at 9.8 or 6.7, the remaining activity of the enzyme is half the maximum activity. In the dry state, alcohol oxidase can be stored over 6 months at 4°C without any significant loss of activity. Solutions at 56°C can't be maintained more than 30 min. [www.aetltd.com]

The comparative alcohol oxidase activity with ethanol (EtOH) is 97.2% of that with methanol (MeOH), ethanol being converted into acetaldehyde and methanol into formaldehyde. Alcohol oxidase encounters a substrate inhibition with methanol, thus explaining the choice of ethanol as substrate for the oncoming studies. [www.brenba.uni-koeln.de]

Alcohol biosensors find many useful applications. They are used for alcohol detection by the police. They are also widely used in the wine industry, to monitor wine fermentation.

b. Acetylcholine esterase

i. Acetylcholine – Ach

Nerves communicate with one another and with muscle cells by using neurotransmitters. These are small molecules that are released from the nerve cell and rapidly diffuse to neighboring cells, stimulating a response once they arrive. Many different neurotransmitters are used for different jobs: glutamate excites nerves into action; GABA (γ-aminobutyrique acid) inhibits the passing of information; dopamine and serotonin are involved in the subtle messages of thought and cognition.

The main job of the neurotransmitter acetylcholine (Ach, structure Fig.13) is to carry the signal from nerve cells to muscle cells. When a motor nerve cell receives the correct signal from the nervous system, it releases acetylcholine into its synapses with muscle cells. There, acetylcholine opens receptors on the muscle cells, triggering the process of contraction. Of course, once the message is passed, the neurotransmitter must be destroyed; otherwise later signals would get mixed up in a jumble of obsolete neurotransmitter molecules. The cleanup of old acetylcholine is the job of acetylcholine esterase.

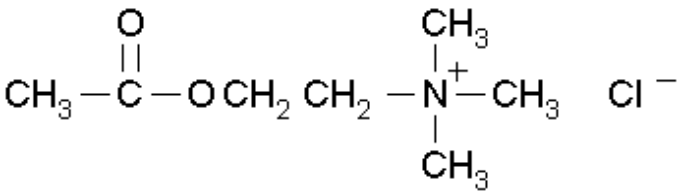


Figure 13: Structure of acetylcholine

Even though it is the main natural substrate for acetylcholine esterase, acetylcholine has been found to be less used for biosensor development compared to acetylthiocholine (ATch). As a result, acetylthiocholine has been chosen for this study as the substrate for acetylcholine esterase, a more extensive literature enabling a better defined starting point for the optimization at hand. [Vogel *and* Angermann (1994) www.pharmacorama.com] The hydrolysis of acetylcholine is shown in Equ. 5.

$$Acetylcholine + Water \xrightarrow{AchE} Choline + Acetate$$

Equation 5: Hydrolysis of acetylcholine in presence of acetylcholine esterase

Electrochemical detection of organophosphates is performed using a derivative of acetylcholine, acetylthiocholine. The thiocholine product is electrochemically active and can then be oxidized on the electrode surface at 400 mV vs Ag/AgCl, thus explaining the choice of acetylthiocholine as a substrate in this study. [Chelsea Monty *et al.* (2007). Larsson *et al.* (1998).]

ii. Acetylcholine esterase

Acetylcholine esterase (AchE, E.C.3.1.1.7: Hydrolases) acts on ester bonds, and is an example of carboxylic ester hydrolase. Acetylcholine esterase is a serine hydrolase that belongs to the esterase family within higher eukaryotes. This family acts on different types of carboxylic esters. Acetylcholine esterase's biological role is the termination of impulse transmissions at cholinergic synapses, within the nervous system, by rapid hydrolysis of the neurotransmitter, acetylcholine.

Acetylcholine esterase is an ellipsoidal molecule approximately 45 x 60 x 65 angstroms and consists of a 12 stranded central mixed beta sheet surrounded by 14 alpha helices (cf. Fig.14). Studies have indicated several major domains within the protein:

- a catalytic active site composed of two subsites, the aromatic gorge in which the catalytic active site lies;
- a peripheral anionic site, distinct from the catalytic active site, which plays a role in the confirmation of the residues within the aromatic gorge and active site.

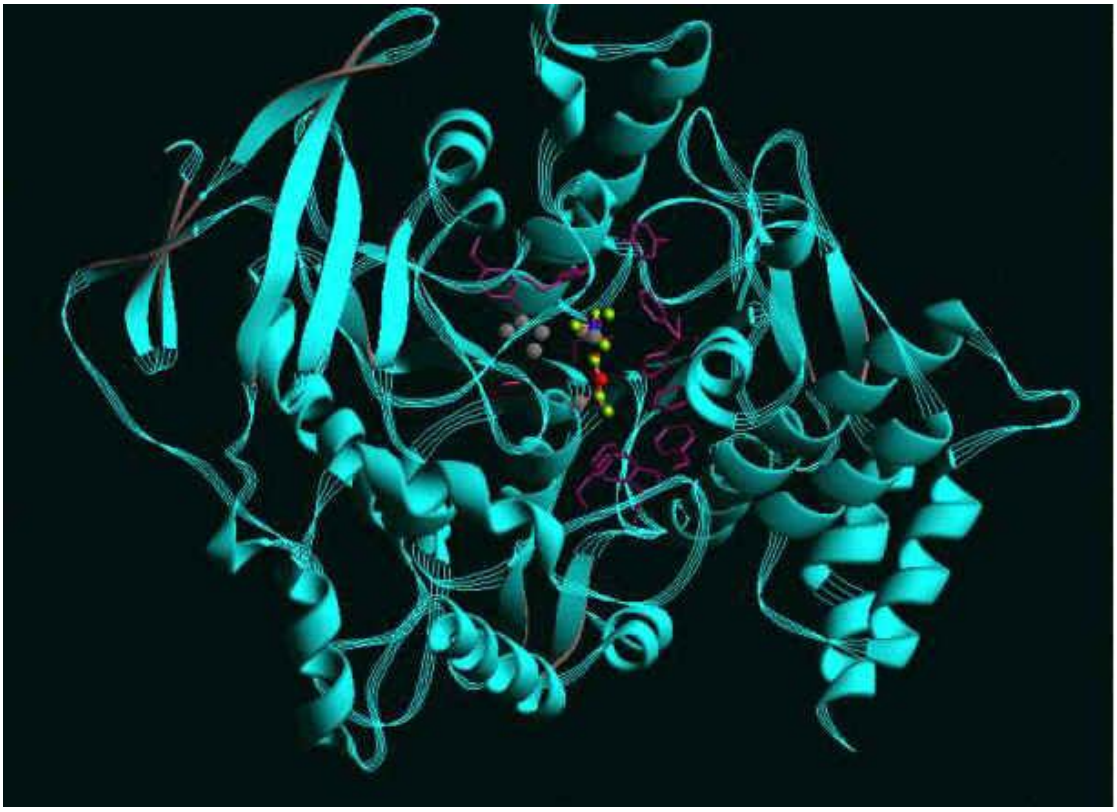


Figure 14: Structure of acetylcholine esterase

The aromatic gorge in the protein is approximately 20 angstroms deep and penetrates halfway into the enzyme. The active site lies at the base of this gorge only 4 angstroms above the base, reported as the active gorge. The aromatic gorge is a more appropriate term, because 40% of its lining is composed of 14 aromatic residues, which are highly conserved from different species of acetylcholine esterase. The high aromatic content of the walls and floor may explain why studies have proposed hydrophobic and anionic binding sites independent of the active site. Only a few acidic residues are present within the gorge. [www.biocyc.org.1555. www.srv2.lycoming.edu. www.neurosci.pharm.utoledo.edu]

Acetylcholine esterase converts acetylthiocholine into acetic acid. An additional reaction with either of the acetylthiocholine degradation product is necessary, which will enable a current variation relative to the reaction of interest. The pathway is presented Equ.6.

$$ATch + H_2O \xrightarrow{AchE} Tch + Acetic\ acid$$

$$Tch_{red} + TCNQ_{ox} \rightarrow Tch_{ox} + TCNQ_{red} + 2H^+$$

$$TCNQ_{red} \xrightarrow{SPE\ surface} TCNQ_{ox} + 2e^-$$

Equation 6: Acetylcholine esterase reaction pathway against acetylthiocholine for electrochemical biosensors

The amperometric detection condition, as found in literature, are, at pH=7.0 (optimum for the enzyme), E=+100mV if the working electrode is bare carbon and the reference electrode Ag/AgCl. This enzyme is however not very stable and its storage stability does not excess 6 months at -20°C and only 76 days at 37°C, both in dry state, which is doubtless the most stable state of the enzyme. [Andreescu *et al.* (2002). Xin *and* Wightman (1997).]

Acetylcholine esterase was first studied by using the form found in electric fish, such as the torpedo ray. These fish have massive arrays of nerve-like structures in the organs that generate electricity, so acetylcholine esterase is particularly abundant. The enzyme used in this study was produced by the electric eel. Acetylcholine esterase from electric eel has been found to provide good results in term of stability, sensibility and sensitivity compared to the other sources available. [Brasil de Olivera Marquez *et al.* (2004).]

Acetylcholine esterase has a major significance in medicine, as it is involved in several hereditary diseases, a variety of neurological and neuromuscular disorders involving a diminution of cholinergic activity. Often the most effective treatments are ligands which inhibit the breakdown of acetylcholine. Acetylcholine esterase is involved in Alzheimer disease [Lenigk *et al.* (2000).], acromegaly, amyotropis sclerosis, Huntington disease or Parkinson disease. In addition, cholinesterase inhibitors are widely utilized as pesticides and, if misused, can produce toxic responses in mammals and man. Acetylcholine esterase is there used in inhibition biosensors. [Vogel *and* Angermann (1994). www.brenba.uni-koeln.de. www.nist.rcsb.org.]

iii. Acetylcholine esterase inhibitors

Some inhibitors of acetylcholinesterase act by competitively blocking hydrolysis, without reacting with the enzyme. Others inhibit by acylating the serine hydroxyl group, forming a carbamyl ester, which is more stable than acetate and less likely to leave the active site. The competitive blocker edrophonium is a quaternary compound that blocks the enzyme by binding to the active site.

The alkaloids physostigmine and neostigmine act as metabolic inhibitors of acetylcholinesterase. The carbamyl ester formed by these compounds is much more stable than acetate (half-life measured in minutes as opposed to microseconds). The cholinesterase inhibitors are widely used to treat glaucoma (a disorder characterized by increased intraocular pressure). Acetylcholine reduces intraocular pressure, and cholinesterase inhibitors such as physostigmine are useful in treating the disease.

The other major use of cholinesterase inhibitors is for treatment of myasthenia gravis, an autoimmune disease in which antibodies are formed against the nicotinic receptor at the neuromuscular junction. The antibodies bind to nicotinic receptors to cause a profound muscle weakness and paralysis. Cholinesterase inhibitors can alleviate the symptoms of myasthenia by increasing muscle strength and endurance.

Recent efforts have been directed towards the development of novel strategies for the treatment of Alzheimer's disease. One strategy for the treatment of Alzheimer's patients has been the use of acetylcholinesterase inhibitors to increase the levels of acetylcholine in the synapse, thereby enhancing cholinergic activity in the affected brain regions. Physostigmine was used in early efforts to enhance cholinergic activity in the central nervous system although results were far from satisfactory.

Tetrahydroaminoacridine (THA, or tacrine) was the first cholinesterase inhibitor approved for use in Alzheimer's patients. Many patients given THA during clinical trials exhibited some alleviation of symptoms and some were able to resume normal activity and personal care. Not all patients respond to tacrine, and side effects include elevation of liver enzymes. Tetrahydroaminoacridine is formed from aminoacridine, an antimicrobial agent by hydrogenation of one of the rings. The resulting structure is no

longer planar, and loses antibiotic activity, but does exert an action as a cholinesterase inhibitor.

Within the past few years, other compounds have become available for clinical use. Donepezil is a cholinesterase inhibitor with improved selectivity for acetylcholinesterase and good CNS penetration. It also exhibits lower toxicity than tacrine. Rivastigmine is another acetylcholinesterase inhibitor that has been approved for use in Europe.

The insecticide carbaryl (Sevin), is uncharged and lipophilic and can penetrate the CNS of insects to act on the insect acetylcholinesterase, although the toxic effects on mammalian Acetylcholine esterase are much lower. Malathion is another effective pesticide which is more effective on insects than on humans because it requires biotransformation to the phosphate form, which can only be carried out by insects.

The molecule pralidoxime is a useful antidote for intoxication with cholinesterase inhibitors such as the organophosphates. The molecule removes the inhibitor from the active site in the form of an oxime phosphonate. Atropine also is used to block muscarinic responses due to excess acetylcholine. In addition, valium often is given as an antidote in conjunction with atropine to counteract seizures which may develop due to elevated levels of acetylcholine.

c. Lactate dehydrogenase

i. Lactate

Lactic acid (CH_3-CHOH-CO_2H) is a fermentation product of lactose; its systematic name is 2-hydroxypropanoic acid. Chemically, lactic acid occurs as two optical isomers, a dextrogyre and a levogyre form; only the levogyre form takes part in animal metabolism. [Vogel and Angermann (1994).]

Lactic acid is present in sour milk, yogurt, and cottage cheese. In addition, lactic acid is also produced in the muscles of mammals during intense activity. A growing body of scientific literature supports the link between lactate and endurance in sports training. Lactate detection is therefore needed in the food industry and also finds application in the study of sport performances.

Calcium lactate, a soluble lactic acid salt, is used as a source of calcium in the diet. Lactic acid is produced commercially for use in pharmaceuticals and foods, in leather tanning and textile dyeing, and in making plastics, solvents, inks, and lacquers. Although it can be prepared by chemical synthesis, production of lactic acid by fermentation is a less extensive method.

During one form of anerobic glycolysis (or fermentation), L-lactate is transformed into pyruvate via the enzyme lactate dehydrogenase. This conversion also oxidizes one molecule of NAD^+ to NADH, and hence: NAD^+ has to be regenerated so that glycolysis can continue.

This lactic acid fermentation occurs in red blood cells since they lack mitochondria and in skeletal muscle during intense exertion when sufficient amounts of oxygen cannot be supplied fast enough. The liver takes up about 60% of the lactate and reoxydizes it to pyruvate, which is then reconverted to glucose in a process known as gluconeogenesis. This glucose → lactate → glucose cycle, originally described by Carl and Gerti Cori, is known as the Cori cycle. Lactic acid is also the result of malolactic fermentation, a process used in winemaking to convert sharp-tasting malic acid into the gentler lactic acid. [www.fact-index.com]

ii. Lactate dehydrogenase

Lactate dehydrogenase (LDH, E.C.1.1.2.3.: Oxidoreductases) acts on the CH-OH group of donors, with NAD^+ or $NADP^+$ as acceptor. Lactate dehydrogenase transforms lactate into pyruvate following the pathway described thus (cf. Equ.7):

$$Lactate + NAD^+ \xrightarrow{LDH} Pyruvate + NADH + H^+$$

$$NADH \xrightarrow{SPE\ surface} NAD^+ + e^-$$

Equation 7: Lactate dehydrogenase reaction pathway for detection using electrochemical biosensors

The amperometric detection conditions are E=+350mV on Rh-C versus Ag/AgCl. The optimal pH for the enzyme is 7.0. [White *et al.* (1994).] The difficulty in detecting NADH and NADPH lies in achieving simultaneous 2-electron transfer between NADH or NADPH and the electrode of a biosensor. Lactate dehydrogenase is also very unstable,

its stability depending upon its origin. For the preliminary study, only one origin was chosen, but a comparative study would have been led, should the result not have been satisfying regarding the stability. At 37°C, lactate dehydrogenase preservation may be satisfactory up to 98 days in dry state, but only 5 days if in solution. Lactate dehydrogenase is preserved correctly less than 200 days at 25°C in dry state. [Vogel *and* Angermann (1994). www.chem.qmul.ac.uk. www.biochem.ucl.ac.uk Anchordoquy *et al.* (2001). Choi (2005).]

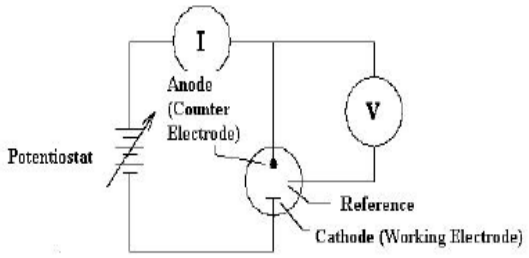
Lactate dehydrogenase is inhibited by metallic cations, such as Cu^{2+}, Co^{2+}, Hg^{2+}. It also is inhibited by AMP (adenine monophosphate), ATP (adenine triphosphate), GTP (guanine triphosphate). It is finally found to be inhibited by NAD^+ and NADH. [www.brenba.uni-koeln.de]

2.3.3. Detection principle: Electrochemistry

In contrast to potentiometry, a process that operates at a null current, other electro-analytical methods, such as amperometry, impose an external energy source to the test solution in order to provoke a electrochemical reaction that otherwise wouldn't have spontaneously occur.[Pan *et al.* (2005). Rishpon and Ivnitski (1997). Santandreu *et al.* (1999). Purvis *et al.* (2003).] Amperometry is a dynamic process in which electron flow at an inert electrode is measured, typically while maintaining a constant applied potential to drive the direction of electron flow from or to the redox molecule to be monitored. The fundamental measurement system uses three electrodes: a working electrode where the desired reaction occurs, a reference electrode to govern the value of the applied potential at this working electrode and a counter-electrode to complete the primary electrode circuit. [Ivnitski and Rishpon (1996). Jawaheer *et al.* (2003).]

As soon as the working electrode potential reaches a value high enough for a species in solution to be reduced or oxidized, the current in the circuit between the two electrodes changes, in proportion to the concentration of the analyte being oxidized or reduced at one electrode surface In order to prevent the flow of current in the reference electrode, another electrode or counter-electrode is added. (cf. Fig.15) The use of a counter-electrode avoids any shift in the reference potential at higher currents. A study of the intensity versus potential curve allows the determination of the optimal working

potential. [Gallardo Soto *et al.* (2001). Padeste *et al.* (1998). Sarkar (2000).] Algorithms have been found, that enable the generating of calibration curve for amperometric conditions. [Wang (1999). Kirstein *et al.* (1985). Kueng *et al.* (2004). Orsquo *et al.* (2002).]



$$i = \frac{nFAC^{b}D^{\frac{1}{2}}}{\pi^{\frac{1}{2}}t^{\frac{1}{2}}}$$

i = current (A)
N = number of electrons involved in the electrode reaction
F = Faraday's constant (
A = Area of the planar electrode (cm^2)
C^b = The bulk concentration of the electroactive substance (ML^{-1})
D = Diffusion coefficient of the electroactive substance (cm^2s^{-1})
t = time (s)

Figure 15: Principle of amperometry

2.3.4. Kinetics

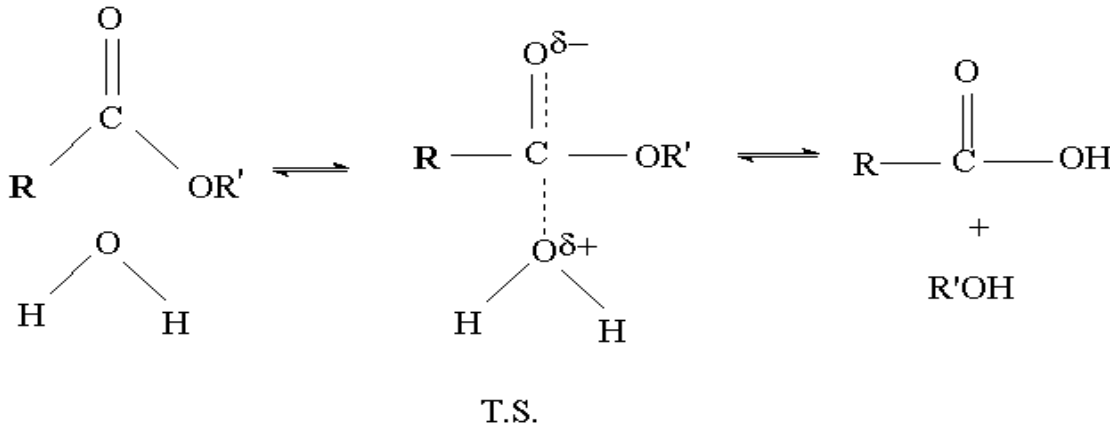
Kinetics are very important in the study of enzymes for many reasons: they are the most obvious reflection of the catalytic reaction, they provide important mechanistic information, they reflect the effect of pH, temperature and other environmental factors on the enzyme and catalytic reaction, they are critical in the function of the enzyme in its physiological environment.

Although enzymes contain hundreds of amino acid residues, only two or three are normally involved in the bond-making and -breaking steps in the transformation of substrate to product. These catalytic groups are involved in redistributing the electron density in going from the ground state to the transition state. [Botkin and Turova (2004). Tang *et al.* (2004). Jianping Li (2005).]

2.3.4.1. *Introduction*

a. Uncatalyzed reaction

In the following uncatalyzed reaction (cf. Equ. 8), the charge build-up in the transition state is unfavourable, i.e. costs energy. Therefore anything which decreases the size of the charge will decrease the energy of the transition state and therefore increase the rate of the reaction. The corresponding energy diagram is shown Fig.16.



Equation 8: Example of uncatalyzed reaction

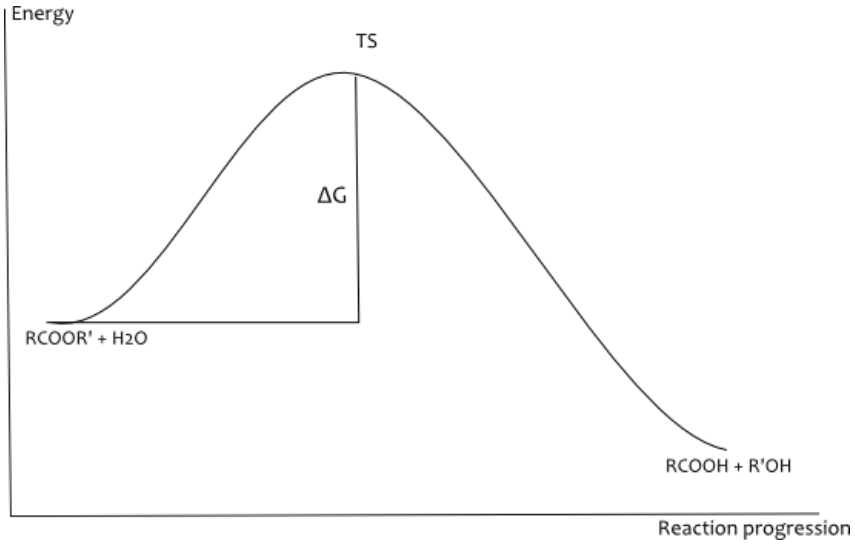


Figure 16: Energy variation diagram during a chemical reaction

The energy of the transition state ΔG corresponds to the minimum free energy barrier which must be overcome for conversion of substrate to product. The rate of the

reaction is given by the transition state theory. It assumes that any molecule which achieves the transition state free energy, i.e. the transition state structure, will break down to product. The entropy, ΔS, will include contributions from solvation, steric and orientation factors, especially changes in the degrees of freedom associated with translational, rotational and vibrational modes. The rate of the reaction therefore is proportional to ΔG, i.e. the difference in free energy between the ground state and the transition state.

The transition state in the case of ester hydrolysis involves charge separation, whereas both ground states are neutral. This is energetically unfavorable and increases the transition state energy. Therefore, anything which decreases the effective charge build-up in the transition state will increase the rate by decreasing ΔG. This could happen if a more polar solvent were used, or if a general base were present, as the base helps to pull off the proton, thereby diffusing the charge build-up.

b. Basic principles

The Bronsted catalysis law states that the stronger the base, or acid, the better it will be as a general catalyst. However, this can be offset if the pH of the reaction is such that the acid is mostly in the form of its conjugate base, or vice versa.

The principle of microscopic reversibility states that a given reaction must proceed by the same pathway in the forward and reverse directions. This is strictly only applicable to systems at equilibrium, but is believed to be valid for kinetics systems at steady state.

The diffusion limit is the fastest rate for an enzyme catalyzed reaction would be where the rate-limiting step is the formation of the initial encounter complex between enzyme and substrate, i.e. the rate at which the substrate and enzyme diffuse together. Such reactions have very low energy barriers (typically 2 kcal/mol). For aqueous solution, 25°C, the value of the rate constant k comes out around 10^9 $M^{-1}.s^{-1}$. For protein-protein interactions the rate is about 10^7 - 10^8 $M^{-1}.s^{-1}$ as larger molecules diffuse more slowly then smaller ones.

The Hammond Postulate states that if there is an unstable intermediate the transition state will resemble it. If the transition state resembles the products the reaction will be faster.

A very important difference between enzymatic and non-enzymatic reactions is that the enzymatic one is effectively intra-molecular. This is because once the substrate is bound to the enzyme, the catalytic events, i.e. the interactions between the enzyme's catalytic groups and the substrate to make and break bonds, all occur within the same molecule. This has profound effects on the entropy of the reaction, compared to the analogous reaction without enzyme catalysis.

c. Review

- Zero-order kinetics: $v = k$
- Uni-molecular reaction: $A \rightarrow P$

  First-order kinetics: $v = k[A]$
- Bimolecular: $A + B \rightarrow P$ or $2A \rightarrow P$

  Second-order kinetics: $v = k[A][B]$ *or* $v = k[A]^2$

  If [B] is constant then becomes pseudo-first-order, i.e. $v = k'[A]$

Consider a first-order reaction:

$$S \rightarrow P; v = \frac{dP}{dt}; \text{but } [S] = [S_o] - [P] \text{ therefore } \frac{dP}{(S_o - P)} = kdt$$

Integrating gives $-\ln(S_o - P) = kt + constant$

At t = 0, P = 0, thus $[P] = S_o(1 - e^{-kt})$

A very useful relation for first order reactions is $t_{1/2}$ = 0.7/k and that $5t_{1/2}$ correspond to the apparent time for completion of the reaction.

For a reaction of the form $A + B \rightarrow C$ the rate expression is likely to be of the form $v = k[A][B]$

However, it depends on the reaction mechanism and the rate limiting step. Also, an important point as far as enzymatic reactions are concerned, if water is a reactant, its concentration is usually so much greater than that of the compounds it reacts with that its concentration effectively does not change, i. e. its constant, and therefore it shows up as part of the rate constant. For example, in the above case, if B was water, and A was millimolar, there would be no change in the water concentration (55.5 M), so the rate expression would be $v = k'[A]$ with $k' = 55.5k$

2.3.4.2. *Enzymatic kinetics*

a. Comparison between catalyzed and uncatalyzed reactions

The kinetics of the enzyme-catalyzed reaction are rather different than those of a typical chemical reaction. For example, for the reaction $A \rightarrow B$ the uncatalyzed reaction will exhibit first-order kinetics, i.e. a plot of rate (v) against [A] is linear since v = k[A]. (cf. Fig.17)

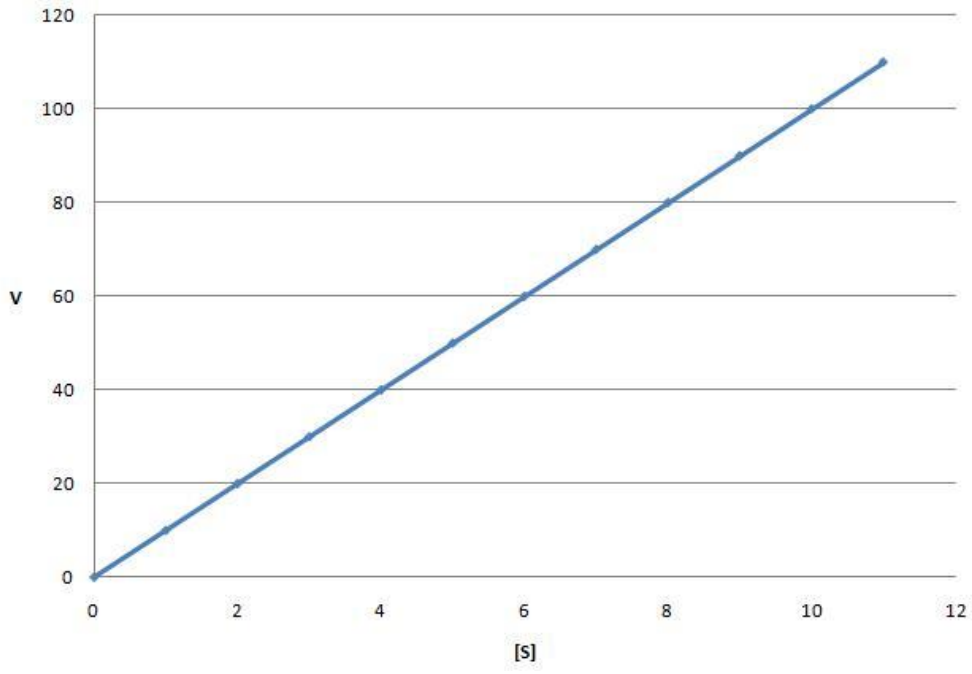


Figure 17: First order kinetics for an uncatalyzed reaction

On the other hand enzyme-catalyzed reactions often show hyperbolic plots of V versus [S] or [A]. Such plots (cf. Fig.18) are known as saturation plots and indicate that

a pre-equilibrium exists on the reaction pathway; along the reaction: $A + E \rightarrow EA \rightarrow E + B$, where E = enzyme, A = substrate and B= product. [Quinn and O'Kennedy (2001).]

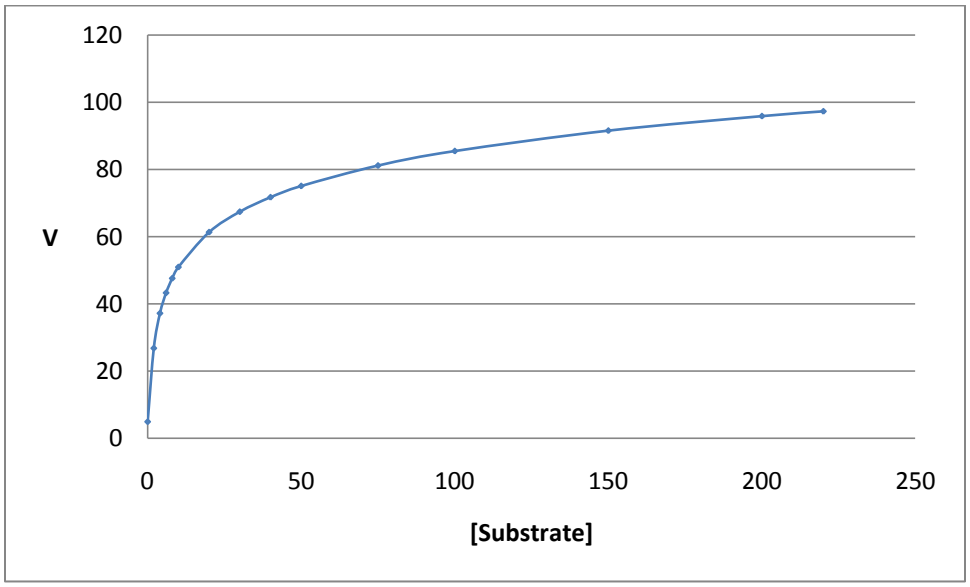


Figure 18: Saturation plot for a catalyzed reaction

b. Michaelis-Menten equation

Typical methods used to monitor enzyme-catalyzed reactions and thereby provide kinetic data include the following:

- Change in spectral property (typically UV or vis. absorbance, fluorescence) with a spectrometer
- Release or uptake of H^+ or OH^- with a pH-stat (a device which automatically adds acid or base to keep the pH constant)
- Chemical analysis by HPLC (chromatography), or NMR, or TLC (ATPase)
- Isotope analysis (e.g. radioactive ^{32}P)
- Coupled reactions - used in cases where there is no easy way of following the reaction of interest. Instead another enzyme is added to react with the product in a reaction which typically generates a spectral signal.

Many enzyme-catalyzed reactions show an initial velocity versus [substrate] relationship of the form shown below, and which can be accounted for by a scheme of the following sort (cf. Equ.9):

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \overset{k_2}{\rightarrow} E + P$$

Equation 9: Principle of enzyme-catalyzed reaction

where the concentration of each elements of the equation varies as shown Fig.19.

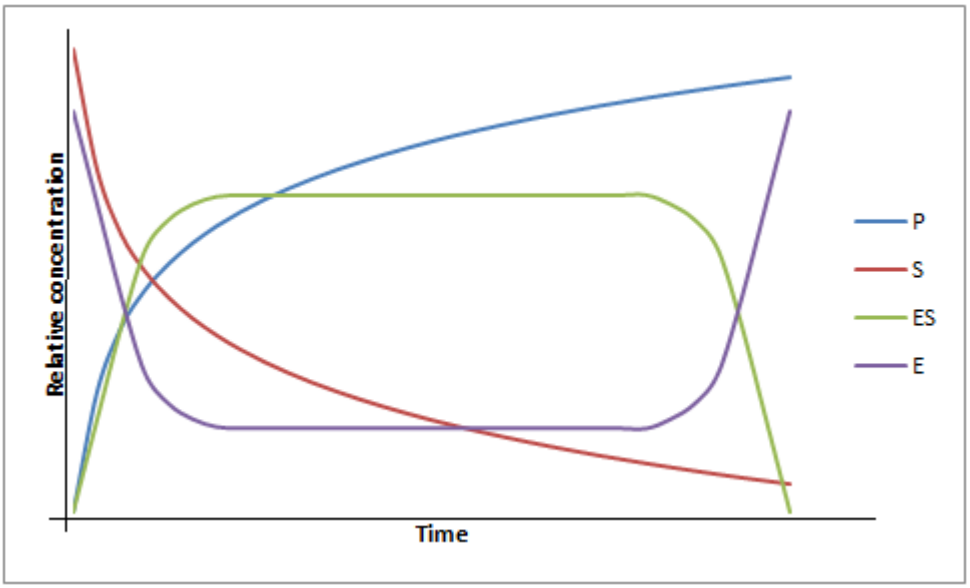


Figure 19: Evolution over time of concentrations in a catalyzed reaction

To determine the relationship between the observed rate and those of the above scheme, the approach is to set up four types of equations:

- The velocity expression
- The conservation of enzyme expression
- The rapid equilibrium assumption where appropriate
- The steady-state approximation

In deriving the steady-state enzyme kinetic equations one assumes that if one measures initial velocities the back reaction is negligible and for the majority of the reaction time the concentration of each of the enzyme-containing species will be essentially constant, whereas the rapid equilibrium assumption is that the equilibration between enzyme, substrate and enzyme-substrate complex is fast

compared to the subsequent reaction of the enzyme-substrate complex. From this, the Michaelis-Menten equation can be obtained: $v = \frac{V_{max}S}{S+K_m}$, with $V_{max} = k_{cat}[E_o]$.

If $S << K_m$, v becomes $V_{max}S/K_m$ i.e. first-order, where the rate constant is V_{max}/K_m. If $S >> K_m$, v becomes V_{max}, i.e. zero order and a saturation is observed.

c. Definitions

k_{cat} is a first-order rate constant corresponding to the slowest step or steps in the overall catalytic pathway. It represents the maximum number of molecules of substrate which can be converted into product per enzyme molecule per unit time, which only happens if the enzyme is saturated with substrate, and thus is often known as the turnover number.

K_m is an apparent dissociation constant, and is related to the enzyme's affinity for the substrate; it is the product of all the dissociation and equilibrium constants prior to the first irreversible step in the pathway. Often it is a close measure of the enzyme-substrate dissociation constant.

k_{cat}/K_m is a second-order rate constant which refers to the free enzyme. It is also a measure of the overall efficiency of the enzyme catalysis and is known as the specificity constant.

The traditional method of linearizing the Michaelis-Menten equation is that of Lineweaver and Burk, involving $1/v$ versus $1/[S]$. The intercepts are $-1/K_m$ with the horizontal axis and $1/V_{max}$ with the vertical axis. The Lineweaver and Burk plot suffers from unequal weighting of the data points, where too much weight is given to those at low substrate concentration which are the least accurate.

d. Pre-steady-state kinetics

The pre-steady-state or transient phase has a kinetic expression of the form $v = Ae^{-kt}$. At early times, v is significant, whereas at large values of t it goes to 0. Since the transient phase is usually complete in milliseconds or less, it is clear that very fast mixing is necessary.

The problem with steady-state kinetics is that they essentially only provide information about the slowest step in the catalytic reaction, whereas for mechanistic studies the intermediates and their transformations are under observation. The amplitude of B (the burst, cf. Fig.20) is a measure of the enzyme concentration. It can be used as a means of obtaining a very precise measure of the concentration of active-enzyme.

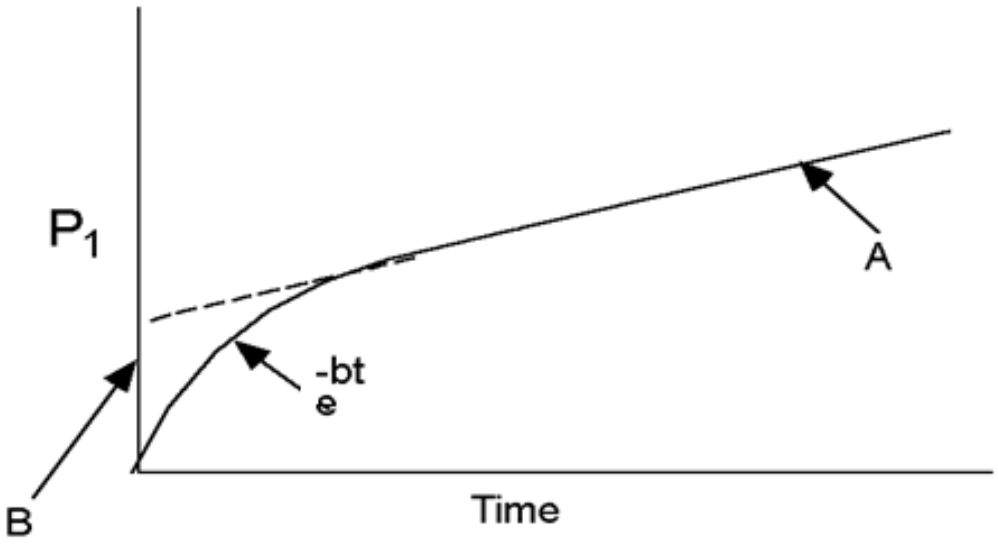


Figure 20: Evolution of product concentration over time in pre-steady-state kinetics

2.3.4.3. *Inhibition*

a. Introduction

There are many different kinds of inhibitors; an inhibitor being any compound which causes a decrease in the catalytic rate. Irreversible inhibitors such as active-site directed modifying reagents will not be considered. [Botrè *et al.* (2000). Mazzei]

There are many possible physical causes of reversible inhibition. Inhibition can be caused by products, substrates, related and unrelated compounds. The general scheme is presented Fig.21. In that figure, L is the ligand. Inhibition occurs if $k'_{ELS}<k_{ES}$, whereas activation occurs if $k'_{ELS}>k_{ES}$. Most types of inhibition are defined empirically by their effects on k_{cat} and K_m: The results are summarized Table 1.

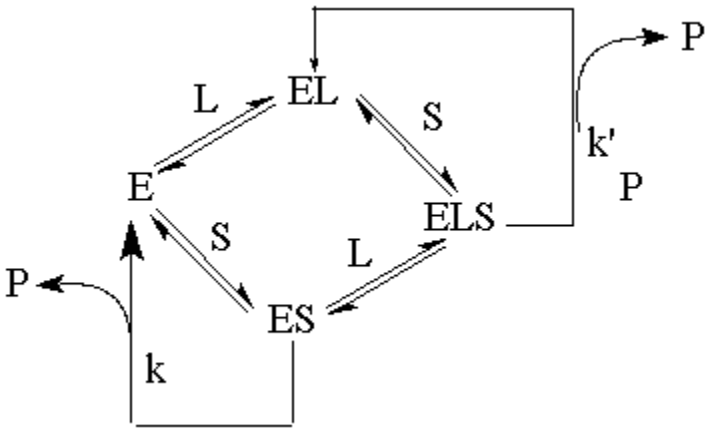


Figure 21: Inhibition general scheme

Type	K_m	V_{max}
Competitive	Increased	Same
Mixed	Increased	Decreased
Uncompetitive	Increased	Decreased
Non-competitive	Same	Decreased

Table 1: Influence of inhibition on Km and Vmax

b. Different types of inhibitions

Competitive inhibition is perhaps the most common form. It: results from the direct competition between the inhibitor and the substrate for the substrate-binding site of the enzyme.

Mixed inhibition is also very common. The inhibitor binds to both free enzyme and enzyme-substrate complex. In that scenario, both K_m and V_{max} are affected, to different extents. If they are affected to the same extent, then the inhibition is called uncompetitive. It implies that the inhibitor binds only to the enzyme-substrate complex.

Another type of inhibition is the non-competitive inhibition. It is usually found in allosteric systems and not so common. The inhibitor binds to the enzyme and the

enzyme-substrate complex with identical inhibition constants. Here, the effect is the opposite of that of competitive inhibition.

In uncompetitive inhibition, the inhibitor binds only to the enzyme-substrate complex. The ration V_{max}/K_m is unchanged, which means that. V_{max} and K_m are both changed to the same extent.

A non-productive binding occurs when the natural substrate is large but a small substrate is being used instead. In that case, the substrate used binds in a bad position. It will then be improperly positioned for catalysis to occur.

Another sort of inhibition is the substrate inhibition. Two substrate molecules bind where normally only one does. It is sometimes observed at high substrate concentrations.

A very common inhibition is the product inhibition. It is often overlooked. It's usually competitive, along the lines presented Equ.10.

$$E + S \underset{\leftarrow}{\rightarrow} ES \underset{\leftarrow}{\rightarrow} E + P \underset{\leftarrow}{\rightarrow} EP$$

Equation 10: Typical reaction pathway for product inhibition

It can be readily missed, as it gives a linear plot which is indistinguishable from that of regular Michaelis-Menten kinetics. Therefore it is very difficult to detect that a product inhibition is occurring, without using several different concentrations of substrate. However, looking at the enzyme activity kinetics, product inhibition becomes obvious as the activity decreases as the product concentration increases.

2.3.4.4. *pH and temperature influence*

The effect of pH on the kinetic parameters of an enzyme-catalyzed reaction is fairly well known. It results most of the time in either sigmoidal or bell-shaped curves, such as shown Fig.22 and 23.

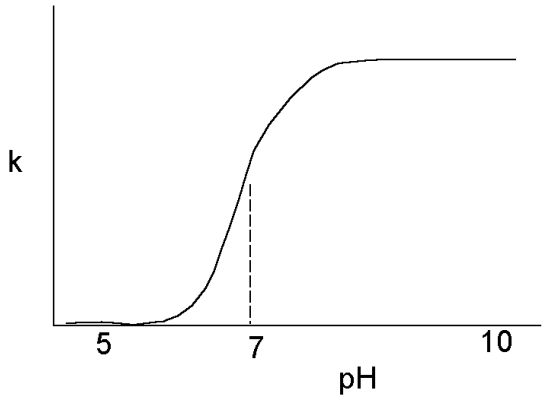


Figure 22: pH effect on enzyme – sigmoidal curve

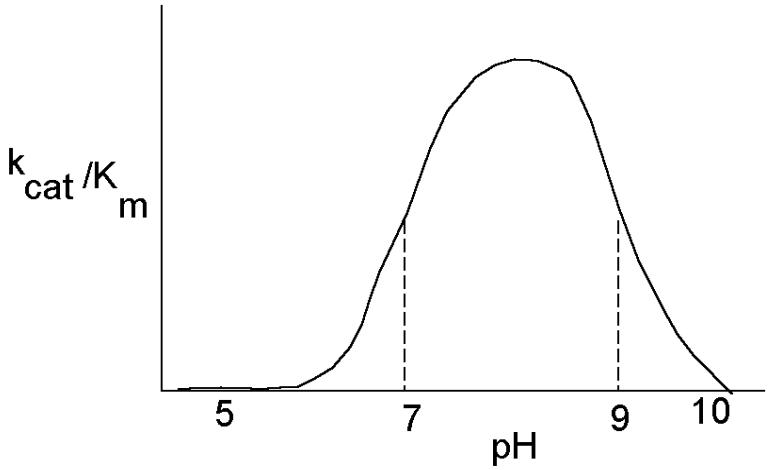


Figure 23: pH effect on enzyme – bell-shaped curve

Understanding the effects of pH on enzyme-catalyzed reactions is fairly simple: the underlying basis is as follows:

- The essential catalytic groups in the active-site are often ionizable groups, which frequently will act as acid, base, nucleophile or electrophile catalysts, and thus are only functional in one of their ionization states.
- The pH-dependence of such ionizations is governed by the Henderson-Hasselbalch equation: $pH = pK + \log {[A]}/{[AH]}$

The effect of temperature against enzyme activity is also well documented. As the enzyme is a protein, when the temperature reaches a certain point, the enzyme is

denatured and can no longer work. This is materialized by a rather sharp drop in reaction rate, as is shown Fig.24.

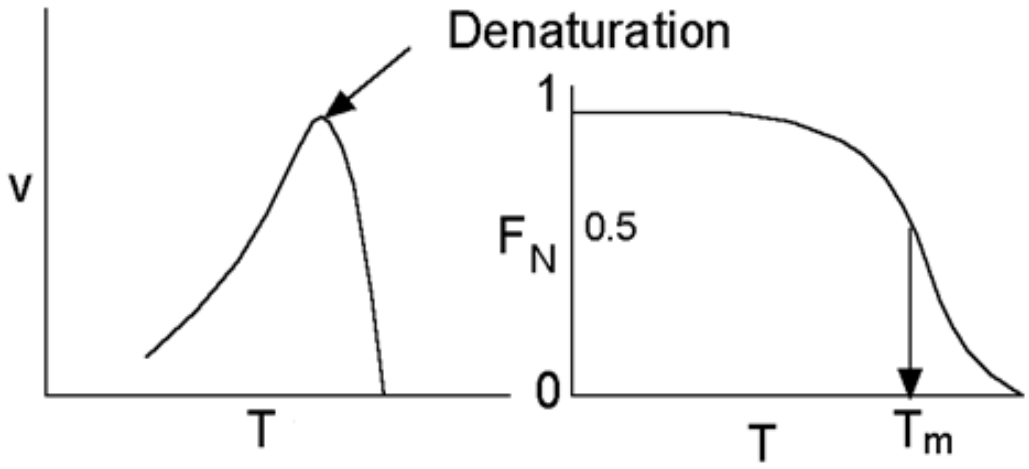


Figure 24: Reaction rate vs. temperature

2.3.4.5. *Theories of enzyme catalysis*

Theories have been proposed to account for the great rate-enhancement brought about by enzymes. Obviously the enzyme must either raise the ground state free energy of the substrate(s) or decrease the free energy of the transition state, or both.

Some of the factors which have been proposed include:

- Proximity
- Orbital steering
- Orientation
- Approximation
- Entropy
- Solvation
- Transition State Complementarity

- Acid/base/nucleophilic catalysis
- Strain/distortion
- Electrostatic stabilization
- Protein mobility
- Intrinsic binding energy

Some of these are very difficult to quantitate, others it is clear that their direct contribution is small, for others it is clear they are major factors. The latter include intrinsic binding energy, approximation, entropy effects, acid/base/nucleophile catalysis, electrostatic stabilization and especially transition state complementarity.

2.4. Immunosensors

The second biosensor system studied was immunosensors, of which there is here an overview, as well as the systems used for detection purposes. [Gizeli and Lowe (1996). Luppa *et al.* (2001). Pathak and Savelkoul (1997).]

2.4.1. Antibodies: a brief overview

An antibody is a protein used by the immune system to identify and neutralize foreign objects like bacteria and viruses. Each antibody recognizes a specific antigen unique to its target. Production of antibodies is referred to as the humoral immune system.

Immunoglobulins are glycoproteins in the immunoglobulin superfamily that function as antibodies. The terms antibody and immunoglobulin are often used interchangeably. They are found in the blood and tissue fluids, as well as many secretions. They are synthesized and secreted by plasma cells which are derived from the B cells of the immune system. [Hock *et al.* (1995).]

2.4.1.1. Structure of the antibody

Immunoglobulins are heavy plasma proteins, often with added sugar chains (see glycosylation) on N-terminal (all antibodies) and occasionally O-terminal (IgA1 and IgD) amino acid residues.

The basic unit of each antibody is a complex of four monomers. It results in an "Y"-shaped molecule that consists of two identical heavy chains and two identical light chains connected by disulfide bonds.

There are five types of heavy chain: γ, δ, α, μ and ε. They define classes of immunoglobulins. Heavy chains α and γ have approximately 450 amino acids, while μ and ε chains have approximately 550 amino acids. Each heavy chain has a constant region, which is the same by all immunoglobulins of the same class, and a variable region which differs between immunoglobulins of different B cells, but is the same for all immunoglobulins produced by the same B cell. Heavy chains γ, α and δ have the

constant region composed of three domains; the constant region of heavy chains μ and ε is composed of four domains. The variable domain of any heavy chain is composed of one domain. These domains are about 110 amino acids long. There are also some amino acids between constant domains.

There are only two types of light chain: λ and κ. In humans they are similar, but only one type is present in each antibody. Each light chain has two successive domains: one constant and one variable domain. The approximate length of a light chain is from 211 to 217 amino acids.

The monomer is composed of two heavy and two light chains. Together this gives six to eight constant domains and four variable domains. If it is cleaved with enzymes papain and pepsin, with two Fab (fragment binding antigen) fragments and an Fc (fragment crystallizable) fragment.

Each half of the forked end of the "Y" shaped monomer is called the Fab fragment. It is composed of one constant and one variable domain of each the heavy and the light chain, which together shape the antigen binding site at the amino terminal end of the monomer. The two variable domains bind the antigens they are specific for and that elicited their production.

The Fc fragment is the stem of the "Y" and is composed from two heavy chains that contribute each to two to three constant domains, depending on the class of the antibody. It binds to various cell receptors and complement proteins. In this way it mediates different physiological effects of antibodies, such as opsonization, cell lysis, mast cell, basophil and eosinophil degranulation and other processes.

2.4.1.2. Isotypes

According to differences in their heavy chain constant domains, immunoglobulins are grouped into five classes or isotypes: IgG, IgA, IgM, IgD, and IgE. The antibodies that a single B lymphocyte produces can differ in their heavy chain and the B cell often expresses different classes of antibodies at the same time.

However, they are identical in their specificity for antigen, conferred by their variable region. The structure of an antibody and its interaction with an antigen is presented Fig. 25.

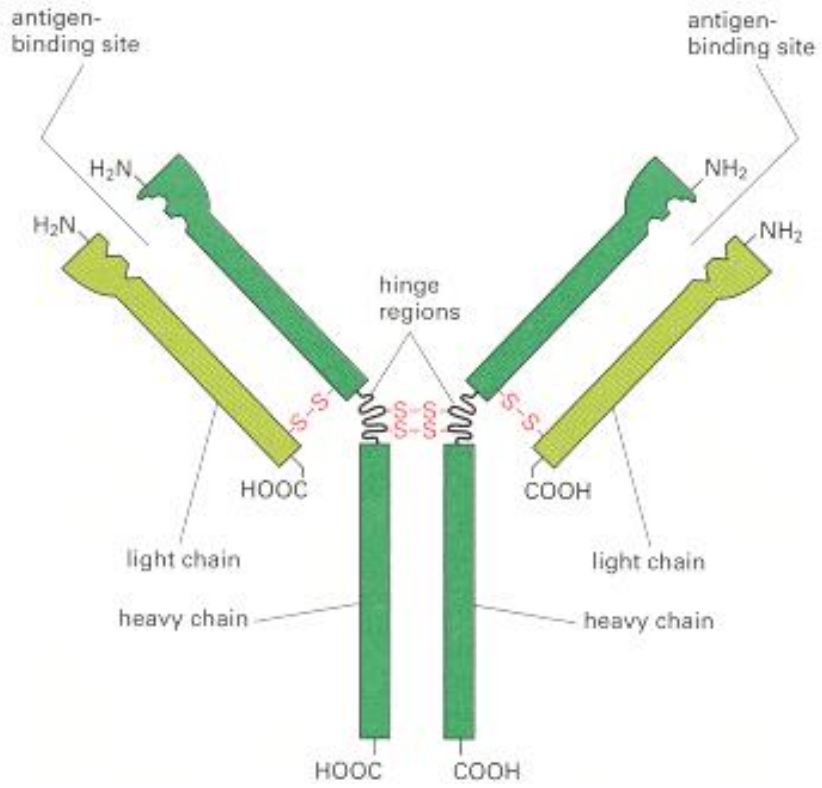


Figure 25: Schematic of an antibody structure

a. IgG

IgG is a monomeric immunoglobulin, built of two heavy chains γ and two light chains. Each molecule has two antigen binding sites. This is the most abundant immunoglobulin and is approximately equally distributed in blood and in tissue liquids. This is the only isotype that can pass through the placenta, thereby providing protection to the fetus in its first weeks of life before its own immune system has developed.

It can bind to many kinds of pathogens, for example viruses, bacteria, and fungi. It protects the body against them by complement activation (classic pathway), opsonization for phagocytosis and neutralisation of their toxins.

b. IgA

IgA represent about 15 to 20% of immunoglobulins in the blood although it is primarily secreted across the mucosal tract into the stomach and intestines. It is also found in maternal milk, tears and saliva. This immunoglobulin helps to fight against pathogens that contact the body surface, ingested, or inhaled.

The IgA found in secretions have a special form. They are dimeric molecules, linked by two additional chains. One of these is the J chain (from join), which is a polypeptide of molecular mass 1,5 kD, rich with cysteine and structurally completely different from other immunoglobulin chains. This chain is formed in the antibodies secreting cells. The dimeric form of IgA in the outer secretions has also a polypeptide of the same molecular mass (1,5 kD) that is called the secretory chain and is produced by the epithelial cells.

c. IgM

IgM forms polymers where multiple immunoglobulins are covalently linked together with disulfide bonds, usually as a pentamer or a hexamer. It has a large molecular mass of approximately 900 kD. The J chain is attached to most pentamers, while hexamers do not possess the J chain due to space constraints in the complex.

Because each monomer has two antigen binding sites, an IgM has 10 of them, however it cannot bind 10 antigens at the same time because they hinder each other. Because it is a large molecule, it cannot diffuse well, and is found in the interstitium only in very low quantities. IgM is primarly found in serum, however of the J chain it is also important as a secretory immunoglobulin. Due to its polymeric nature, IgM possesses high avidity, and is particularly effective at complement activation. It is also a so-called “natural antibody”: it is found in the serum without any evidence of prior contact with antigen.

d. Other immunoglobulins

IgD makes up about 1% in the plasma membranes in B-lymphocytes. It is monomeric with the δ heavy chain. While IgD's function is not yet completely understood, it is often coexpressed with IgM and is used as a marker of mature, naive

B cells. It may also be involved in the differentiation of B cells into plasma and memory cells.

IgE is a monomeric immunoglobulin with the heavy chain ε. It contains a high proportion of carbohydrates. Its molecular mass is 190 kD. It can be found on the surface of the plasma membrane of basophils and mast cells of connective tissue. IgE plays a role in immediate hypersensitivity and the defense against parasites such as worms. The IgE antibodies are present also in outer excretions. Only IgE is heat labile.

2.4.1.3. *The humoral immune response*

When a macrophage ingests a pathogen, it attaches parts of the pathogen's proteins to a class II MHC protein. This complex is moved to the outside of the cell membrane, where it can be recognized by a T lymphocyte, which compares it to similar structures on the cell membrane of a B lymphocyte. If it finds a matching pair, the T lymphocyte activates the B lymphocyte, which starts producing antibodies. A B lymphocyte can only produce antibodies against the structure it presents on its surface.

Antibodies exist freely in the bloodstream or bound to cell membranes. They are part of the humoral immune system. Antibodies exist in clonal lines that are specific to only one antigen, e.g., a virus hull protein. In binding to such antigens, they can cause agglutination and precipitation of antibody-antigen products prime for phagocytosis by macrophages and other cells, block viral receptors and stimulate other immune responses such as the complement pathway.

Antibodies that recognize viruses can block these directly by their sheer size. The virus will be unable to dock to a cell and infect it, hindered by the antibody. They can also agglutinate them so the phagocytes can capture them. Antibodies that recognize bacteria mark them for ingestion by macrophages. Together with the plasma component complement, antibodies can kill bacteria directly. They neutralize toxins by binding with them.

It is important to note that antibodies cannot attack pathogens within cells, and certain viruses hide inside cells (as part of the lysogenic cycle) for long periods of time

to avoid them. This is the reason for the chronic nature of many minor skin diseases (such as cold sores); any given outbreak is quickly suppressed by the immune system, but the infection is never truly eradicated because some cells retain viruses that will resume it later.

In biochemistry, antibodies are used for immunological identification of proteins, using the Western blot method. A similar technique is used in ELISPOT and ELISA assays, in which detection antibodies are used to detect cell secretions such as cytokines or antibodies. Antibodies are also used to separate proteins (and anything bound to them) from the other molecules in a cell lysate. [Stocklein *et al.* (1998).]

2.4.2. Immunoassays

2.4.2.1. Presentation

Evolution of diagnostic tests began in the 1940's with colorimetric measurements of the enzymes and metabolites found in biological fluids using classical chemistry methods and agglutination reactions. Immunoassays have been in use since the early 1950's when radio-immunoassays (RIA) were used to quantify insulin in plasma samples. It was developed by Rosalyn Yalow and Solomon Berson, who were later awarded the Nobel Prize in 1977 for developing an RIA to detect and measure blood glucose levels in diabetic patients. In the 1960s, immunoassay technology was enhanced by replacing radio-isotopes with enzymes for color generation. [Brecht *et al.* (1995). Ferreira (2004). Hudson (1999).]

Since their introduction, immunoassays have been used to detect and quantify hundreds of types of molecules both native to living organisms, such as hormones, and foreign molecules, such as pharmaceuticals. The molecules detected by immunoassays vary widely in size, chemical and physical properties, and biological activity. The ability of an antibody to discriminate between the millions of naturally occurring molecules in a living organism is critical to the use of immunoassays in environmental analysis. [Crowley *et al.* (1999). Dai *et al.* (2004).]

While a virus is a very small physical object, it is larger than most macromolecules. Macro molecules such as proteins, polysaccharides, and nucleic

acids (like RNA) are usually discernable by immunoassays. And while some immunoassays may be limited in distinguishing between small differences in molecular structure within a family of compounds, they do have the distinct advantage of being highly selective even in the the midst of obscuring material, like humic acids, fulvic acids, petroleum spills etc. A sample that might require days of clean up in the lab can in some cases be checked in the field by immunoassay in about an hour without excessive cost or bulky equipment. [Angel Gonzalez-Martinez *et al.* (1999). Corry *et al.* (2003). Diaz-Gonzalez *et al.* (2005). Hock *et al.* (1999). Hock *et al.* (2002).]

2.4.2.2. ELISA testing

The Enzyme-Linked Immunosorbent Assay (ELISA or EIA for short) is a biochemical technique used in immunology to detect the presence of an antibody or an antigen in a sample. It was first tested in the lab in 1970 and appeared in publications in 1971. The first published enzyme immunoassay (EIA) and ELISA systems differed in assay design, but both techniques are based on the principle of immunoassay with an enzyme rather than radioactivity as the reporter label. Two scientific research groups independently and simultaneously developed this idea and executed the necessary experiments to demonstrate its feasibility. [Engvall (1971)]

ELISA utilizes two antibodies, one of which is specific to the antigen and the other of which is coupled to an enzyme. This second antibody gives the assay its "enzyme-linked" name, and will cause a chromogenic or fluorogenic substrate to produce a signal. Because the ELISA can be performed to evaluate either the presence of antigen or the presence of antibody in a sample, it is a useful tool both for determining serum antibody concentrations (such as with the HIV test or West Nile Virus) and also for detecting the presence of antigen. [Fu(2004; 2005).]

ELISA tests are generally highly sensitive and specific and compare favorably with radio-immunoassay (RIA) tests. They have the added advantages of not needing radioisotopes or a radiation-counting apparatus. [Hennion and Barcelo (1998). Kim *et al.* (2003).]

a. Indirect ELISA

The steps of the general, "indirect", ELISA for determining serum antibody concentrations are as follow. A schematic is drawn Fig.26.

- Apply a sample of known antigen to a surface, often the well of a microtiter plate. The antigen is fixed to the surface to render it immobile.
- The plate wells or other surface are then coated with serum samples of unknown antibody concentration, usually diluted in another species' serum. The use of non-human serum prevents non-specific antibodies in the patient's blood from binding to the antigen.
- The plate is washed, so that unbound antibody is removed. After this wash, only the antibody-antigen complexes remain attached to the well.
- The second antibodies are added to the wells, which will bind to any antigen-antibody complexes. These second antibodies are coupled to the substrate-modifying enzyme.
- Wash the plate, so that excess unbound antibodies are removed.
- Apply a substrate which is converted by the enzyme to elicit a chromogenic or fluorescent signal.
- View/quantify the result using a spectrophotometer or other optical device.

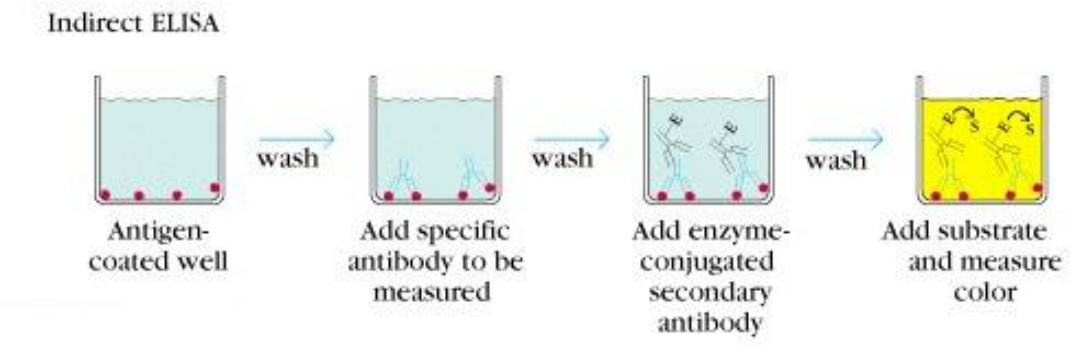


Figure 26: Indirect ELISA principle

The enzyme acts as an amplifier: even if only few enzyme-linked antibodies remain bound, the enzyme molecules will produce many signal molecules.

ELISA may be run in a qualitative or quantitative format. Qualitative results provide a simple positive or negative result for a sample. The cutoff between positive

and negative is determined by the analyst and may be statistical. Two or three times the standard deviation is often used to distinguish positive and negative samples. In quantitative ELISA, the optical density or fluorescent units of the sample is interpolated into a standard curve which is typically a serial dilution of the target.

b. Sandwich ELISA

Another variant of this technique, called "sandwich" ELISA, is used to detect sample antigen. The steps are as follows, summarized Fig.27:

- Prepare a surface to which a known quantity of antibody is bound.
- Apply the antigen-containing sample to the plate.
- Wash the plate, so that unbound antigen is removed.
- Apply the enzyme-linked antibodies which are also specific to the antigen.
- Wash the plate, so that unbound enzyme-linked antibodies are removed.
- Apply a chemical which is converted by the enzyme into a fluorescent signal.
- View the result: if it fluoresces, then the sample contained antigen.

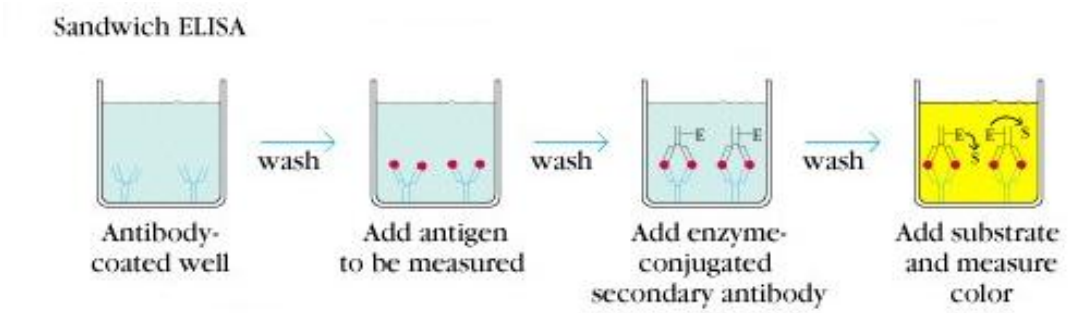


Figure 27: Sandwich ELISA principle

c. Competitive ELISA

A third use of ELISA is through competitive binding. The steps for this ELISA are somewhat different than the first two examples, as presented Fig.28:

- Unlabeled antibody is incubated in the presence of its antigen.
- These bound antibody/antigen complexes are then added to an antigen coated well.
- The plate is washed, so that unbound antibody is removed. (The more antigen in the sample, the less antibody will be able to bind to the antigen in the well, hence "competition.")
- The secondary antibody, specific to the primary antibody is added. This second antibody is coupled to the enzyme.
- A substrate is added, and remaining enzymes elicit a chromogenic or fluorescent signal.

For competitive ELISA, the higher the original antigen concentration, the weaker the eventual signal.

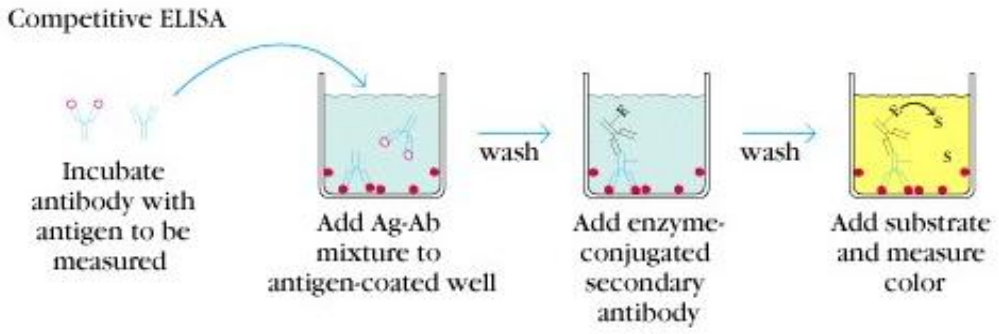


Figure 28: Competitive ELISA principle

2.4.3. BIAcore system

The BIAcore technology (cf. Fig.29) was also used in this study to assess both immunosensors and DNA-based biosensors. BIAcore is based on surface plasmon resonance (SPR) to monitor the interaction between molecules in real time. Although there are several SPR-based systems, by far the most widely used one is the BIAcore. [Rich and Myszka (2004). Moreno-Bondi (2002).]

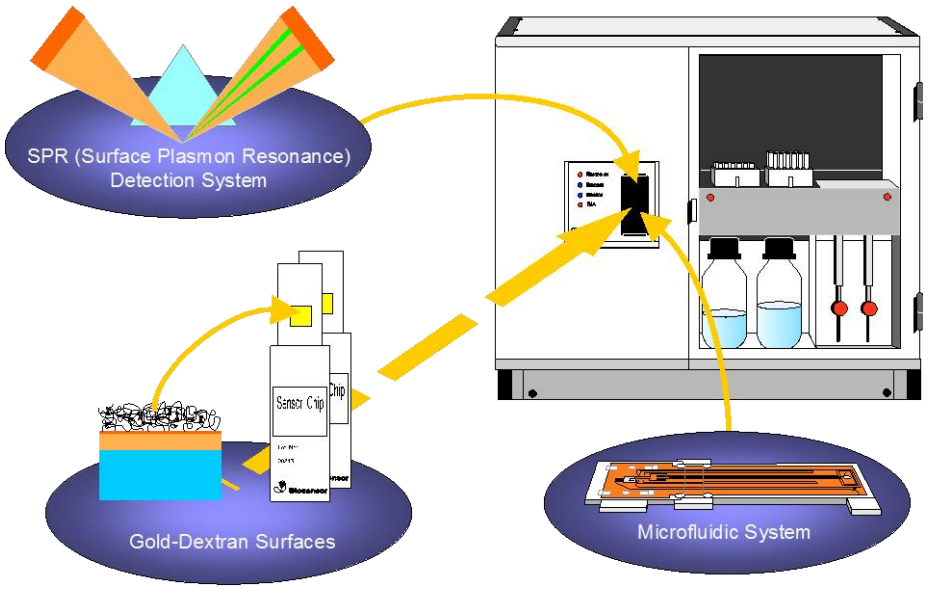


Figure 29: BIAcore system [BIAcore©]

2.4.3.1. *SPR and evanescent wave*

Evanescent waves are formed when sinusoidal waves are internally reflected off an interface at an angle greater than the critical angle so that total internal reflection occurs. "Evanescent" means "tending to vanish", which is appropriate because the intensity of evanescent waves decays exponentially with distance from the interface at which they are formed (see Fig.30). In this case, at least one component of the wavevector k becomes imaginary or complex and the wave experiences exponential damping when propagating in this region. When light experience total internal reflection at the core-cladding interface, some of the energy of the light waves in the core of the fibre penetrate into the cladding for a very short distance. The energy flow of this evanescent wave is parallel to the surface of the core and in the same direction as the main flow of energy within the core. [Karp *et al.* (2005). Rousseau *and* Rousseau (2000). Tschmelak *et al.*. (2005).]

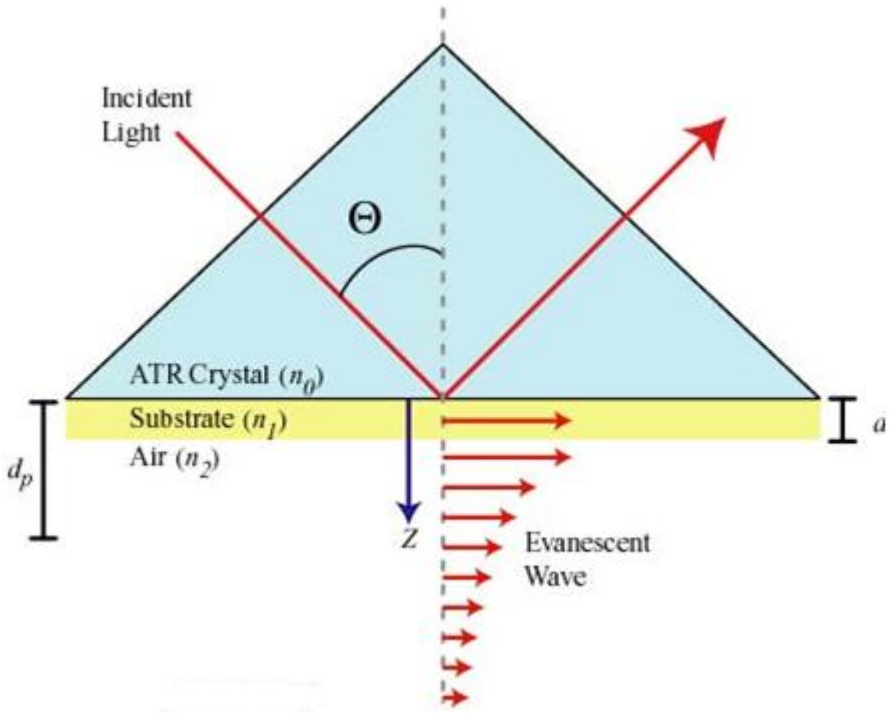


Figure 30: Evanescent wave

In the case of surface plasmon resonance not a guiding film is necessary, but a metal-dielectric interface typically located on a substrate for handling purposes. In this geometry, only one resonance exists. Therefore one needs to know more details about the system under consideration to measure specific quantities. Nevertheless the strength of the evanescent field in the case of a surface plasmon is larger than for waveguide modes. Therefore the sensitivity is typically larger with surface plasmon resonances in comparison to waveguide modes. Also a surface plasmon is a guided wave. It also has a propagation constant beta. The energy is not carried very far, because the metal dissipates it in about one micrometer. [Chou *et al.* (2004). Stocklein *et al.* (2000). Homola (2003). Karp *et al.* (2005).]

2.4.3.2. *The BIAcore*

SPR-based instruments use an optical method to measure the refractive index near a sensor surface, within about 300nm. In the BIAcore this surface forms the floor of a small flow cell, 20 to 60nL in volume, through which an aqueous solution, called the running buffer passes under continuous flow, ranging from 1 to 100µL/min. In order to detect an interaction one molecule, the ligand, is immobilised onto the sensor

surface. Its binding partner, the analyte, is injected in aqueous solution through the flow cell, also under continuous flow. As the analyte binds to the ligand, the accumulation of protein on the surface results in an increase in the refractive index. This change in refractive index is measured in real time, and the result plotted as response or resonance units (RU) versus time, drawing a sensorgram. (cf. Fig.31). Importantly, a background response will also be generated if there is a difference in the refractive indices of the running and sample buffers. This background response must be subtracted from the sensorgram to obtain the actual binding response. The background response is recorded by injecting the analyte through a control or reference flow cell, which has no ligand or an irrelevant ligand immobilized to the sensor surface.

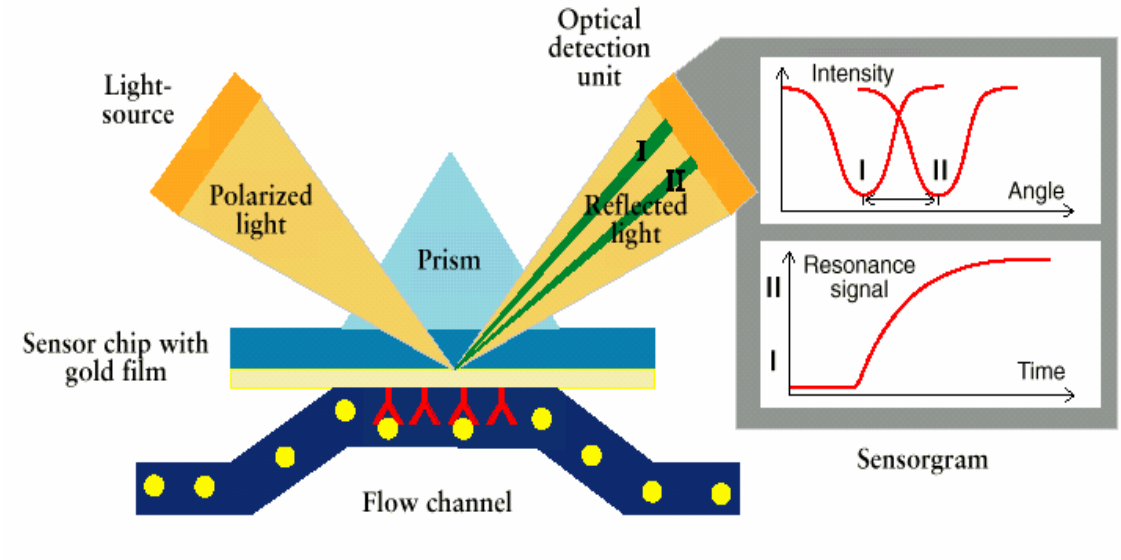


Figure 31: BIAcore system and fluidics [BIAcore©]

One resonance unit represents the binding of approximately 1 pg protein/mm2. In practice over 50pg/mm^2 of analyte binding is needed. Because is it very difficult to immobilize a sufficiently high density of ligand onto a surface to achieve this level of analyte binding, BIAcore have developed sensor surfaces with a 100 to 200nm thick carboxymethylated dextran matrix attached. By effectively adding a third dimension to the surface, much higher levels of ligand immobilization are possible.

The chip that was extensively used during this study is the Biacore CM5 chip. The sensor chip consists of a glass surface, coated with a thin layer of gold. The gold

surface is modified with a carboxymethylated dextran layer. This forms the basis for a range of specialized surfaces designed to optimize the binding of a variety of molecules. This dextran hydrogel layer forms a hydrophilic environment for attached biomolecules, preserving them in a non-denatured state. The chip surface is detailed Fig.32.

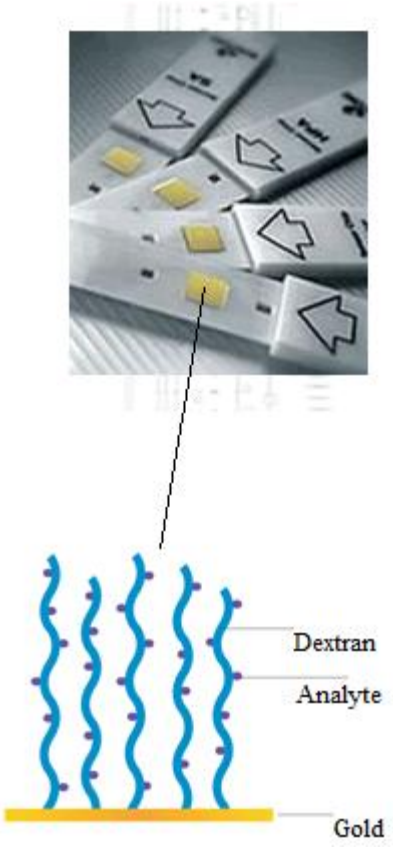


Figure 32: CM5 chip surface chemistry [BIAcore©]

The CM5 chip is the most polyvalent chip available from Biacore, with applications ranging from basic research to quality control. It allows interactions involving all types of biomolecules such as proteins, lipids, carbohydrates, and nucleic acids to be studied, through to large molecular assemblies or whole viruses. A high binding capacity gives a high response, advantageous for capture assays and for interactions involving small molecules. High surface stability provides accuracy and precision and allows repeated analysis on the same surface. The binding of the ligand onto the chip surface is most commonly done through amine coupling, as shown Fig.33. The chip surface is activated with a 1:1 mixture of NHS:EDC, to give reactive

succinimide esters. The ligand, in an appropriate buffer giving a positive charge, is passed over the surface and the esters react spontaneously with amino groups. Any free amine group can react with the surface. [www.biacore.com Mitchell *et al.* (2005). Myszka *et al.* (1998). Velge-Roussel *et al.* (1995).]

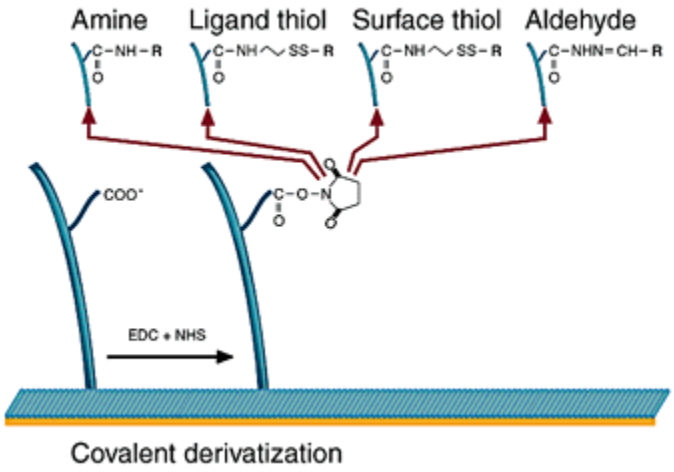


Figure 33: CM5 chip surface possible binding reactions [BIAcore©]

2.4.3.3. *Data analysis*

a. Optical detection

When a beam of light passes from material with a high refractive index (e.g. glass) into material with a low refractive index, for example water, some light is reflected from the interface. When the angle at which the light strikes the interface, the angle of incidence (θ), is greater than the critical angle (θ_c), the light is completely reflected. This phenomenon is called total internal reflection.

If the surface of the glass is coated with a thin film of a noble metal like gold, this reflection is not total as some of the light is "lost" into the metallic film. There then exists a second angle greater than the critical angle at which this loss is greatest and at which the intensity of reflected light reaches a minimum or "dip". This angle is called the surface plasmon resonance angle (θ_{spr}). It is a consequence of the oscillation of mobile electrons (or "plasma") at the surface of the metal film. These oscillating plasma waves are called surface plasmons. When the wave vector of the incident light

matches the wavelength of the surface plasmons, the electrons resonate, hence the term surface plasmon resonance.

The coupling of the incident light to the surface plasmons results in a loss of energy and therefore a reduction in the intensity of the reflected light. It is because the amplitude of the wave vector in the plane of the metallic film depends on the angle at which it strikes the interface that a θ_{spr} is observed. An evanescent or decaying electrical field associated with the plasma wave travels for a short distance, about 300nm into the medium from the metallic film. Because of this, the resonant frequency of the surface plasma wave and thus θ_{spr} depends on the refractive index of this medium.

If the surface is immersed in an aqueous buffer (refractive index or μ ~1.0) and protein (μ ~1.33) binds to the surface, this results in an increase in refractive index which is detected by a shift in the θ_{spr}. The instrument uses a photo-detector array to measure very small changes in θ_{spr}. The readout from this array can be viewed on the BIAcore. The change is quantified in resonance units or response units (RU) with 1 RU equivalent to a shift of 10^{-4}degrees. Empirical measurements have shown that the binding of 1ng/mm^2 of protein to the sensor surface leads to a response of ~1000 RU. Since the matrix is around 100nm thick, this represents a protein concentration within the matrix of 10mg/mL. Apart from the refractive index, the other physical parameter which affects θ_{spr} is temperature. Thus a crucial feature of any SPR instrument is precise temperature control. [Casper *et al.* (2004). Khalifa *et al.* (2001). Lee *et al.* (2004).]

b. Affinity

There are a number of ways to represent the affinity of an interaction.

- The association constant (K_A) or affinity constant is simply the ratio at equilibrium of the product and reactant concentrations. Thus, for the interaction A+B↔AB, the association constant is (cf. Equ.11)

$$K_A = \frac{C_{AB}}{C_A \times C_B}$$

Equation 11: Association constant for expressing affinity

Note that K_A has units M-1 (i.e. $L.mol^{-1}$)

- Many prefer to express affinity as the dissociation constant or K_D, which is simply the inverse of the K_A, and therefore has the units M.
- Affinity can also be expressed as the binding energy or, more correctly, the standard state molar free energy ($\Delta G°$). This can be calculated from the dissociation constant as follows Equ.12.

$$\Delta G^o = RT\ ln\frac{K_D}{C^o}$$

Equation 12: Standard state molar free energy for expressing affinity

where T is the absolute temperature in Kelvin (298.15K = 25°C),R is the Universal Gas Constant ($1.987 cal.K^{-1}.mol^{-1}$) and C° is the standard state concentration (i.e. 1M).

In order to derive an affinity constant from the data a particular binding model must be used. The simplest model, Langmuir model: A+L↔AL is applicable in the vast majority of cases. It assumes that the analyte A is both monovalent and homogenous, that the ligand L is homogeneous, and that all binding events are independent. Under these conditions data should conform to the Langmuir binding isotherm, presented Equ.13.

$$Bound = \frac{C_A \times Max}{C_A + K_D}$$

Equation 13: Expression of the Langmuir binding isotherm

where "Bound" is measured in resonance units (RU) and "Max" is the maximum response (RU), C_A is the concentration of injected analyte and K_D is in the same units as C_A, usually M.

The K_D and Max values are best obtained by non-linear curve fitting of the equation to the data using suitable computer software. [Liu *et al.* (2004).]

c. Kinetics

The period during which analyte is being injected is termed the 'association phase' whereas the period following the end of the injection is termed the 'dissociation phase'. During the association phase there is simultaneous association and dissociation. Equilibrium is reached when the association rate equals the dissociation rate. (cf. Fig.34)

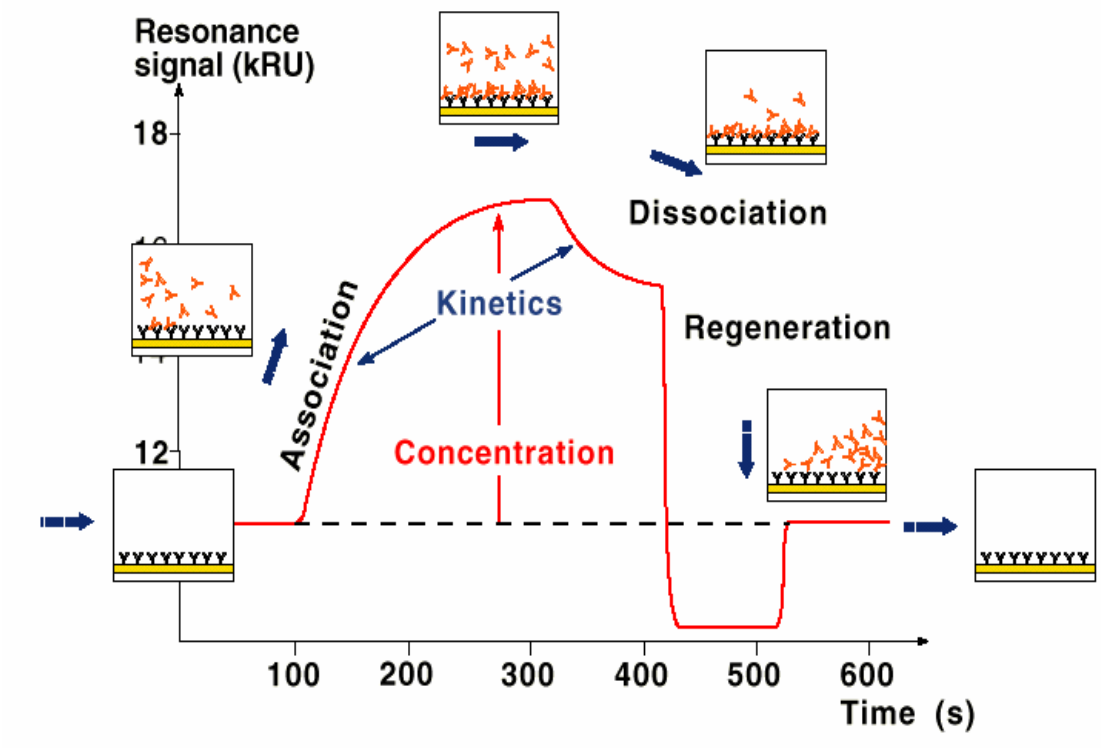


Figure 34: BIAcore sensogram [BIAcore©]

The main factors affecting the association rate are the concentration of analyte near the ligand (C_A), the concentration of ligand (C_L), and the association rate constant (k_{on}). Because of the high surface density of ligand on the sensor surface, the rate at which analyte binds ligand can exceed the rate at which it is delivered to the surface, referred to as mass transport. The main factors affecting the analyte dissociation rate are the surface density of bound analyte, the dissociation rate constant (k_{off}), and the extent to which dissociated analyte rebinds to ligand before leaving the sensor surface.

Mass transport limitations, which lead to an underestimation of the intrinsic kinetics, are aggravated by low flow rates, high levels of immobilized ligand, and high

intrinsic association rate constants. They can be reduced by increasing the flow rate and, most importantly, lowering the level of immobilized ligand.

Analysis of kinetic data is best performed using the BIAevaluation software supplied with the instruments as this has been designed especially for the purpose. After subtracting the background responses, an attempt should be made to fit the simple 1:1 Langmuir binding model to the data. For any particular sensogram, as much of the data as possible should be included in the fit. This normally includes the entire association and dissociation phases, omitting only the noisy few seconds at the beginning and end of the analyte injection.

The association phase cannot be analysed if equilibrium is attained within 2 to 4s, which is usually the case if the k_{off} is over $1s^{-1}$. In contrast, the dissociation phase can analyzed even if the k_{off} is over $1s^{-1}$. This global fitting establishes whether a single global k_{on} and k_{off} provide a good fit to all the data. An important internal test of the validity of the kinetic constants is to determine whether the calculated K_D (K_{Dcalc}= k_{off}/k_{on}) is equal to the K_D determined by equilibrium analysis. When a poor fit is obtained to the data using the simple 1:1 binding model, the binding kinetics are considered complex. [Gooding *et al.* (2004). Stocklein *et al.* (2000). Haseley *et al.* (1999). Katsamba *et al.* (2002). Lipschultz *et al.* (2000).]

2.4.4. Akubio system

This system is in many aspects parallel if not similar to the BIAcore, although its detection principle is based on acoustics rather than optics. The Akubio system, in a few words, is an acoustic sensor exploiting resonating quartz crystals to detect the binding of an analyte to a receptor. Its principle is presented Fig.35.

Many molecules are polarized, and when an electric field is applied, the molecules will align themselves with it, producing induced dipoles within the molecular structure of the material. A permanently polarized material, like quartz (SiO_2), will produce an electric field when the material changes dimensions as a result of an imposed mechanical force. This material is called piezoelectric and the phenomenon known as piezoelectric effect. Quartz resonator became of interest when it was demonstrated that there is a linear relationship between the mass adsorbed at the

surface and the resonant frequency of the crystal. Application to biological samples became possible when oscillator circuits in liquids were developed.

The molecule interacts with immobilized receptors on the surface of a quartz crystal, resulting in a variation in the acoustic resonance of the crystal. This in turns produces an electrical signal that can be analyzed. The signal indicates not only the presence of the analyte, but also its affinity and specificity to a surface-bound receptor. Real-time monitoring of changes in the resonance properties of the crystal allows the label free determination of interaction affinities and kinetics in a large variety of buffers. [Zhou and Muthuswamy (2004). Heising (1946). Mason (1950)]

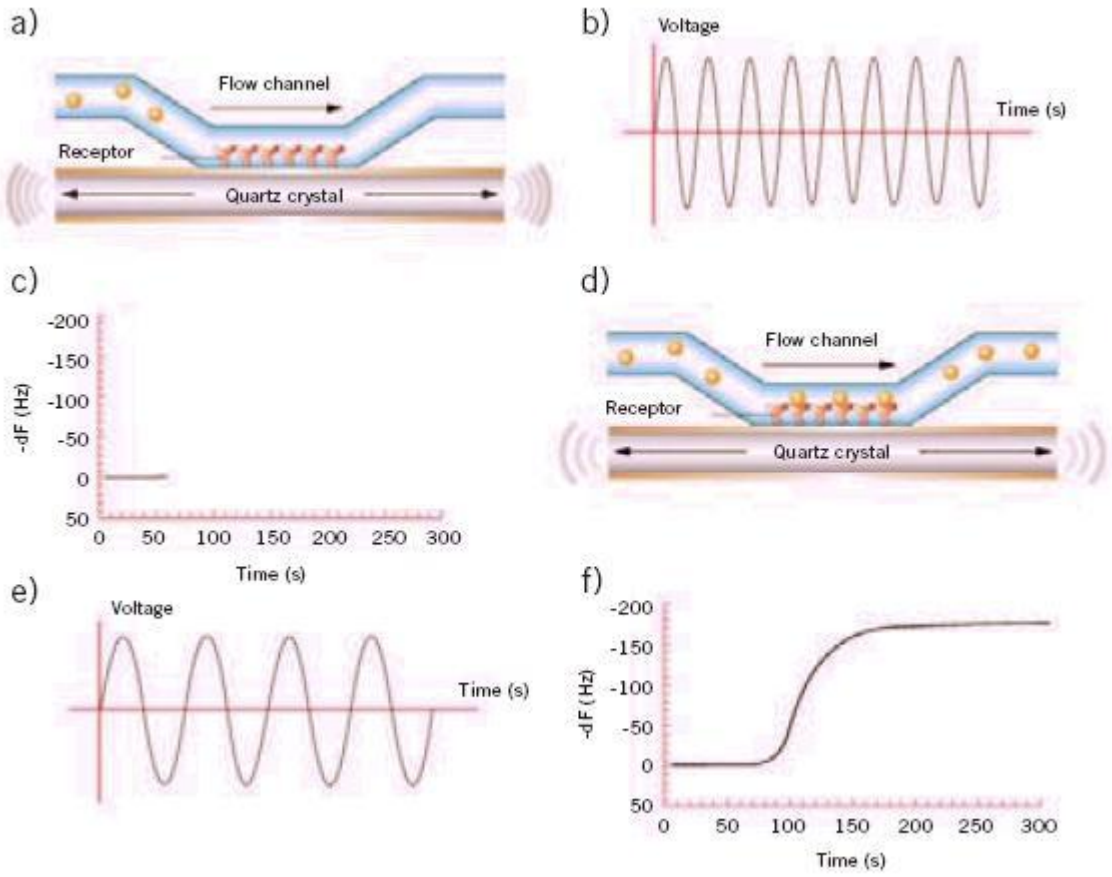


Figure 35: Principles of Resonant Acoustic Profiling [Akubio©]

a) A quartz resonator coated with target receptor is integrated with a liquid handling system for sample delivery

b,c) While buffer is passed over the sensor surface the crystal is oscillated and its resonant frequency measured in real-time

d) Sample is then passed across the sensor surface

e) Binding of material to the surface results in a change in the resonance profile of the resonator

f) The change in frequency is proportional to the amount of sample bound to the sensor surface

Although the detection systems are based on very different principles, the fluidics and binding mechanisms of the Akubio system are very close to those of the BIAcore, as is the information collected, thus allowing the carefully optimized system developed for the BIAcore system easily transferable to the Akubio system, but over a different range of applications.

2.5. DNA-based biosensors

The last system used in this study is DNA-based biosensors. This section will provide with a short background on this type of biosensors. [Karlsson *et al.* (1999).]

2.5.1. DNA: a brief overview

2.5.1.1. Presentation

Deoxyribonucleic acid (DNA) is a nucleic acid that contains the genetic instructions used in the development and functioning of all known living organisms and some viruses. The main role of DNA molecules is the long-term storage of information. Chemically, DNA is a long polymer of simple units called nucleotides, with a backbone made of sugars and phosphate groups joined by ester bonds. Attached to each sugar is one of four types of molecules called bases. It is the sequence of these four bases along the backbone that encodes information. A schematic of the DNA structure is presented Fig.36.

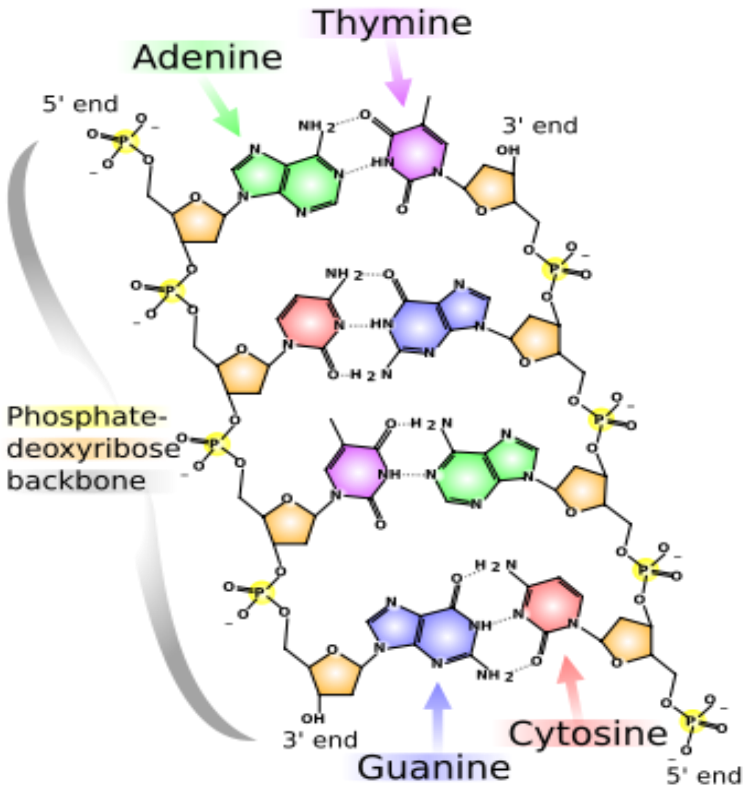


Figure 36: Chemical structure of DNA

The DNA chain is 22 to 26Å wide, and one nucleotide unit is 3.3Å long. Although each individual repeating unit is very small, DNA polymers can be enormous molecules containing millions of nucleotides. For instance, the largest human chromosome, chromosome number 1, is approximately 220 million base pairs long. In living organisms, DNA does not usually exist as a single molecule, but instead as a tightly-associated pair of molecules. These two long strands entwine like vines, in the shape of a double helix. If multiple nucleotides are linked together, as in DNA, this polymer is called a polynucleotide

2.5.1.2. *Structure*

The backbone of the DNA strand is made from alternating phosphate and sugar residues. The sugar in DNA is 2-deoxyribose, which is a pentose sugar. The sugars are joined together by phosphate groups that form phosphodiester bonds between the third and fifth carbon atoms of adjacent sugar rings. These asymmetric bonds mean a strand of DNA has a direction. In a double helix the direction of the nucleotides in one

strand is opposite to their direction in the other strand. This arrangement of DNA strands is called antiparallel. The asymmetric ends of DNA strands are referred to as the 5′ and 3′ ends, with the 5' end being that with a terminal phosphate group and the 3' end that with a terminal hydroxyl group.

The DNA double helix is stabilized by hydrogen bonds between the bases attached to the two strands. The four bases found in DNA are adenine (A), cytosine (C), guanine (G) and thymine (T). These four bases are attached to the sugar/phosphate to form the complete nucleotide These bases are classified into two types; adenine and guanine are purines, while cytosine and thymine are pyrimidines Each type of base on one strand forms a bond with just one type of base on the other strand: This is called complementary base pairing. Purines form hydrogen bonds to pyrimidines, with adenine bonding only to thymine, and cytosine bonding only to guanine (cf. Fig.37). The double helix is also stabilized by the hydrophobic effect and pi stacking, which are not influenced by the sequence of the DNA. As hydrogen bonds are not covalent, they can be broken and rejoined relatively easily. The two strands of DNA in a double helix can therefore be pulled apart like a zipper, either by a mechanical force or high temperature.

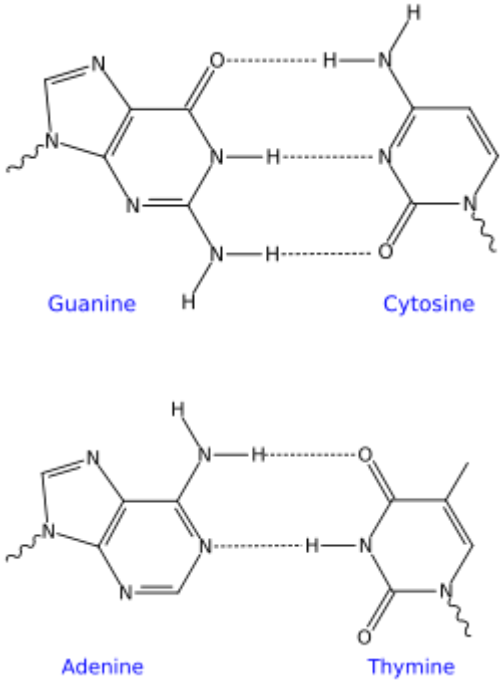


Figure 37: Complementary base pairing

2.5.1.3. *Applications*

DNA finds many applications: Modern biology and biochemistry make intensive use of recombinant DNA technology, while forensic scientists can use DNA in DNA profiling. Also, bioinformatics involves the manipulation, searching, and data mining of DNA sequence data.

DNA nanotechnology uses the unique molecular recognition properties of DNA and other nucleic acids to create self-assembling branched DNA complexes with useful properties. DNA is thus used as a structural material rather than as a carrier of biological information.

Finally, because DNA collects mutations over time, which are then inherited, it contains historical information and by comparing DNA sequences, geneticists can infer the evolutionary history of organisms, their phylogeny. This field of phylogenetics is a powerful tool in evolutionary biology. If DNA sequences within a species are compared, population geneticists can learn the history of particular populations.

2.5.2. DNA-based biosensors

DNA biosensors and gene chips are of major interest due to their tremendous promise for obtaining sequence-specific information in a faster, simpler and cheaper manner compared to the traditional hybridization. Recent advances in developing such devices open new opportunities for DNA diagnostics. [Zhai Junhui *et al.* (1997). Zhu *et al.* (2002) Meadows (1996). Meadows (1996).]

DNA biosensors, based on nucleic acid recognition processes, are rapidly being developed towards the assay of rapid, simple and economical testing of genetic and infectious diseases. Unlike enzyme or antibodies, nucleic acid recognition layers can be readily synthesized and regenerated for multiple uses. DNA sensors can be made by immobilizing single stranded (ss) DNA probes on different electrodes using electroactive indicators to measure the hybridization between DNA probes and their complementary DNA strands. [Del Pozo *et al.* (2005). Mannelli *et al.* (2005). Wang *et al.* (2004).]

Recent advances in the field of biomolecular techniques can be used to fabricate a new generation of miniaturized biosensor. The Table 2 summarizes the advantage and disadvantages of different types of DNA biosensors. [Donaldson *et al.* (2004) Marrazza *et al.* (1999). Minunni *et al.* Oliveira-Brett *et al.* (2004). Wang *et al.* (2004). Jianrong *et al.* (2004). Meric *et al.* (2004).]

Type	Principle	Advantage	Disadvantage
1/ Optical			
- Fiber optics	Evanescent wave based, allows measurement of binding at the fiber surface	Remote in-situ measurement, inherent sensitivity of optical approached	Costly equipment and not portable
- Laser interferometry	Planar waveguides have evanescent field responsive to changes in index of refraction	Highly sensitive, detect up to one cell	Susceptibility to turbidity interference
2/ Electrochemical		Fast, low cost	Highly buffered solutions may interfere
- Conductimetric	Change in conductance		
- Potentiometric	Electric potential		
- Amperometric	Oxidation/reduction		
3/ Piezoelectric	Quartz crystals oscillations at defined frequencies, binding of an analyte to it changes the mass of crystal, hence the oscillation frequency	Highly sensitive, fast	Sensitivity levels up to one cell have not been demonstrated
4/ Colorimetric / Strip	Color development	No instruments required	Not quantitative
5/ DNA biochip	Array based	Instrument required	Quantitative

Table 2: Different types of DNA sensors

2.6. Biosensor stabilization

Lack of stability of the biological component, in this case enzyme, is one of the most important drawbacks for biosensors application, especially where reusable electrodes are required. Enzymes have to fulfill two important characteristics: to be stable and to be functional

In order to be stable, enzymes have to form a hydrophobic core and optimize their intra-molecular H-bonds. In order to be functional, they must carve out their activity site, exposing hydrophilic areas. [Beadle *and* Soichet (2002).]A model has been calculated, that allows predicting shelf-life of biological products, for first degree enzyme deactivation. [McAteer *et al.* (1999).]

To increase the stability of the enzyme without hindering its activity, various attempts have been made. Both shelf life and operational stability may be enhanced through several ways: [Fagain (2003). Dankwardt *et al.* (1998). Sotiropoulou *et al.* (2005). Ramos *et al.* (1997). Hinrichs *et al.* (2001).]

- Immobilization to a supporting structure,
- Chemical modification,
- Protein engineering,
- Additives.

2.6.1. Immobilization

While immobilization can give notable stability gains, it is generally undertaken to prevent loss of a biocatalyst, or to improve bioreactor operation. It may affect the protein conformation. It may also be responsible for non-uniform distribution, parasite reaction or diffusion.

The entrapment of the enzyme, glucose oxidase, for example, in liposome is highly dependant of the liposome composition. It allows the constitution of a diffusion membrane with low permeation for glucose, for instance, and high permeation for O_2. [Memoli *et al.* (2002).] The enzyme is more often entrapped in polymers. [Andreescu *et al.* (2002). Pan *et al* (2004).]

The use of a membrane protects the enzymes and reduces interferences in a similar way to enzyme entrapment. The membrane is usually permselective and enhances the sensibility of the biosensor. [Kok *and* Hasirci (2004).] The presence of inert peptides or polymers in the membrane lowers the detection limit and increases the sensitivity, pointing out the importance of the enzyme matrix on analytical performances. [Ivanov *et al.* (2000).]

The enzyme can be immobilized within a composite compound:

- Sol-gel carbon composite, by covalent bonding, where the composite comprises graphite powder, ferrocene, and an amino- and methyl-silicate backbone. The graphite powder provides the conductivity for the electrode and ferrocene acts as the mediator for signal transduction from the active center of the enzyme to the electron conductive surface. The presence of amine groups in the sol–gel silicate network allows for the covalent bonding sites for the enzyme via the carbodiimide reaction. [Yang *et al.* (2003).]
- Or on Prussian blue modified glassy carbon with a silica sol-gel outlayer with the enzyme immobilized on a flow-through working electrode, which was prepared from reticulated vitreous carbon (RVC) or from a composite material consisting of RVC and superporous agarose. [Li *et al.* (2004).]

- On composite films, for example the immobilization of lactate dehydrogenase (LDH) on poly(aniline)/poly(acrylate) [PANi/PAA] and poly(aniline)/poly(vinylsulfonate) [PANi/PVS] composite films. [Simon *et al.* (2002).]
- Or composite paste, for example pyrrole-based: The composite carbon paste electrodes were prepared by in situ generation of polypyrrole (PPy) within a paste containing the enzyme polyphenol oxidase (PPO). The in situ electrogenerated polypyrrole improves the enzyme immobilisation within the paste [Mailley *et al.* (2004]

- On hydrogel [Patel *et al.* (2001). Castillo *et al.* (2003).], for example in a Clark-type oxygen electrode, the enzyme, lactate dehydorgenase, is

entrapped on a PCS (poly(carbamoyl) sulfonate) hydogel on a Teflon membrane, allowing a completely different detection system. [Kwan *et al.* (2004).]

- In organic films, within a layer-by-layer system, such as glucose oxidase / polyethylenimine bilayers on modified pyrolytic graphite electrodes [Zhang *et al.* (2004).]
- In biocomposites, for example, alcohol oxidase - graphite-epoxy resin system, that greatly enhances alcohol oxidase stability [Morales *et al.* (1998).]
- In or on graphite Teflon composite, for instance by physical insertion of the enzyme in the bulk: The bienzyme electrodes are constructed by simple physical inclusion of the enzymes and the mediator (alcohol oxidase (AOD) and horseradish peroxidase (HRP), as well as the mediator ferrocene) in the bulk of graphite/70% Teflon rigid cylindrical pellets. The composite biosensors are robust and reusable because of the renewability of the electrode surface by polishing. [Guzman-Vasuez de Prada *et al.* (2003).]

Indium-tin oxide (ITO) electrodes were used in several immobilization systems, either by direct covalent immobilization of the enzyme on indium-tin oxide sputtered platinum electrode [Fang *et al.* (2003).], or in a layer-by-layer system: for example, coupling glucose oxidase with poly(allylamine) hydrochloride, onto indium-tin oxide modified with a prussian blue electrode. [Ferreira *et al.* (2004).].

The enzyme can be contained in or located at the surface of a cell, which is immobilized at the electrode surface. [Ito *et al.* (2002). Yu *et al.* (2004). Katrlik *et al.* (1997] The cell may be genetically engineered to produce the enzyme of interest at a precise location. [Akyilmaz *and* Dinckaya (2004).]

The electrode surface may be modified in order to allow the immobilization. The addition of an electrocatalyst whose structure would facilitate the enzyme adsorption is an example. Fullerene, carbon nanotubes, porous carbon and porous glassy carbon are now used in glucose oxidase biosensors as electrochemical mediators, stabilizing agents and transducers. [Sotiropoulou *et al.* (2003).]. Glucose oxidase has also been

successfully immobilized on colloidal gold modified carbon electrodes and on Teflon-carbon electrodes, and associated for the latter with high performance liquid chromatography (HPLC) systems. [Liu *and* Ju (2003). Guzman-Vasquez de Prada *et al.* (2004).]

A few other techniques of immobilization have been put to test, like plasma polymerization technique and affinity immobilization based on bio-recognition mechanisms, avoiding chemical cross-linking. [Cokeliler *and* Mutlu (2002). Bucur *et al.* (2004).]

Different immobilization techniques have been compared, from bio-encapsulation in sol-gel composite to immobilization by metal-chelate affinity, to entrapment in polymer. [Andreescu *et al.* (2002] However, physical adsorption of the enzyme at the surface electrode is the easiest, less denaturing, cheapest and quickest method. [Bonnet *et al.* (2003).] One drawback is the involvement higher amount of enzyme; the other is the gradual leaching observed under stirring conditions. A membrane, for example acetate membrane, is an easy way to prevent the latter. Mediators and stabilizing agents, if needed, are easily added either at the Carbon-ink or adsorbed at the electrode surface together with the enzyme. [Jawaheer *et al.* Ohfuji *et al.* (2003).]

2.6.2. Chemical modification

This remains a useful solution, despite having been overshadowed by genetic strategies. Several ways have been used, of which:

- Cross-linked enzymes
- Covalent attachment to polymers
- Surface modification (by chemical modification of charged groups at the molecule surface)

Cross-linking and covalent attachment are the most widely used technique within the chemical modifications. Enzymes can be cross-linked to one another [Xin *and* Wightman (1997).] by the use of bifuntional reagents such as glutaraldehyde [Nguyen *et al.* (2003). Wu *et al.* (2004).] When an enzyme is cross-linked with

glutaraldehyde, other compounds are also added as glutaraldehyde is a strong bifunctional reagent that modifies the enzyme in such a way as to lead to conformational change and loss of activity. This effect, however, may be minimized by the addition of inert proteins, such as Bovine Serum Albumine or gelatine, as these proteins may have a stabilizing effect. [Armada *et al.* (2003). Gouda *et al.* (2001).] Other compounds may be added, such as Nafion [Ricci *et al.* (2003).]. The cross-linked enzyme can also be contained within a polymer, for example chitosan, a biopolylmer. [Yang *et al.* (2004).]

Covalent immobilization is also used to improve thermal stability. It enhances as well the biosensor shelf life. However, it may cause great losses in enzyme activity. [Appleton *et al.* (1997). Razumiene *et al.* (2003).]

Other systems have been successfully tested, but they remain seldom used compared to immobilization techniques. One of those systems uses the avidin/biotin interactions on biotinilated glucose oxidase: Conjugates of avidin with ferrocene and with microperoxidase have been used as electrochemically active molecular building blocks. Assemblies of the conjugates with biotinylated glucose oxidase on gold electrodes were then used as enzyme sensors. [Padeste *et al.* (2003] Another is the silanization of glucose: Immobilization of the enzyme is carried out using glass beads as support and the effect of silane concentration during the silanization step on the thermal stability of glucose oxidase has been investigated. The increased stability of the enzyme in the presence of high silane concentrations may be attributed to the increase in the surface hydrophobicity of the support. [Sarath Babu *et al.* (2004).]

The lasts are using charges: the first being the use of glucose oxidase as a doped anion in ethacridine polymer [Xu *and* Chen (2000).] and the second the addition of charged electrolyte onto the glucose oxidase biocrystal surface within a nanoscale of polyelectrolyte film of polyalylamine/polystyrene sulfonate, achieved by the sequential adsorption of oppositely charged polyelectrolytes onto the glucose oxidase biocrystal surface. The polyelectrolyte system polyallylamine/polystyrene sulfonate was being used under high salt conditions to preserve the solid state of the highly water soluble glucose oxidase biocrystals during the encapsulation process. The resulting polymer multilayer capsule of about 15 nm wall thickness is permeable for small molecules

such as glucose, but non-permeable for macromolecules thus preventing the enzyme from leakage and at the same time shielding it from the outer environment e.g., from protease or microbial activity. [Trau *and* Renneberg (2003).]

2.6.3. Protein engineering

It is the manipulation of the protein at the genetic level. Genetically modified proteins offer enhanced capacities compared to their native forms. [Brasil de Olivera Marquez *et al.* (2004).] Protein engineering occur under 3 main forms:

- It can be performed as a direct evolution, through the recombination of beneficial point mutations, with a selection in order to obtain further improved properties. The major drawback of the direct evolution is its leading to noteworthy alterations of the baseline properties. On the other hand, no previous knowledge of the target protein molecular structure is required.
- Another way is a polypeptide chain extension. It is the attachment to either the C- or N- terminal extremity of an enzyme to a polypeptide chain. This often leads to enhanced thermo-stability. [Chen *et al.* (2002).]
- The last option is site specific mutagenesis. It offers a great scope for protein stability improvement where the structure f the target protein is known. [Schulze *et al.* (2003). Gulla *et al.* (2004).]

2.6.4. Additives

They involve the use of salts, polyols, divalent metal ions and sugars. These additives are believed to influence the microenvironment of the enzyme and modify the hydrophobic or hydrophilic interactions by disrupting the enzyme/water interactions.

A range of low molecular weight additives, sugar derivatives, exert stabilizing effect, such as trehalose. [Nuyen *et al.* (1997).] The study of protein and enzymes from extremophilic organisms can give insight to protein stability as well as providing ready-made stable proteins and stabilizing agents for biotechnological applications.

The use of additives, as well as protein engineering, is often coupled with immobilization techniques. Additives and protein engineering allow the enzyme to be more stable whereas immobilization also prevents the enzyme from leaking and in some cases (the use of membranes, for example) protects the enzyme from interferences.

The case that have been here presented are however simple, as they only utilize one enzyme type per biosensor. Several systems involved the association of two or more enzymes, in addition to co-enzymes and mediators, within the stabilization systems detailed earlier. A simple example would be the association of lactate dehydrogenase to lactate oxidase on a screen-printed electrode, with a mediator (e.g. cobalt phtalocyanine). The association of both allows the detection of hydrogen peroxide at E=+300mV, versus Ag/AgCl. The further addition of glucose dehydrogenase (GDH) enables the recycling of lactate dehydrogenase cofactor, NADPH, lowering its concentration. Another simple system is the association of glucose oxidase and hexokinase (HEX) for the detection of ATP, using a pH shift induced. The constitution of bienzyme systems, allows simpler detection means. For example oxidase/peroxidase systems are very efficient.

2.7. Hypersolutes

2.7.1. Origin and production

2.7.1.1. Compatible solutes from hyperthermophiles

Thermophiles and hyperthermophiles accumulate compatible solutes that have not been found, or have been rarely encountered in mesophilic organisms leading to the view that the compatible solutes of hyperthermophiles are specifically associated with life at high temperatures. These compatible solutes are generally negatively charged, while other microorganisms generally accumulate neutral or zwitterionic compatible solutes. The term “Hypersolutes” was coined to designate compatible solutes derived from hyperthermophiles. [Fields (2001). Fujiwara (2002).]

Thus far, di-myo-inositol-1,1'-phosphate (DIP) is the most widespread small molecular weight solute of hyperthermophiles and has never been found in mesophilic organisms. In most of those organisms large increases in the levels of DIP are observed at growth temperatures above the optimum. [Goncalves *et al.* (2003)]

Another, mannosylglycerate (MG) is a compatible solute widely distributed among thermophilic and hyperthermophilic organisms. Mannosylglycerate together with the corresponding amide derivative, mannosylglyceramide (MGA), was identified in the thermophilic bacterium Rhodothermus marinus. [Alarico *et al.* (2005)] In contrast to di-myo-inositol-1,1'-phosphate, the concentration of Mannosylglycerate increases concomitantly with the Sodium Chloride concentration of the medium and serves, therefore, as a compatible solute under salt stress.

Very recently, a novel compatible solute was discovered, mannosyl glucosyl glycerate in the thermophilic bacterium Petrotoga miotherma. It is interesting that the molecular structure of this compatible solute comprises both mannosyl and glyceryl present in mannosylglycerate. Another hypersolute, di-glycerol-phosphate (DGP), has only been found in Archaeoglobus fulgidus, where it is by far the major compatible solute during salt stress. [Stetter (1999). Kengen *et al.* (1996) Jaenicke and Bohm (1998). Scandurra *et al.* (1998).]

2.7.1.2. *Synthesis of hypersolutes*

As an example of the different synthesis solutions for Hypersolutes, two pathways for the synthesis of Mannosylglycerate have been found in R. marinus. One pathway is identical to the one found in other hyperthermophiles, while the other is a one-step pathway in which GDP-mannose is condensed with D-glycerate to produce mannosylglycerate.

Some of these genes were recently cloned in an E. coli strain engineered to produce Mannosylglycerate from mannose. [Madigan and Oren (1999).] Although the accumulation of Mannosylglycerate in E. coli was very low, this strategy allowed the production of labeled mannosylglycerate, essential to investigate transport of this solute in bacterial as well as mammalian cells. The accumulation of mannosylglycerate by T. thermophilus was also enhanced single mutant strains lacking the genetic

information for the synthesis of trehalose, which in these organisms is the major compatible solute.

With these mutants, or natural variants, the accumulation of mannosylglycerate could be several folds increased. Moreover, this organism can be subjected to several cycles of hypo-osmotic shock leading to the secretion of mannosylglycerate that facilitates downstream processing, and to the re-utilization of the cell mass to produce more mannosylglycerate upon osmotic shock. The patent of this process, designated "bacterial milking" is owned by one of partners of the consortium. [Rusterholtz and Pohlschroder (1999). Schiraldi and De Rosa (2002). Rontein *et al.* (2002).]

2.7.1.3. *Present applications*

A number of hypersolutes have been shown to be superior to their mesophilic counterparts in the preservation of the performance of test enzymes. The protecting properties of compatible solutes from mesophiles, trehalose in particular, have been demonstrated in a variety of biomaterials, ranging from enzymes, viruses, living cells, skin, and artificial tissues, against damage caused by heat, dehydration, freezing, and UV radiation.

Among hypersolutes, mannosylglycerate and diglycerol phosphate have been studied to a greater extent and have been shown to protect enzymes and proteins in vitro better than compatible solutes from mesophiles. Several industrial applications have been proposed for Mannosylglycerate, but the most encouraging to date is as a skin moisturizer since this compound has been found to be superior to ectoine, which is already used for skin protection. Other applications studied recently have been the protection of vaccines against inactivation caused by storage and transportation. [Faria *et al.* (2004). Zeder-Lutz *et al.* (1997). Miroliaei and Nemat-Gorgani (2001). Navratilova *et al.* (2005).]

2.7.2. The HotSolutes project

Compatible solutes from hyperthermophilic bacteria, called hypersolutes, are superior to their mesophilic counterparts in the preservation of the performance of a wide variety of biomaterials, ranging from proteins to whole cells, skin, and artificial

tissues. In both an economical and scientific points of view, the rapid development and discovery of new such compounds makes it urgent to unravel their potentialities. The goal of the HotSolutes project is to find hypersolutes applications in old and emerging areas of research, ranging from classical Molecular Genetics to emerging approaches such as Genomics and Proteomics. The intent of the HotSolutes project is to find applications within the areas of protein stabilization and aggregation, DNA and protein microarrays, molecular biology enzyme performance, biosensor technology, heterologous protein-production systems, and animal cell line stabilization. [Aguilar (1996). HOTsolutes project (2004).]

2.7.2.1. *Biosensors*

For most commercial biosensor systems, enzymes constitute the core component. Hence, enzyme stability is at the forefront of producing a stable and reproducible biosensor. At present, stabilization products are added to formulations used to mass-produce biosensors; they provide thermal and chemical stabilization. In addition these compounds help to prevent proteolytic degradation after processing.

Despite the use of these stabilizers, many enzyme based sensor systems still suffer significant instability. The introduction of new, more effective stabilization compounds would greatly enhance the biosensor industry. This would apply to the existing commercially available biosensors (e.g. glucose, lactate, alcohol etc.) and would permit the development of hitherto unknown available biosensors.

2.7.2.2. *Preservation of biological material upon storage*

The last few years have witnessed a significant expansion of human DNA, tissue or cell collections in order to exploit and study the genetic information collected. This activity has strategic implications for genetic research, clinical care and future treatments. Thus, the stability of biological materials during long periods of time is of the utmost importance in molecular biology. The non-destructive preservation of biological materials is dependent upon suspending spontaneous and enzymatic degradation while maintaining form and function. Not only is the integrity of the biological material required, but also the maintenance of spatial arrangement (e.g., protein folding) and biological activity.

Basically, both the macroscopic and molecular aspects of the preserved material must be maintained. Several means are available for preservation, and these include low temperatures, the reduction of water activity and the addition of chemical protectants. These methods can be used alone or in combination. The nature of the biological material, whether it is a protein, nucleic acid or tissue sample, will determine the methods used for its preservation.

2.7.2.3. *Hypersolutes to prevent misfolding/misassembly and protein aggregation*

Protein aggregation and precipitation is a commonplace observation in biotechnology, in both the inclusion body formation during the heterologous expression of DNA and in attempts to refold these proteins in vitro. In a different field, medical sciences, for many decades, clinicians have been aware of the formation of insoluble protein aggregates or amyloid fibrils in a variety of human disease states.

The aggregation step is triggered by misfolding of an intermediate state during the protein folding process. Therefore, osmolytes, also called chemical chaperones, which are known to stabilize native structures and probably assist folding, could be a useful strategy to prevent protein aggregation in vitro as well as in vivo.

Trehalose, the most used solute of mesophiles, has been shown to be required for conformational repair of heat-denatured proteins in the yeast endoplasmic reticulum after severe heat stress. Moreover, mannosylglycerate was found to be better than trehalose in preventing aggregation of Lactate Dehydrogenase upon heating, but the data available are very limited. [Crowe *et al.* (2001).]

2.7.3. Hypersolutes and stabilization process

2.7.3.1. *Stabilizers used in this study*

The stabilizing agents considered for this study were provided by the German partner of the project, Bitop AG (Witten, D) and the Portuguese partner (StabVida). There were 5 different stabilizing agents: Firoin, Ectoine®, Hydroxyectoine, Diglycerol Phosphate (DGP) and Potassium Mannosyl-Lactate (PML). [Park *et al.* (2003).]

There is little information regarding the compounds studied and their mechanism of action is not yet fully understood, but their protein-stabilizing activity has already been thoroughly investigated. In one model, the hypersolutes act by being excluded from the hydration shell of proteins, forcing the latter to adopt a more compact, therefore more stable, conformation. All the corresponding structures are presented Appendix 1, along that of trehalose.

Firoin is one of the less known of the five hypersolutes available. Its name is αD-mannosglycerate (MG). It has a molecular weight of 306.2g/mol and solubility in water of 537g/L at ambient temperature, e.g. 1.75M.

Ectoine and Hydroxyectoine are compounds from the same family, Ectoines. They are produced as predominant osmolytes by heterotrophic aerobic bacteria and are isolated from Halomonas elongata. They are superior stabilizing agents for proteins, nucleic acids, membranes and whole cells as well as osmolytes. They may be obtained from halophilic bacteria, where they act as osmolytes and as desiccation protectants [Bitop Product Catalog 2004] Ectoine and Hydroxyectoine are chemically pure substances; zwitterionic molecules and biologically inert, i.e. they don't interfere with most enzymatic and binding reactions. However, Hydroxyectoine as been proven to slightly lessen Lactate Dehydrogenase maximal activity while shifting its activity curve towards higher temperature. They are highly compatible with cell metabolism and tolerated up to 1M concentrations. However, the optimum concentration for both compounds, as well as for Firoin, has to be determined empirically, within the range of 50 to 100mM. [Manzanera *et al.*. (2004).]

Ectoine, or (S)-2-Methyl-1,4,5,6-tetrahydropyrimidine-4-carboxylic acid has a molecular weight of 142.2g/mol and a high solubility in water of 569g/L at ambient temperature, e.g. 4M, whereas Hydroxyectoine, or(S)-2-Methyl-5-hydroxy-1,4,5,6-tetrahydropyrimidine-4-carboxylic acid, has a MW of 158.2g/mol and a slightly higher solubility in water of 664g/L at ambient temperature, e.g. 4.2M.

As for the two other hypersolutes, Diglycerol Phosphate (DGP) and Potassium Mannosyl Lactate (PML), they are new compounds, little is known about them, never tested. Due to their similarities with mannosyl glycerate (MG), they may function as stabilizers. Within the HotSolutes project partners, StabVida and Cranfield University

are the first to have access to these solutes. The Potassium Mannosyl Lactate, which structure isn't available at present, is the less well known of the hypersolutes. It has a molecular weight of 290g/mL and was provided in solution at pH=4.6. Diglycerol Phosphate has a molecular weight of 284.2g/mol and its solubility in water is of 12.9M. It is the major compatible solute accumulated in Archeglobus fulgidus under supra-optimal salinity. It also accumulates, though without being predominant, for supra-optimal temperatures.

All these compounds are currently being studied by other partners of the HotSolutes project, concerning their physico-chemical characteristics. There is no further information available regarding their characteristics.

2.7.3.2. *Stabilization mechanism*

It has been suggested by STABvida and Bitop partners that the hypersolutes used might be presumed to be similar to trehalose in their stabilization mechanisms, although there isn't yet any hard evidence or any detailed study of it. Trehalose is widely used as stabilizing agent of notably unstable protein, such as S-adenosyl L-methionine (SAM). It has also be shown to stabilize immobilized Acetylcholine Esterase, allowing the enzyme to resist long exposures to acidic pH or temperatures over 50°C. Trehalose is composed of two α-glucose molecules linked together. It helps maintaining the protein structure during changes in temperature and humidity, for example during freeze-drying, thanks to an alteration of the protein microenvironment. Its intracellular concentration is found to increase when the cells are under stress; similarly the hypersolute concentrations increase within the hyperthermophilic bacteria. Trehalose has already been thoroughly studied and two mechanisms have been proven to play a part in protein stabilization. The first is the formation of a glassy matrix and the second, the formation of H-bonds to replace of water molecules. Neither mechanism can however explain stabilization.

Sugar glasses are thoroughly used to stabilize protein during drying. They require a high Tg (glassy transition temperature), a poor hygroscopicity, a low crystallization rate and must contain no reducing group to be efficient stabilizing agents. The formation of a glassy matrix leads to the restriction of the molecule

motion, thereby limiting degradation, as this process is thought to be directly linked to protein mobility i.e. its ability to have its conformation modified. According to this model, the glassy transition temperature (Tg) greatly influences stabilization. The higher a Tg the solute has the more stable the protein will be. However, the hydration levels of both protein and sugar influences their respective Tg. In addition, proteins in a dry state do have higher Tg than sugar. This system alone therefore can't explain stabilization.

An additional explanation that completes the model is the formation of H-bonds to replace water molecules. In the absence of either water or sugar, H-bonding may occur between sites inside the protein, thereby eventually compromising its activity through a change of conformation. The remaining native protein conformation is the key to the remaining activity, the active site untouched losing none of its activity: the sugar-protein interaction being less labile than the water-protein one, leading to an increased stability for the conformation. Obviously, the greater the levels of interaction are between the sugar and the protein, the more effective the preservation will be. Sugars have been proven to preserve the native αα-helix. They also inhibit the dissociation of the protein into subunits, preserving its quaternary structure. [Xie and Timasheff (1997). Morana *et al.* (2002). Patist *and* Zoerb (2004). Richards *et al.* (2002). Schiraldi *et al.* (2002). Silva *et al.* (2005). Katakis and Dominguez (1995). Nuyen *et al.* (1997). Pais *et al.*]

3. EXPERIMENTAL

3.1. Materials and Methods

3.1.1. Solutions and various products

The Reverse Osmosis (RO) Water used for the dilutions was produced by an Elgastat system (The Elgastat Group, Buckingham, UK). All biological components were prepared in a 0.1M phosphate buffer pH=7.5 with 0.1M KCl, else otherwise stated.

All chemicals used to prepare the buffer (NaP tablets, KCl solution) and set its pH (NaOH, HCl) are provided by Sigma-Aldrich (Gillingham, Dorset, UK), as well as the hydrogen peroxide, the enzymes used, their co-enzymes and their substrate. Acetylcholine esterase is of Type VI-S, from electric eel, alcohol oxidase from Hansulena sp. and lactate dehydrogenase comes from a Bacillus Stearothermophilus recombinant expressed in E. Coli.

The enzymes specific activity are as following : for glucose oxidase, 220U/mg, for alcohol oxidase, 7.7U/mg, for acetylcholine esterase, 64U/mg and for lactate dehydrogenase, 62.5U/mg. The stabilizing agents were provided by other members of the HotSolutes project, namely Bitop AG and StabVida. Antibodies were obtained from Sigma-Aldrich (Gillingham, Dorset, UK), anti-human IgG, grown in goat as the primary (immobilized) antibody and anti-goat IgG conjugated to horseradish peroxidase enzyme as secondary antibody (I2136 – A9452). Two complementary single strands of DNA, 18 bases long were used in the DNA-biosensor part of the study. The model system involved a first strand of poly-adenine (poly-A) conjugated to biotin at the 5' end and a second complimentary strand of poly-thymine (poly-T) that contained fluorescent Cy3 label, again attached to the 5' end of the single strand. Both were provided by Thermo Electron GmbH (Ulm, Germany).

For the immunosensors and DNA-biosensors, all buffers, sensor chips, regeneration solutions, coupling solutions were provided by BIAcore (BIAcore UK, Eyeworth, Bedfordshire, UK). CM5 sensor chips as well as SA sensor chips were used. EDC (N-ethyl-N'-(3-dimethyl aminopropyl)-carbodiimide hydrochloride), and NHS (N-hydroxysuccinimide) were provided as part of an immobilization package, while the regeneration buffers were part of another package.

3.1.2. Screen-printing process

Screen-printing is the controlled application of ink layers through specifically designed screens onto a supporting base material substance, using thick-film technology to produce screen-printed electrodes (SPE). The applicability of these cheap, mass-producible electrochemical devices as alternative, sensitive, rapid and reproducible devices for more traditional biosensors is an accepted practice throughout the industry. One of their major advantages is their being disposable hence avoiding the problem of electrode fouling.

Two types of backing substrate are commonly used:

- Aluminium oxide ceramic band materials combined with binder glass (boro- or alumino-silicates) vinyl
- Plastic-based materials combined with epoxy resin-based ink.

Whereas the first set requires the drying to be followed by firing at high temperature and the working electrode to be of a noble metal in binder glass ink, the latter only requires drying at low temperature using polymeric carbon ink. [White *et al.* (1994).]

The screen-printed electrodes (Fig.38) were manufactured using an automated screen-printing machine (DEK 248 Machine, DEK Printing Machines Ltd, Weymouth, UK). The printing process is shown Fig.39. The squeegee pushes the ink over the screen onto the base material maintained under a vacuum. The printing parameters are detailed Table 3.

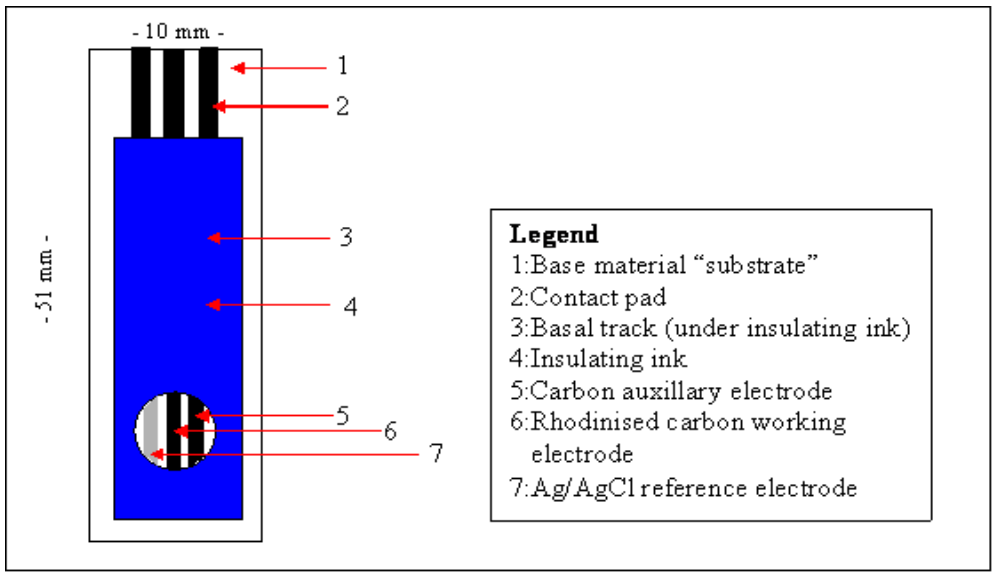


Figure 38: Screen printed electrode (SPE) schematic

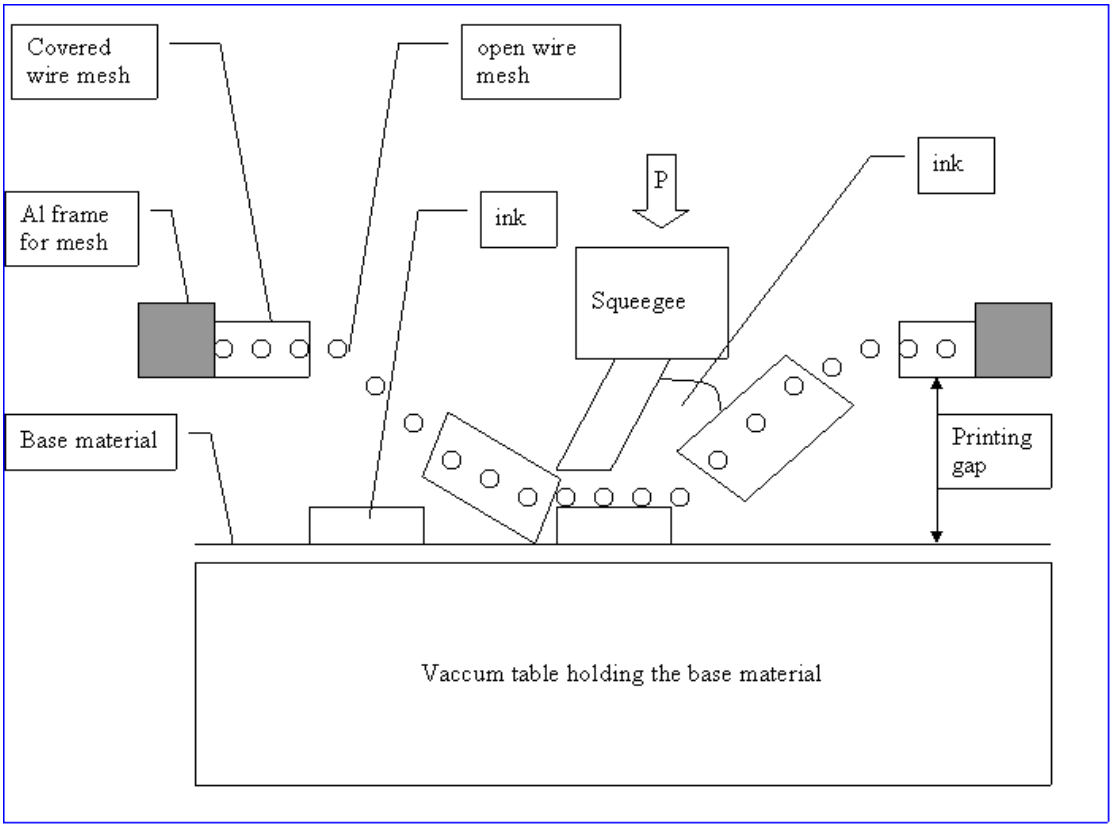


Figure 39: Printing process for the screen-printed electrodes

Print parameters	Settings
Print mode	Print/Flood
Squeegee pressure	4 kg
Print gap	2.5 mm
Deposit #	1
Forward carrier speed	50 mm/s
Reverse carrier speed	50 mm/s
Front limit	40 mm
Rear limit	400 mm
Separation speed	70%

Table 3: Printing parameters for the screen-printing process

The screen-printing process is based on the sequential deposition of ink layers. Between the printing steps, the different layers were dried at ambient temperature. The insulation layer was dried at 120°C for 2h. This heat treatment helps stabilize the rhodinized carbon electrode allowing then to operate in aqueous solutions.

If operated at high temperatures, the rhodinized carbon electrode response to hydrogen peroxide decreases, but this can be restored by anionic preconditioning. However, even without this preconditioning, the screen-printed electrode current response to hydrogen peroxide is high enough for this additional step not to be performed. In addition, the heat treatment disables the addition of enzyme to the working electrode ink before its application on the electrode.

A membrane, e.g. cellulose acetate, can be added after the deposit of enzyme at the electrode surface. This will protect the electrode coating from desorption (in flow injection analysis (FIA) systems, for example), and from interferences. [O'Halloran *et al.* (2001). O'Regan *et al.* (2002).]

The base sheet material was acetate (VT Plastics, Bedford, UK).The basal carbon tracks were made of a carbon ink, Electrodag 423 SS (Acheson, Scheemda, D). The reference electrode was silver / silver chloride Electrodag 6038 SS (Acheson, Scheemda, D) and comprises 15% silver chloride, requiring chloride ions as supporting

electrolyte and to enable the reference potential to hold. The last layer deposited was the insulation layer, a vinyl matt insulation ink 242 SB (ESL Europe, Reading, UK) diluted down to the correct viscosity using a thinner (402 Thinner for (242 SB), ESL Europe, Reading, UK).

The working electrode comprised rhodinized-carbon (Rh-C), MCA 4a (MCA, Melbourne, UK). This ink was prepared by a 1:4 dilution of the paste in 2.5% hydroxyethyl cellulose (HEC) in Reverse Osmosis (RO) water.

The working ink is composed of rhodium (up to 5%) in a carbon paste, with added promoters for the favorable oxidation of the peroxide species at the reduced potential. The open porous structure and physico-chemical properties of the electrocatalyst has proven to be an excellent medium for the simple physical immobilization of proteins [Silva Nunes *et al.*. (2004). Kröger *and* Turner (1998).]. The matrix lowers the potential for amperometric detection and renders the use of mediator unnecessary. A lower potential decreases the possible interferences, increasing the biosensor specificity.

The reactions between the electrocatalyst and hydrogen peroxides are presented Equ.14.

$$Me(OH)_2 + H_2O_2 \rightarrow Me + 2H_2O + O_2$$

$$Me + 2H_2O \rightarrow Me(OH)_2 + 2e^- + 2H^+$$

Equation 14: Reaction between the electrocatalyst and hydrogen peroxide

The formation of an oxide layer is therefore essential before the modified electrode can display activity. However, it is automatically formed during the electrode production. [Honeychurch and Hart (2003). Hong *et al.* (2002). Susmel *et al.* (2003). Kröger *and* Turner (1997).]

3.1.3. Electrochemical systems

Tests were carried out on a laboratory potentiostat (Autolab) incorporating a general purpose electrochemical software operating system (GPES3, Ecochemie,

Utrecht, NL) and on a field-portable electrochemical analyzer (PalmSens, Palm Instruments BV, Utrecht, NL) (cf. Fig.40).

Figure 40: PalmSens analyzer

The electrodes were connected to the potentiostat by specifically adapted electrical connectors (cf. Fig.41) and were immerged into an unstirred measurement solution (10mL) or a droplet of sample was deposited at the circular aperture within the insulation layer on the electrode surface. The sampling time were of 1/sec for the Autolab system and of 2/sec for the PalmSens device.

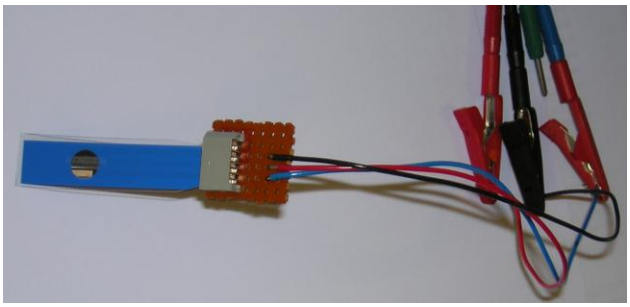


Figure 41: Electrical connector for the screen-printed electrode

Raw data were transferred into Matlab for further treatment and analysis and processed using Excel. As the electrodes are disposable, the data analysis required some statistic background study.

The use of an electrocatalyst amplifies the signal, giving a degree of specificity to the system: e.g. a system would receive no current signal at E=0.6V without electrocatalyst, but would produce significant signal at less than E=0.3V with the catalyst. Increasing the potential would increase the response, but this would again allow interferences to occur.

Stirring was avoided during measurement process to avoid removal of hydrogen peroxide produced from the electrode surface and to prevent enzyme desorption. [Parellada *et al.* (1998). Pearson *et al.* (2000).]

3.2. Enzyme-based biosensors

The first type of biosensor we considered was enzyme-based biosensor, using amperometry on screen-printed electrodes. The different systems that were studied were using four enzymes: Glucose oxidase, alcohol oxidase, acetylcholine esterase and lactate dehydrogenase. The first system to be characterized, also the main system studied, was based on the detection of glucose using the glucose 1-oxidase.

Glucose oxidase based biosensors represent the vast majority of biosensors market and publications. The other three enzymes came as a support, when looking for differences in the influence of the Hypersolutes over enzyme-based biosensors, as the enzyme inner stability or reaction pathway vary: Glucose oxidase and alcohol oxidase have a similar reaction mechanism, producing hydrogen peroxide as they degrade their substrate, glucose and ethanol respectively. However, while glucose oxidase is quite stable, alcohol oxidase is notably unstable. As for acetylcholine esterase and lactate dehydrogenase, they both require a mediator. On top of that, lactate dehydrogenase requires a co-enzyme, nicotinamide adenine dinucleotide (NAD), which is notably problematic. Whereas lactate dehydrogenase is still quite stable, acetylcholine esterase is almost as unstable as alcohol oxidase.

3.2.1. Testing the screen-printed electrodes using hydrogen peroxide

The screen-printed electrodes are used as disposable systems. Whilst this is not an environmentally friendly system, and also increases inter-experiences variation, it is by far the simplest system at our disposal. It was tried, as much as possible, to use systems that are easy to set up, fast, reliable and reproducible.

Because the internal variations between the screen-printed electrodes, at worse coming from different batches, was to have repercussions on all ulterior experiments, it was imperative to investigate the screen-printed electrodes characteristics prior to any other analysis. Since for glucose oxidase-based biosensors, the biosensor response is based on the oxidation of enzymatically generated hydrogen peroxide at the electrode

surface, the use of hydrogen peroxide for characterizing the screen-printed electrode was an obvious choice.

3.2.1.1. *Parameters*

a. Assay time

The first parameter we investigated was the measurement time. The experimental conditions were thus: A 30µL droplet of 2.5mM H_2O_2 solution in aqueous buffer (0.1M NaH_2PO_4, 0.1M KCl) was deposited on a screen-printed electrode. A potential of E=+350mV was applied on the rhodinized carbon (Rh-C, an electrocatalyst used to lower the working potential and reduce interferences) working electrode against a Ag/AgCl reference electrode. The electrochemical transduction system was amperometry; the potential being held constant during the experiment as the resulting current is measured. The experiment lasted 400s, the result is presented Fig.42.

The plateau was reached at 100s. In order to have enough data points to observe the plateau, and taking in account a likely diffusion effect when using the enzyme as detection system, the assay time was set up at 150s. The points obtained between 120s and 150s, forming the plane of equilibrium, were used to determine an average value of the current at the plateau.

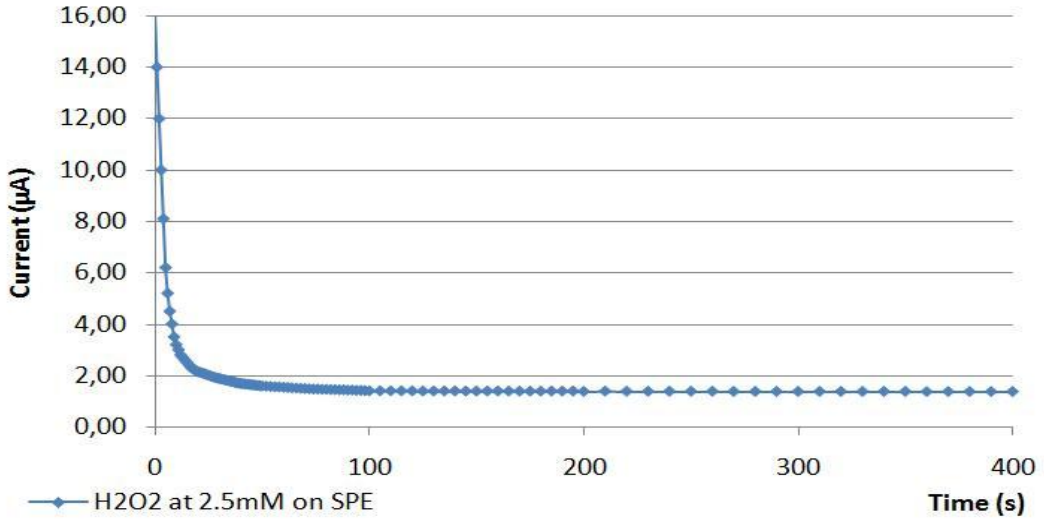


Figure 42: Optimization of the assay time for the detection of H_2O_2 2.5mM on screen-printed electrodes (Rh-C vs. Ag/AgCl)

b. Volume

The second parameter to be optimized was the volume of sample deposited at the electrode surface. Different amounts of the hydrogen peroxide solution, used in the previous experiment, were deposited on the electrode surface, varying from 7.5µL to 30µL. As for the previous experiment, the working electrode was rhodinized carbon, while the reference electrode was Ag/AgCl and the potential applied E=+350mV.

Under 7.5µL, it was not possible to cover the whole operational surface of the electrode and results were therefore not reproducible nor could one have any confidence in them. Equally, deposits over 30µL proved to be too large and overlapped on the electrode surface, resulting in a loss of material and unreliable results.

Between 7.5µL and 30µL, the response was found to increase with the volume up until an 18µL deposit, at which point the response stabilizes. A loading between 20 and 28µL was therefore found to be optimal. Any subsequent study was done depositing a sample no smaller than 20µL and no larger than 30µL. For all the studies realized on the bare electrode system, without enzyme, the volume of sample (hydrogen peroxide) deposited was 25µL.

3.2.1.2. Amperometric detection conditions

For both previous experiments, the potential applied was E=+350mV, as it is the commonly used potential for detecting hydrogen peroxide on rhodinized carbon working electrode against a Ag/AgCl reference. In order to confirm this potential to be the optimal one on that electrode configuration, a study of current versus potential (i=f(E)) was carried out using the same hydrogen peroxide solution at 2.5mM as previously used. The result is presented Fig.43.

For potentials lower than E=+300mV or higher than E=+400mV, the slope of the curve i=f(E) is quite important, whereas it is almost null between E=+300mV and E=+400mV. Therefore, any minor fluctuation in potential that would significantly affect the current response outside of the range [E=+300;+400mV], will not affect the response within that range. The medium value of that range, E=+350mV, is thus confirmed as the optimal transduction potential for the amperometric detection of

hydrogen peroxide using the screen-printed electrodes with rhodinized carbon as working electrode and Ag/AgCl as reference electrode.

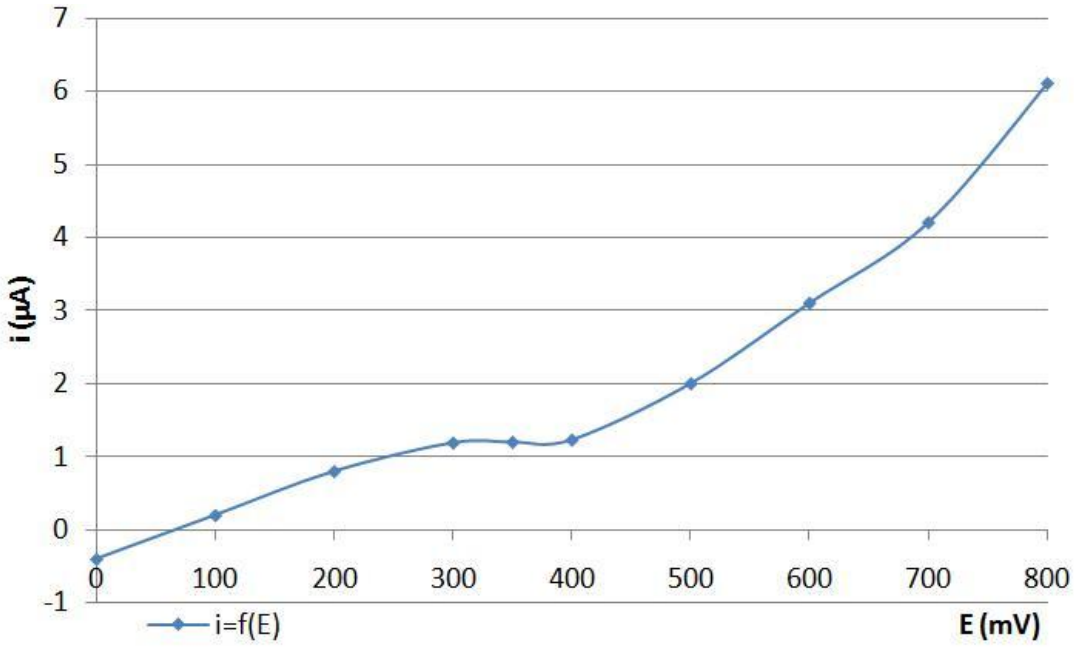


Figure 43: i=f(E) on Rh-C vs. Ag/AgCl screen-printed electrode for 2.5mM H_2O_2 to determine the optimal electrochemical parameters

3.2.1.3. *General characterization*

a. Statistics on one concentration

The screen-printed electrode are disposable, namely one electrode is used to obtain one measurement value, and then discarded. It becomes compulsory to determine the repeatability of that basal system. The response obtained for the detection of hydrogen peroxide 2.5mM in aqueous buffer was measured on 20 different electrodes. The distribution of the responses is presented Fig.44.

The statistic characteristics of this distribution were calculated. The results were found to follow a Gaussian distribution. At 95%, the average response was $1.11\mu A \leq m \leq 1.17\mu A$. For the standard deviation (STD), $9.2\times10^{-2}\mu A \leq \sigma \leq 5.3\times10^{-2}\mu A$. The average is therefore $i=1.14\pm(6.7\times10^{-2})\mu A$ with an inter-electrode relative standard deviation (RSD) of 5.8%.

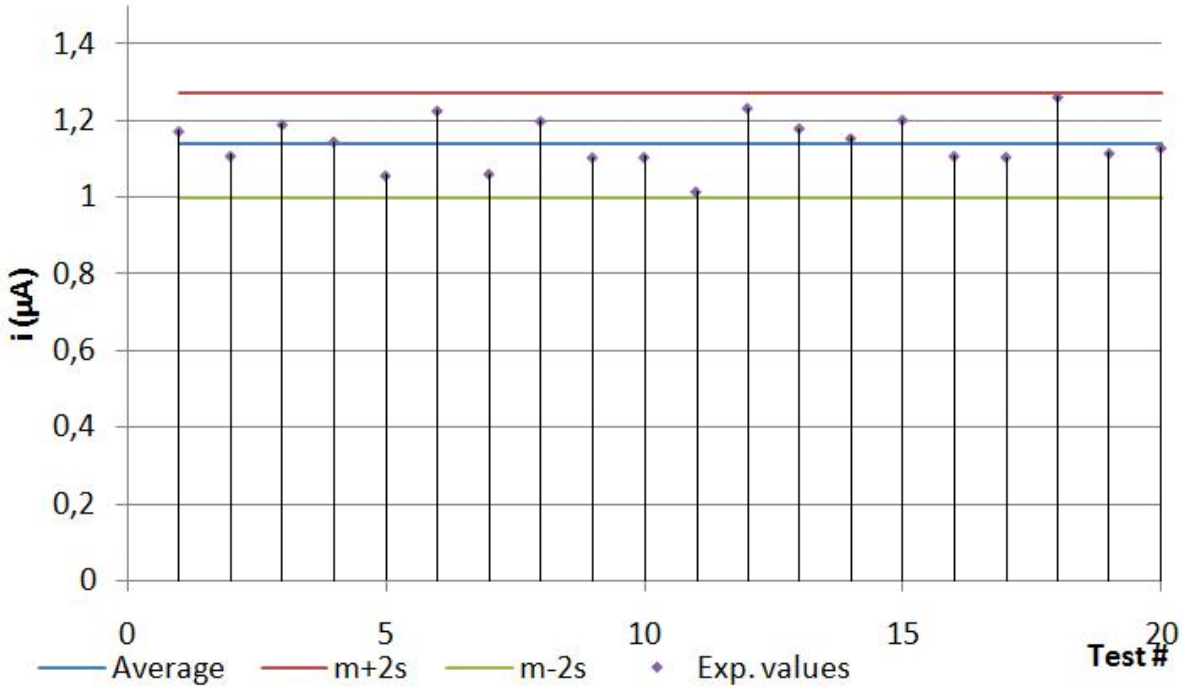


Figure 44: Distribution of responses and standard deviation for 2.5mM H_2O_2 on screen-printed electrodes

The hydrogen peroxide concentration of 2.5mM was used as a reference and the relative standard deviation value obtained here was used for all forthcoming experiments using hydrogen peroxide on the bare electrode system. Also, as the current value we use as a data point is in fact the average of all the points obtained over a 30s period, the intra-electrode relative standard deviation attached to that value was calculated to be compared to the inter-electrode relative standard deviation (RSD). The intra-electrode relative standard deviation was found to be less than 1%, thereby not significant compared to the inter-electrode relative standard deviation (RSD). Any relative standard deviation considered thereafter is inter-electrode and will be simply quoted as 'relative standard deviation' or RSD.

b. Calibration curve

The specifications of the screen-printed electrodes were to be determined for hydrogen peroxide: The linear range of the sensor was investigated, as well as its limit of detection (LOD) and its saturation limit. [Mocak *et al.*] The experimental parameters were set as previously optimized; a 30µL droplet of H_20_2 in aqueous buffer (0.1M NaH_2PO_4, 0.1M KCl) was deposited at the electrode surface. The working electrode was

rhodinized carbon against Ag/AgCl reference. The potential E=+350mV was applied for 150s. The range studied was 0-88mM H202. The results are presented Fig.45.

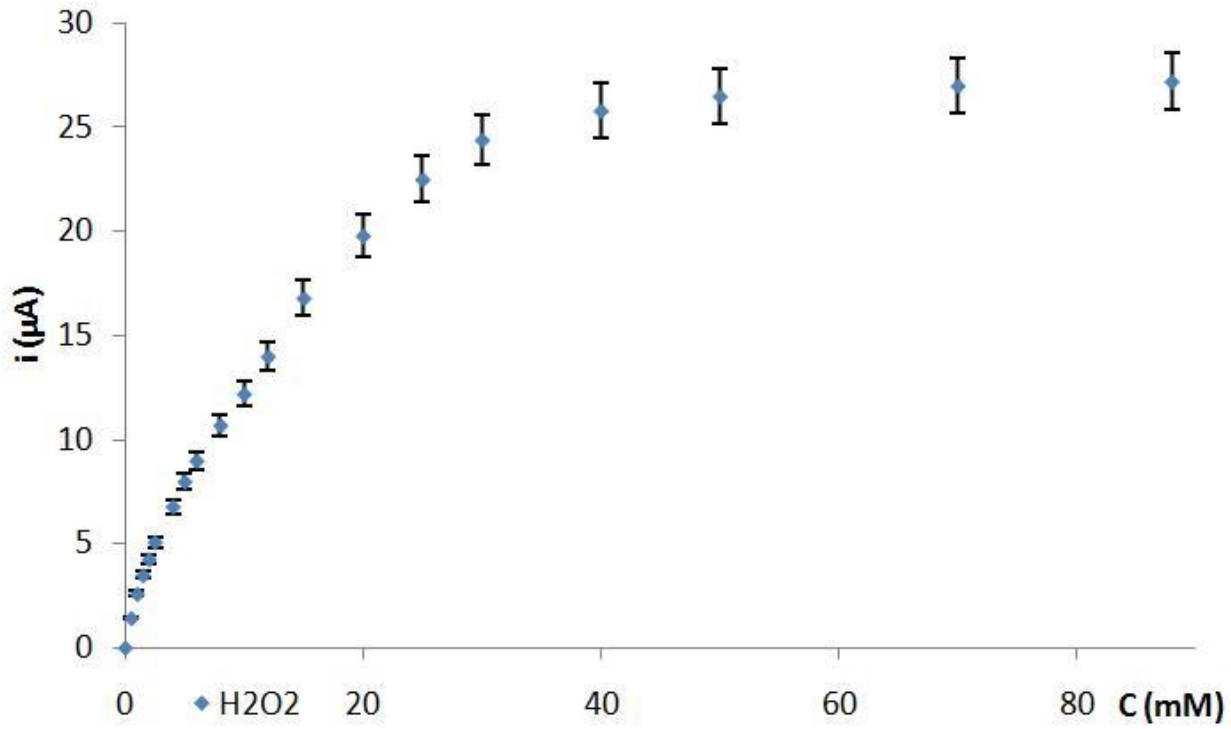


Figure 45: Calibration curve of H_2O_2 over the range 0-88mM on screen-printed electrodes

Over 20mM, the curve starts to flatten and the saturation limit is found to be 55mM. Over that concentration, the signal has reached its plateau and any concentration increase won't change noticeably the resulting current. Observing low hydrogen peroxide concentrations, in order to determine the limit of detection, the linearity of the response is obvious over the range 0-2.5mM, as is shown Fig.46, and even more so under 0.5mM, as is shown Fig.47.

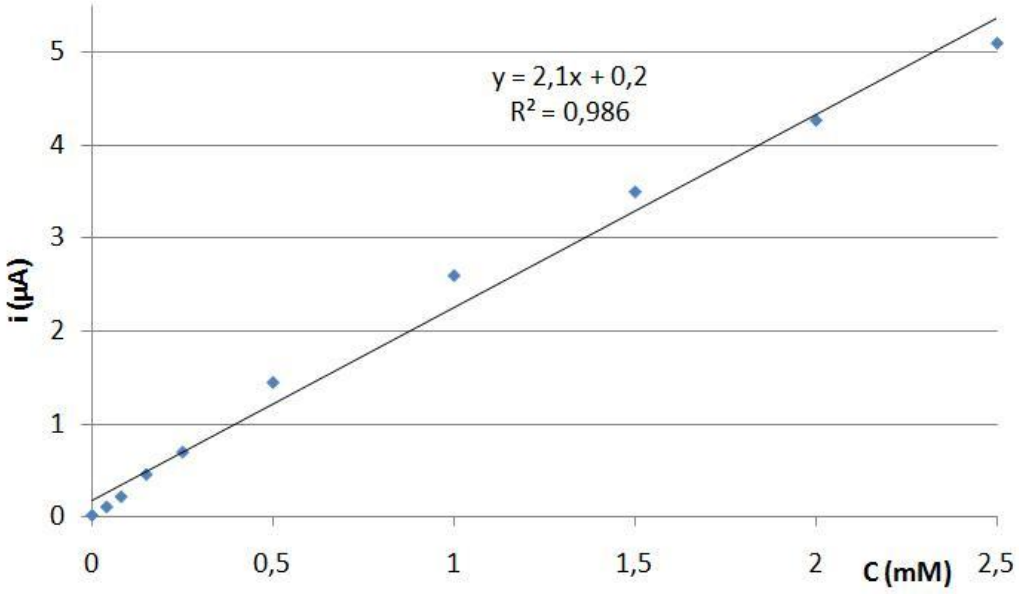


Figure 46: Linearity of the current response over the range 0-2.5mM H_2O_2 on screen-printed electrodes

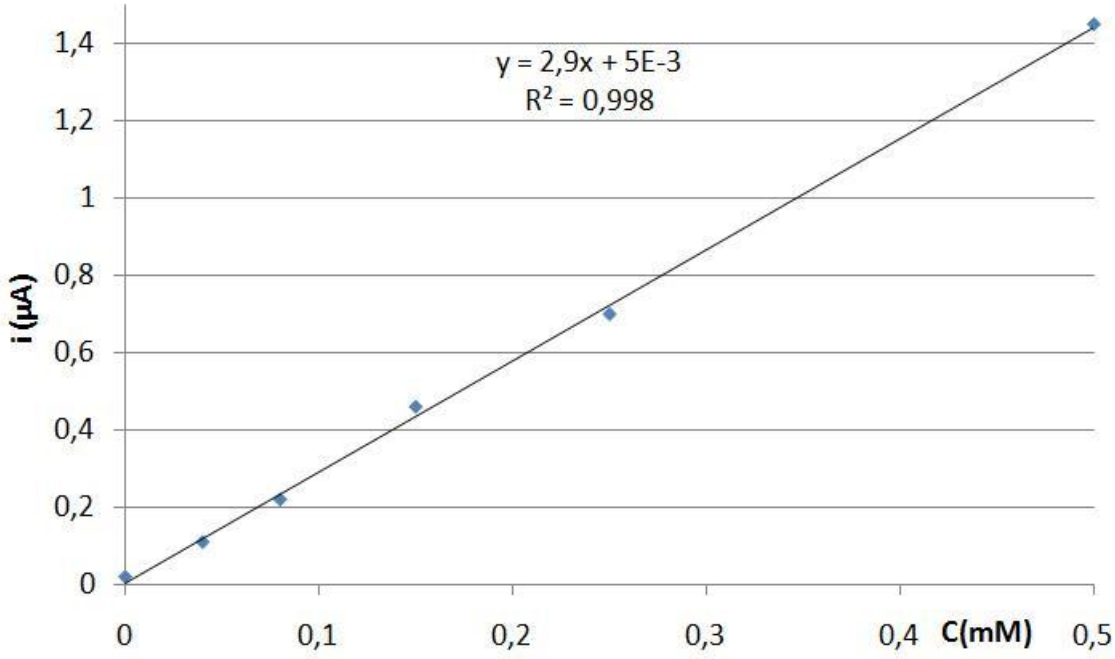


Figure 47: Linearity of the current response over the range 0-0.5mM H_2O_2 on screen-printed electrodes

The linearity of the response was determined over the range 0-2.5mM H_2O_2 as R^2=0.9852. Over the lowest concentration range of 0-0.5mM H_2O_2, the linearity is of R^2=0.9985. Studying these very low concentrations allowed us to determine the limit of

detection of the sensor. The limit of detection is the lowest concentration of an analyte that the analytical process can reliably detect, whereas the limit of quantification is the smallest concentration which can be quantitatively analyzed with reasonable reliability by a given procedure The limit of detection (LOD) is defined as LOD=3σ, where σ is the standard deviation and the limit of quantification (LOQ) is defined as LOQ=10σ by the American Chemical Society (ACS). The limit of detection of the screen-printed electrode for hydrogen peroxide is LOD=0.05mM and the limit of quantification is LOQ=0.17mM.

3.2.1.4. *Effect of ethanol on basal electrode performance*

One of the enzymes studied, alcohol oxidase, has for substrate ethanol (EtOH). The by-product of enzymatic activity that is used for generating the signal is hydrogen peroxide. It was therefore compulsory to assess the behavior of the screen-printed electrode for hydrogen peroxide in alcoholic solutions. The response of the screen-printed electrode was studied in different ethanol concentrations and a calibration curve for hydrogen peroxide in ethanol was then characterized.

Up to 50% ethanol, the presence of alcohol did not influence the sensor, which is coherent with the information found in literature. Over 50% ethanol, the signal generated was unstable and overall not acceptable as valid. For ethanol amounts below 50%, the relative standard deviation was calculated: For 2.5mM H_2O_2 in 20% ethanol, the relative standard deviation was found to be close to 6%, very similar to the relative standard deviation on the screen-printed electrode for hydrogen peroxide in aqueous buffer.

In real-life applications, in the wine industry for example, an alcohol oxidase-based biosensor would be used with ethanol concentrations up to 20%. However, the range that we studied for our alcohol oxidase-based biosensor is broader, up to 35% ethanol. The calibration curve was therefore determined in 30% ethanol, over the range 0-10mM H_2O_2. The results are presented Fig.48. The curve is very similar to the calibration curve observed in aqueous buffer. A good linear correlation was found over the range 0-1.7mM H_2O_2, with R^2=0.987, as shown Fig.49.

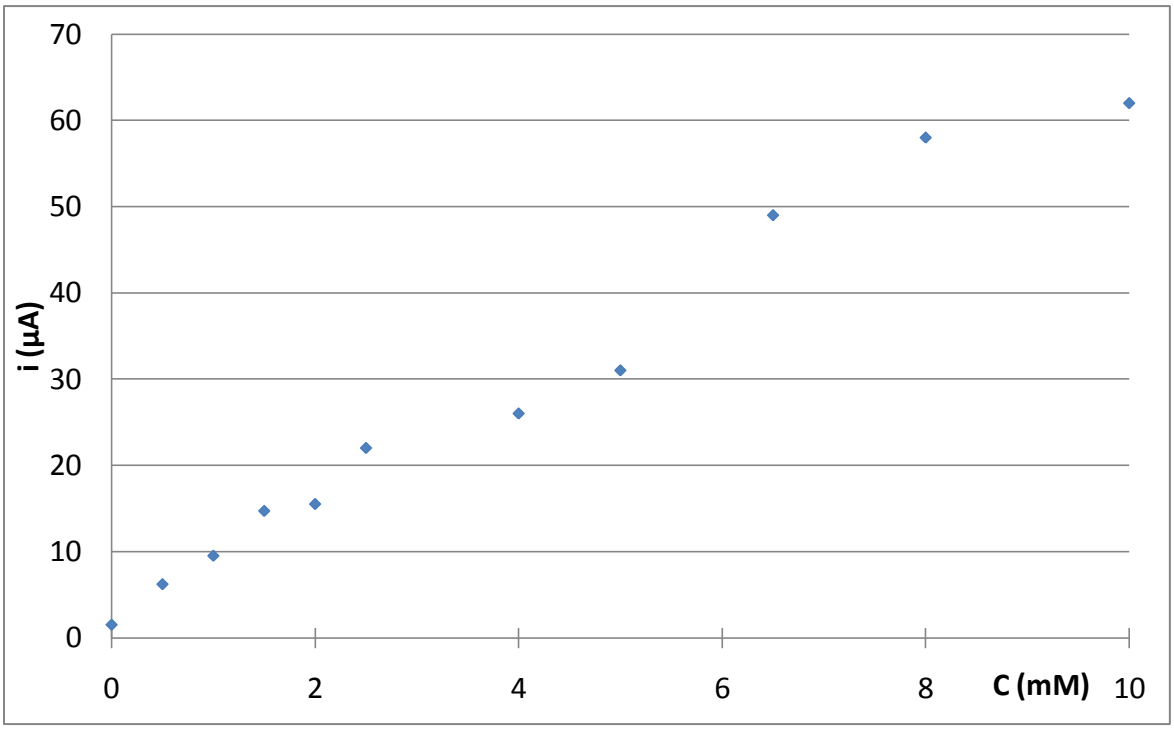


Figure 48: Calibration curve for H_2O_2 in 30% ethanol on screen-printed electrodes

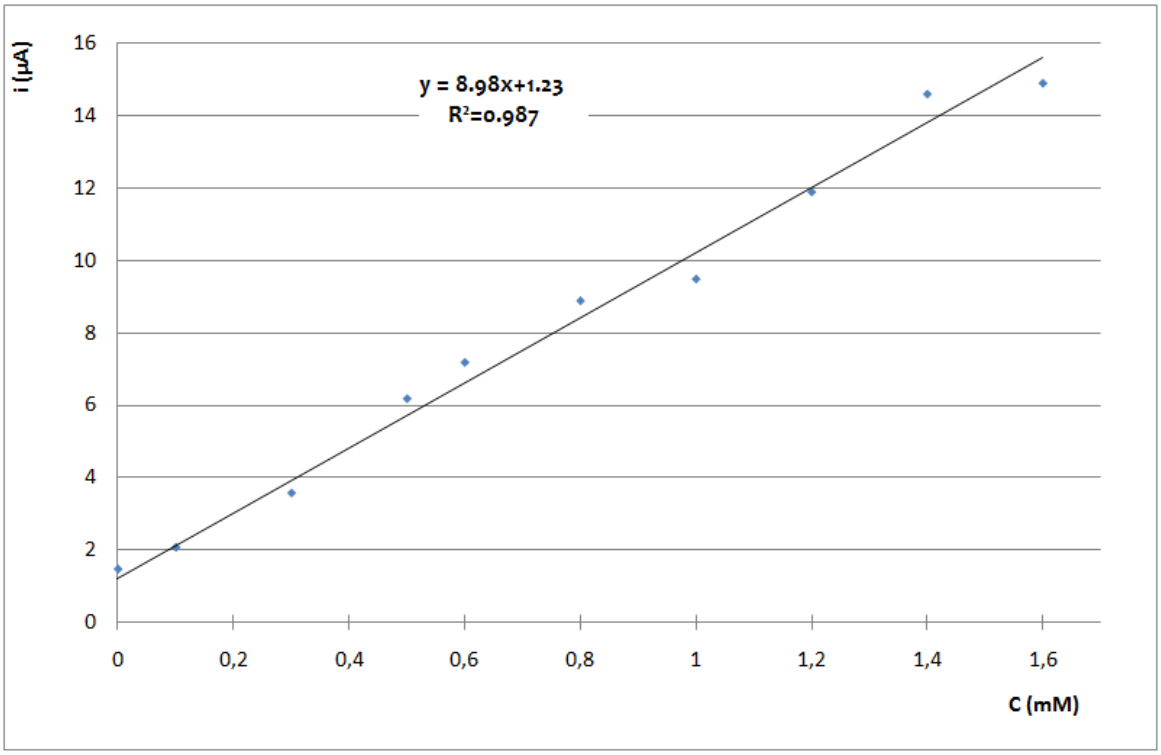


Figure 49: Linearity of the current response over the range 0-1.7mM H_2O_2 on screen-printed electrodes in 30% ethanol

3.2.2. Enzyme on the sensor

The basal system having thus been characterized and its working conditions optimize, the biological part could be then added, in this case enzymes, in order to form a biosensor. The first enzyme studied was glucose oxidase. Alcohol oxidase and acetylcholine esterase were then optimized. Lactate dehydrogenase, however, proved true to its reputation and was a problematic system to set up.

3.2.2.1. Glucose oxidase

Glucose oxidase is the most studied enzyme in biosensors. It concerns 80% of yearly publications, represents the majority of commercialized biosensors and has many applications. One of its major attributes is its stability. Also, its reaction pathway is quite simple, producing hydrogen peroxide

c. Loading of glucose oxidase

A series of experiment were performed to optimize the loading of glucose oxidase onto the electrode surface. The loading method and parameters were studied; the amount of glucose oxidase immobilized being then optimized. The influence of the immobilization method on the enzyme activity was assessed later on. [De Prada *et al.*. (2003).]

i. Deposition of glucose oxidase – Volume and manner thereof

It was tried, as much as possible, to keep the systems used as simple as may be, so that very few factors would take part in the variation of the biosensor responses. Also, it was tried to aim for systems which settings and analyzing would be fast as well as reproducible. The immobilization of the enzyme onto the electrode surface was therefore realized by simple physical adsorption at the electrode surface. That adsorption was facilitated by the porous nature of the electrocatalyst (rhodinized carbon, Rh-C) present on the working electrode surface.

Based on previous experimentation, it was clear that the optimal volume to work with would be in the range 10-30µL. However, since the enzyme deposit would ideally be done on the working electrode surface, rather than on the whole biosensor surface available, the optimal volume would belong to the lower part of that range. The optimal volume of enzyme deposited was found to be 10µL. Under 7µL deposit, the volume was too small for the deposit to appear homogeneous on the working electrode surface. Over 12µL, the droplet deposited didn't dry completely at the electrode surface within 16h at room temperature (RT, 20°C), the droplet having dried out defining the biosensor as ready-to-use.

The immobilization method was chosen to be thus:

- A 10µL droplet of Glucose Oxidase at a given concentration in phosphate buffered saline (PBS) 10mM buffer was deposited over the working electrode with the rhodinized carbon (Rh-C) electrocatalyst.
- The droplet was left to dry over night (OVN, about 16h) at room temperature (RT, 20°C), the biosensor being thereafter ready for use.

ii. Optimization of enzyme activity deposit

A study of the influence of glucose oxidase activity deposited at the electrode surface on the current response was carried out in order to assess the optimal loading of enzyme on the working electrode surface. The optimal loading represents a balance between the maximum signal to noise ratio(S/N) and the prevention of the insulation of the working electrode by an excess of biological material. The result over the range 0-60U of glucose oxidase deposited at the working electrode surface is shown Fig.50.

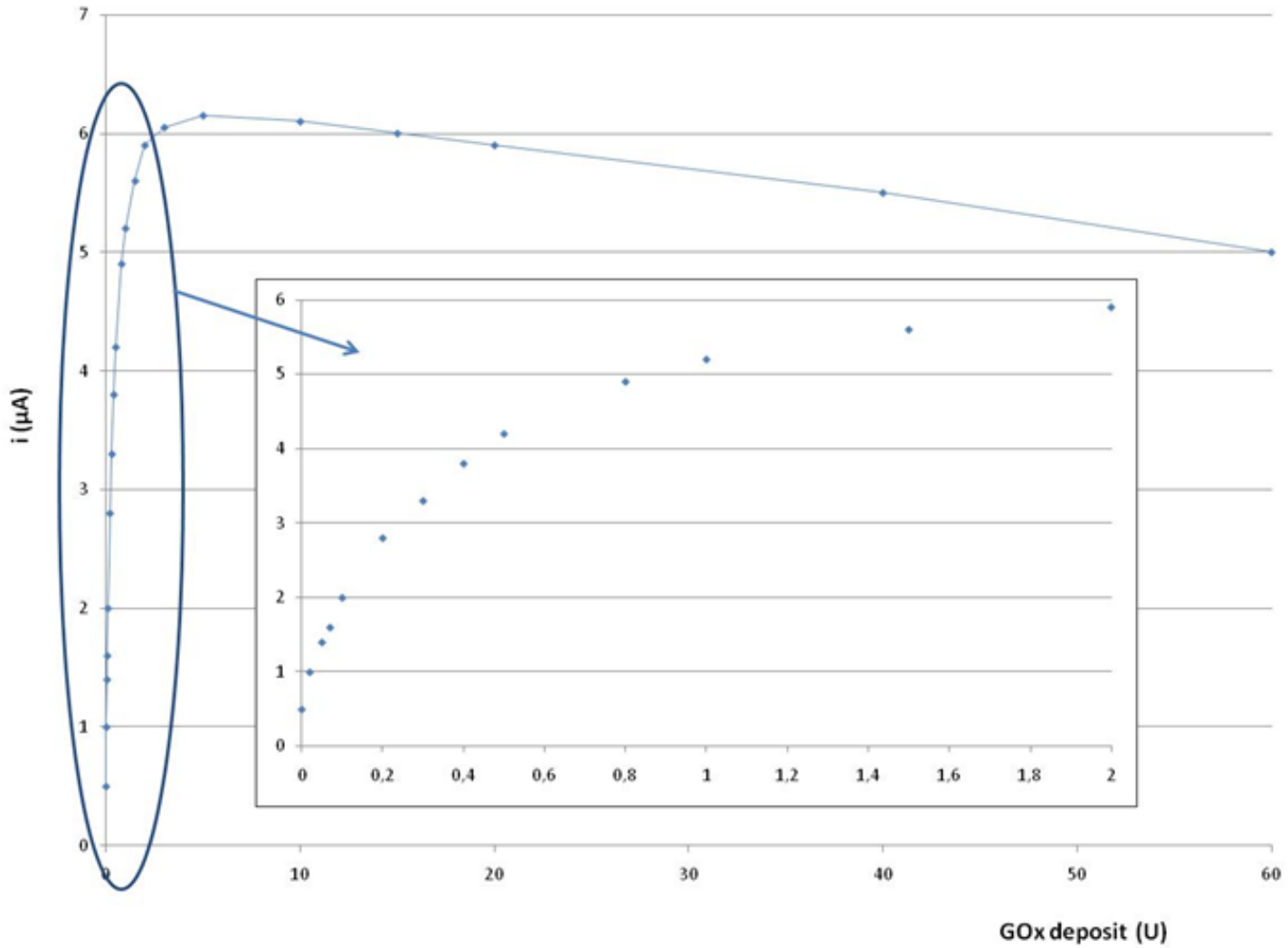


Figure 50: Optimal loading of glucose oxidase – sensogram for glucose oxidase loading between 0 and 60U deposit against glucose 20mM on screen-printed electrodes

Between 5 and 60U deposit, the response follows a near-zero order relationship, the electrode being overloaded at those concentrations. Also, over 20U deposit, a diffusion phenomenon appears at the electrode surface, leading to a signal decrease. Consequently, at those concentrations, any change in the enzyme activity would remain undetected, until the remaining enzyme activity at the electrode surface becomes lower than 5U. Under 2U deposit, the current response decreases sharply with the enzyme activity present at the electrode surface.

The optimal loading of glucose oxidase was hence found to be 2U per electrode. As the volume of enzyme deposited is optimal at 10µL, the glucose oxidase solution used is 200U/mL in phosphate buffered saline (PBS) 10mM buffer. A decrease of enzyme activity in these conditions would be marked by a significant decrease of the current response, the remaining signal being still important and with a satisfactory signal to noise ratio (S/N).

d. Analysis parameters

Because we added a biological component onto the screen-printed electrode (SPE), the measurement parameters were influenced and had to be characterized again. It was then put into light that although the reproducibility was high between electrodes within one batch, the reproducibility inter-batch was in contrast quite low: The operator building the batch as well as the ink lot used and the age of the electrode sheet were found to have a significant impact. It was therefore vital to use electrodes from a same batch in conducting a series of experiment.

A time assay of 150s was confirmed to be satisfactory, being fairly short yet long enough to allow the averaging of the last 20s to determine the current response at the steady-state. Also, several volumes of sample were tested, of glucose 2.5mM in phosphate buffered saline (PBS) 10mM buffer. As previously observed, a volume of 25µL was found to be optimal.

Finally, a short study was realized to confirm that the amperometric conditions applied for the experiment were indeed optimal. A study was realized on a glucose oxidase coated screen-printed electrode against glucose. The electrode was prepared by depositing a 10µL droplet of a 200U/mL solution of glucose oxidase on the electrode and subsequently left to dry overnight at room temperature. A 25µL sample of a 20mM

solution of Glucose solution was then deposited at the electrode surface and a current was applied, over the range E=0mV to E=+600mV. The result obtained was very similar to that of the bare electrode and a potential of E=+350mV was confirmed to be optimal.

e. Statistic study on one concentration

The reproducibility of measurements was tested for the screen-printed electrodes with glucose oxidase deposited against glucose and compared to that of the bare electrode against hydrogen peroxide. The reproducibility of the sensor with the enzyme was found to be less consistent than that of the bare electrode. This was to be expected, because of the addition of a biological component included in the sensor matrix.

10µL droplets of a glucose oxidase solution at 200U/mL were deposited onto the sensor surface and left to dry overnight (OVN) at room temperature (RT). A 25µL sample of glucose solution at 2.5mM was then deposited on the working surface of the electrode. A potential of E=+350mV was then applied for 150s and the response current observed. The repartition of the result over 20 measurements is presented Fig.51.

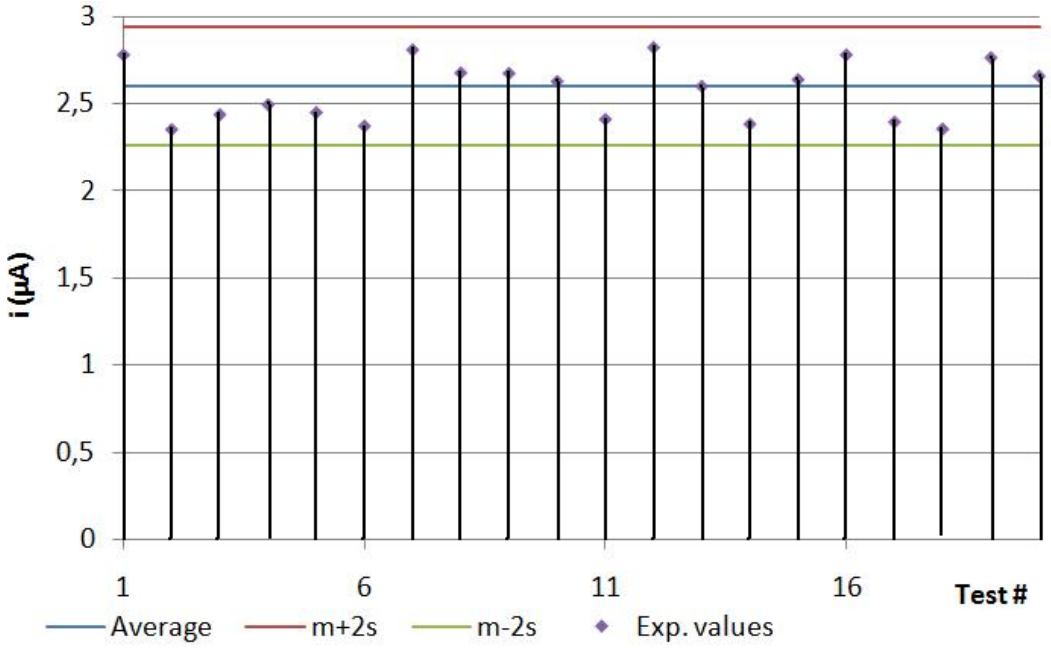


Figure 51: Standard deviation (STD) of glucose oxidase coated screen-printed electrodes against 2.5mM glucose

The response average was determined m=2.60±0.17µA. The superior limit (Sup. Limit, S) on the graph is defined as S=m+2σ, where m is the average and σ the standard deviation (STD). Accordingly, the inferior limit (Inf. Limit, I) is defined as I=m-2σ. The interval defined within those limits contains the experimental values at α=97.5%. The relative standard deviation (RSD) for these Glucose Oxidase biosensors was hence found to be RSD=6.8%.

f. Calibration curve

The glucose oxidase used on the screen-printed electrode was further characterized by the construction of calibration curves, with respect to the sensor response to glucose in phosphate buffered saline (PBS) 10mM buffer. As was optimized, the biosensor was prepared beforehand by the deposition of a 10µL droplet of glucose oxidase at 200U/mL and left to dry at room temperature (RT) overnight (OVN). Then 25µL sample of glucose at various concentrations were applied on the surface of the electrode and a potential E=+350mV was applied for 150s. The resulting calibration curve, over the range 0-10mM, is presented Fig.52.

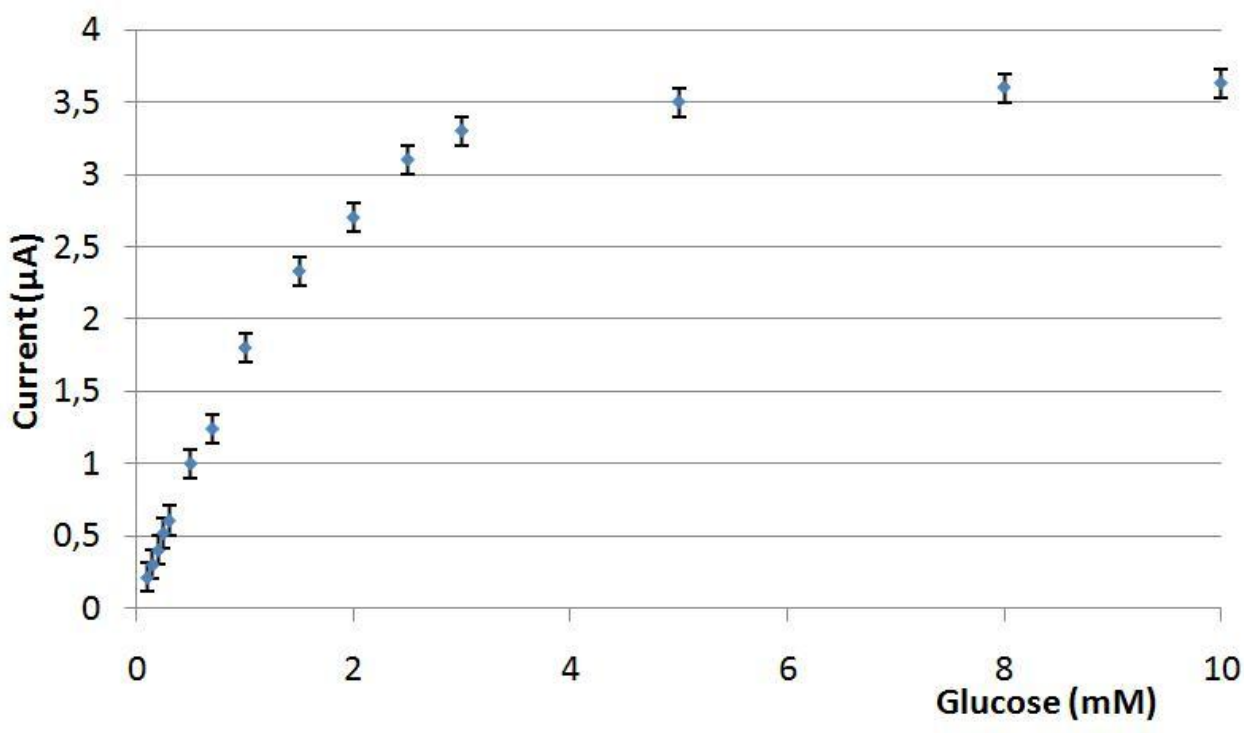


Figure 52: Calibration curve for the glucose oxidase biosensor against glucose over the range 0-10mM

Glucose oxidase follows a classical enzyme kinetic profile: a typical Michaelis-Menten substrate reaction response is observed over the range 0-10mM, with a first order relationship obvious at low glucose concentrations, for C<2mM, tending to a zero order response. A Line-Weaver-Burke plot of the response is presented Fig.53. The kinetics values of the glucose oxidase used in this study were thus determined.

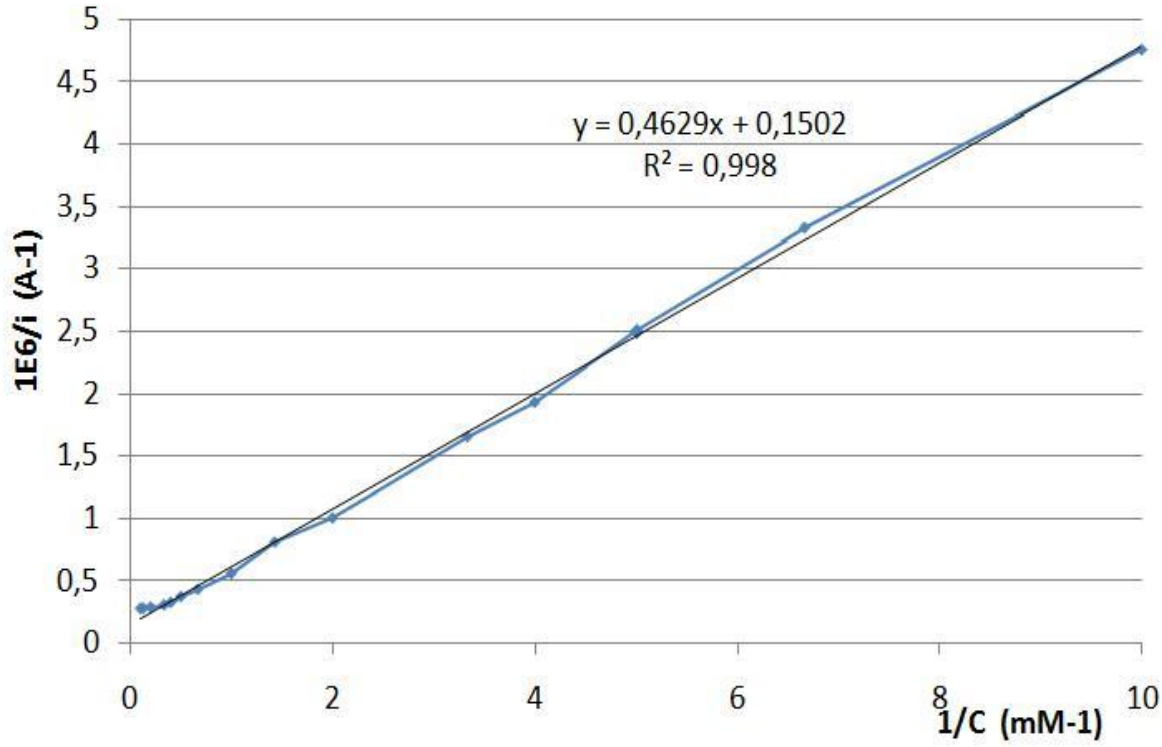


Figure 53: Line-Weaver-Burke plot for glucose oxidasebiosensor on screen-printed electrodes against glucose over the range 0-10mM

The glucose oxidase Michaelis constant was calculated to be K_m=4.4mM and the maximum current value to be i_{max}=9.5µA. The Michaelis constant value for immobilized glucose oxidase from Aspergillus Niger was found to be $(K_m)_{lit.}$=4mM in literature. Thus, the experimental value compared well with the value reported in literature. As the Michaelis constant of glucose oxidase was close to K_m=4mM, the subsequent studies involving the investigation of the influence of stabilizing agents on the biosensor were performed at a glucose concentration C=20mM. This concentration value of C=5K_m is being empirically proven to be located in the plateau of the Michaelis-Menten profile.

g. Influence of the immobilization process on enzyme activity

Glucose oxidase is immobilized by simple physical adsorption on the electrode surface. The influence of this immobilization on the enzyme kinetic characteristics was determined by comparing the activity of free glucose oxidase in solution with the immobilized enzyme activity.

For the immobilized enzyme, the screen-printed electrode was coated with 10µL of a 1kU/mL Glucose Oxidase solution and left to dry overnight. The measurement was realized against 25µL of glucose solutions at different concentrations. A potential of E=+350mV was then applied for 150s, and the resulting current recorded. For the free enzyme measurements, the same stock solutions were used and the same proportion retained. An aliquot of 25µL of the solution containing glucose and glucose oxidase was deposited on a bare screen-printed electrode, after being left unstirred for 3h at room temperature.

Calibration curves were established for both systems, displaying classic Michaelis-Menten profiles. Line-Weaver-Burke plots were then drawn to determine the kinetic characteristics of the enzyme, either free or immobilized. The results obtained are presented Table 4.

Enzyme	Immobilized GOx	Free GOx
i_{max} (µA)	18.7	9.2
K_m (mM)	4.4	5.1

Table 4: Influence of the immobilization process on Glucose Oxidase activity

The Michaelis constant values obtained were again related to those recorded in literature. As expected, the Michaelis constant (K_m) value was higher for the free enzyme, and the maximum current (i_{max}) value was higher for the immobilized enzyme.

The i_{max} value is higher for the immobilized enzyme as the enzyme is bound directly at the sensor surface. Therefore, the reaction products are situated closer to the electrode, and more hydrogen peroxide is likely to be detected for the same period of time, which in turn is translated in the output signal by a higher current value.

The K_m value, on the other hand, is lower when the enzyme is immobilized. The K_m value relates directly to the enzyme activity. Thus, we can conclude from the lesser K_m value of the immobilized enzyme that the immobilization process does affect the enzyme configuration, lessening the activity of the enzyme. However, the difference between both K_m for free and immobilized enzyme is not found to be very significant enough, with a relative variation of less than 8%, to justify looking for other, less affecting, means of immobilization. The physical adsorption of the enzyme at the electrode surface was found to be a fast, reproducible, simple and reliable method whilst preserving the enzyme activity in a satisfactory manner.

Most subsequent studies involving glucose pxidase were performed against glucose at a 20mM concentration, unless stated otherwise. The K_m value for glucose pxidase was found to be around 4mM, and, as stated earlier, a substrate concentration of $5K_m$ has empirically being proven to be situated in the plateau part of the Michaelis-Menten kinetic profile for enzymes. In that range, a small variation in substrate concentration will not lead to a significant variation of the signal output. As the ultimate aim of this study is to determine the influence of hypersolutes on biosensors, it is essential to limit and reduce as much as possible any potential interference that could cause variations in the signal output.

h. Effect of pH on glucose oxidase activity

One area where a positive influence of the Hypersolutes on biosensor stability seemed very likely was against pH variations. Two main options were available to assess pH influence on biosensor response:

- The first, which was pursued, consisted in considering the biosensor as a previously prepared system and limit pH variations to the sample solution. Extrapolating this system, it would assess whether adding hypersolutes as part of the industrial fabrication process of the biosensor would lead to an increased stability and reliability of its response when facing sample of different pH.
- The other solution was not investigated, but it was looked into for a different system by one of our partner in the European project. It consisted in changing the pH of the enzyme solution, while the sample

pH would remain constant at its optimal working value, pH=7.5 for Glucose Oxidase. In that case, then enzyme would be more exposed to pH variations and to potential stabilizing effects of the hypersolutes.

However, in the perspective of finding potentially interesting applications for the hypersolutes in biosensors and the biosensor industry, the first solution was more likely to be relevant. Also, if no notable effect could be detected in those less sensitive conditions, then there would be no potential applications to be pursued in this particular area. It was decided to follow the first option, where the biosensor is prepared by absorbing glucose oxidase from a solution at pH=7.5 and testing that biosensor against glucose solutions at different pH.

The glucose oxidase biosensor used was thus characterized against pH variations and its optimal working pH determined. The biosensor was prepared as described earlier, by depositing a 10µL droplet of a glucose oxidase solution at a 200U/mL concentration and pH=7.5 and left to dry overnight at room temperature, namely 20°C. The influence of pH on the biosensor response against 25µL droplets of glucose solutions at 2.5mM concentration over the range pH=2 to pH=11 is shown Fig.54.

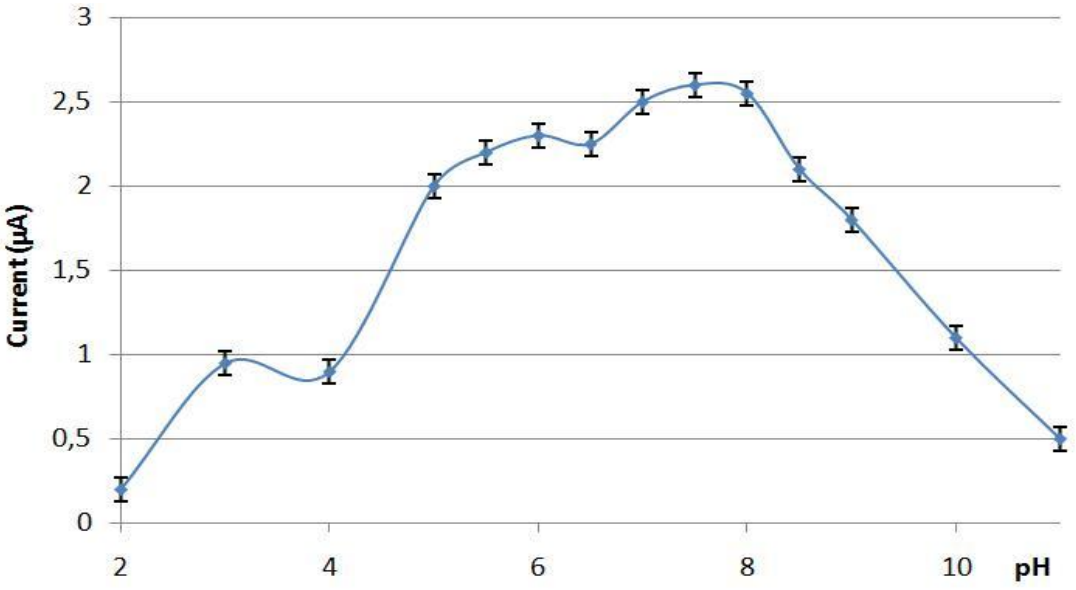


Figure 54: Influence of pH variations over the range 2-11 on glucose oxidase biosensors against glucose 2.5mM

At low pH, pH≤4, the signal is very low because the enzyme is completely inhibited. The optimal pH value for glucose oxidase was found to be pH=5.6 in literature, but in mediated systems, the optimal pH is slightly higher. Also, as the system used in this study relies on hydrogen peroxide detection to generate a signal, the sensor response increases with pH. The optimal working pH for our glucose oxidase biosensor is therefore closer to neutral pH, around pH=7.0. These observations explain the curve's aspect, close to a plateau between pH=5.5 and pH=8.0, with a slight elevation from pH=7.0. The pH of phosphate buffered saline (PBS) buffer at a 10mM concentration is pH=7.5, close to physiological conditions, thus justifying its choice as an appropriate buffer for both enzyme and substrate solutions. For higher pH, when pH≥8.0, the signal decreases as the enzyme becomes inhibited again.

A preliminary study on glucose oxidase was realized very early on, that strongly emphasized the importance of optimizing the biosensor, and more specifically enzyme loading at the electrode surface. In that study, the enzyme was deposited in massive excess and the resulting biosensors were used against glucose solutions at various pH. For higher pH, over pH=8.0, the signal kept increasing, as the enzyme was partially desorbing from the surface. This created a preferential pathway to the electrode surface for the hydrogen peroxide generated by the enzyme still adsorbed at the electrode surface. The concentration of hydrogen peroxide detected at the sensor surface was therefore artificially increased, hence the signal. This in turn led to the detailed optimization of the biosensor presented in this section.

3.2.2.2. *Alcohol oxidase, acetylcholine esterase*

Other enzyme-based biosensors were also studied. They were chosen because of their inherent stability, pathways and commercial importance.

The fist enzyme to be selected was alcohol oxidase. Although its pathway is very similar to that of glucose oxidase generating hydrogen peroxide as a by-product, this enzyme is notably instable. One other of its drawbacks is that its characteristics depend widely of its origin, making it a highly versatile enzyme. It finds many applications in the wine industry, as well as in breath control tests [Azevedo *et al.* (2005).].

The second enzyme we selected was acetylcholine esterase. This enzyme is not very stable and requires a co-enzyme to degrade its substrate. It has a more complex pathway than both glucose oxidase and alcohol oxidase, and therefore might be affected by hypersolutes in a different way. It is mostly used in organophosphate pesticide monitoring.

a. Enzyme loading

This experiment was conducted in a very similar manner to the glucose oxidase loading optimization; however, the manner of loading was not investigated again and physical adsorption of the enzyme at the electrode surface was the chosen immobilization process. Different volumes of 1kU/mL solutions of alcohol oxidase and acetylcholine esterase respectively were deposited on the electrode surface and left to dry overnight at room temperature. These volumes varied in the range 5mL to 20mL as it had been proven earlier to be that of interest. As with glucose oxidase, an enzyme deposit of 10µL was found to be optimal.

The enzyme activity deposited at the electrode surface was then optimized for both alcohol oxidase and acetylcholine esterase. The enzyme solution was deposited at the electrode surface, the activity deposit varying within the range 1mU to 5U deposit. The electrodes were then tested against 2.5% ethanol (EtOH, corresponding to a concentration of 0.43M) for alcohol oxidase and 2.5mM acetylthiocholine (ATch) for acetylcholine esterase. The cofactor for acetylcholine esterase, TCNQ (7,7',8,8'-tetracyanoquinodimethane), was present in the enzyme solution deposited at the electrode surface to prepare the biosensor. The TCNQ concentration in that solution was 100mg/mL.

An optimal enzyme loading is below saturation conditions. Also, any decrease in enzyme activity should relate to a significant decrease of the signal. With this in mind, and in a similar fashion to glucose oxidase, a 500mU deposit was found optimal for alcohol oxidase and a 1U deposit optimal for acetylcholine esterase. In both cases, up to the optimal loading, an increase in enzyme activity lead to an increase in signal, whereas after these optimal activity deposit, the slope of the curve signal vs. activity was greatly reduced. The optimal loading conditions for glucose oxidase, alcohol oxidase and acetylcholine esterase are summarized Table 5.

Enzyme	Substrate	Activity deposited	Substrate concentration
GOx	Glucose	2U	6.2mM
AOx	Ethanol	500mU	2.5%
AchE	Acetylthiocholine	1U	14.7mM

Table 5: Optimal enzyme loading conditions for glucose oxidase, alcohol oxidase and acetylcholine esterase on screen-printed electrodes

b. Amperometric conditions

As had previously been done for glucose oxidase, assay parameters were confirmed for alcohol oxidase and acetylcholine esterase. An analysis time of 150s was confirmed for both enzymes, the last 20s of the assay being used to determine an average value of the steady state. Also, a sample volume of 25µL was found to be optimal to test these enzyme-based biosensors.

Finally, the amperometric conditions for alcohol oxidase as well as acetylcholine esterase were tested. Alcohol oxidase based electrodes, like for glucose oxidase, rely on the detection of one by-product, hydrogen peroxide, at the electrode surface. As was observed for glucose oxidase, a potential of E=+350mV was confirmed to be optimal. The test was realized against ethanol at 2.5% in PBS buffer 10mM, at pH=7.5, the ethanol concentration being 0.43M. The curve is very similar in aspect to that of the hydrogen peroxide detection on the bare screen-printed electrode. Again, the curve is close to a plateau between E=+300mV and E=+400mV. [Boujtita *et al.* (2000).]

Acetylcholine based biosensors, on the other hand, detect the oxidation of their cofactor, TCNQ (7,7',8,8'-tetracyanoquinodimethane) at the electrode surface. In literature, recommended amperometric conditions were found to be E=+100mV on bare carbon working electrodes, at pH=7.0. As with the other enzymes, a biosensor was prepared by depositing 10µL of an enzyme solution, here acetylcholine esterase at a 10U/mL concentration, and with a 100mg/mL concentration of TCNQ, the enzyme co-factor. Such a TCNQ concentration was in large excess compared to that of the enzyme, therefore ensuring there would be no signal loss due to a lack of co-enzyme. The slow step of the acetylcholine esterase system is the degradation of the

acetylthiocholine by the enzyme, and not the oxidation of the co-enzyme at the electrode surface. The acetylcholine esterase biosensor thus manufactured was then tested against an acetylthiocholine solution of 2.5mM in PBS buffer 10mM at different potentials between E=-100mV and E=+500mV. A potential of E=+100mv was confirmed to be optimal.

c. Statistic study on one concentration

Again, as had previously been done for glucose oxidase, the reproducibility of the systems we designed was tested. For each enzyme the systems that had been optimized were used: 500mU of alcohol oxidase and 1U of acetylcholine esterase were deposited on each sensor surface respectively. These biosensors were left to dry overnight at room temperature. They were then tested against ethanol at a 2.5% concentration and acetylthiocholine at a 2.5mM concentration respectively, in a PBS buffer 10mM at pH=7.5.

The experiment was repeated over twenty screen-printed electrode per enzyme. The response average was determined for each enzyme, as well as the standard deviation (STD) and relative standard deviation (RSD). The results are displayed Table 6. The results obtained for both hydrogen peroxide on bare screen-printed electrode as well as for the glucose oxidase based biosensor are added in order to point out the differences in stability and reproducibility between the enzymes.

	H_2O_2	GOx vs. Glucose	AOx vs. Ethanol	AchE vs. ATch
Substrate	2.5mM	2.5mM	2.5% - 0.43M	2.5mM
i (μA)	1.14	2.60	5.73	7.25
STD	0.07	0.17	0.53	0.30
RSD	5.8%	6.8%	11.6%	8.3%

Table 6: Statistical study on one substrate concentration for enzyme based biosensors

The results corroborate what was expected, namely that the addition of a biological component increases the relative standard deviation associated with the

system, as does a higher instability of the enzyme. The latest is being clearly demonstrated by alcohol oxidase based biosensors.

d. Calibration curves

Following the pattern established for the characterization of the glucose oxidase based biosensor, calibration curves for both alcohol oxidase and acetylcholine esterase were established. Their respective Line-Burke-Weaver curves were then calculated and the kinetics characteristics for either enzyme determined. Both systems were prepared according to the parameters previously optimized, with a 500mU activity deposit for alcohol oxidase and a 1U activity deposit for acetylcholine esterase.

i. Alcohol oxidase based biosensors

The calibration curve of alcohol oxidase is shown Fig.55. The range studied was 0% to 50% ethanol in PBS buffer 10mM. The range was determined by an earlier study, as the sensor response to hydrogen peroxide remained constant within that range, being noticeably affected over a concentration of 50% ethanol.

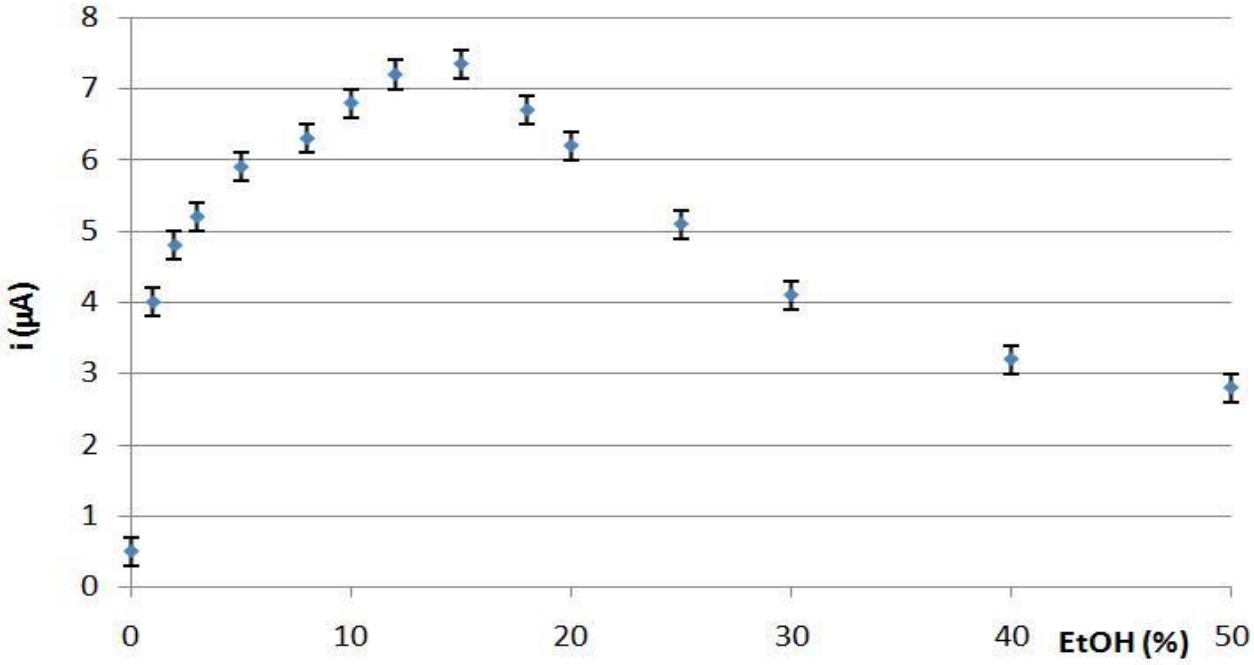


Figure 55: Calibration curve for alcohol oxidase on screen-printed electrodes against ethanol over the range 0-50%

The response follows a classic kinetic display up to a concentration of ethanol of 15%, with a first order relationship between concentration and response current. Over 15% of ethanol, substrate inhibition occurs, causing the response to drop with the increase of ethanol. A Line-Burke-Weaver curve was calculated using the experimental results from the first part of the calibration curve and the kinetic values for alcohol oxidase were determined. The maximal current value obtained thus was i_{max}=5.8µA and the Michaelis-Menten constant was calculated to be K_m=0.026, i.e. a 2.6% ethanol concentration. The high instability of alcohol oxidase was again observed during these experiments, with the error on the measure of the triplicates confirming the relative standard deviation determined earlier for that enzyme. The values found in literature for the Michaelis-Menten constant were on too large a range, partly depending on the originof the alcohol oxidase to allow any reliable comment on how our experimental value relate to them.

ii. Acetylcholine esterase based biosensors

The calibration curve of acetylcholine esterase against acetylthiocholine was studied over the concentration range 0mM to 10mM of substrate. As for alcohol oxidase, a first order relationship, of a classic kinetic response, can be observed up to 2.7mM of acetylthiocholine. Over that concentration, however, a moderate substrate inhibition can be observed. It is less pronounced than that observed in alcohol oxidase in presence of more than 15% ethanol.

Up to 2mM of acetylthiocholine, the linear range of the response is very clear, and the corresponding experimental values were used to determine the kinetic values of acetylcholine esterase. Its Line-Burke-Weaver curve is shown Fig.56. The maximum response current obtained was i_{max}=128µA and the Michaelis-Menten constant was calculated to be K_m=7.3mM. It relates very nicely to that found in the literature: K_m(acetylcholine esterase)=7.0mM. [Liu *et al.* (2005). Snejdarkova *et al.* (2004). Vakurov *et al.* Khayyami *et al.* (1998).]

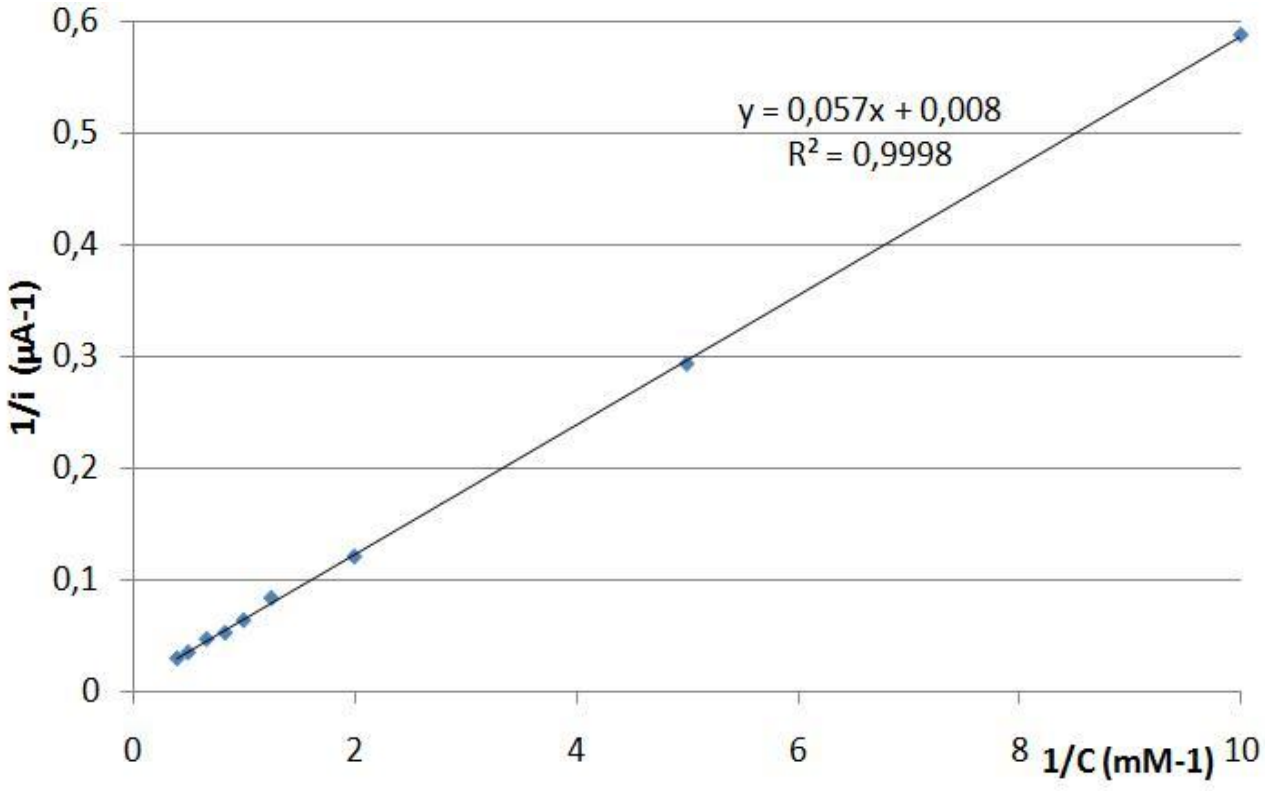


Figure 56: Line-Burke-Weaver curve for acetylcholine esterase on screen-printed electrodes against acetylthiocholine over the range 0-2mM, with 100mg/mL TCNQ

e. Influence of the immobilization process

As with glucose oxidase, the enzymes were immobilized onto the surface of the screen-printed electrode using simple physical adsorption. The influence of the immobilization process on the enzyme activity was realized for both alcohol oxidase and acetylcholine esterase. The activity of the enzyme was studied, whether the enzyme was free in solution or physically immobilized at the electrode surface. Calibration curves were determined in both cases for each enzyme, in similar conditions to those studied before, as has been done for glucose oxidase.

For the immobilized enzyme, the screen-printed electrode was coated with 10µL of enzyme solution to deliver the appropriate amount of activity, 500mU for alcohol oxidase and 1U for acetylcholine esterase, with 100mg/mL of TCNQ in the acetylcholine esterase solution. The electrodes were left to dry overnight at room temperature and then tested against 25µL of substrate.

For the free enzyme activity measurement, the same stock solutions were used and the same proportions retained. An aliquot of 25µL of the solution containing the enzyme, the substrate and the co-enzyme when applicable was deposited on the screen-printed electrode. The resulting current was then recorded over 150s.

Calibration curves were established and Line-Weaver-Burke plots drawn. The kinetics characteristics of the enzymes, either free or immobilized were determined. The results are presented Table 7. As we had observed in glucose oxidase, the Michaelis-Menten constant value is higher for the free enzymes, whereas the i_{max} is higher for the immobilized enzymes. There is a diminution of the enzymes activity and an increase of the signal measured due to the immobilization process. As physical adsorption is a simple, fast and quite reliable method, with few parameters interfering, it was thus confirmed as the chosen immobilization method for our subsequent studies.

Enzyme	AOx		AchE	
	Free	Immobilized	Free	Immobilized
i_{max} (µA)	2.76	5.8	76.9	128
K_m	4.6% EtOH	2.6% EtOH	14.7mM ATch	7.3mM ATch

Table 7: Influence of the immobilization process on alcohol oxidase and acetylcholine esterase

3.2.2.3. *Lactate dehydrogenase*

The last enzyme we interested ourselves in was lactate dehydrogenase. While it is relatively stable, although less so than glucose oxidase, it requires a co-enzyme to work properly, the nicotinamide adenine dinucleotide (NAD^+). Lactate dehydrogenase finds many applications, particularly in the food industry. However, its coenzyme, the nicotinamide adenine dinucleotide, has proven to be rather difficult to detect using electrochemical and a mediator is often used to improve that condition, complicating the pathway of the detection reaction. This might in turn be further affected by the use

of hypersolutes than the simpler systems we considered thus far. [Mazzei *et al.* (1996). Serra *et al.* (1999). Smutok *et al.* (2005). Tap *et al.* (2000). Goller. and Galinski (1999)]

a. Amperometric conditions

The first step taken here was to look in literature for working amperometric conditions using lactate dehydrogenase and nicotinamide adenine dinucleotide against lactate. The conditions were found to be, at pH=7.5, a potential of E=+350mV on a working electrode made of rhodinized carbon against a reference electrode of Ag/AgCl, over an enzyme range 5mU to 4U, with a cofactor concentration of 1mM. [Avramescu *et al.* (2001).]

Based on the experiment realized for the other enzymes, a batch for cross-testing these parameters was set up. Electrodes were prepared by depositing 10µL of lactate dehydrogenase over the range 0mU to 4U, with in solution nicotinamide adenine dinucleotide at 1mM, as well as a completely blank electrode used as reference point. These sensors were then tested against lactate over the range 0 to 100mM. No significant difference was noted between the different enzyme loadings or the substrate concentrations.

New solutions batches were made, but the response currents remained negatives and the results obtained similar to that of the previous test. The amperometric conditions used were suspected not to be accurate and a cyclic voltammetry study was carried out. An electrode was coated with 10mU of lactate dehydrogenase and 1mM of co-enzyme, and tested against 20µL of lactate at a 20mM concentration, over the range of potential E=0mV to E=+1V. It proved the potential used previously to be too low and a new potential was selected for subsequent studies, E=+750mV. The cross-testing assay was realized again, using these new amperometric conditions. The resulting signal varied with the enzyme loading, but wasn't reproducible or very reliable. The relative standard deviation associated with a result was over 30%, which was not acceptable enough for this system to be used as such, but could be related to the high potential used, due to the detection of the NADH at the electrode surface.

b. Use of a mediator

It was therefore necessary to optimize the amperometric conditions of the assay, studying the coenzyme alone before any further study on lactate dehydrogenase could be carried out. The obvious solution was to use a mediator, given the particular of the difficulties associated with the electrochemical detection of nicotinamide adenine dinucleotide.

i. Nicotinamide adenine dinucleotide detection issues

Nicotinamide adenine dinucleotide is an expensive chemical regenerating by reoxidation to its original state, this regeneration being imperative if it is to be economically used in low cost, disposable, analytical devices. NAD^+ is a natural mediator with one of the most negative potential found in aerobic organisms, with E=-560mV vs. saturated calomel electrode (SCE) at pH 7. It is not surprising that nature discovered how to prevent oxidation of its reduced form in aerobic conditions. As a result, regeneration of NADH on bare electrodes requires an extremely high overvoltage. The NADH is oxidized directly at different base electrode materials only with a high overvoltage on the order of 1V. [Gao *et al.* (2004).]

This high overvoltage has several problematic consequences. Therefore, there is a great need to drastically decrease it for the direct electrochemical oxidation of nicotinamide adenine dinucleotide at all conventional electrodes. One of the main issues is that this high overvoltage is accompanied by electrode fouling. It also causes strong adsorption of nicotinamide adenine dinucleotide, and interfering background currents in real samples. Furthermore, the overvoltage leads to the formation of enzymatically inactive forms of nicotinamide adenine dinucleotide, as noted by several authors [Grundig *et al.* (1995). Jaraba *et al.* (1998). Nagy *et al.*. (1995).]

ii. Mediators

However, a decrease in this over-voltage can be obtained by the immobilization of functionalities on the electrode surface which mediate the electron transfer from NADH to the electrode. Such mediators are typically selected from materials which may be reoxidized electrochemically without excessive overvoltages rendering them

useful as an auxiliary system for electrochemical regeneration. Various mediator compounds suitable for this purpose are known.

Different families of compounds may be used for such purposes such as nitrofluorenone derivatives or such as phenazine methosulphate (PMS),thionine and 1,2-benzoquinone. In certain cases, the electron exchange with the co-enzyme is realized by structural elements comprising one of either alkyl-phenazinium ions, phenazinium ions, phenazinones, phenoxazinium ions, phenoxazinones, phenothiazinium ions or phenothiazinones. The graphite electrode can also be chemically modified for oxidation of the reduced form of nicotinamide adenine dinucleotide based on the phenothiazine derivative 3-β-naphthoyl-toluidine blue [Kubota *et al.* (1996). Munteanu *et al.* (2002). Gao *et al.* (2004).]

Some mediators, which could be described as "electron shuttles", provide redox coupling between the electrode and the redox center of the co-enzyme. Perhaps the best known mediators for use on graphite electrodes are phenothiazinium and phenoxazinium salts such as Meldola's Blue (MB). It is the latest that was chosen to be used in this study. [Prieto-Simon and Fabregas (2004). Schuhmann *et al.*. (1993).]

iii. Using Meldola's blue

As the lactate dehydrogenase and nicotinamide adenine dinucleotide system had been found to have unreliable responses, due to the lack of stability of the amperometric detection of the NADH, a redox mediator was used, Meldola's blue. This molecule's exact chemical formula is 8-Dimethylamino-2,3-benzophenoxazine hemi(zinc chloride) salt and its structure is presented Fig.57. [Sprules *et al.* (1995). Vasilescu *et al.* (2003).]

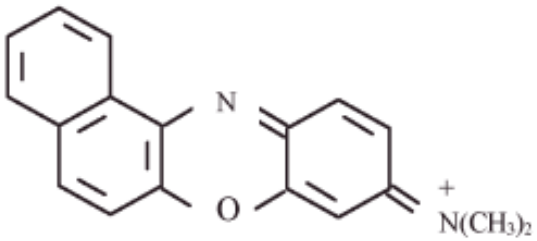


Figure 57: Meldola's blue structure

Meldola's blue can be oxidized at the electrode surface at a considerably lower potential than that of nicotinamide adenine dinucleotide. The new reaction pathway for lactate dehydrogenase, incorporating Meldola's blue, is as follows in Equ.15, with the detection at the electrode surface being through the mediator instead of the co-enzyme.

$$Lactate + NAD^+ \overset{LDH}{\underset{\longleftarrow}{\longrightarrow}} Pyruvate + NADH + H^+$$

$$NADH + MB^+ \underset{\leftarrow}{\rightarrow} NADH \cdot MB^+$$

$$NADH \cdot MB^+ \rightarrow NAD^+ + MBH$$

$$MBH \rightarrow MB^+ + H^+ + 2e^-$$

Equation 15: Lactate dehydrogenase reaction pathway against lactate, using Meldola's blue

c. Optimization

i. Optimal amperometric conditions

The detection of NADH with Meldola's blue was first characterized. Solutions containing Meldola's blue at a 6.6mM concentration with nicotinamide adenine dinucleotide over the concentration range 0.5mM to 50mM were studied, at pH=7.5 and with a potential of E=+350mV. It was observed that the optimal volume to be deposited at the electrode surface was 20µL and the assay duration for the amperometric test was 150s. The lowest concentration of nicotinamide adenine dinucleotide that gave a quantifiable response in those conditions, and with an acceptable relative standard deviation (RSD≤20%) was C=25mM.

Using these new parameters, a cyclic voltammetry study was then realized. Electrodes were coated with 25mM NADH and Meldola's blue at 6.6mM. The potential applied varied between E=-200mV and E=+750mV. The optimal working potential was thus determined to be E=+100mV. This experiment proved the system reproducibility to be correct, with a relative standard deviation, calculated over 15 tests, for the response, of 9%.

ii. Optimal enzyme loading and statistic study on one concentration

Lactate dehydrogenase deposition at the electrode surface was then optimized, using the expertise gained on the other enzymatic systems. Several deposition methods were considered:

- 10µL droplets of lactate dehydrogenase at 100mU/mL were deposited over the working electrode, either alone,
- or along with 10µL of nicotinamide adenine dinucleotide at 25mM only,
- or along with 10µL of nicotinamide adenine dinucleotide at 25mM and 10µL of Meldola's blue at 6.6mM.

These electrodes were then tested against a 30µL sample droplet containing a solution of lactate at a 20mM concentration, nicotinamide adenine dinucleotide at 25mM and Meldola's blue at 3.3mM. All three systems gave similar results, so a statistical study of all three systems over 20 samples each was realized to identify the most reproducible. The electrodes where lactate dehydrogenase, nicotinamide adenine dinucleotide and Meldola's blue were deposited simultaneously presented the lowest relative standard deviation with only 12%RSD.

Using this deposition method, different enzyme loading were investigated, over the range 50mU to 5U. As for the other enzymes studied, so that, while below saturation conditions, any decrease in enzyme activity relates to a significant decrease of the signal. A loading of 1U for lactate dehydrogenase was found to be optimal in the conditions described here.

iii. Confirmation of the amperometric conditions

A cyclic voltammetry study of this system was then realized to confirm the validity of the amperometric parameters used. Electrodes were prepared depositing all three elements simultaneously and then were left to dry overnight at room temperature. They were tested against the same sample solution as before, over the potential range E=-200mV to E=+300mV. The result is presented Fig.58.

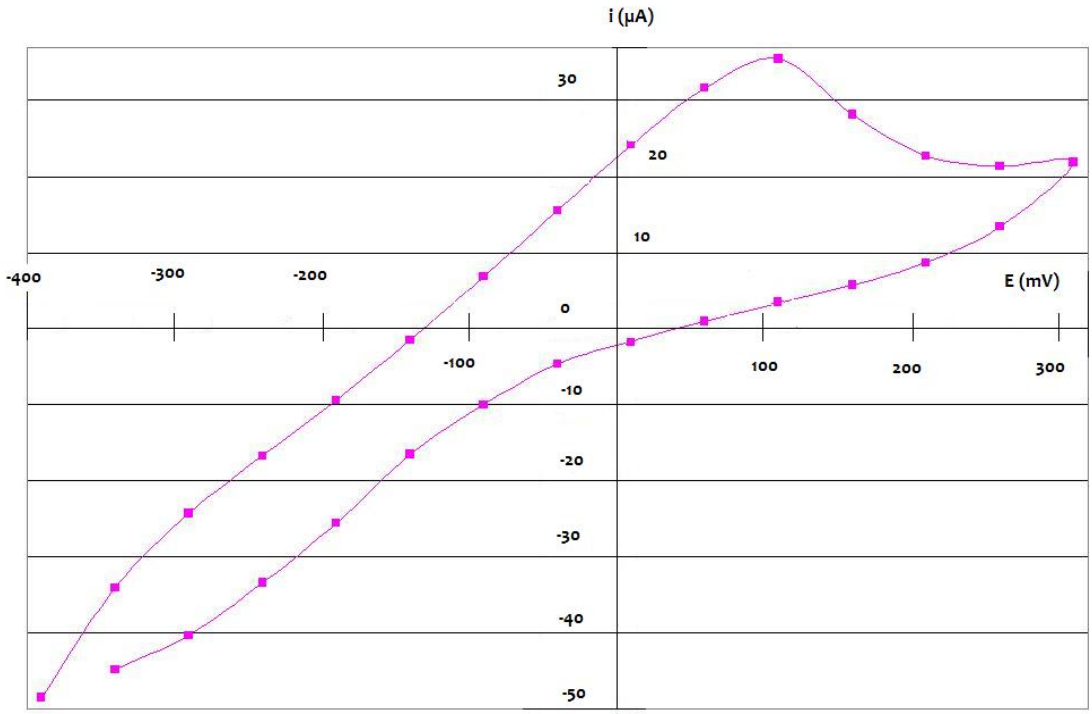


Figure 58: Cyclic voltammetry of lactate dehydrogenase with NAD^+ and Meldola's blue against lactate over the range E=[-200;+300]mV

The oxidation of the Meldola's blue occurs for a potential of E=+90mV. The detection potential chosen for the amperometric studies is higher than this oxidation potential, therefore allowing for the oxidation to take place, but is still low in order to minimize both interferences and other complications associated with a high potential and nicotinamide adenine dinucleotide. The signal obtained is thus related to changes in the redox state of the Meldola's blue, which in turn relates to lactate degradation. The relation between the oxidation of the Meldola's blue and the degradation of the lactate into pyruvate is proportional; the electrochemical signal measured is thus directly related to the lactate concentration deposited at the electrode surface.

d. Calibration curve

Using the biosensor system described earlier, where the enzyme, the co-enzyme and the mediator are deposited together at the working electrode surface and then tested against a sample containing the substrate along with the co-enzyme and the mediator, a calibration curve for lactate dehydrogenase was drawn. The substrate,

lactate, was introduced over the concentration range 0mM to 100mM, in solution in PBS buffer 10mM at pH=7.5. The result is presented Fig.59.

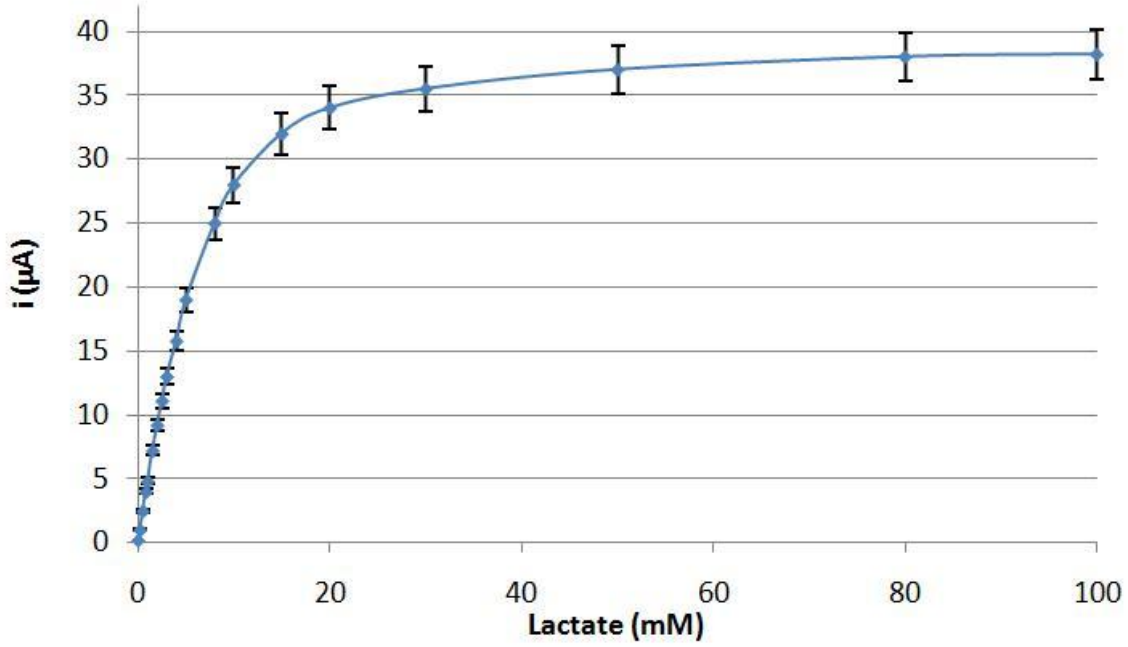


Figure 59: Calibration curve for lactate dehydrogenase on screen-printed electrodes, using Meldola's blue, against lactate over the range 0-100mM

The curve followed a classical Michaelis-Menten display with a first degree relationship between substrate concentration and response current over the range 0mM to 5mM. The corresponding Line-Burke-Weaver curve was obtained and kinetic parameters associated with the lactate dehydrogenase used in this study with Meldola's blue were determined. The maximum current value was i_{max}=64µA and the Michaelis-Menten constant was K_m=14mM.

3.2.3. Adding the hypersolutes

The different enzyme systems used in this study having been thus characterized, we now could add the different hypersolutes provided by our European partners for us to study on biosensors. As the deposition parameters of all the enzymes considered had been previously optimized, the manner of deposition of the different Hypersolutes at our disposal was then considered. Six different Hypersolutes were available; some of them part of the same family: Ectoine (Ect) and hydroxyectoine

(HydE), firoin (Fir) and firoin A (FirA), diglycerol phosphate (DGP) and potassium mannosyl-lactate (PML).

3.2.3.1. *Deposition of the hypersolutes onto the biosensor*

In preparing the biosensors, several ways of adding the hypersolutes to the enzyme at the electrode surface were identified. Four possibilities were compared, using glucose oxidase in association with every hypersolutes available one at a time. The result obtained for glucose oxidase was then transferred onto the other enzymes.

- One of the hypersolutes was deposited at the electrode surface and left to dry at room temperature overnight, followed by the application of the enzyme, and left again to dry.
- On the opposite, the enzyme was deposited at the electrode surface and left to dry at room temperature for 16h, before one of the hypersolutes was added, and left again to dry.
- A solution containing both the enzyme and one of the hypersolutes was prepared before hand, and a droplet of this solution was then deposited onto the electrode and left to dry at room temperature overnight
- The enzyme was deposited onto the electrode surface, closely followed by one of the hypersolutes, the resulting droplet being carefully stirred before being left to dry overnight at room temperature.

The first two possibilities were rejected as placing the hypersolute and the enzyme separately on the electrode surface lessened the interaction between enzyme and hypersolute. As the aim of this overall study is the stabilizing effect, or lack thereof, of the hypersolutes on enzyme activity against different types of stress, these methods weren't appropriate. In addition, the significant preparation time was also a drawback, as it is twice as long with either these two methods as with the other. The third method considered wasn't selected either: whilst no significant difference in results was found with the last method during these preliminary tests, the liquid handling steps required in preparation were much heavier and time consuming than for the last method.

A study was carried out to compare all four methods, resulting in the deposition of a glucose oxidase activity of 1U and a hypersolute concentration of 100mM in PBS 10mM at pH=7.5. The biosensors thus created were then tested against glucose 20mM. The last two methods gave similar results, while the first two methods resulted in a significantly lower response current, with a signal decrease averaging 30%. As the last method was proven to be the most effective and simplest method, it was selected for the preparation of all subsequent enzyme-based biosensors containing hypersolutes.

- The deposition of both enzyme and stabilizing agent at the screen-printed electrode surface was thus done following the steps described here:
- 10µL of enzyme, for example glucose oxidase, at the appropriate concentration, is deposited at the electrode surface,
- 10µL of hypersolute, at a concentration to be optimized, is applied immediately after.
- The resulting droplet is carefully stirred and homogenized at the electrode surface using a pipette tip.
- The coated electrode is then left to dry at room temperature for 16h.

3.2.3.2. *Optimization of the hypersolutes deposition*

The next parameter to be optimized was the amount of hypersolutes to be loaded at the electrode surface. A first study was carried out using glucose oxidase, before testing the other enzymes. This was done to have a first assessment of the influence of the hypersolutes on the enzyme-based sensor as well as allow us to determine the optimum concentration of hypersolute to add at the electrode surface for the subsequent studies.

The first study we carried out was realized at room temperature over a 21 days period, using glucose oxidase with the different hypersolutes over the range 50mM to 1M. That range was given to us by our European partner from Bitop as it is the range they recommend for using those of the hypersolutes that are being commercialized already. The stress factor we tested was storing time, as it is the application advertised for these commercialized hypersolutes. The biosensors were tested every day for a week, then every other day against glucose at a 20mM concentration. However, over

the time period of this study, no difference between the electrodes was noticed, regardless of the amount of hypersolutes added, be it 1M, 50mM or none at all, due to the inherent stability of the glucose oxidase.

The study was therefore redesigned, with an increase of the stress factor by storing the biosensors at 37°C. Again, the biosensors were tested every other day over the assay against glucose 20mM. There is a direct correlation that can be made between length of storage and temperature and this was used to artificially age our biosensors. The higher temperature encourages enzyme destabilization and after 14 days, significant differences between the biosensors were apparent. The result obtained for glucose oxidase with hydroxyectoine is shown Fig.60. A graph summarizing the results for glucose oxidase with every stabilizing agent can be found Appendix 2.

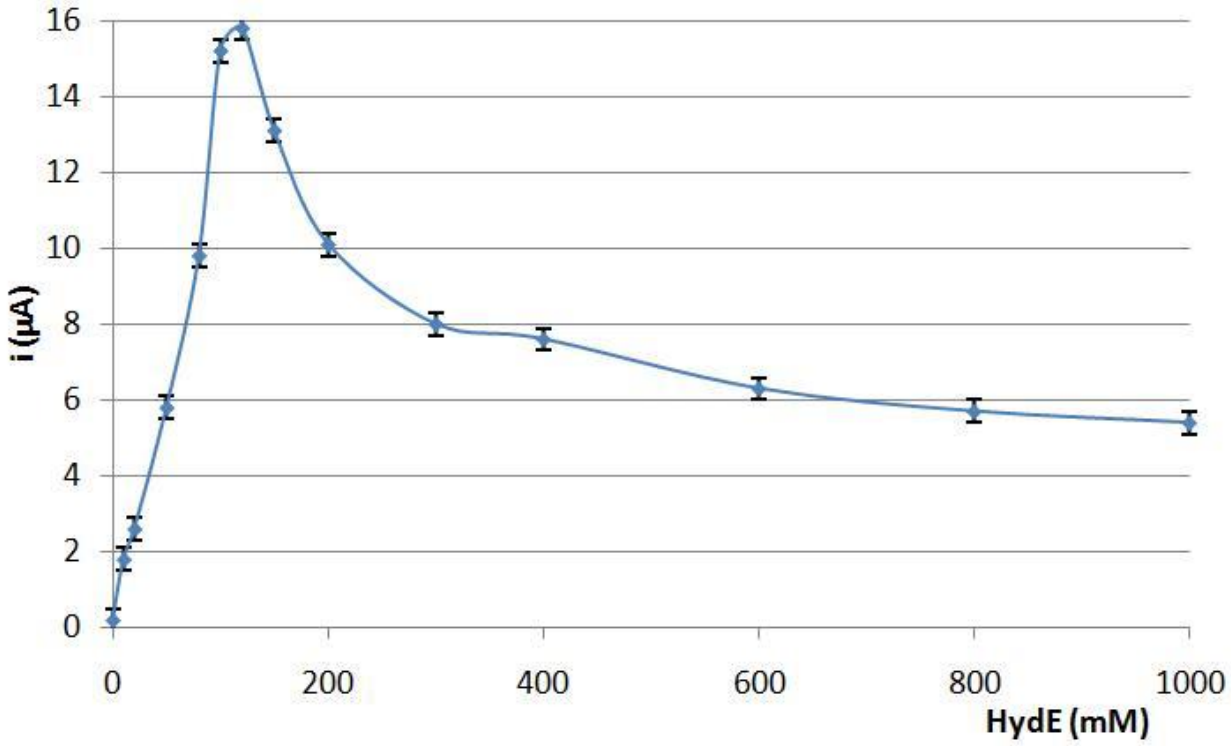


Figure 60: Glucose oxidase biosensor with hydroxyectoine on screen-printed electrodes against 20mM glucose after 14 days of storage at 37°C

In parallel, enzyme-based biosensors using respectively all the enzymes tested were prepared and stored at room temperature, namely glucose oxidase, alcohol oxidase, acetylcholine esterase and lactate dehydrogenase, with each of the solutes we

considered, namely ectoine, hydroxyectoine, firoin, firoin A, diglycerol phosphate (DGP) and potassium mannosyl-lactate (PML), over the range 50mM to 1M. These sensors were tested every 4 days over a 2 months period, against their respective substrate. A summary of the enzyme loading and substrate concentration is presented Table 8.

Enzyme	Activity deposited	Substrate	Substrate concentration	RSD associated
GOx	2U	Glucose	2.5mM	6.8%
AOx	500mU	Ethanol	2.5%	11.6%
AchE	1U	ATch	2.5mM	8.3%
LDH	1U	Lactate	20mM	12.5%

Table 8: Summary of enzyme-based biosensor characteristics

A significant difference in response for the biosensors with regard to the hypersolute concentration was noticeable after 1 month storage for alcohol oxidase, acetylcholine esterase and lactate dehydrogenase. After 2 months of storage, there was no activity left for alcohol oxidase, whether with or without hypersolutes. For glucose oxidase, significant differences were observed after 2 months storage. The curves obtained were all of similar shape, regardless of the enzyme or the hypersolute considered. The results obtained for glucose oxidase in this test were identical to those of the test realized storing the biosensors at a higher temperature. The result obtained for glucose oxidase with hydroxyectoine storing at room temperature is shown Fig.61. The results for each enzyme with every hypersolute against their substrate can be found Appendix 3.

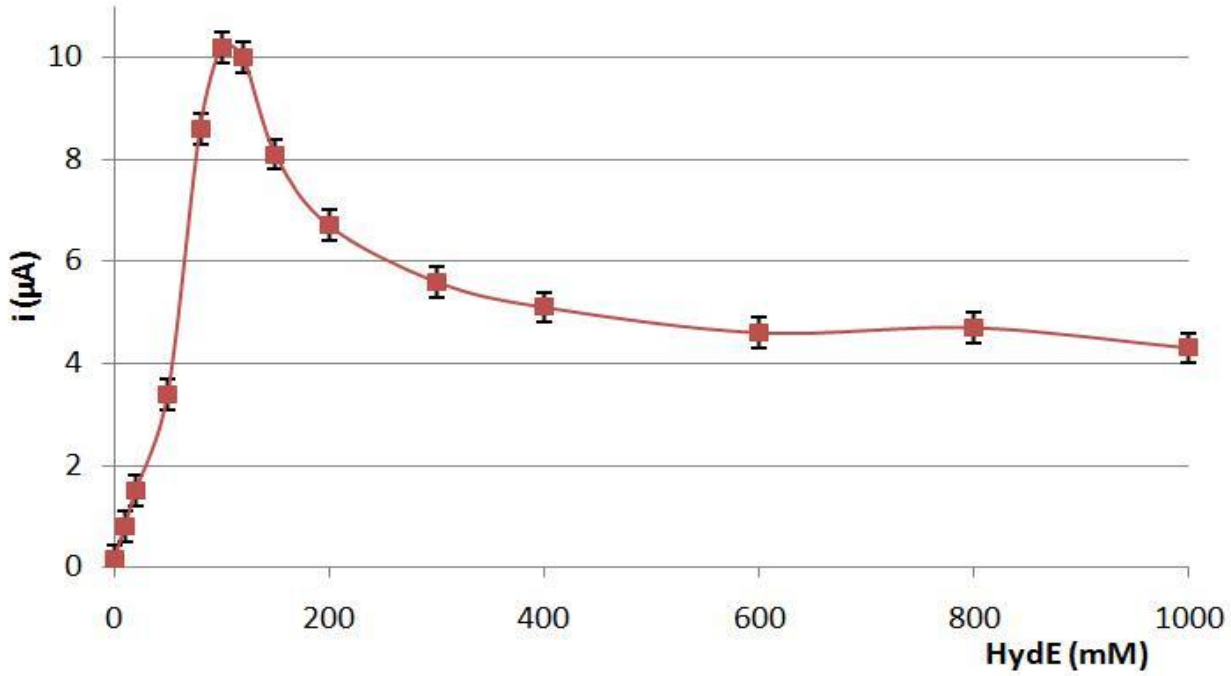


Figure 61: Glucose oxidase biosensor with hydroxyectoine on screen-printed electrodes against 20mM glucose after 2 months of storage at room temperature

The optimum concentration of hypersolutes with an enzyme-based biosensor is the lowest concentration associated with a high sensor response after storage. The hypersolutes were available in limited quantities only for one, and potential enzyme inhibition with higher hypersolutes concentrations was also a concern. This aspect of the hypersolute interaction with the enzyme is discussed in a further section. The results for all enzymes is summarized Table 9. The overall average for all the optimum hypersolutes concentration was found to be around 70mM. Therefore, in order to limit the varying parameters in subsequent studies that might influence the stabilization process, all Hypersolutes were deposited with the enzyme at this concentration.

	Fir	FirA	Ect	HydE	PML	DGP	Average
GOx	60mM	50mM	100mM	80mM	70mM	100mM	77mM
AOx	50mM	100mM	60mM	100mM	55mM	55mM	70mM
AchE	55mM	70mM	50mM	80mM	80mM	80mM	69mM
Average	55mM	73mM	70mM	87mM	68mM	78mM	72mM

Table 9: Optimum loading of hypersolutes on enzyme-based biosensors

3.2.4. Influence of the hypersolutes on the enzyme-based biosensor

All main aspects of the enzyme-based biosensor were now characterized and documented, and the addition of the stabilizing agent optimized. This in turn allowed for the assessment of the stabilizing effect of the Hypersolutes on the enzyme-based biosensor thus defined.

3.2.4.1. Enzyme inhibition

The first aspect of the influence of the hypersolutes on the enzyme-based biosensor to need assessing is the evaluation of any inhibition of the enzyme activity by the hypersolutes. The possibility of significant inhibition of the enzyme activity by the hypersolutes became apparent in the hypersolute loading optimization study.

Looking back, the optimum concentration was found to be where the peak of signal is, around 70mM. The higher signal indicates the enzyme activity has been better preserved. However, this peak is followed by a dip in current response, one that is attributed to the inhibition of the enzyme by the hypersolutes, between 85mM and 450mM hypersolute concentration. Over a concentration of hypersolute of 450mM, for example with hydroxyectoine against glucose oxidase based biosensor, as was shown Fig.61, a steady state is achieved. One possible explanation for this concentration related inhibition is a change in the predominant stabilization mechanism occurring as the hypersolute loading increases. It is important here to emphasize the hypothetic character of this assertion though, as it is based on the supposition that the stabilization mechanism of the hypersolutes on the enzyme is following a similar pathway to that of trehalose, the only solute whose interaction mechanism with enzyme has been studied in detail so far.

3.2.4.2. Enzyme desorption

The stabilizing agents have been found to have a stabilizing effect on enzyme desorption using PBS-T (phosphate buffer saline-tween). A variation in the loss of enzyme material deposited at the electrode surface was investigated. The electrode with

immobilized enzyme with or without stabilizing agent was stirred for 5 seconds in a solution of PBS-T, and was then interrogated amperometrically. Without stabilizing agent, a loss of signal of 33% was observed, this loss being reduced to 18% in presence of a stabilizing agent. The RSD of the electrode with physically immobilized enzyme was found to vary between 8 and 15%. For Glucose Oxidase, whose results are presented Fig.62, the RSD was 8%. The stability of the enzyme preparation at the electrode surface was significantly increased by the presence of a stabilizing agent.

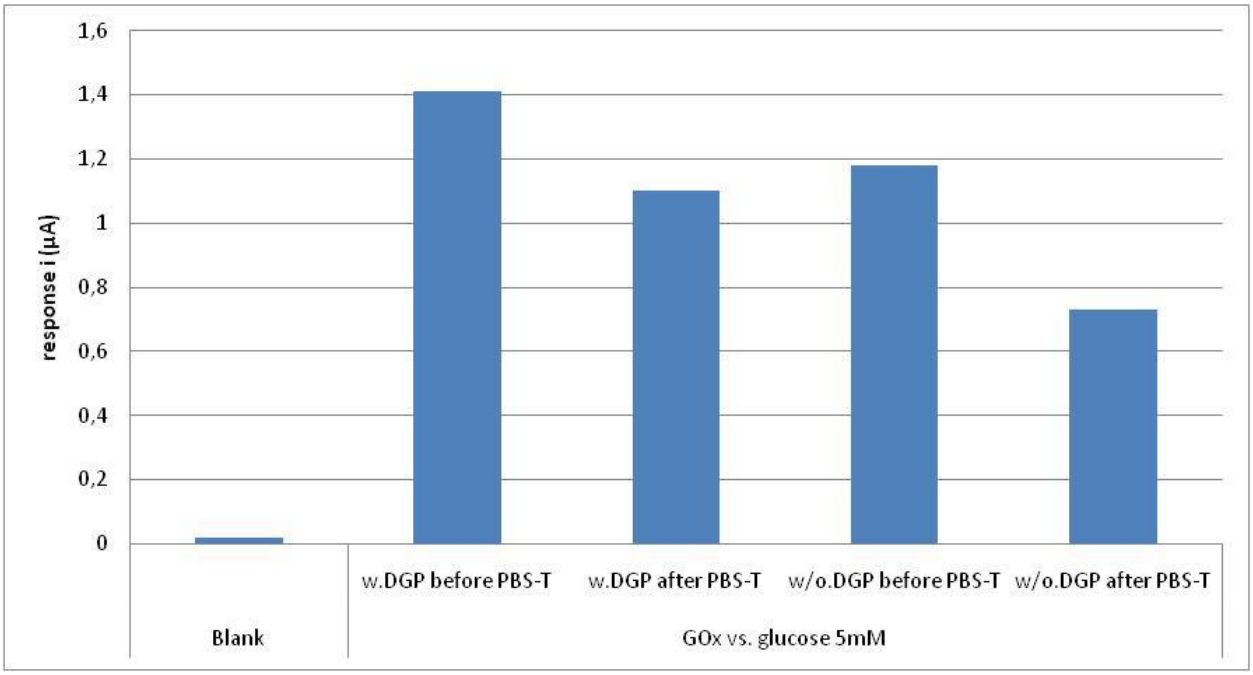


Figure 62: Desorption test of glucose oxidase biosensors on screen-printed electrodes with or without DGP in PBST after 5s against 5mM glucose

3.2.4.3. *pH variations*

The second stress parameter the enhanced enzyme-based biosensors were tested against was pH variation. The effect of pH upon the enzyme with/without stabilizing agent has been assessed within the range 3-11. No significant difference between the electrodes has been found. The hypersolutes hence don't appear to have any significant stabilizing influence on the biological components employed within the biosensor devices developed here.

The alcohol oxidase was found more sensitive to acid pH than the other enzymes, as there is almost no response for pH≤5. The results obtained for

acetylcholine esterase are shown Fig.63. The curves have a very similar aspect whether acetylcholine esterase is alone or with a hypersolute, pH affecting the enzyme and the substrate detection in a similar way, as for manner or effect. The results obtained for the other enzymes are very close to that of acetylcholine esterase and can be found Appendix 4.

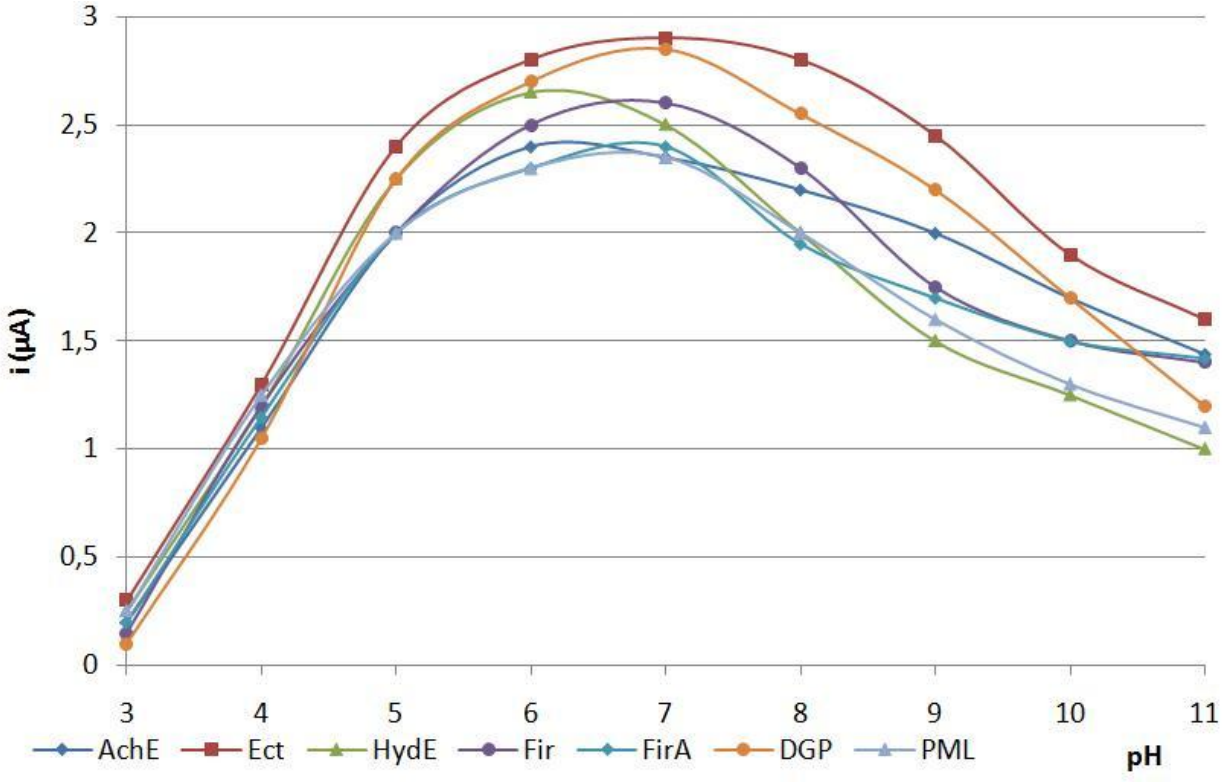


Figure 63: pH influence on acetylcholine esterase biosensor on screen-printed electrodes, with or without stabilizing agent, against acetylthiocholine 2.5mM

3.2.4.4. *Ionic strength variations*

The influence of stabilizing agents on salt variation was tested. Results for Glucose oxidase are presented Fig.64. Ectoine and firoin A showed a stabilizing effect, whereas the other stabilizing agents did not have any significant effect on glucose oxidase activity. Similar results were obtained for alcohol oxidase and acetylcholine esterase. DGP did have a small stabilizing effect with alcohol oxidase, even though it was less than firoin A. These results are presented Appendix 5.

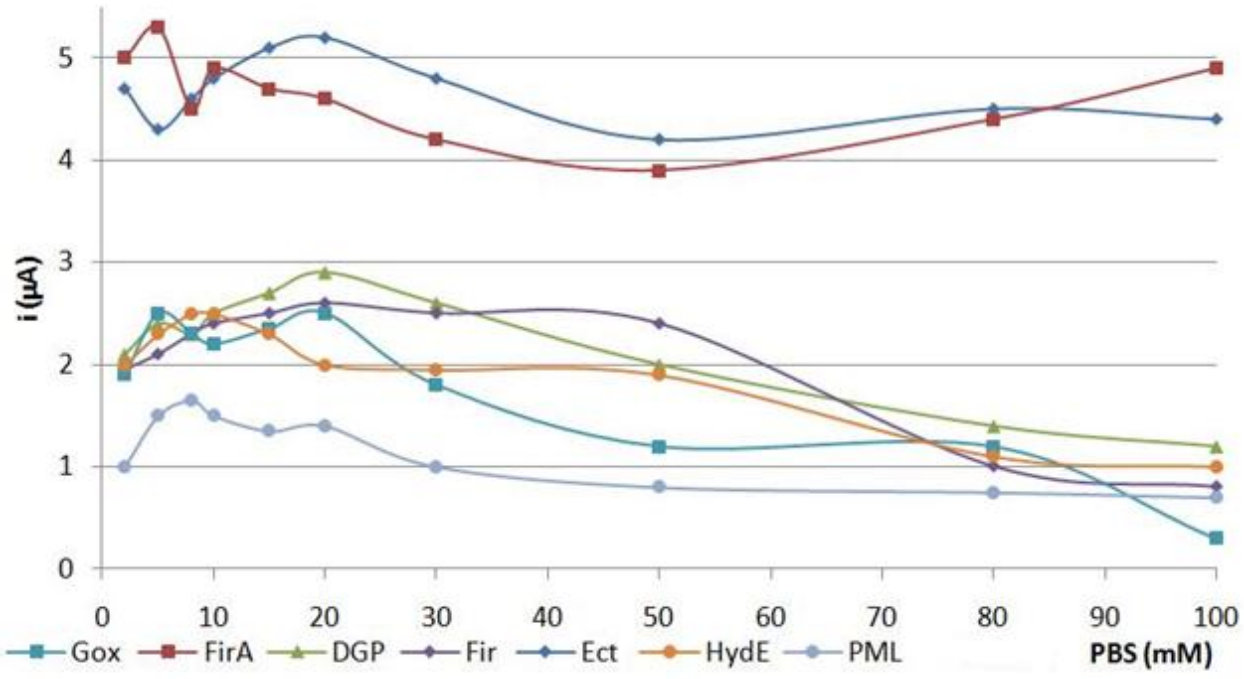


Figure 64: Influence of hypersolutes under salt variations on glucose oxidase biosensor against glucose 2.5mM

3.2.4.5. *Storing*

All enzymes were being stored, with and without stabilizing agent, at different temperatures (4°C, room temperature [20°C] and 36°C). The range of temperature studied is that of non-aberrant use. They were tested at regular time intervals in order to assess the effect of the different stabilizing agent on the storing stability of the biosensors and the influence of temperature.

The preliminary results obtained, after 2 months of storage at room temperature, indicated a difference of effect of the stabilizing agents on the enzymes. For glucose oxidase, hydroxyectoine and DGP were the most efficient of the hypersolutes, whereas ectoine and PML showed the most significant loss of activity over a two month period. With alcohol oxidase, PML and DGP proved to be the most efficient, while hydroxyectoine, PML and DGP associated with acetylcholine esterase preserved best its activity over one month of storage. As for lactate dehydrogenase, DGP and PML proved to be the most interesting stabilizing agents. Overall, DGP gave the best results in the preliminary study and ectoine the worse. The enzyme activity was still important enough in all cases to generate a significant signal.

All enzymes were stored up to 8 months at different temperatures. Storing at 36°C for any length of time proved to be highly impractical and was not pursued in this study. However, the enzymes were stored at room temperature and 4°C respectively. The electrodes were prepared according to the optimized protocol, but the concentration of hypersolute with the enzyme varied over the range 0-1000mM. The electrodes were then tested every month over an 8 months period. The results were highly coherent for the different enzymes, independently of the temperature of storage.

Alcohol oxidase was true to its reputation of instability. It was completely degraded after 3 months of storage at room temperature and 4 months of storage at 4°C. There was no significant signal to be obtained, whether with or without any stabilizing agent. The enzyme had been completely degraded.

As for the stabilizing agents, firoin formed, very early on, a white deposit with the different enzymes at the electrode surface. This might have been the cause of its rather poor results as a stabilizing agent, with signals even lower than those of the enzyme alone.

The optimum hypersolute concentration with respect of enzyme stabilization was once again found to be in the range of 70 to 100mM. The results obtained for glucose oxidase biosensor stored with hydroxyectoine over a month at room temperature are presented Fig.65. The maximum response is a peak at about 100mM of hypersolute.

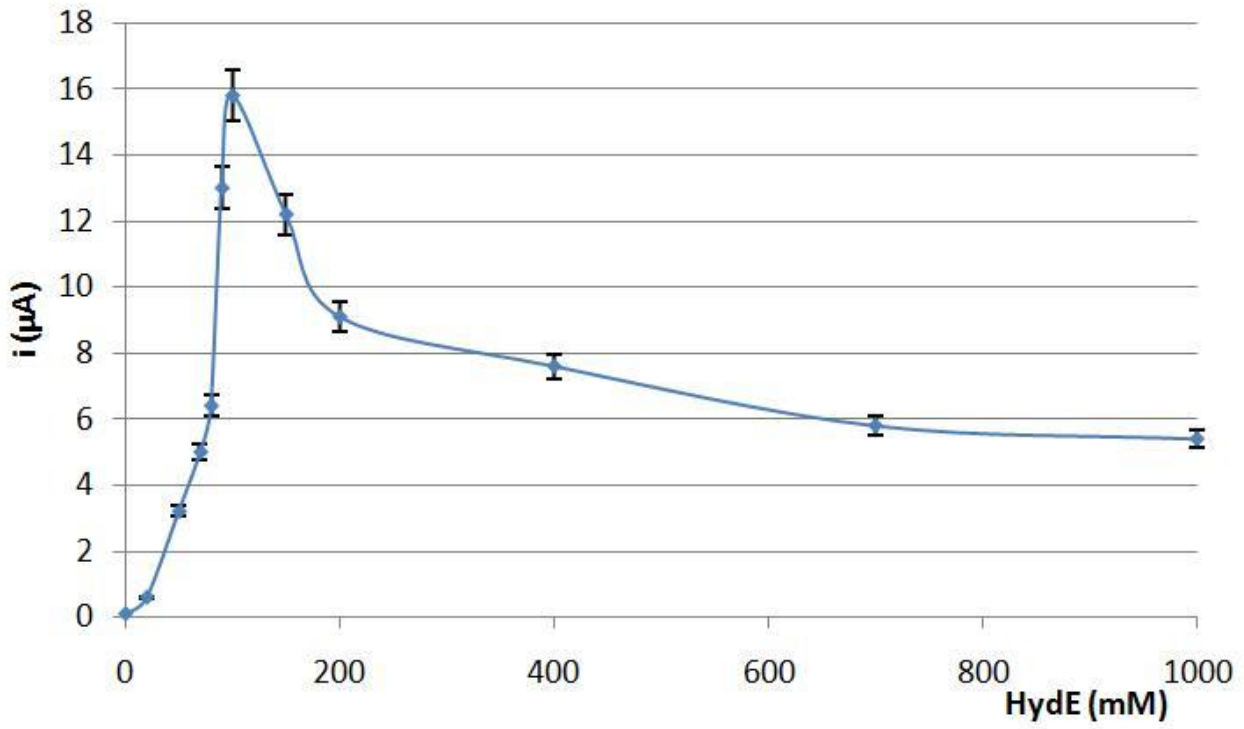


Figure 65: Influence of hydroxyectoine concentration on glucose oxidase activity preservation after 1 month of storage – on glucose oxidase biosensor against glucose 2.5mM

Overall, ectoine, with the exception of firoin, was the least effective stabilizing agent. DGP, hydroxyectoine and to a certain extent firoin A proved to provide stabilization satisfactorily. The enzyme activity of glucose oxidase after 8 months of storage at 4°C with 80mM of hypersolutes is presented Fig.66.

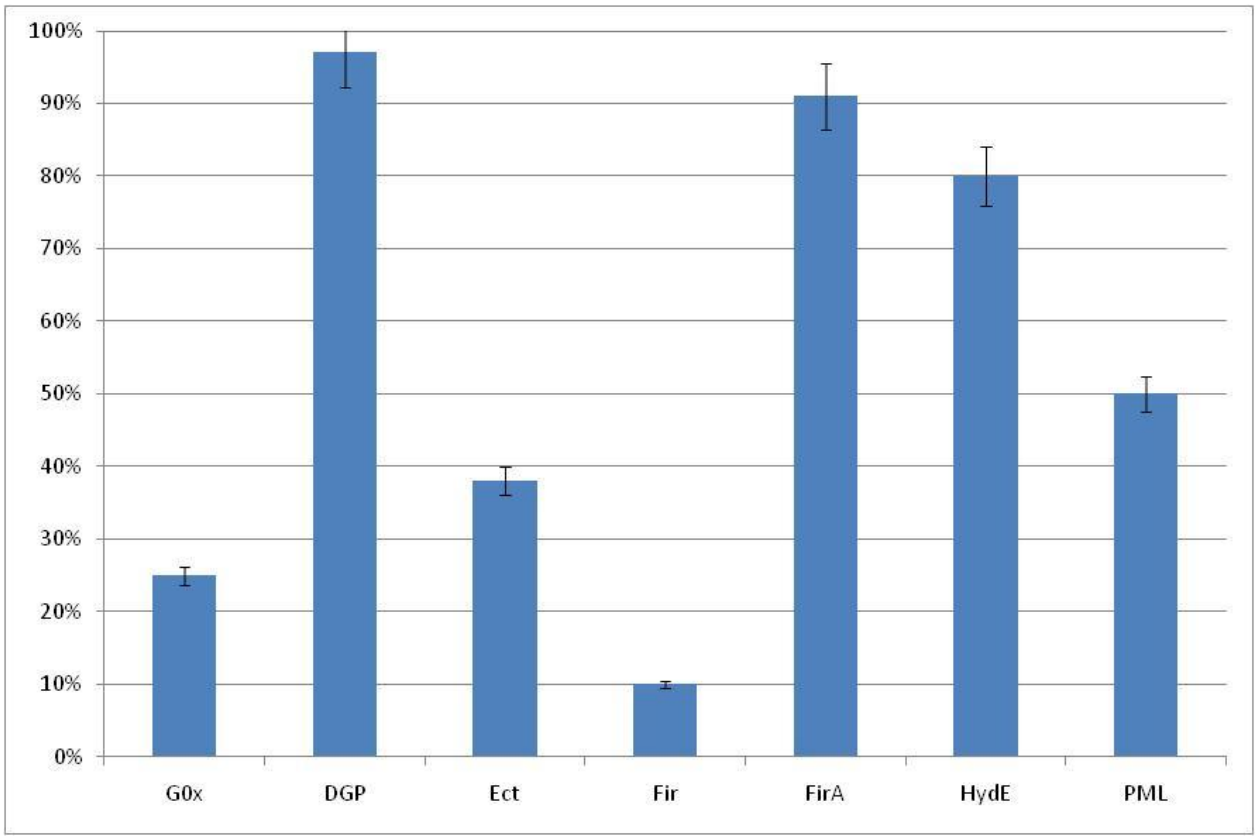


Figure 66: Comparative enzyme activity for glucose oxidase biosensors with or without hypersolutes stored 8 months at 4°C against glucose 20mM

The results obtained after 14 weeks of storing at 4°C for each enzyme, with or without stabilizing agent can be found Appendix 6. The results after 2 months of storing at room temperature are also presented there. The results obtained with all enzymes in those different conditions were concordant with those detailed here.

When comparing the results of 8 months of storage to the results of the preliminary test, it can be observed that DGP is quite consistent in the way it preserved the enzyme, compared to the enzyme alone. PML, as well as ectoine, are also quite consistent, although neither is nearly as efficient as DGP. Hydroxyectoine loses in efficiency overtime, but still gives very satisfactory results over a long period of time. Firoin also loses in efficiency, in a rather more drastic manner since the result obtained for enzyme activity preservation is lower than for the enzyme stored alone. Finally, firoin A gave pretty much enzyme-dependant results, but still overall acceptable. A comparative summary for all enzymes after 8 weeks of storage is presented Fig.67.

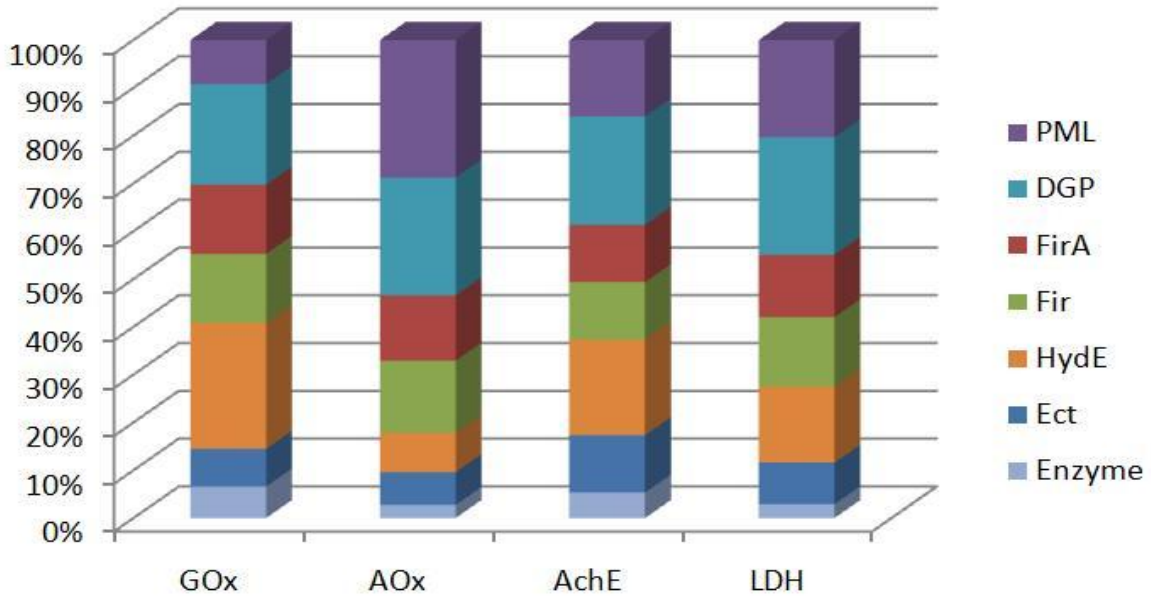


Figure 67: Relative preserved enzyme activity after 10 weeks of storage at room temperature with or without the hypersolutes

3.2.4.6. *Operational stability*

a. Designing the system

The fast and intensive development of the flow injection analysis methodology used in this study was due to several factors considered important when routine analytical determinations. Considerations included minimal sample consumption, short analysis times based on a transient signal measurement in a flow-through detector and deployment of the system on-line for performing difficult unit operations such as separation, pre-concentration or physicochemical conversion of analytes into detectable species. [Van Es *et al.* (2001). White *et al.* (1996). Shichiri *et al.* (1982).]

Flow injection analysis is an analytical technique based on microfluidic manipulation of samples and reagents. Samples are injected into a carrier/reagent solution which transports the sample zone into a detector while desired (bio)-chemical reactions take place. Detector response (absorbance, fluorescence, mass spectra, etc) yield a calibration curve quantifying the target analyte (cf. Fig.68).

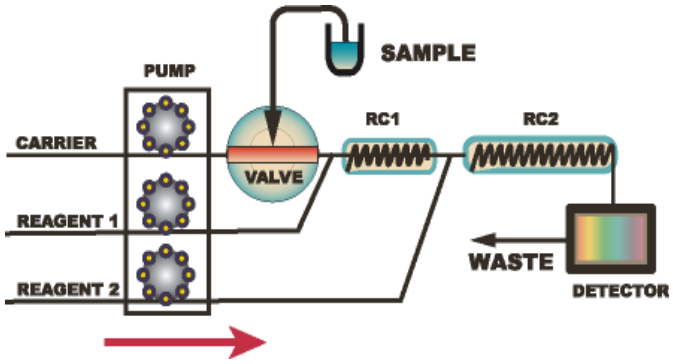


Figure 68: Principle of the Flow Injection Analysis (FIA)

The modern flow injection analysis system usually consists of a high quality multi-channel peristaltic pump, an injection valve, a coiled reactor, a detector such as a photometric flow cell, and an auto-sampler. Additional components may include a flow through heater to increase the speed of chemical reactions, columns for sample reduction, de-bubblers, and filters for particulate removal.

The typical FIA flow rate is 1mL/min, typical sample volume consumption is 100µL per sample, and typical sampling frequency is 2 samples per minute. FIA assays usually result in sample concentration accuracies of a few percent. [Guan *et al.* (2004). Male and Luong (1993). Tothill *et al.* (1997).]

b. Setting up the system

FIA (Flow-Injection Analysis) is a system that allows near-real-time on-line or at-line measurements, with minimal sample consumption and its analysis time are short. It is based on a transient signal measurement in a flow-through detector. In addition, as this system can be automated, operator related errors are minimized.

This system was to be used to test the influence of the stabilizing agents on the enzyme-based biosensors upon repeated use. A relative decrease of the signal on each electrode would be related to a loss of enzyme activity. Internal standards were to be analyzed at regular intervals to assess any eventual signal loss related to the electrochemical system. The system was designed to test in similar conditions one enzyme with the hypersolute as a parameter, as is shown Fig.69.

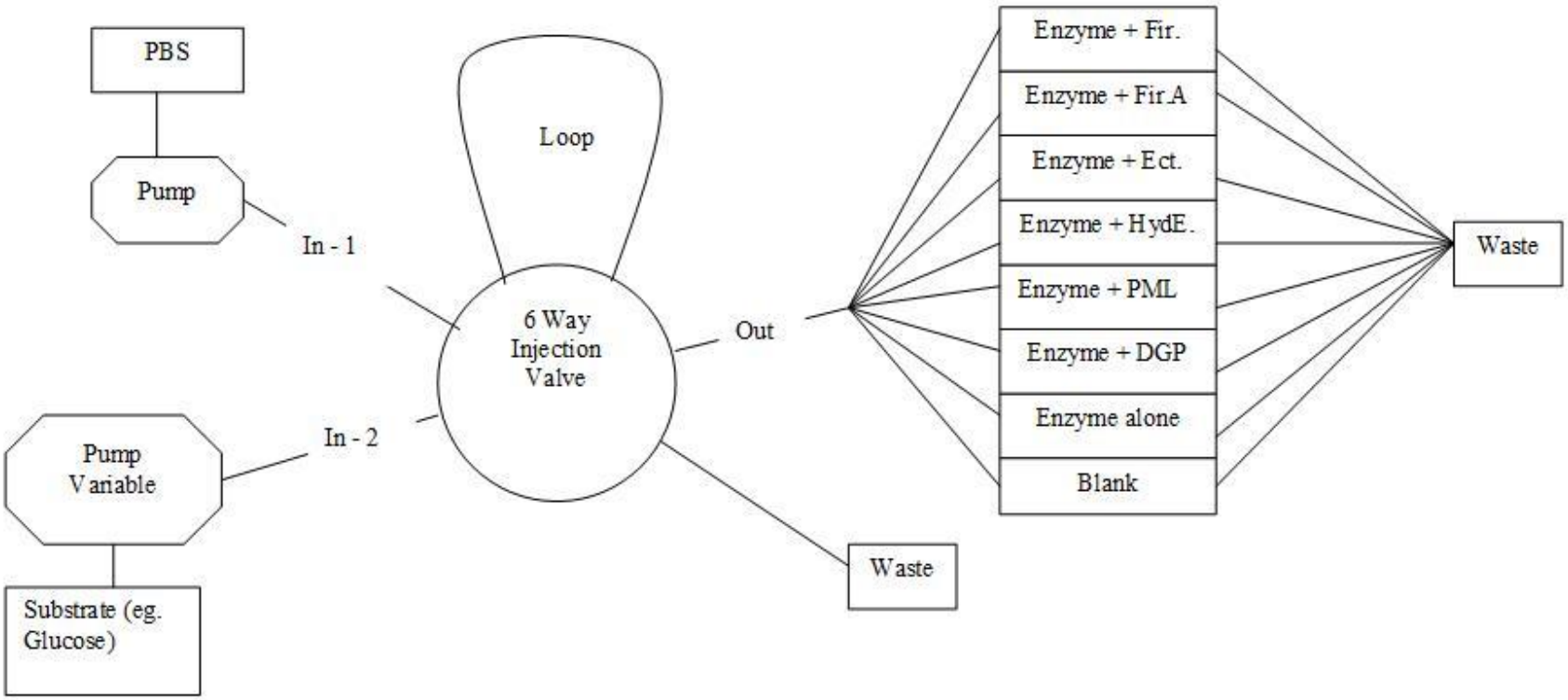


Figure 69: Design of the FIA System

A 3-electrode configuration has been designed, associated with a custom detector cell (cf. Fig.70). Enzymes, stabilizing agents and cellulose acetate membrane, in order to prevent desorption and loss of the enzyme and stabilizing agent in the flowing process stream, are immobilized on the working electrode.

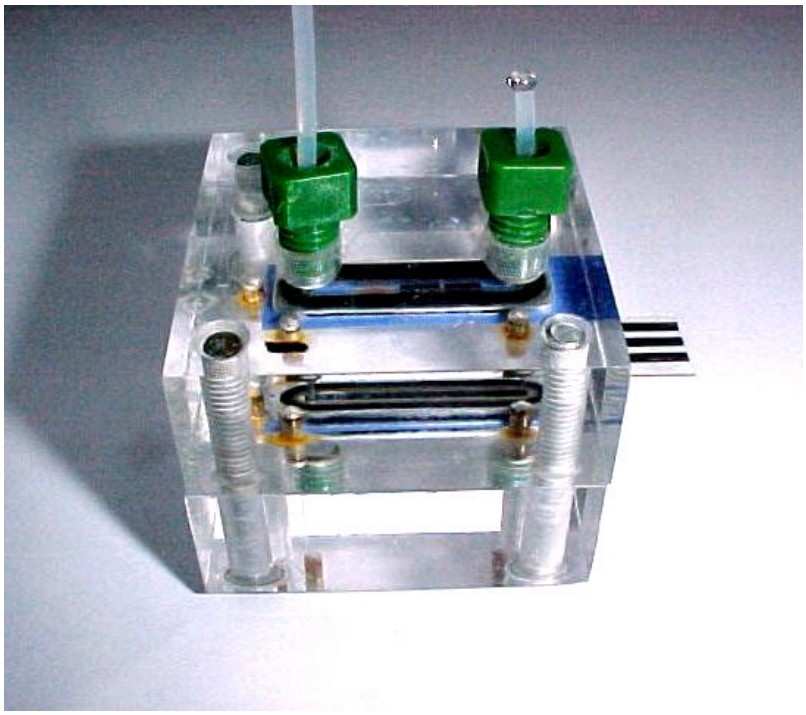


Figure 70: Detector cell, screen-printed 3-electrodes design

In order to facilitate the multiple biosensor analysis programme using the FIA system Cranfield developed a four-channel multi-potentiostat module for electrochemical analysis. The battery powered module features an onboard microcontroller, together with analogue-to digital and digital-to-analogue converters, and programmable gain on each input channel. Sampled data may be recorded to internal memory, or transmitted serially to a pc and monitored in real time using a specially developed software analysis tool. The prototype instrument is shown below in Fig.71.

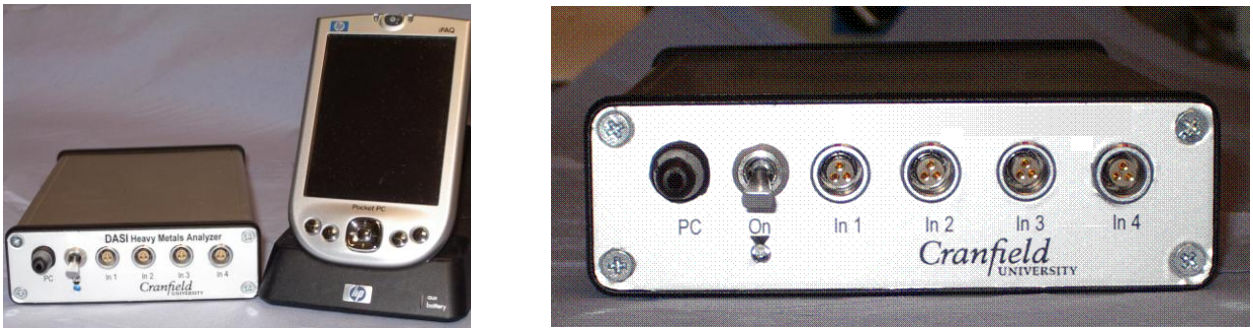


Figure 71: Multiplex potentiostat developed by Cranfield University to allow simultaneous measurement of biosensor strips

Unfortunately, the FIA pumps proved themselves to be not quite powerful enough. Even the slightest variation in height for one of the measuring cell was enough to cause a noticeable change in flow rate through that cell, compared to the others. We were unable to repeatedly obtain a consistent and identical flow rate through all the cells during any length of time. The system proved to be highly sensitive to vibrations, and any impact on the table on which it was set was enough to disrupt the system, if it was balanced before. This part of the study was therefore discontinued as the instrument instability and a lack of time made the experiment planed impossible to realize.

3.2.5. Summary on enzyme-based biosensors

- Tests were performed on screen-printed sensors, using a 3-electrode system (working, counter and reference electrode). An electrocatalyst (based on rhodinised carbon) was added to the working electrode material to lower the potential required for the working electrode to oxidize products of the enzymatic reaction. The reference electrode was Ag/AgCl.

- Electrodes were characterized using the electroactive material hydrogen peroxide.

- The optimum working conditions for the enzymes (glucose oxidase, alcohol oxidase, acetylcholine esterase, lactate dehydrogenase) were determined. Addition of stabilizing agent was optimized on the sensors.

- The hypersolutes have proven to lessen enzyme desorption from the electrode surface.
- The impact of pH variations on the enzyme activity was determined. No significant influence of the stabilizing agent was observed.
- The influence of ionic strength on the enzyme was determined: Ectoine and firoin A were found to have a small stabilizing effect.
- Storage stability has been studied over a 8 months period:
    - The best enzyme activity preservation was observed with DGP, hydroxyectoine.
    - Firoin formed a white deposit after 4 month storage.

- A FIA (Flow injection analysis) system to test biosensor stability upon repetitive use, with/without stabilizing agents was designed. However, it could not be successfully translated into a practical experiment.

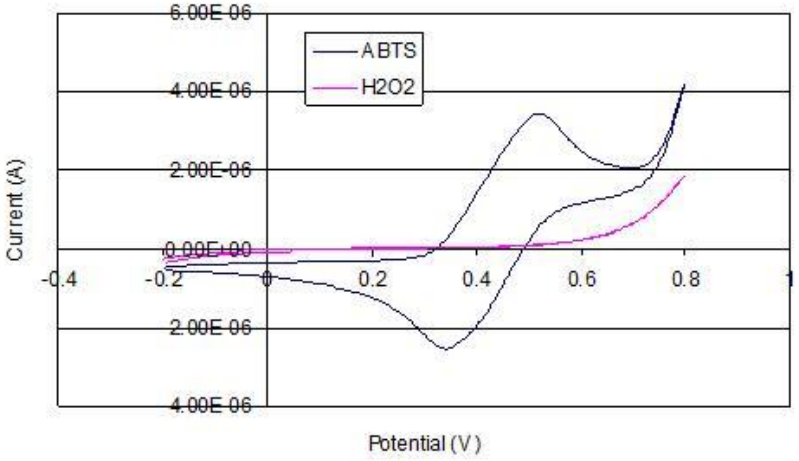
3.3. Immunosensors

3.3.1. Designing a model system – ELISA tests

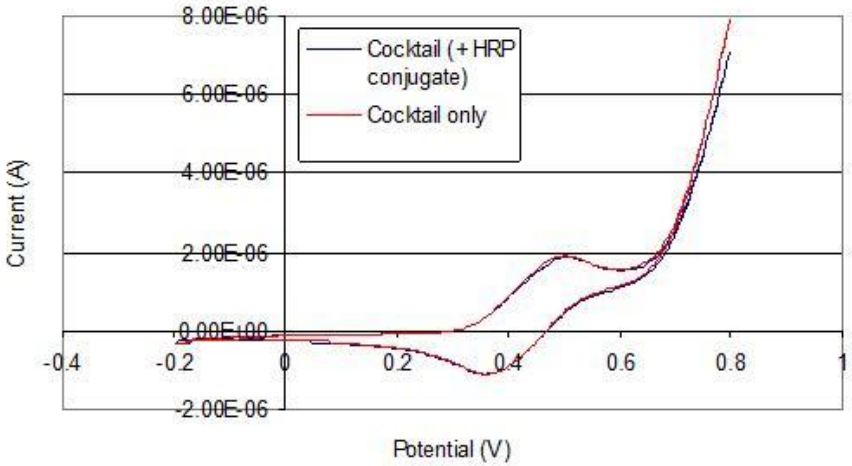
In order to test the effect of stabilizing agent activity on ELISA performance, a model system using inexpensive immunoreagents was selected, namely the binding of horseradish peroxidase (HRP) labeled IgG (human) to anti-human IgG. Initial tests were performed in microtitre wells, to allow simple and rapid assay optimisation. [Lin and Ju (2005).]

3.3.1.1. Detection system: ABTS vs. TMB

The detection process utilised the enzyme horseradish peroxidase (HRP) as the label. Two enzyme substrates were available, ABTS (2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) and TMB (3,3'5,5'-tetramethyl benzidine). A comparative study was performed, the results being shown Fig.72.



5mM ABTS; 9 mM H_2O_2; 0.1M KCl; 70 mM phosphate buffer, pH 7.4; Scan rate 20 mV/s; step: 5 mV; scan 5



Cocktail: 5mM TMB; 9 mM H_2O_2; 0.1M KCl; 70mM phosphate buffer, pH 7.4; Equal volume atrazine-HRP conjugate; Scan rate 20 mV/s; step: 5 mV; scan 5

Figure 72: Comparative study of ABTS and TMB as enzyme substrates for horseradish peroxidase enzyme with amperometric detection at screen-printed carbon electrodes

5mM ABTS or TMB were tested with 9 mM H2O2 and 0.1M KCl in70 mM phosphate buffer at pH 7.4 with a scan rate of 20mV/s. ABTS was found to be more sensitive a substrate than TMB, with a 5 unit HRP activity leading to a current change from 0 to -1.2µA for ABTS, compared to 0 to -0.2µA for TMB as is shown Fig.73 *and* 74: 48ml at 1% v/v of H_2O_2 were tested against 192ml 0.6%v/v of ABTS or TMB in 40mM buffer at -100 and -400mV respectively versus Ag/AgCl at 1 ml/min flow rate during 3 min of reaction time without any acidification. ABTS was therefore used for detection purposes, whether amperometric or optical.

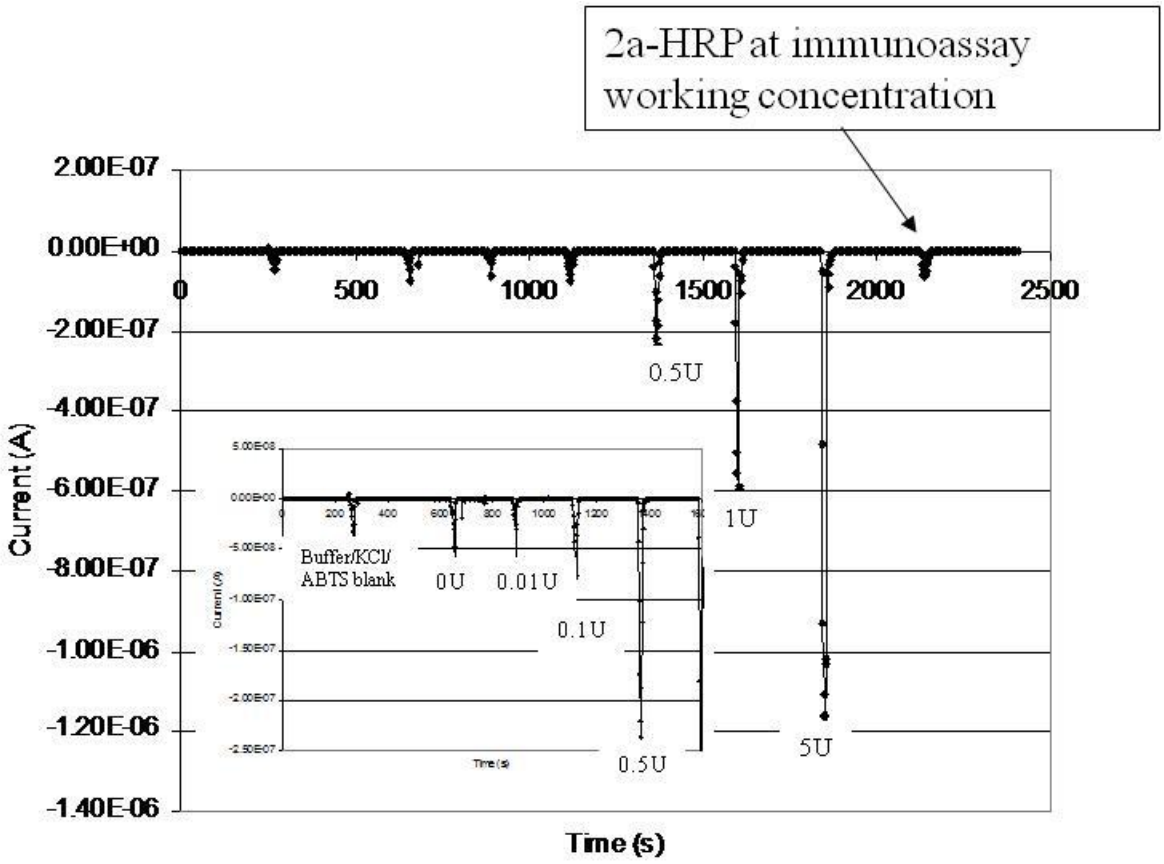


Figure 73: HRP calibration against ABTS substrate

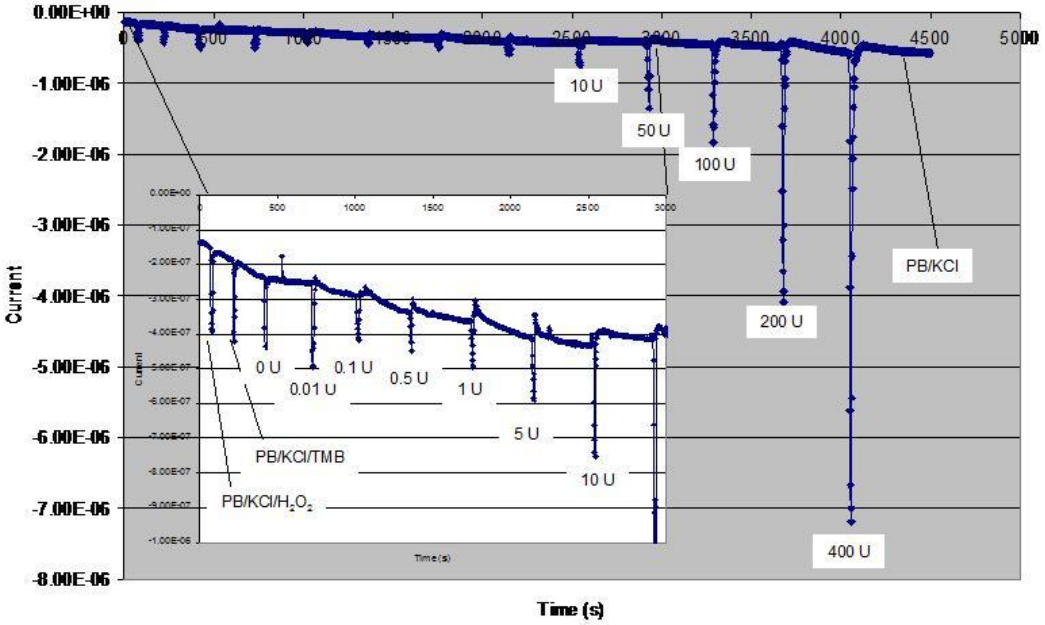


Figure 74: HRP calibration against TMB substrate

3.3.1.2. *ELISA test*

The process required: a coating step (to immobilise anti-human IgG, grown in goat, to the microtitre well walls), where the immobilization is done by simple physical adsorption on the wells walls; an incubation step to allow the binding of anti-goat IgG, with peroxidase label, to the immobilised anti-human IgG; a reaction step in which peroxidase substrate is added to the wells to allow biocatalytic generation of optically or electrochemically active product for subsequent determination. The size of the signal generated can be directly related to the extent of immunoglobulin-immunoglobulin binding. The ELISA test was designed and optimized as follow:

- Microtitre Well Coating step:
  - Anti-Human IgG 0.70µg/mL, 100µL in PBS 10mM
  - Left overhight at 4°C
  - Washed in Phosphate buffer saline-Tween (PBST 20%)
- Incubation step:
  - HRP-IgG 0.80µg/mL, 100µL in PBS
  - Left 2h at RT
  - Wash in PBST

- Reaction:
    - Optical:
        - ABTS cocktail (5mM ABTS; 9 mM H_2O_2; 0.1M KCl; 70 mM phosphate buffer, pH 7.4), 100µL
        - Left 40min at RT in dark
        - Reaction stopped by H_2SO_4 4N, 50µL
        - Measurement at λ = 405nm
    - Electrochemical: at +150mV
        - ABTS cocktail (5mM ABTS; 9 mM H_2O_2; 0.1M KCl; 70 mM phosphate buffer, pH 7.4) added, 100µL for 100s. Hydrogen peroxide is to be added to the ABTS cocktail at the last minute.
        - Solution transferred to 3-electrode screen-printed electrode assembly
        - Measure response at 400sec

This system was then used as a model system to initially assess the feasibility of the selected primary antibody-secondary antibody approach. A basic set of tests proved the fact that primary antibody-secondary antibody binding could be achieved with this system, and hence this approach was transferred to the SPR-based BIAcore approach. [Darain *et al.* (2005). Diaz-Gonzalez *et al.*. (2005).]

3.3.2. Testing the BIAcore using BSA

An alternative method for immunosensor testing is the BIAcore system, based on the surface plasmon resonance (SPR) phenomenon. Immobilization conditions are a key factor in immunosensor performance and have to be optimized as a small variation may lead to a significant difference in the amount of material bound to the sensor (chip) surface.

3.3.2.1. Immobilization conditions

A first test to gain an understanding of system performance was realized using the model protein BSA (bovine serum albumin) using a range of immobilization buffers.

The surface chemistry of the sensor was such that the physical adsorption of the protein to the chip could be observed in real time. The immobilization buffers used were:

- 10mM acetate pH=4.8
- 5mM malate pH=6.0
- 5mM malate pH=7.0

The results are shown in Fig.75. The highest immobilization rate was obtained at pH 4.8, as evidenced by the increase in the baseline from about 20,000 to 20,500 AU (absorption units) or RU (Response Unit). These conditions were therefore applied in subsequent experiments.

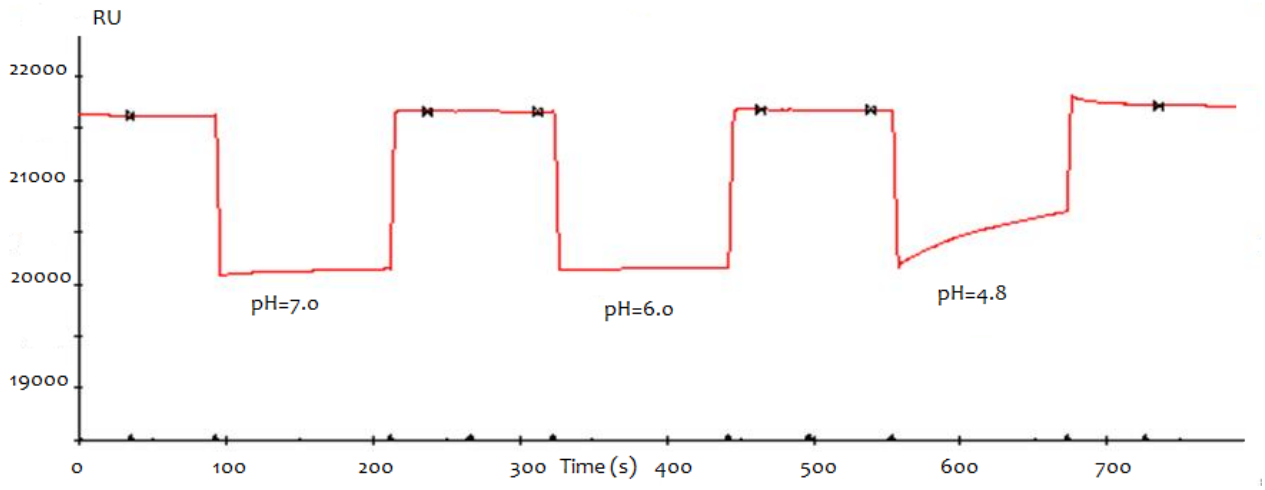


Figure 75: BIAcore sensogram, examination of the effect of different immobilization buffers on the immobilization of the model protein bovine serum albumin to the sensor surface

3.3.2.2. *Interaction analysis*

a. **Binding**

Series of tests were performed to further characterize the interactions on the BIAcore chip surface. Fig.76 shows the interaction analysis between BSA and its conjuguate. 100µg/mL of protein in acetate buffer, pH 4.5, was injected on the BSA coated CM5-chip surface and run against a blank.

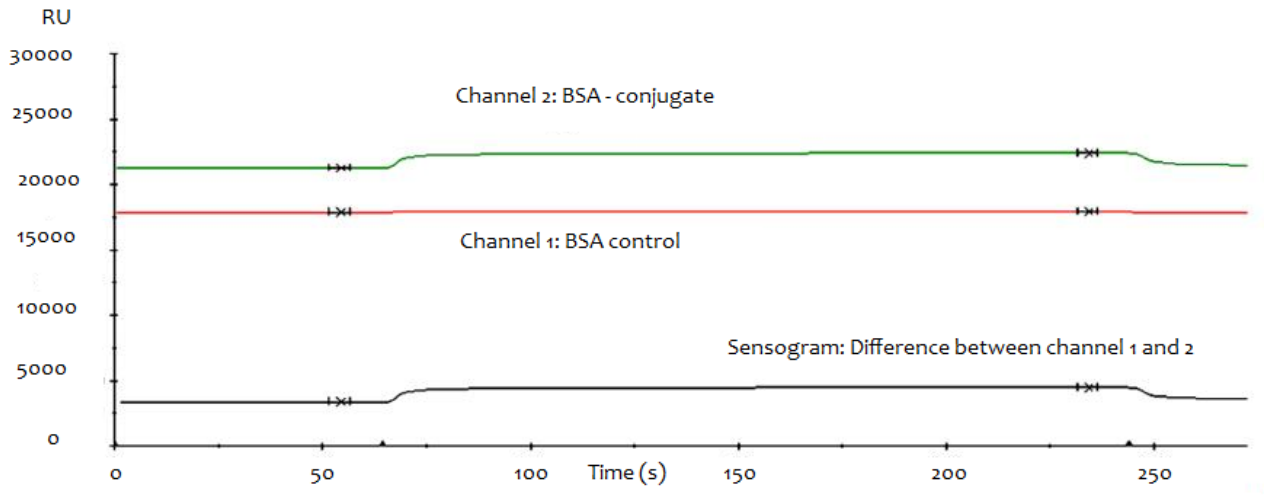


Figure 76: Interaction analysis on the BIAcore, specific vs. non-specific binding

The resulting binding was found to be 1.25kRU for the interaction. The control test allows us to determine non-specific binding. The analysis software can thus determine the specific binding, by subtracting the blank to the test in real-time.

b. Calibration of the immobilized material at the chip surface

The signal in Response Units (RU) or Absorbance Units (AU) can be linked to a concentration of molecule interacting with the chip surface. A study was realized that linked the BIAcore signal to the molecule surface concentration. The results are presented Fig.77. There is a clear linear correlation between both. Therefore an increase in the signal can be correlated to an increase of quantity of material attached to the chip surface. [www.biacore.com]

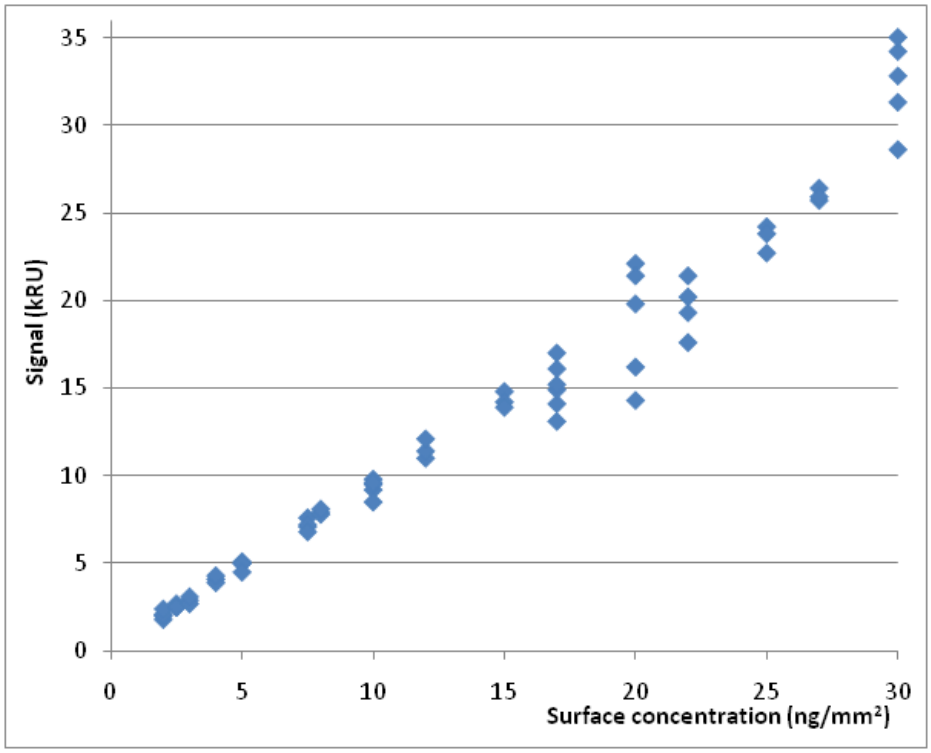


Figure 77: Signal / concentration ratio on the BIAcore signal [BIAcore©]

It is also to be noted that the BIAcore is a highly sensitive system and that its working parameters must be clearly defined, especially when writing one's own running cycle program. The solvent, for example, must be filtered and consistent throughout any series of tests. Any change could influence the results and the BIAcore ability to generate valid results, as it could be perturbed by impurities or bubbles. In such cases, the experiment must be stopped, however far along they might be, so that the instrumentation may be cleaned. It is therefore advisable to use BIAcore-produced buffers and solutions rather than home-made ones.

Also, temperature is a very important parameter, as a small variation can influence the solvent properties and the interaction to be studied. It can be set in the BIAcore system and it is then very precisely defined, with variations of less than 0.2°C. It was set at 20°C throughout all of the experiments that we performed on the BIAcore system.

3.3.3. Immunosensor characterization on the Biacore without hypersolutes

3.3.3.1. Primary antibody immobilization

The model system selected for examination of the effect of the stabilisation agents on antibody binding stability was identical for that reported in the indirect electrochemical immunosensor (ELISA) approach reported above, namely: anti-human IgG, grown in goat and monoclonal anti-goat IgG, grown in mouse. The anti-goat IgG ('secondary antibody') recognises the Fc fragment of the anti-human IgG ('primary antibody') since the latter antibody was raised in a goat host.

It should be noted here that this approach is direct in nature, and hence does not require the use of a label to visualise the binding process. Therefore, no 'activation' of the peroxidase portion of the secondary antibody is required. However, the presence of the enzyme label does actually enhance the device response since detection is based on mass differences and the presence of the conjugated enzyme increases the mass of the binding component.

Anti-human IgG was immobilised to CM5 sensor chips (BIAcore UK, Eyeworth, Bedfordshire, UK) according to the manufacturers instructions. Several combinations of buffer pH and antibody concentration were tested in order to validate the immobilization protocol we chose. The result is presented Fig.78. Reg. indicates a regeneration step to restore the chip surface. The higher the signal is, the better the interaction between the antibody and the chip surface.

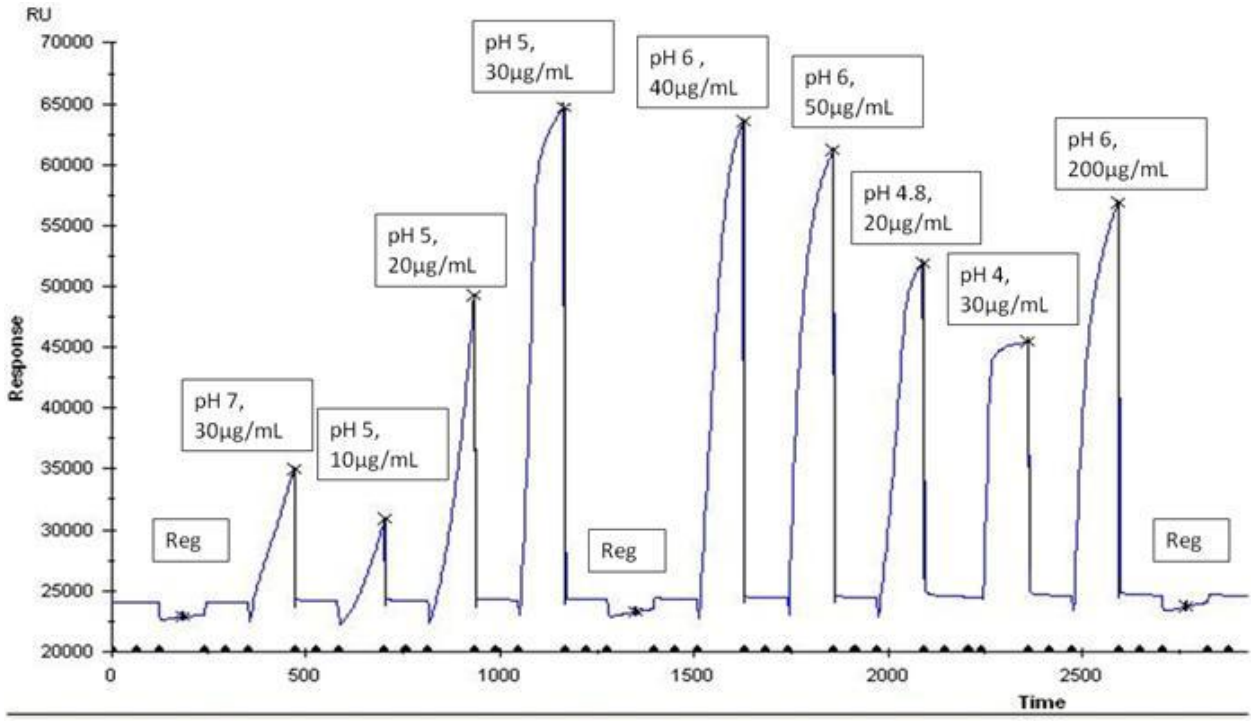


Figure 78: Optimization of the parameters for the primary antibody injection, testing different pH and concentrations

The CM5 chips incorporate a carboxymethyl (CM) dextran matrix that allows the covalent coupling of compounds containing amino-, thiol- and aldehyde groups. Coupling of the antibody to the chip surfaces required CM dextran activation using the well known procedure of EDC (N-ethyl-N'-(3-dimethyl aminopropyl)-carbodiimide hydrochloride), and NHS (N-hydroxysuccinimide) treatment. Following activation, a 40µg.ml^{-1} solution of primary antibody (acetate buffer, pH=5.0), was injected into the system over a 10 min. time period at a flow rate of 5µg.min^{-1}, the resulting sensorgram indicating that antibody material had been successfully immobilized at the surface of the transducer chip (Fig.79). Ethanolamine is then injected to neutralize any remaining active site on the chip surface, preventing any further binding. The materiel adsorbed on the surface corresponds to a signal variation of 15000 RU.

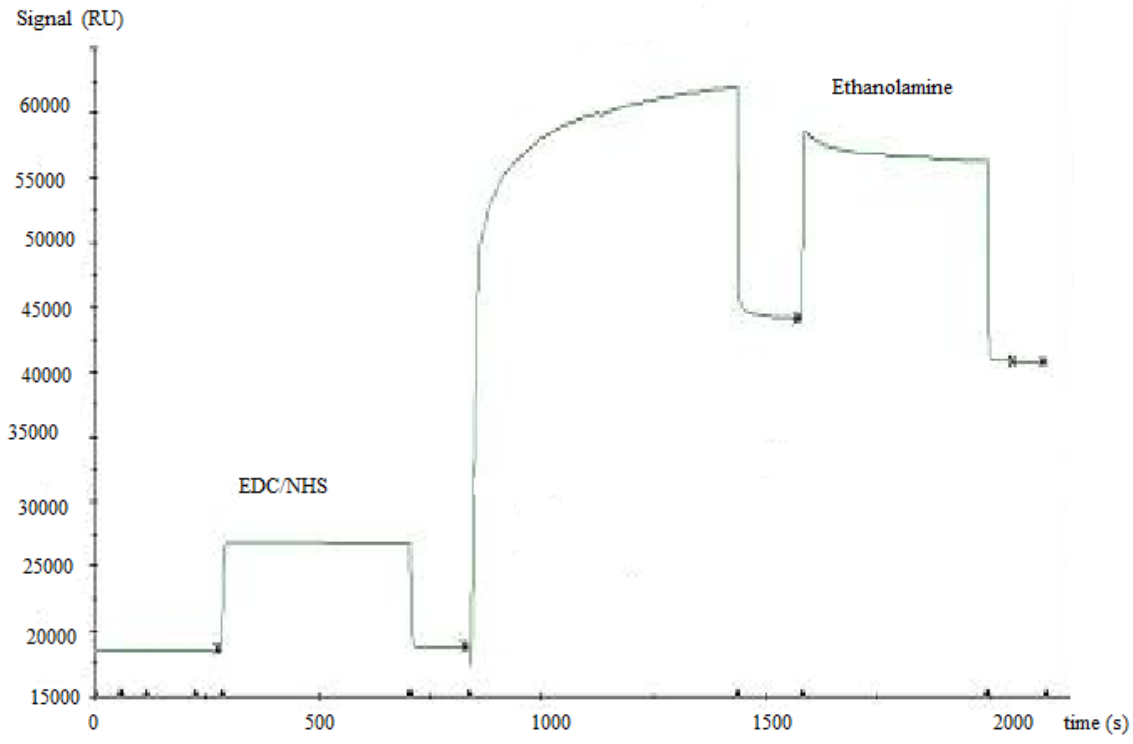


Figure 79: Sensogram indicating successful immobilisation of primary antibody (anti-human IgG) to CM5 sensor chips

3.3.3.2. *Addition of the secondary antibody*

a. Regeneration

Methods of regeneration of the sensor surface (removal of secondary antibody) were examined using a number of methods recommended by the chip manufacturer. They are based on the use of glycine/HCl, sodium hydroxide, acetic acid or SDS (sodium dodecylsulphate – at various concentrations and pH values).

The results are presented Table 10. The regeneration buffer chosen for the following studies is glycine pH=2.0.It was found to be the most suitable since it was harsh enough to lead to the slowest accumulation of material at the electrode surface, namely the secondary antibody, but no so harsh as to remove the immobilized primary antibody. None of the systems were entirely satisfactory and glycine pH=2.0 was found to be the lesser evil.

Buffer	Result
Glycine/HCl, 10mM, pH 2.0	Poor regeneration, lead to slow accumulation on sensor surface
Glycine/HCl, 10mM, pH 3.0	Poor regeneration, lead to slow accumulation on sensor surface
Glycine/HCl, 10mM, pH 8.5	Poor regeneration, lead to slow accumulation on sensor surface
Glycine/HCl, 10mM, pH 10.0	Poor regeneration, lead to slow accumulation on sensor surface
NaOH, 50mM, pH 12.5	Harsh regeneration,, removed part of the immobilized analyte
Acetic Acid, 2%, pH 2.4	Poor regeneration, lead to slow accumulation on sensor surface
SDS 2mM	Harsh regeneration,, removed part of the immobilized analyte
SDS 10mM	Harsh regeneration,, removed part of the immobilized analyte

Table 10: Biacore regeneration systems

b. Interaction analysis

Secondary antibody was introduced at varying concentrations, over the range 0 to 4500nM. Specific binding profiles were obtained (cf. Fig.80), indicating the quantitative nature of the method.

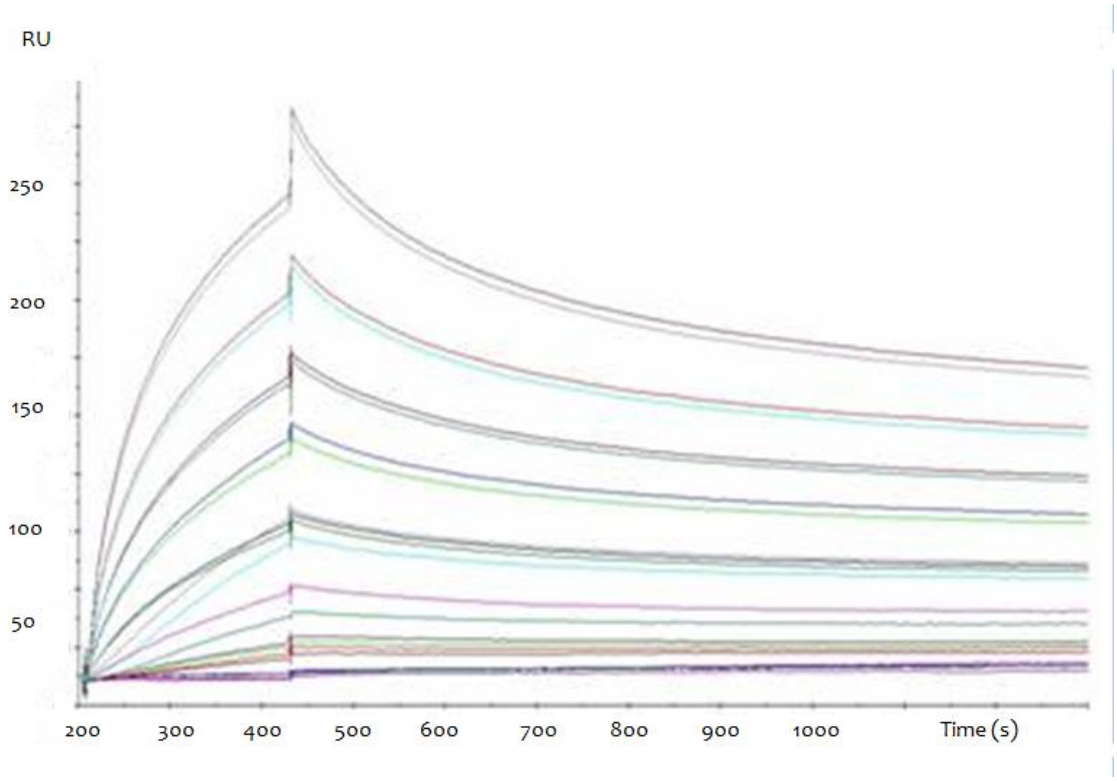


Figure 80: Sensorgram indicating binding of secondary antibody (anti-goat IgG) to primary antibody (anti-human IgG, grown in goat) across the concentration range 0-4500 nm secondary antibody

The relative standard deviation (RSD) of the maximum peak response at the end of injection was also analyzed. It is representative of the interaction between the immobilized primary antibody with 2250nM of secondary antibody, and was found to be less than 1% over 50 injections.

The calibration curve for the antibody-antibody interaction was obtained. The result is presented Fig.81. The primary antibody was first immobilized onto the chip surface. The secondary antibody was then injected at various concentrations on the chip, with regeneration rounds between the injections.

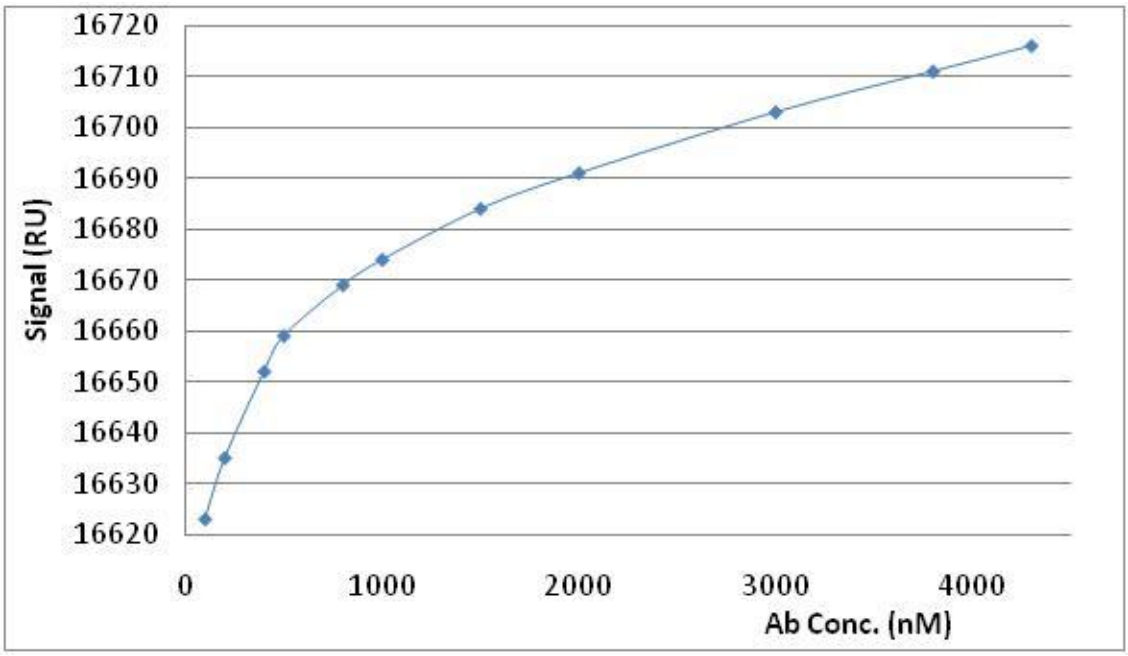


Figure 81: Secondary antibody binding response to the primary antibody bound on the CM5 chip surface using the BIAcore, over the range 0-4500nM

3.3.4. Influence of adding the hypersolutes on the immunosensor

The influence of Hypersolutes on primary antibody binding was assessed. 40µg.ml^{-1} solution of primary antibody with 35mM of hypersolutes (in acetate buffer, at pH=5.0), was injected into the system over a 10min time period at a flow rate of 5µg.min^{-1}.

3.3.4.1. Antibody affinity

With the exception of DGP, in which no signal deterioration was noted, the primary antibody binding in presence of the solutes was totally inhibited. At lower solute concentrations (17.5 and 8.75mM), the extent of secondary antibody binding increased, except for DGP where a binding decrease was observed, presumably related to the decrease in DGP concentration. The binding of secondary antibody was found to be similar for all solutes at the 8.75mM concentration (for ectoine, hydroxyectoine, firoin, firoin A and PML) as shown Fig.82. The injection sequence for this test was as follow: the antibody alone was injected, then again with the hypersolutes, in turn,

firoin, firoin A, ectoine, hydroxyectoine, DGP and finally PML, followed by a regeneration step on the chip surface. It is likely that DGP is being bound on the chip surface along with the antibody, explaining the very high peak obtained for a hypersolute concentration of 35mM. The hypersolute concentration that was chosen to assess their influence on the immunosensor was thus slightly lower than 8.75mM, as there was a noticeable loss of binding affinity of the antibody at that concentration. It was chosen at 7.5mM.

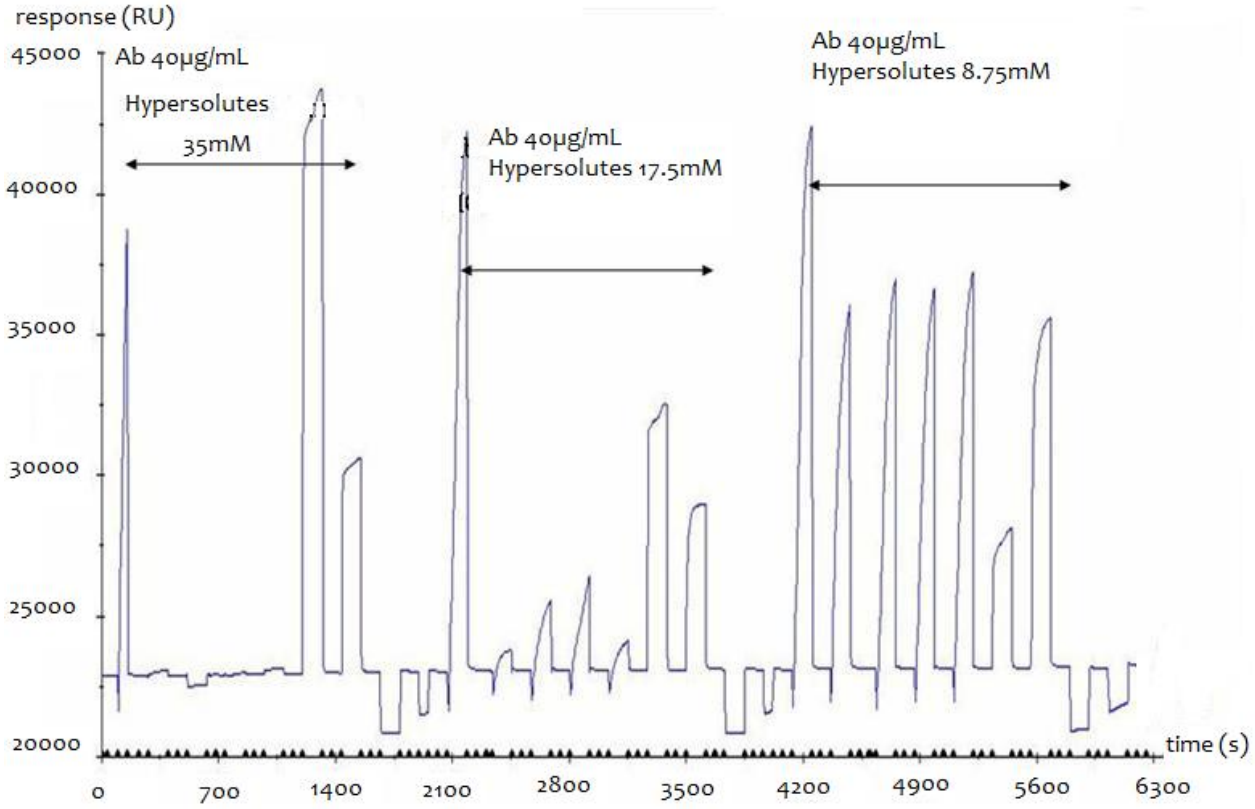


Figure 82: Stabilizing agent addition (From left to right: Antibody alone, with Fir, with Fir A, with Ect, with HydE, with DGP, with PML, regeneration step)

The buffer used in all the BIAcore experiment, unless stated otherwise, is HBS-EP (10 mM Hepes/150 mM NaCl/3 mM EDTA/0.005% polysorbate 20), pH 7.0. The primary antibody was immobilized onto the chip surface, and the secondary antibody, HRP labelled, was then injected at 920nM, for 3min at 10µL/min. A loss of binding affinity was still observed when the stabilizing agents were added at a 7.5mM concentration. This loss was quantified by comparing the signal obtained when injecting the secondary antibody alone or with one of the hypersolutes. The results are

shown Fig.83. The reference in this case (100% binding) is the response for the secondary antibody injected alone. The most significant loss was observed with DGP with only 78% retention of activity. Ectoine, hydroxyectoine, firoin and firoin A gave very similar results with about 92% of activity retained. PML was not studied any further, due to reagent availability. It was replaced in most cases by trehalose, an already studied and commercially available stabilizing agent, similar to the Hypersolutes to a certain extent.

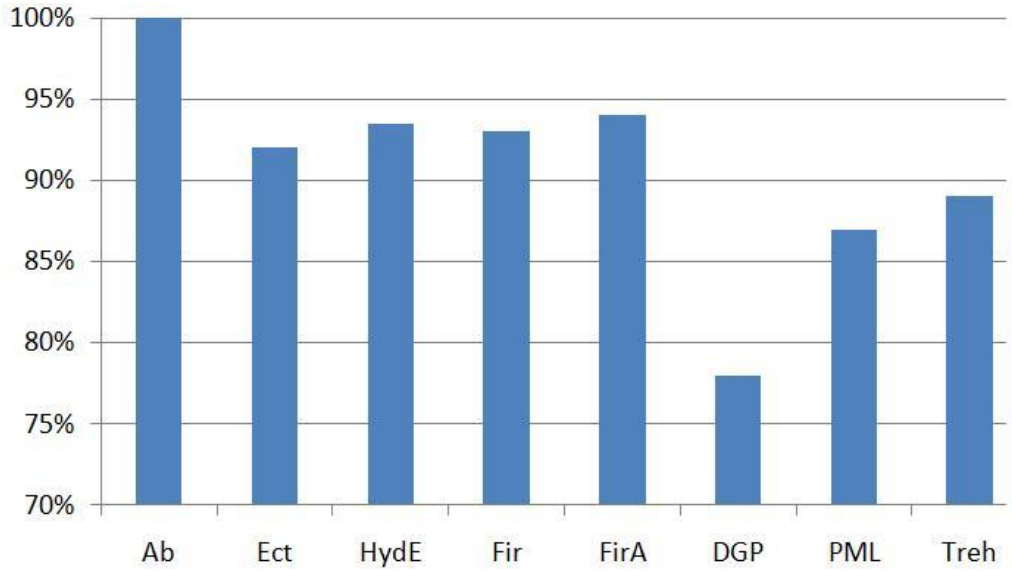


Figure 83: Loss of affinity (in %) of binding of secondary antibody to chip-immobilised primary antibody due to the introduction of stabilizing agent (7.5mM) at pH 7.0

3.3.4.2. *pH variations*

The influence of pH was studied over the range 3-11. For pH under 4 and over 10, no binding was observed, the conditions being too far removed from optimal pH for the antibodies to display any significant binding activity. The optimum working pH was found to be 5.0, but the variation in response was found to be minimal at pH=7.0. As pH=7.0 is also close to the optimum pH for antibody binding, it was chosen as working pH for the following studies. A statistical study was performed using F tests in order to assess the significance of the influence of the stabilizing agents against pH. The results are presented Table 11.

An F-test is used to test if the standard deviations of two populations are equal. This test can be a two-tailed test or a one-tailed test. The two-tailed version tests against the alternative that the standard deviations are not equal. The one-tailed version, that was used here, only tests in one direction, that being that the standard deviation from the first population is either greater than or less than the second population standard deviation. The F-test is used as the Student's t test, only testing for significant differences in the variances. First, the null hypothesis is invoked, that states that the two variances we are comparing are from the same population. (i.e., they are not statistically different, the null hypothesis proposes a general or default position, such as that there is no relationship between two measured phenomena, or that a potential treatment has no effect.) The F value is then calculated (the ratio of the two variances) and compared to the table value of F for the degrees of freedom used to calculate both variances and for a given confidence level. If the calculated F is greater than the table value, then the null hypothesis is not correct.

Source	d.lib.	SS	Variance	F (calc.)	F (tab.)	α
Hotsolute	5	484042	96808	0.799	2.773	0.05
pH	2	1694658	847329	6.997	3.555	
Hot.• pH	10	106688	10669	0.088	2.412	
Error	18	2179871	121104			
Total	35	4465259				

Table 11: Statistical study on stabilizing agents vs. pH using F-test

If the F calculated value is higher than the F tabulated value, then the corresponding parameter has a significant influence on the data set. If it is lower, no significant influence was found. In this study, the only factor that has a statistically significant impact on the study is the pH value, which was to be expected. Neither the stabilizing agents (Hot) nor the combination of the stabilizing agent and the pH value (Hot*pH) were found to have an influence.

3.3.4.3. *Ionic strength variations*

The same study was realized with respect to ionic strength variation across the ionic strength range 0.1-1000mM PBS. The primary antibody was immobilized at the CM5 chip surface (a 40 $\mu g.ml^{-1}$ solution in acetate buffer, pH 5.0 was injected into the system over a 10min. time period at a flowrate of $5\mu g.min^{-1}$). The secondary antibody, HRP labelled was then added (a 920 $\mu g.ml^{-1}$ solution in HBS-EP buffer, pH 7.0 was injected into the system over a 3min. time period at a flowrate of $10\mu g.min^{-1}$), with or without hypersolutes 7mM, in various PBS concentration solutions. The results are presented Fig.84.

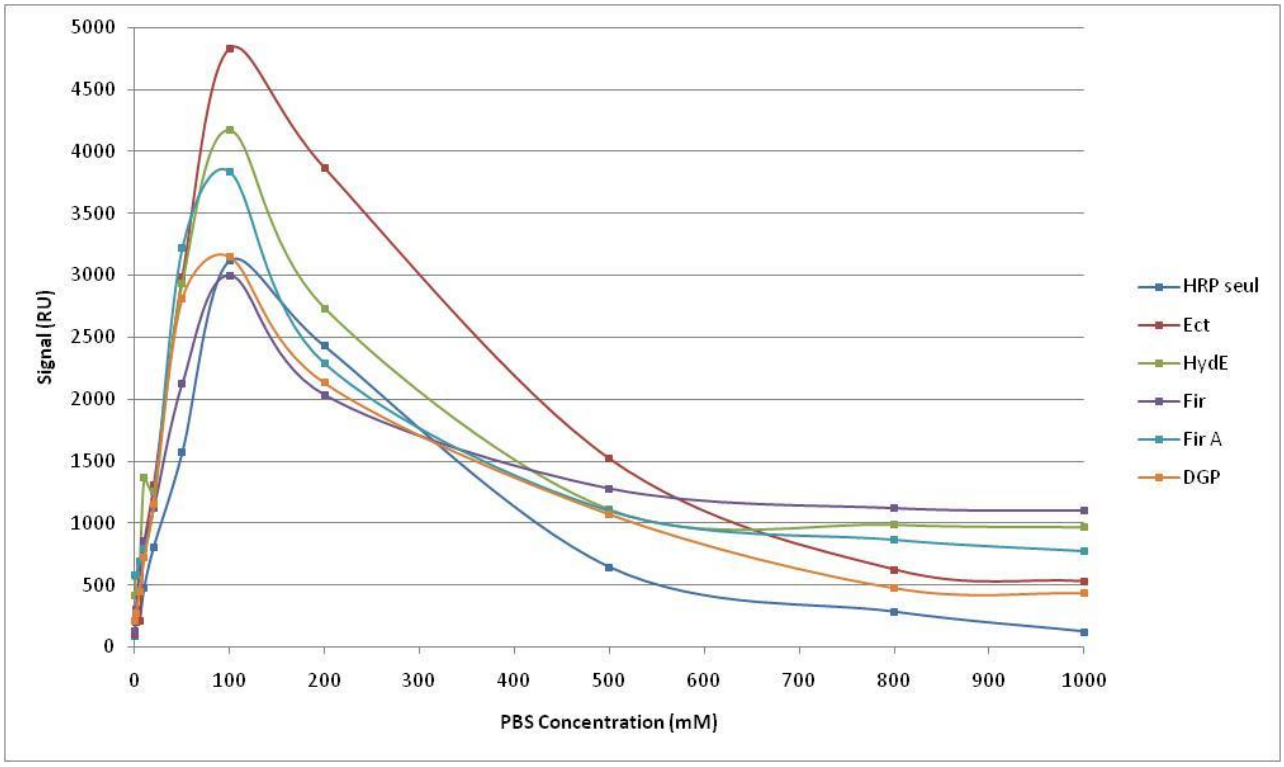


Figure 84: Response signal on secondary (920µg/mL) to primary antibody binding on BIAcore, with or without hypersolutes, in PBS over the range 0.1-1000mM

Both factors, namely the stabilizing agent and the PBS concentration, were found to have a statistically significant influence on the binding of the antibodies. Ectoine and Hydroxyectoine had the most important stabilizing effect, with an affinity preservation of 161 and 145% respectively, compared to the secondary HRP-labelled antibody alone. They preserved the enzyme activity more satisfactorily than Trehalose,

which only registered a 115% affinity preservation. DGP also gave a positive result, in light of the signal loss suffered upon addition of the stabilizing agent in optimum conditions. The activity preservation was found to be close to 125% of its value with DGP in standard conditions. An average of the signal variation was calculated for each of the hypersolutes, compared to the HRP labelled antibody alone. These results showing the impact of the stabilizing agents are presented Fig.85. That value was then translated in percentage, with the secondary antibody alone as reference, namely 100%. This was finally counterbalanced with the signal loss associated with using each hypersolutes on the antibodies binding.

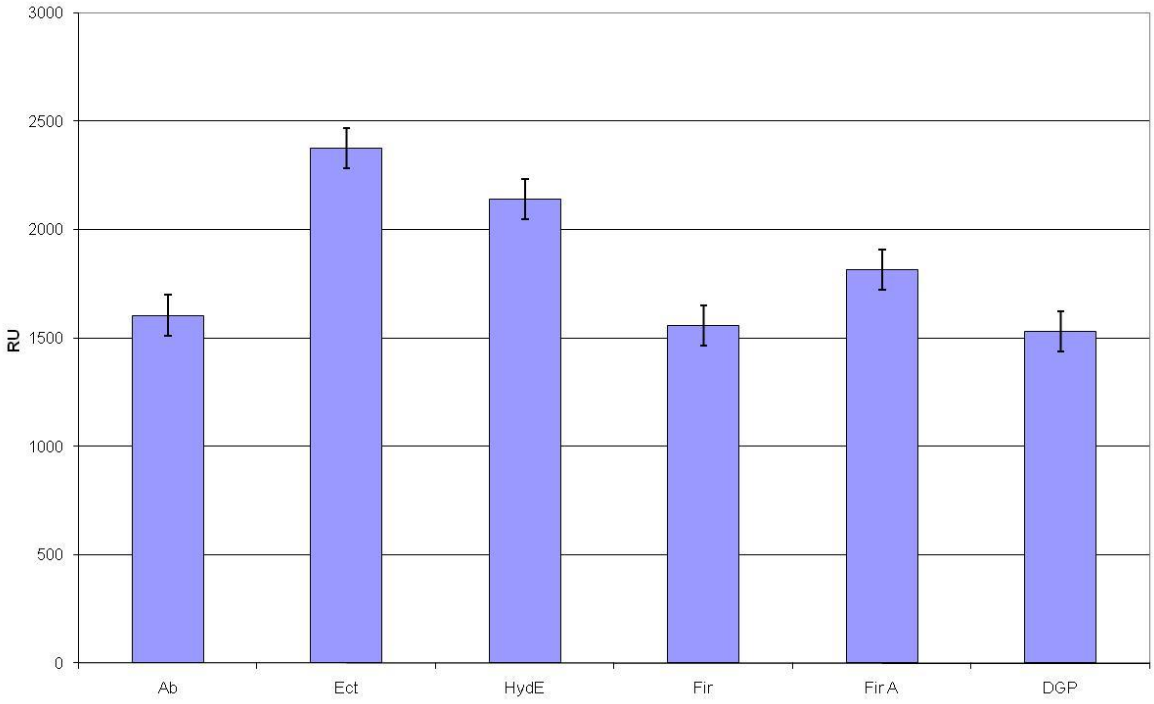


Figure 85: Summary of the influence of the stabilizing agent on ionic strength variation

3.3.4.4. *Versus storage*

The secondary antibody (138µg/mL) was stored at RT over a 2 months period, with or without hypersolutes (7mM). It was tested after its complementary antibody from a fresh batch was immobilized onto the chip surface. The results obtained are presented Fig.86.

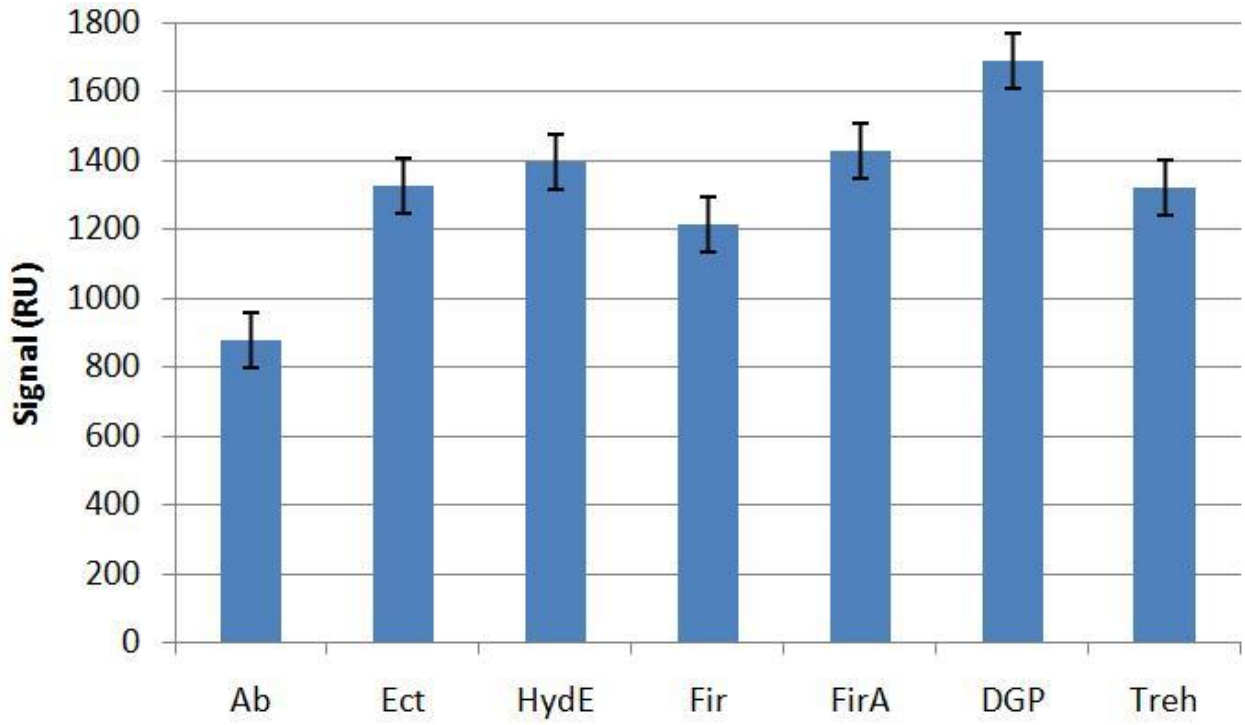


Figure 86: Antibody binding response after 2 months of storage of a 920nM antibody solution containing 7mM stabilizing agent

The signal obtained with the antibody in presence of a stabilizing agent is compared to that of the antibodies alone. The signal obtained without any stabilizing agent serves as reference, at 100%. The result obtained in presence of a hypersolute is then corrected according to the loss of signal determined in an earlier study. DGP gave the most promising result with a stabilization of 244%, followed by Firoin A (178%) and Hydroxyectoine (170%) compared to the antibody stored alone, and in light of the signal loss due to the hypersolutes.

3.3.4.5. *Repetitive use*

As the antibody binding process had proved very stable, several approaches were tested on the antibodies alone in order to find an experimental set-up that would allow the testing of repetitive use effect in a short-term experiment. Experimental conditions such as temperature, organic solvent concentration, and alternative regeneration buffers were examined in these tests. Two sets of experimental conditions were selected for usage in these comparative studies.

a. Designing the experiment

The first involved the injection of a low acetonitrile concentration (0.4% v/v) along with the secondary antibody and the stabilizing agent. The presence of the organic solvent would be expected to interfere with the antibody-binding process and hence significantly reduce the extent of binding of the secondary antibody, HRP labeled on the primary antibody immobilized at the chip surface.

The results for this test, on the HRP-labeled antibody without any Hypersolutes, are presented Fig.87. A program was written and the experiment designed to minimize interferences. Further experiments were planned to be preformed at the elevated temperature of 37.5°C.

The first step here was to find experimental conditions were the binding of the secondary antibody on the primary would be impaired upon repetitive use within a reasonable time scale (not exceeding a day). Tests were performed without any hypersolutes, considering what ought to be the less stable system available. The systems chosen are to be used in presence of hypersolutes as well to assess the influence of the latest on immunosensor stability.

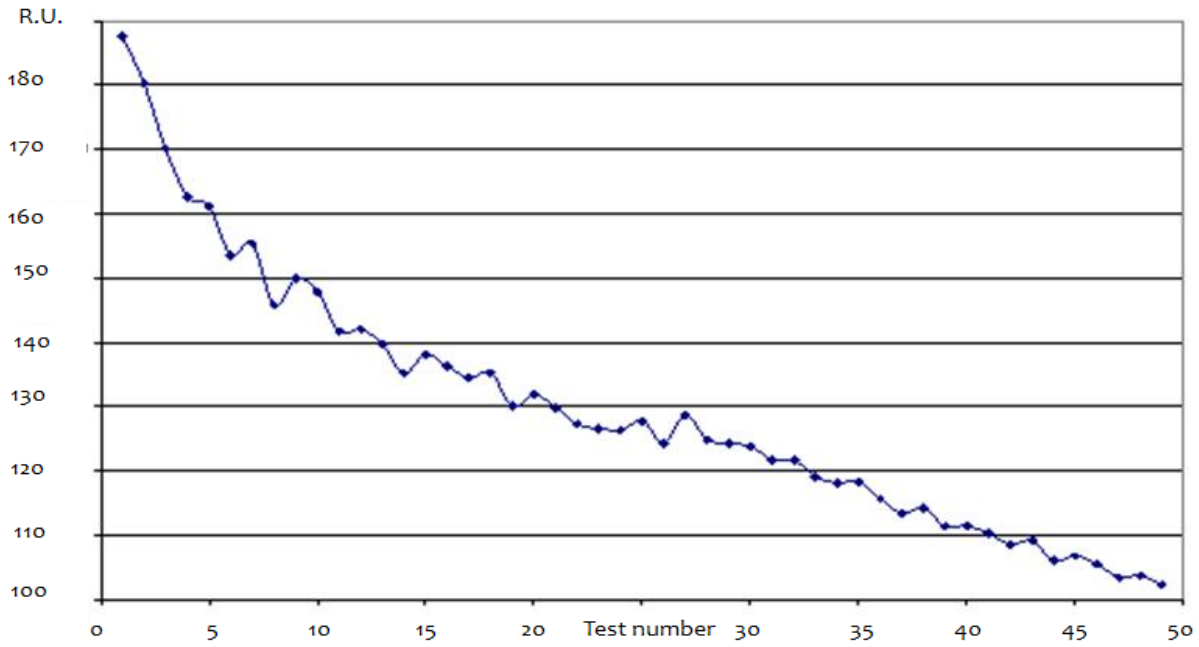


Figure 87: Test over 50 cycles, IgG HRP 130µg/mL with acetonitrile 0.4%

b. Vs. acetonitrile 0.4%

The first system tested consisted in observing the decrease of antibody affinity upon repetitive injections of the secondary antibody at 250µg/mL with or without stabilizing agent (6mM) in a HBS-EP buffer containing 0.4% v/v acetonitrile. The primary antibody was bound onto the chip surface prior to the experiment and the running buffer was HBS-EP.

All 5 hypersolutes available were tested, along with the well known sugar-alcohol stabilizer trehalose. Trehalose is a commercially available stabilizing agent and judging from its structure and molecular weight, its stabilization mechanism may well be similar to that of the hypersolutes. The results obtained after a run of 50 cycles for each configuration are presented Fig.88 and 89. Fig.88 presents the results obtained for the runs on the antibody alone or with a hypersolute. Fig.89 presents the percentage of remaining affinity after these runs, with 100% being the secondary antibody binding response at the first cycle being run.

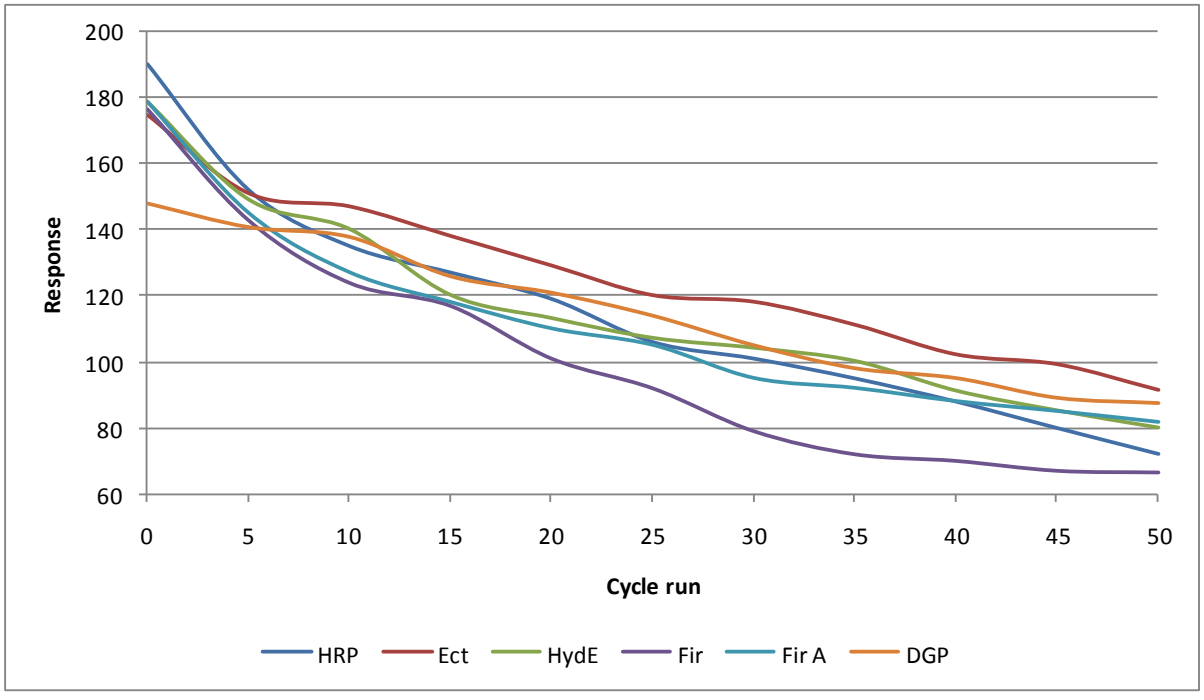


Figure 88: Response over 50 cycles, secondary antibody binding, with or without stabilizing agents

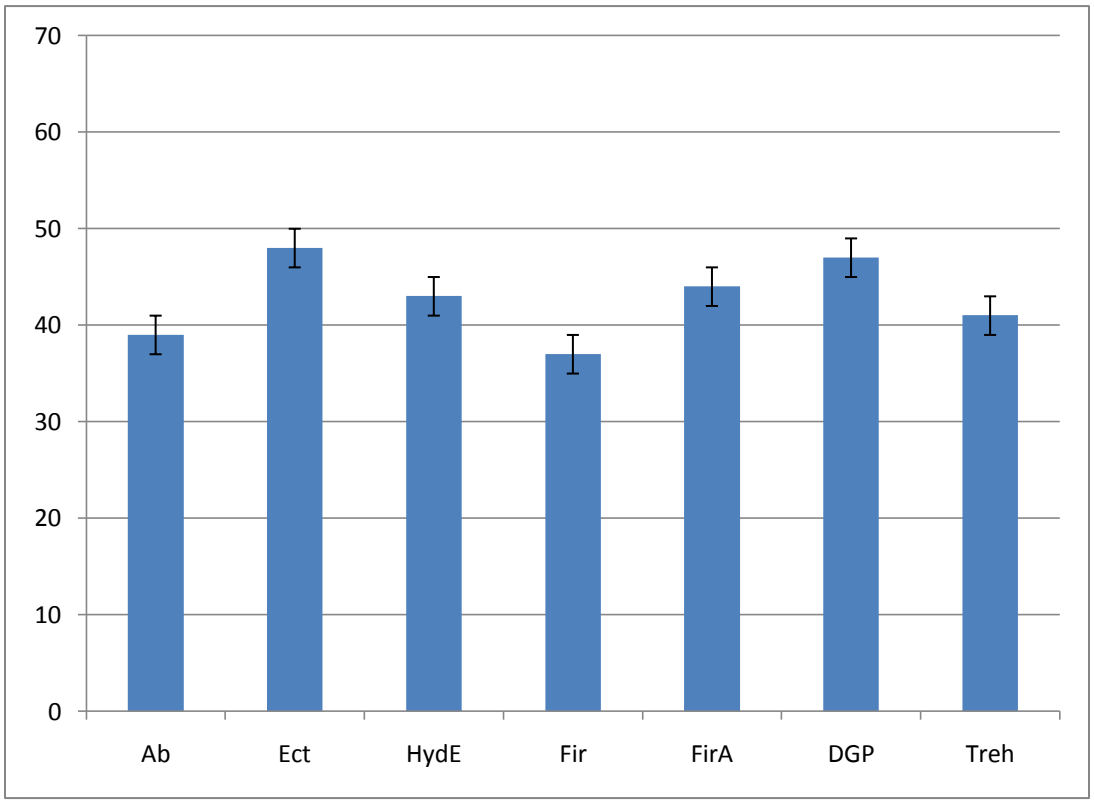


Figure 89: Preservation of affinity (%) against repetitive use with acetonitrile 0.4%

Addition of DGP and Ectoine did result in a better preservation of the antibody affinity compared to the antibody alone (about 125% each). The other solutes, along with trehalose, did not present any significant improvement compared to the secondary antibody injected alone.

c. At 37.5°C

The second system consisted in observing the decrease of antibody affinity upon repetitive injections of the secondary antibody at 250µg/mL with or without stabilizing agent (6mM) in a HBS-EP buffer, working at 37.5°C. Again, the primary antibody was bound onto the chip surface prior to the experiment and the running buffer was HBS-EP and all 5 hypersolutes available were tested, along with trehalose.

The results are presented in Fig.90. Against repeated injections at 37.5°C, the best affinity preservation was obtained with hydroxyectoine, with 160% of affinity compared to the antibody alone. Ectoine did also present a decent affinity preservation (120%).

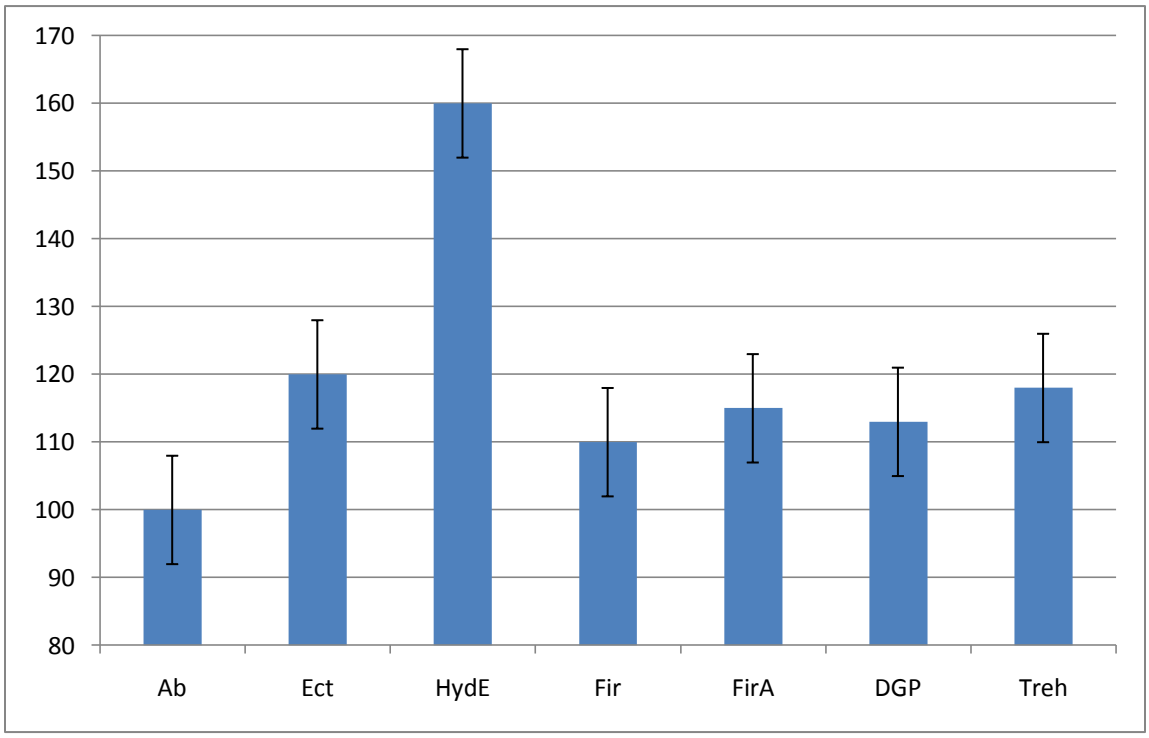


Figure 90: Preservation of affinity (%) against repetitive use at 37.5°C compared to the antibody alone (%)

3.3.5. Influence of the hypersolutes using Akubio

3.3.5.1. Exporting the BIAcore parameters

The characteristics of the Akubio system allowed us to run a couple of tests that would have been impossible or more difficult to run on the BIAcore. The first test realized took advantage of the 4 parallels channels of the Akubio system compared to the 4 serial channels of the BIAcore. Immobilizations were realized in parallel on the 4 channels and repeated, the immobilization levels allowing then a statistic study on immobilization levels. After optimization, the immobilization parameters were thus:

- Flow rate of 25µL/min, HBS-EP buffer
- Channel 1 to 4: EDC/NHS 3min injection (to activate the surface)
- Channel 1 to 4: Goat anti-human IgG 50 µg/ml in 10 mM Na Acetate pH 4.5
- Channel 1 to 4: Mouse IgG 50 µg/ml in 10 mM Na Acetate pH 4.5 (to prevent non specific binding)

- Channel 1 to 4: 1M Ethanolamine pH 8.5 (to deactivate all remaining active sites)
- Channel 1 to 4: 100 mM HCl for 1.0 min (to eliminate all non-covalently bound material and equilibrate the pH for the first injection)

3.3.5.2. Statistic studies

The RSD associated to the primary anti-body immobilization was found to be about 10%. However, the repercussion onto the RSD of the biosensor response to the secondary antibody injection is not that important as that RSD value was found to be between 2 to 3%, which is coherent with the result obtained on the BIAcore.

With a deficient regeneration, this RSD associated with the substrate injection can go up to 8%. This test was performed onto the Akubio system by injecting in all 4 channels the secondary antibody at 150µg/mL and 75µg/mL, and observing the response distribution.

3.3.5.3. In DMSO

The second test realized was the injection of the secondary antibody, with or without stabilizing agent, in solution with DMSO from 0 to 10%. As opposed to the BIAcore system, which doesn't allow for the use of DMSO in the solvent, the Akubio detection system is less sensitive to solvent effect. Thus, we were able to test the influence of DMSO on the immunosensor with or without stabilization. The results are presented Fig.91. The signal value was corrected to take in account the loss suffered on the binding response when in presence of hypersolutes.

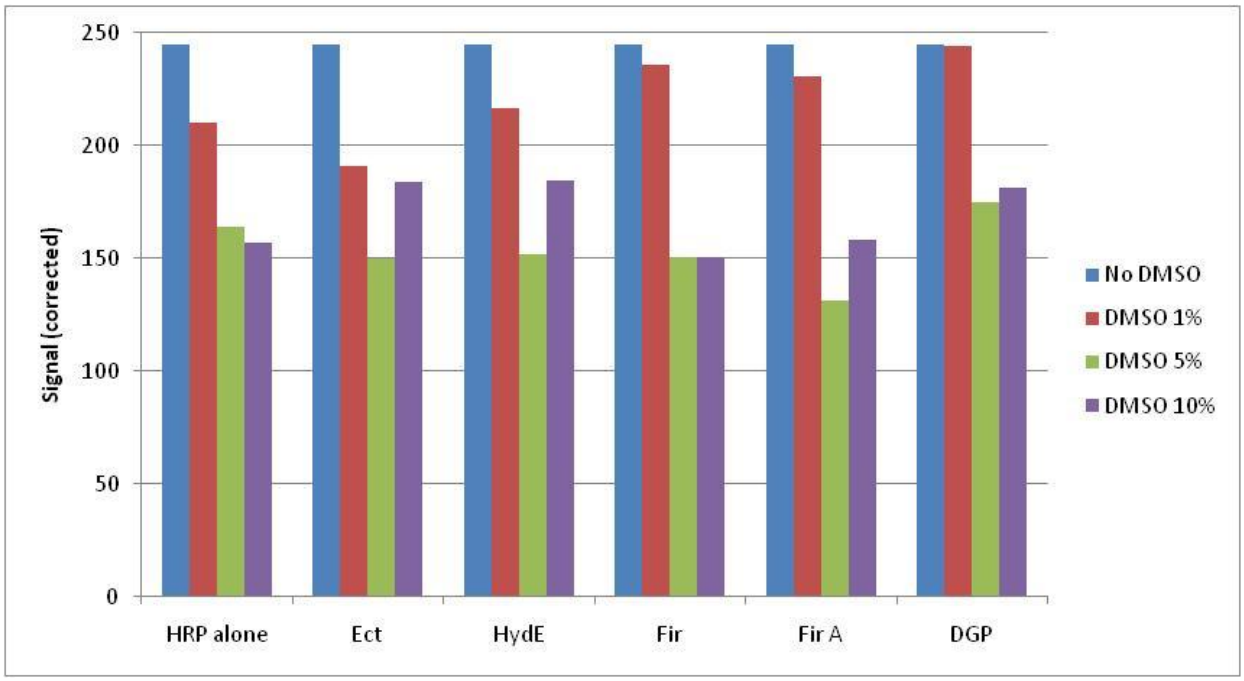


Figure 91: Antibody binding preservation in DMSO 0 to 10%, with or without hypersolutes

Up to 1%, whereas all system presented a decrease of the affinity, DGP preserved it intact: at 1%, DGP was presenting 100% of affinity for 85% only for the secondary antibody alone, compared to the test run with no DMSO in the buffer. For higher DMSO concentration, Ectoine and Hydroxyectoine did preserve the affinity best, with 75% preserved compared to 64% for the antibody alone at 10%DMSO.

3.3.6. Summary on immunosensors

- A model system for immunosensors using the indirect ELISA test format has been designed and optimized. This approach has made use of an anti-human IgG, grown in goat as the primary (immobilized) antibody and anti-goat IgG conjugated to horseradish peroxidase enzyme as secondary antibody.

- A direct immunoassay format was also developed using the same antibody test system using the SPR-based BIAcore system.

Immobilization conditions for the primary antibody were optimized (pH, concentration, flow rate, injection time).

- Interaction with the secondary antibody was characterized (kinetic values and calibration curve). An RSD of less than 1% was reported for with respect to primary antibody-secondary antibody binding.
- Different regeneration conditions were studied and an optimal system using glycine/HCl (2%v/v) selected.

- Influence of the stabilizing agents on primary antibody immobilization to the BIAcore gold chips was determined. All solutes tested acted to reduce the extent of primary antibody binding. Optimum concentration of stabilizing agents and secondary antibody were thus determined.

- The influence of stabilizing agent on antibodies binding kinetics was be studied with the BIAcore with respect to pH and ionic strength variation:
    - No influence on pH;
    - Ectoine and hydroxyectoine binding was stabilized with respect to ionic strength variation.
    - Experiments for assessing the influence of stabilizing agent upon repetitive use have been designed:
        - For repetitive use with 0.4% v/v acetonitrile, DGP and ectoine provided a stabilizing effect;
        - At 37.5°C, DGP also yielded a notable stabilizing effect.
    - Results were obtained on storage stability:
        - DGP gave very good results;
        - Firoin A and hydroxyectoine also exhibited a noticeable stabilizing effect.
    - Statistic parameters associated with the immunosensor studied were also determined using a method based on 'Resonant Acoustic

Profiling' or 'RAP' using a prototype commercial system in conjunction with the instrument manufacturer – Akubio.

- The influence of stabilizing agent on the immunosensor performance in DMSO was also studied using the Akubio system.
  - In up to 1% v/v DMSO, DGP had a stabilizing effect;
  - For higher percentages, (up to 10% v/v DMSO), ectoine and hydroxyectoine were found to create a stabilizing effect

3.4. DNA-based biosensors

3.4.1. Sensor chips and immobilization strategy

3.4.1.1. Introduction

BIAcore produce a number of commercially available sensor chips to allow study of an interaction. In a typical procedure, one of the interaction partners is immobilized onto the sensor surface of a BIAcore sensor chip. Immobilization occurs by direct coupling to the surface or via a suitable molecule already coupled to the surface. [Bianchi *et al.* (1997).]

A range of sensor chips ensures that the most suitable sensor surface can be chosen according to the nature of the molecule to be coupled and the requirements for the analysis. In this study, two types of chip were evaluated the BIAcore 'sensor chip CM5' and 'Sensor Chip SA' chips.

a. CM5 sensor chips

The sensor Chip CM5 chips are described by BIAcore as the most versatile chip available the first choice for immobilization via -NH_2, -SH, -CHO, -OH or -COOH groups, suitable for ligand fishing, high capacity capture and supporting a wide range of immobilization levels. The chips may be used for attach proteins, nucleic acids, carbohydrates or small molecules. Coupling is via carboxyl groups on the sensor surface via -NH_2, -SH, -CHO, -OH or –COOH (as shown Fig.92).

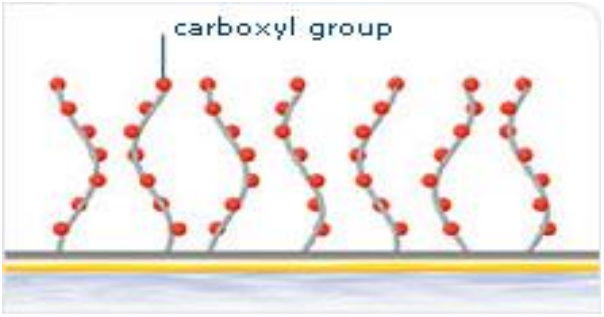


Figure 92: Carboxymethyl-coated BIAcore sensor chips surface

The immobilization matrix is composed of carboxymethylated dextran covalently attached to a gold surface. Molecules are covalently coupled to the sensor surface via amine, thiol, aldehyde or carboxyl groups. Interactions involving small organic molecules, such as drug candidates, through to large molecular assemblies or whole viruses can be studied. A high binding capacity gives a high response, advantageous for capture assays and for interactions involving small molecules. BIAcore claim that the high surface stability provides accuracy and precision and allows repeated analysis on the same surface.

b. Sensor chip SA

These chips are routinely used for immobilization of biotinylated peptides, proteins, nucleic acids or carbohydrates and facilitate high affinity capture of biotinylated ligands such as proteins, peptides, nucleic acids or carbohydrates. Controlled biotinylation enables orientated immobilization (as shown Fig.93).

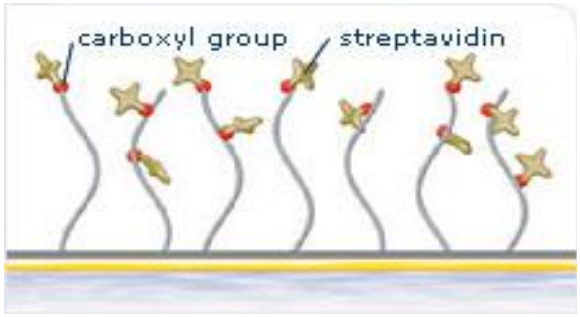
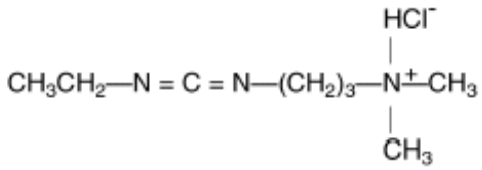


Figure 93: Streptavidin-coated BIAcore sensor chips surface

3.4.1.2. *Binding of proteins to CM chips*

A portion of the work performed focused on alternative methods of nucleic acid immobilization to the CM chips. In this case, the use of anti-biotin antibody to strategically immobilize the nucleic acid fragments to the CM5 chips was examined. It was hoped that this approach would improve the binding efficiency of the capture DNA strand to the sensor chips. In essence, the surface chemistry of the CM5 chips can be activated to bind to the anti-biotin antibody, thus leaving the binding pocket of the antibody free to bind to the biotinylated nucleic acid. This can be achieved by the well known EDC-NHS method as now described:

1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC; presented Fig.94) is a water-soluble derivative of carbodiimide. Carbodiimide catalyzes the formation of amide bonds between carboxylic acids or phosphates and amines by activating carboxyl or phosphate to form an O-urea derivative. This derivative reacts readily with nucleophiles (as shown Fig.95). The reagent can be used to make ether links from alcohol groups and ester links from acid and alcohols or phenols, and peptide bonds from acid and amines. Carbodiimide is often used in the synthesis of peptides as the water-soluble derivative EDC or as the organic soluble derivative, N,N'-dicyclohexyl-carbodiimide (DCC).



EDC
MW 191.7

Figure 94: Water soluble carbodiimide hydrochloride (C8H17N3 • HCl) for amide formation and protein modification

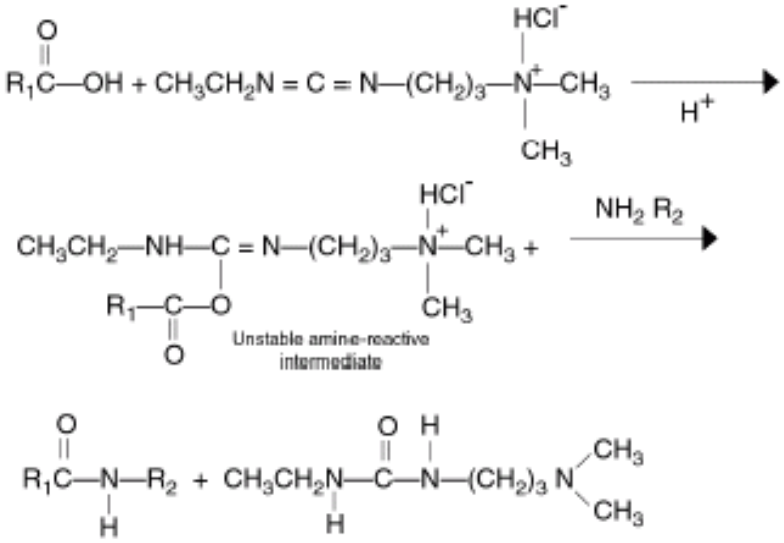


Figure 95: Mechanism of activation of carboxylic groups by EDC and further reaction with amines resulting in the amide bond formation

N-Hydroxysuccinimide (NHS; presented Fig.96) is often used to assist the carbodiimide coupling in the presence of EDC. The reaction includes formation of the intermediate active ester (the product of condensation of the carboxylic group and N-hydroxysuccinimide) that further reacts with the amine function to yield finally the amide bond.

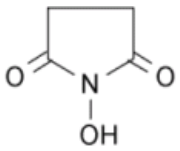


Figure 96: Structure of N-Hydroxysuccinimide (NHS)

An example of the carbodiimide coupling reaction provided by EDC and promoted by NHS is presented Fig.97. This reaction was extensively applied to couple covalently protein/enzyme molecules to self-assemble monolayers of thiolcarboxylic acids. In the reaction EDC converts the carboxylic acid into a reactive intermediate which is susceptible to attack by amines. In some cases EDC and N-hydroxysuccinimide (NHS) are used as the NHS produces a more stable reactive intermediate which has been shown to give a greater reaction yield.

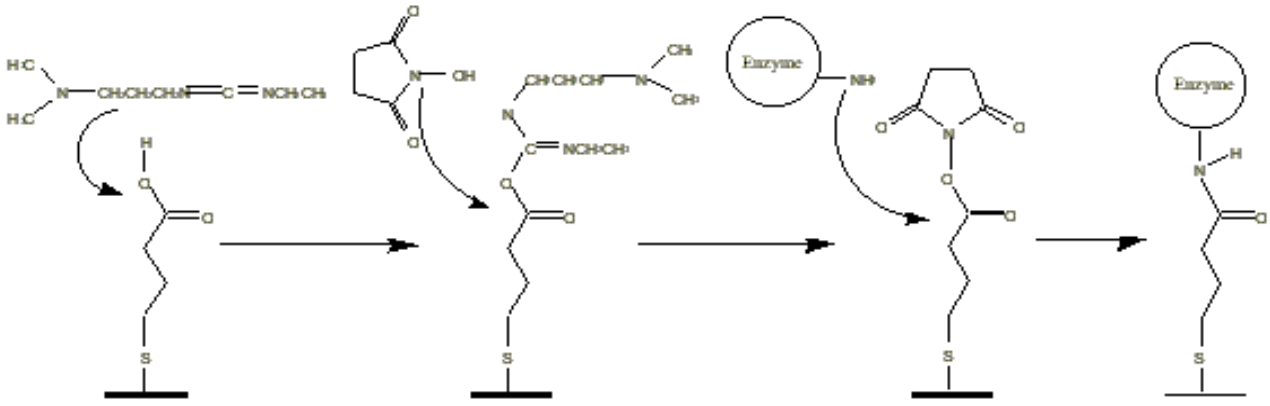


Figure 97: Schematic diagram showing the covalent attachment of an enzyme to a self-assembled monolayer of thiol-carboxylic acid using EDC and NHS

3.4.1.3. System selected

a. Model system

A model system is being used to assess the influence of the stabilizing agent on DNA hybridization. It is composed of two complementary single strands of DNA, 18 bases long. The lower the C-G base composition of a strand, the higher the affinity of hybridization, since three hydrogen bonds are involved in formation of the adenine-thymine bond, compared to 2 with the cytosine guanine. Therefore, the model system involved a first strand of poly-adenine (poly-A) and a second complimentary strand of poly-thymine (poly-T).

The poly-A sequence was conjugated to biotin at the 5' end, whereas the poly-T strand contained fluorescent Cy3 label, again attached to the 5' end of the single strand. The biotin moiety served to anchor the poly-A nucleic acid strand to the sensor surface, and the Cy3 label served to confirm that hybridization had occurred with the model system. Whilst the bulk of these studies were performed on the BIAcore system and thus did not require a label for visualization, the Cy3 fluorophore proved useful for confirming the efficacy of the hybridization process using conventional optical interrogation means.

b. Streptavidin-biotin binding

Streptavidin is a tetrameric protein purified from Streptomyces avidinii that binds very tightly to the vitamin biotin with a K_d of about 10 to 14 mol/L. Biotin, also known as vitamin H or B7 is a water-soluble B-complex vitamin. The streptavidin-biotin interaction is is one of the strongest biochemical interactions known, and is widely taken advantage of for conjugation of biological molecules to other entities, such as other biological molecules or surfaces.

In this work, the streptavidin-biotin system was used to immobilize the single stranded poly-A nucleic acid fragments to BIAcore chip surfaces.

3.4.2. Results

3.4.2.1. Streptavidin immobilisation to chip surfaces

The first immobilization process studied was the immobilization of poly-A onto the BIAcore streptavidin CM5 chips (BIAcore UK, Eyeworth, Bedfordshire, UK). The Poly-A single strand DNA is attached to the chip surface by activating the streptavidin with HABA (2-(4'-hydroxyazobenzene) benzoic acid) followed by binding the biotinylated nucleic acid.

The binding of the streptavidin to the biotinylated poly-A nucleic acid strands was first studied. A range of parameters were evaluated: pH, concentration of the streptavidin as well as flow-rate and injection time, based on a method example provided by BIAcore engineers. The method suggested was as follows:

- Set Flow rate to 5µl min
- Inject 35µl mixture of EDC (0.2M) and NHS (0.05M) for 7min to activate the carboxyl groups on the surface.
- Then inject 35µl streptavidin (over the range 200 to 400µg/ml) in 10mM sodium acetate (pH=4.5) for 7min.
- Finally, inject 35µl ethanolamine to deactivate the excessive carboxyl groups.

This method proved unsatisfactory. Consequently, the parameters were optimized for the system used in this study.

3.4.2.2. Optimization of the immobilization

First, pH was studied over the range 4 to 7, the highest response was obtained for pH of 5.0. The results are presented Fig.98.

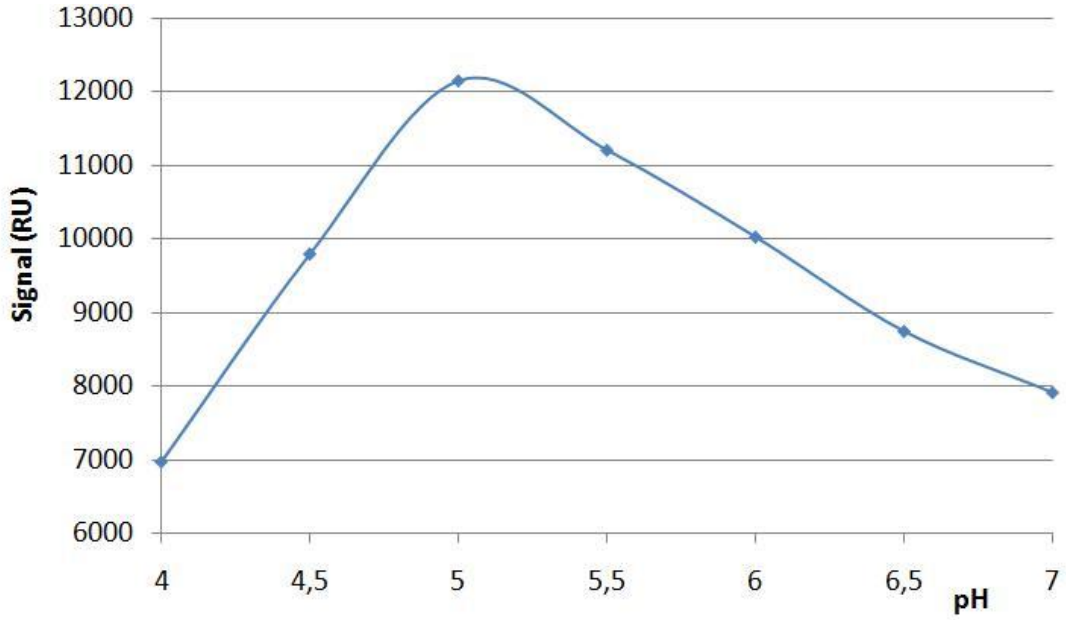


Figure 98: pH influence on the streptavidin immobilization on BIAcore CM5 chip

Different concentrations of streptavidin at pH 5.0 were injected over the range 50 to 500µg/mL and the optimum concentration was found to be 200µg/mL. The results obtained are presented Fig.99.

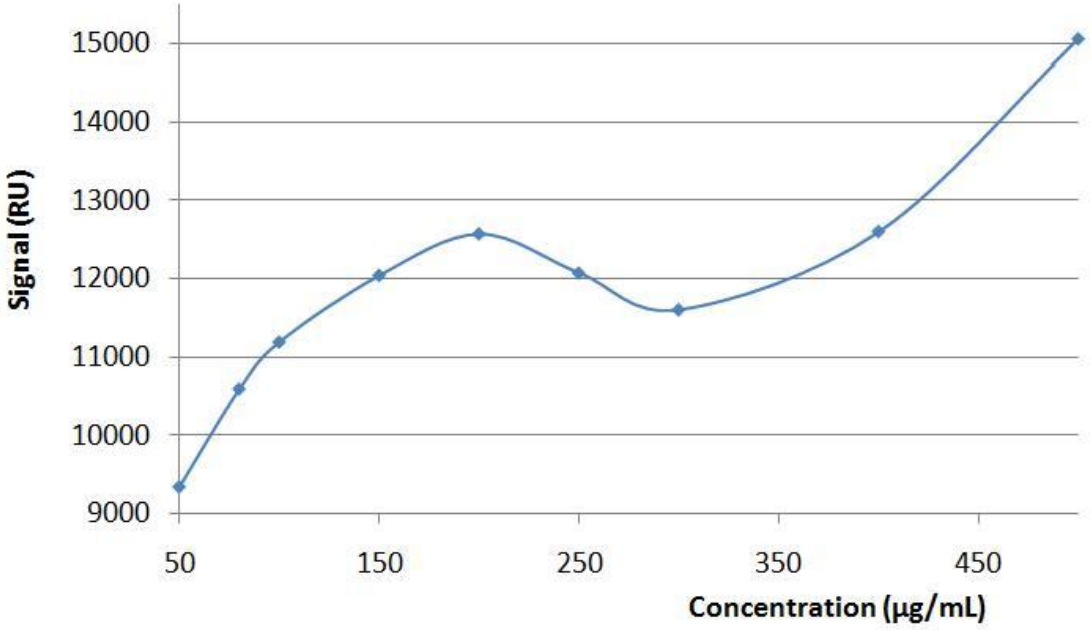


Figure 99: Optimization of streptavidin binding agent loading onto the BIAcore CM5 chips

The optimum injection parameters were also studied, over the range 5 to 15µL/min for the flow rate and duration varying between 2 and 10min. The optimum conditions were determined to be for a flow-rate of 7µL/min over 10min. Streptavidin was thus successfully immobilized at the chip surface, as presented in Fig. 100.

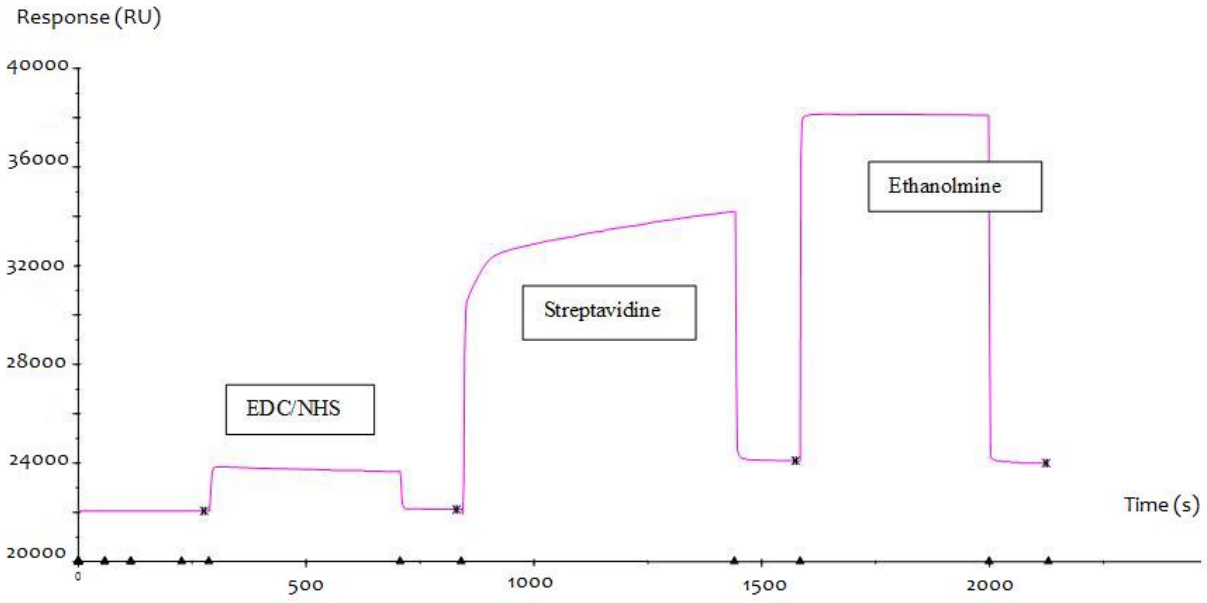


Figure 100: Amine coupling, streptavidin immobilization on the CM5 chip surface using BIAcore

As previously stated, the CM5 chips incorporate a carboxymethyl (CM) dextran matrix that allows the covalent coupling of compounds containing amino-, thiol- and aldehyde groups. Coupling of the streptavidin to the chip surfaces required CM dextran activation using the procedure of EDC (N-ethyl-N'-(3-dimethyl aminopropyl)-carbodiimide hydrochloride), and NHS (N-hydroxysuccinimide) treatment. Following activation, a 200 µg.ml^{-1} solution of streptavidin (acetate buffer, pH 5), was injected into the system over a 10min. time period at a flow rate of 7µg.ml^{-1}, the resulting sensorgram indicating that material had been immobilized at the surface of the transducer chip. The subsequent immobilization of the DNA onto the chip surface was tested, but only very low immobilization levels were obtained and so an alternative immobilization system was required.

3.4.2.3. *Anti-biotin antibody immobilisation to BIAcore chips*

An alternative method for binding the first ss-DNA (poly-A) onto the chip surface was to use an anti-biotin anti-body, since the poly-A sequence is biotinylated at the 5' end. The immobilization of the antibody is more straightforward to set up, as previous work performed within the HotSolutes project on antibody binding onto CM5 surfaces was performed as part of the immunosensor studies. In these studies, IgG antibody was immobilized to CM5 sensor chips according to the manufacturers instructions. Following activation, a 100µg.ml^{-1} solution of antibody (acetate buffer, pH 5), was injected into the system over a 15min. time period at a flowrate of 10 µg.min^{-1}, the resulting sensorgram indicating that antibody material had been successfully immobilized at the surface of the transducer chip with an adsorption of 29000 Response Units (RU). The sensogram for that experiment is presented Fig.101.

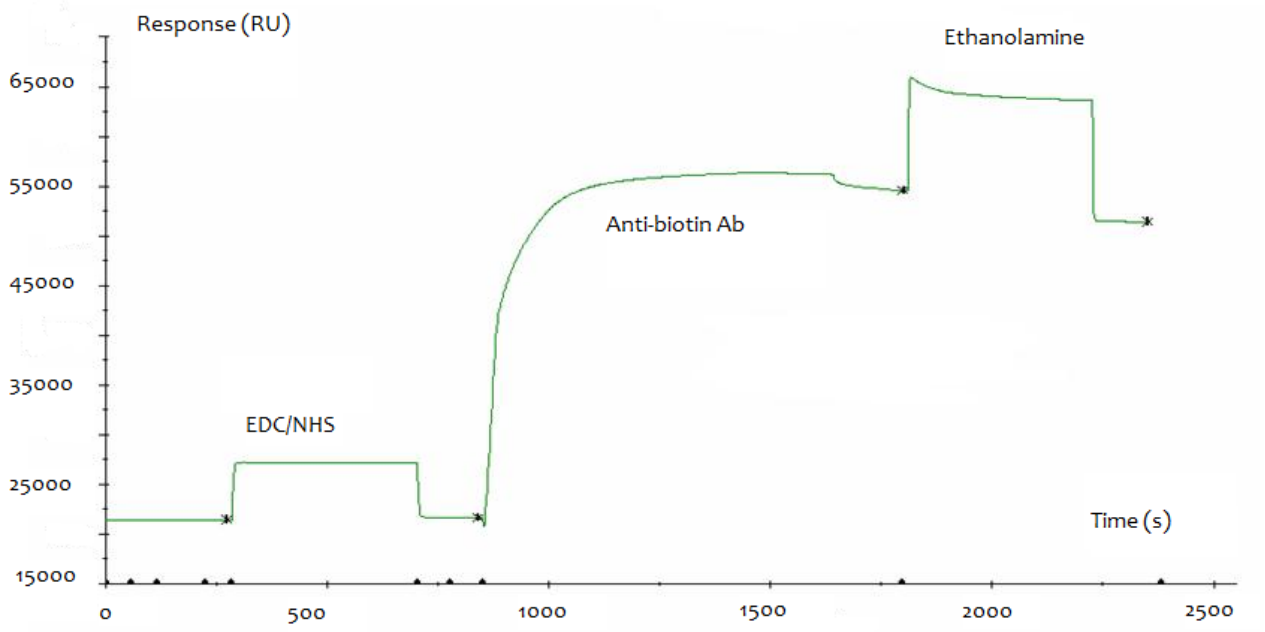


Figure 101: Anti-biotin antibody immobilization using amine coupling on CM5 chip surface using BIAcore

The binding of the biotinylated DNA to the antibody, that of the second ss DNA onto the primary poly-A sequence and subsequent regeneration was then to be studied, using a number of methods recommended by the chip manufacturer, based on the use of glycine/HCl, sodium hydroxide, acetic acid or SDS (sodium dodecylsulphate – at various concentrations and pH values).

3.4.2.4. *Preliminary results with the hypersolutes*

A short preliminary study was conducted, to assess the DNA system chosen and the influence of the stabilizing agents. As the time allocated for the experimental part of this thesis as well as of the European project was drawing to close, the influence of the hypersolutes was investigated straight away rather than after a more detailed study of the system alone.

a. Adding the poly A strand

As the anti-biotin antibody immobilized on a CM5 chip was deemed the most efficient way to bind the primary DNA strand, namely the biotinylated poly-A single strand, a BIAcore chip was prepared according to the protocol described earlier. The poly-A single strand of DNA was then added on the chip surface, injecting 40µL using a 20nM solution at pH 7.0, with a flow rate of $10\mu L.min^{-1}$. The regeneration solution used was Glycine HCl pH 2.0. As the Poly-A strand is bound via the antibody-antigen binding, the regeneration allows the antibody to release the DNA strand. The surface is therefore regenerated and subsequent tests could thus be carried out. A mix of Poly-A DNA with various hypersolutes (8mM concentration solution) was injected following the same protocol. A mix of two hypersolutes, DGP and hydroxyectoine (1:1), was also tested as they had proved in the previous studies, using either enzymes or antibodies, that they were the most promising of the hypersolutes. Their association could therefore prove also very potent, maybe even more so as they could be complementary in their stabilizing effect. The results obtained are presented Fig.102.

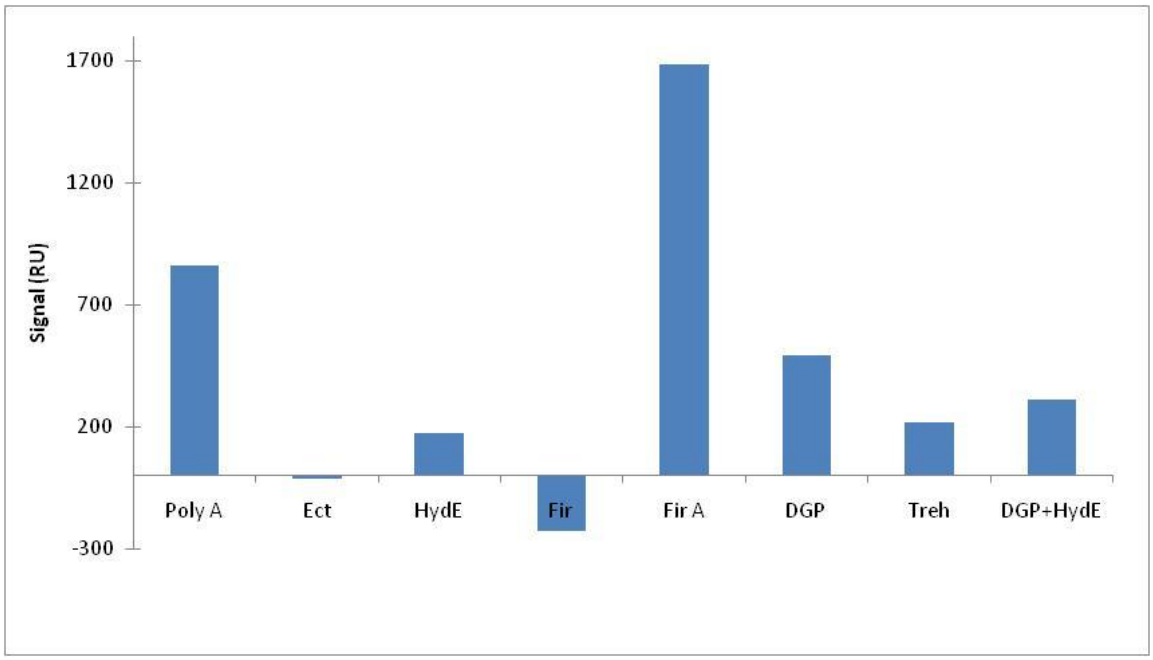


Figure 102: Binding on a CM5 chip coated with anti-biotin antibody of a biotinylated poly-A single strand DNA with or without hypersolutes

Both ectoine and firoin were disappointing, as a loss of signal was observed in each case, and no binding could be measured. Firoin A also gave puzzling results as it seemed the hypersolute bound itself on the chip surface, along the DNA and no exploitable result could therefore be obtained. As for the remaining hypersolutes, a binding could be observed with DGP, hydroxyectoine, a mix of both these stabilizing agents and trehalose. The signal loss was most important with hydroxyectoine and trehalose, with about 25% left. DGP showed the least loss with a signal around 60% of its original strength, when the DNA was injected alone. It was interesting to notice that the loss of signal for the mix of hydroxyectoine and DGP was an average of the values for the corresponding hypersolutes alone.

b. Adding a solution with both strands of DNA

As the interaction that was to be studied was that of DNA to DNA binding, a study was realized using a solution with both strands of DNA. Using the same protocol as before, the binding on the anti-biotin antibody of the poly-A DNA strand associated with its poly-T counterpart was studied and compared to the previous results. The signals obtained for this test are presented Fig.103.

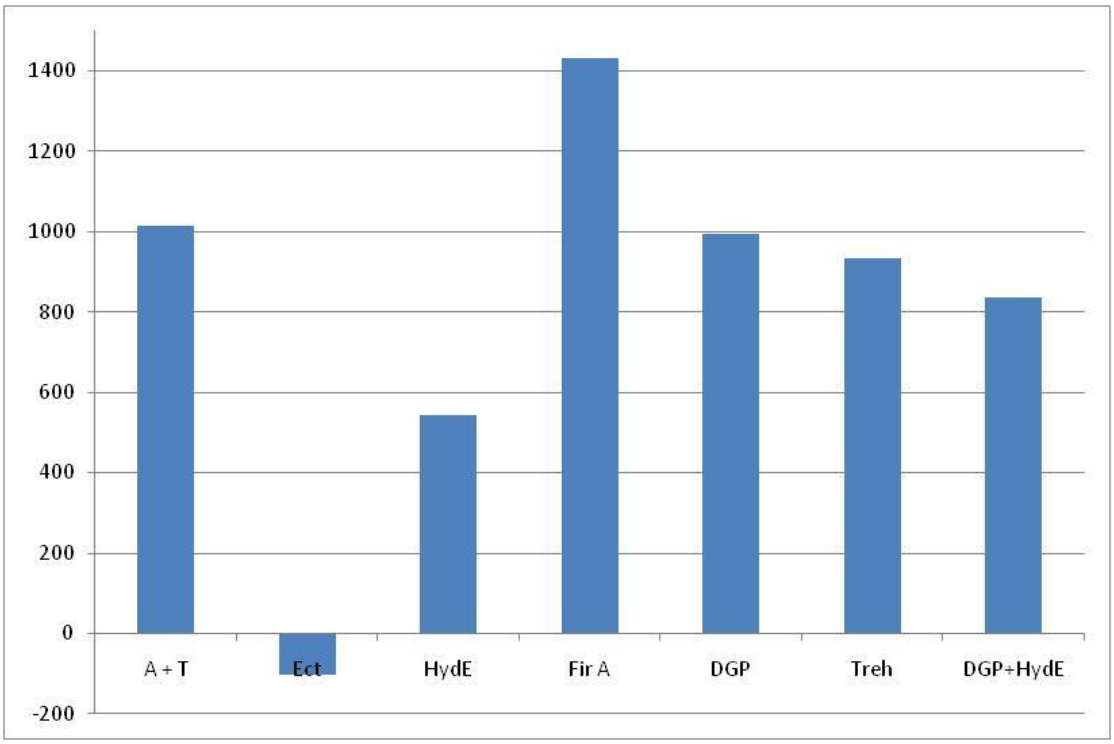


Figure 103: Binding on a CM5 chip coated with anti-biotin antibody of a biotinylated poly-A single strand DNA associated with a poly-T single strand DNA in solution with or without hypersolutes

Again, ectoine and firoin A gave results that weren't exploitable. As for firoin, there was a shortage of this product that wasn't remedied in time by our partners, given the time left to the end of the European project. As for DGP, hydroxyectoine, their mix and trehalose, the results obtained here were consistent with those obtained in the previous study. When comparing the signals in order to assess the poly-A to poly-T binding, trehalose was the most satisfying with 84% of added signal, compared to the poly-A to poly-T binding without any hypersolutes. This poly-A to poly-T binding was roughly evaluated by subtracting the signals for poly-A alone to that of the poly-A/poly-T solution. The poly-A to poly-T binding in absence of stabilizing agent served as reference at 100% of added signal. The addition of hypersolutes led to a decrease of binding, which wasn't unexpected given the loss of signal observed for either the enzyme-based biosensor or the immunosensor. DGP gave about 61% of added signal, while hydroxyectoine gave 45% and their 1:1 mix gave 49% of added signal.

c. Adding the poly T strand

As the previous system was considered to be rather unsatisfactory to precisely evaluate the impact of the stabilizing agents on the poly-A to poly-T binding, another test was realized. On CM5 chip, an anti-biotin antibody was immobilized. In each subsequent cycle using this surface, a first step was to bind the poly-A strand of DNA. A poly-T strand solution was then injected onto the chip surface, with or without hypersolutes and the increase of signal measured. A correction was applied to this result in order to negate any difference of poly-A strand binding that might have occurred. The same injection conditions and concentrations were used as before. The surface was then regenerated to leave the antibody bare of antigen. The results for this study are presented Fig.104.

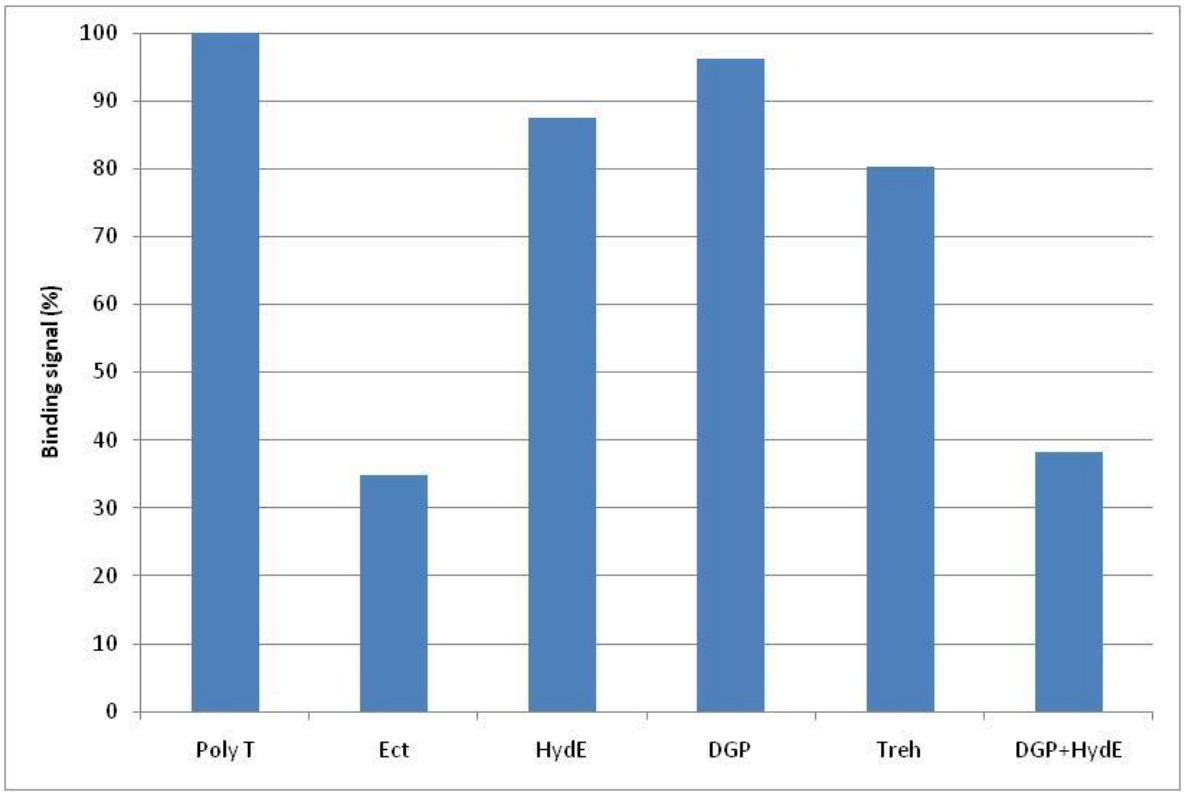


Figure 104: Binding on a CM5 chip coated with anti-biotin antibody of a poly-T single strand DNA in solution with or without hypersolutes

As there was no stabilizing agent when binding the poly-A strand, the binding was similar for each cycle and the influence of ectoine on the DNA pairing could be observed. DGP seemed the most promising, along with hydroxyectoine, with over 85%

of binding maintained, compared to the DNA pairing without any stabilizing agent. Trehalose gave also decent results. Ectoine, as well as the mix of hydroxyectoine and DGP were interfering rather severely with the pairing, as the remaining binding was led than 40% of its reference value.

At this point, the experimental time limit set was reached and these studies could not be led any further. It is thought the DNA-biosensors might work in a similar manner to that of the immunosensor; however, there wasn't enough time to validate that postulate. The system used would have needed to be characterized in more precise details, in the absence of any hypersolutes for their impact to be more accurately defined. Also, the stabilizing effect of the hypersolutes wasn't studied, due to this lack of time and would have needed to be experimented upon, especially in regard to storing.

3.4.3. Summary on DNA-based biosensors

- The model system designed has been tested on a surface plasmon resonance (SPR) biosensor system to allow the label free determination of single stranded nucleic acid hybridisation. In this case the well known and commercially available BIAcore SPR biosensor system has been employed.
- The model system is composed of poly-A nucleic acid, containing biotin at the 5' end to aid immobilisation to the BIAcore sensor chips, and poly-T nucleic acid with a Cy5 fluorophore attached at the 5' end. This fluorophore can be used to determine hybridisation by an alternative method to that of the SPR process for validation purposes.

- Different immobilization methods of the DNA onto the chip surface have been investigated. Methods employed have included use of streptavidin coated chips and CM5 (carboxymethyl dextran) coated chips.
- The CM5 chips have been examined using 2 alternative nucleic acid immobilisation strategies:

- (1) streptavidin immobilisation to the CM5 chips followed by streptavidin-biotin-ss-DNA immobilisation;
- (2) immobilisation of anti-biotin antibody to the chips, following chip activation using the well-established carbodiimide-succinimide ester approach, followed by antibody-biotin-ssDNA immobilisation. This later method is proving more suitable for the efficient immobilisation of the poly-A ssDNA capture material.

- Regeneration systems were being studied. Similar regeneration conditions to those employed for the BIAcore-based immunosensor systems were being examined.
- The DNA hybridisation process was assessed in the presence and absence of Hotsolute stabilizing agents. Parameters under evaluation were to include: pH stability, effect of ionic strength, temperature factors, repeated device usage and long-term device storage stability. The experimental time limit being reached, these experiments couldn't be pursued to maturity.

- It was planned at the start of the HotSolutes program to work collaboratively with Ic DNA biosensors (Moscow, Russia) to develop the DNA based biosensors but issues arose during the progress of the project that resulted in the non-availability of Ic DNA biosensors, due to the departure of Ic DNA biosensors from the project.

4. SUMMARY AND CONCLUSIONS

As part of the HotSolutes project, the aim of the study was to assess the influence of hypersolutes on three types of biosensors: enzyme-based biosensors, immunosensors and DNA-based biosensors. In all three systems, the development process undertook was fairly similar.

First the detection system to be used was determined. It had to be rather cheap if possible, reproducible and reliable. Mostly, it had to be fast and with as little parameters as possible, in order to limit interferences. One model system was then chosen, with the exception of the enzyme-based biosensors, were several were studied.

Each of these systems was then characterized, before the hypersolutes could even enter the picture. This step accomplished, only then would the hypersolutes be added, in normal working conditions, to determine their impact on the system studied. This would then serve as a starting point for the study. Each system was placed in various stressful conditions and looked at for changes in their behavior. This allowed the study of the influence the Hypersolutes on stabilizing these systems and therefore allowing them to keep a decent activity level.

On all systems, no stabilizing effect of the hypersolutes was observed against pH variations. Against ionic strength variation, ectoine was the one that proved to have a significant stabilizing effect in all systems. On storing, both DGP and hydroxyectoine had a stabilizing effect on enzyme-based biosensors as well as immunosensors.

For enzyme-based biosensors, data pertaining to repetitive use weren't obtained. However, a stabilizing effect of all Hypersolutes against enzyme desorption at the electrode surface was observed.

For immunosensors, a strong inhibition of antibody-antigen binding due to the hypersolutes at higher concentrations was observed. Even so, the hypersolutes gave interesting results. Upon repetitive use, and in stringent conditions, DGP and ectoine proved to have a good stabilizing effect.

Finally, on DNA-based biosensors, the study mainly concentrated on optimizing the system. The influence of stabilizing agent did not go beyond early testing.

Overall, the hypersolutes proved to have stabilizing effect. However, only a couple stood out and have been as such recommended for development. DGP consistently gave interesting results and in average is the most promising of all the hypersolutes we studied. Ectoine and hydroxyectoine were found to be promising as well and are actually already commercialized by Bitop Ag as such. However, it is DGP that was felt to warrant further studies the most.

5. DISCUSSION AND FURTHER WORK

The overall aim of this thesis was to assess the influence of novel stabilizing agents on different types of biosensor, in order to determine which components might be interesting to further study and develop, industrially. It was part of the HotSolutes European project. Three main types of biosensors were investigated: enzyme-based biosensors, immunosensors and DNA-based biosensors. The stabilizing agents used are hypersolutes that extremophiles bacteria produce to resist stress and survive in extreme conditions. Several such compounds were produced by partners in the project, namely Bitop AG. (Germany) and StabVida (Portugal). The hypersolutes studied were ectoine ©, hydroxyectoine, firoin, firoin A, DGP and PML.

These hypersolutes are novel compounds. They are therefore not thoroughly studied: their structure is known, as well as their stabilizing effect within the extremophiles that produce them. However, the mechanism through which they do stabilize the bacteria is neither known, nor has it been studied. It is the first and foremost point that would require a thorough investigation before any further study on these hypersolutes is conducted. It is to be understood that the aim of this project was to narrow down the list of hypersolutes to those who might be interesting to produce at an industrial scale and then commercialize. It seems vital that the stabilizing mechanisms of those compounds, which make it to the short list, are investigated.

It has been suggested by some partners within the HotSolutes project that this mechanism might be similar to trehalose. However, this statement isn't, at present, more than an educated guess with no study or data to fully back it up. It wasn't possible within the time frame to realize the study of these mechanisms, as it would be a thesis of its own. Also, no partners within the HotSolutes project were tasked with this study.

The first part of this thesis was centered on enzyme-based biosensor. Several enzymes were tested: Glucose oxidase, alcohol oxidase, acetylcholine esterase and lactate dehydrogenase. Glucose oxidase was an obvious choice, as over 90% of publications on biosensors concern this enzyme. However, because it is inherently stable, it isn't necessarily the most interesting choice for this study, where we try to improve the stability of biosensors. This was the motivation behind studying other enzymes as well.

These systems were designed and tested using electrochemical detection. It might be worth looking into other detection systems for enzyme-based biosensors, and not only amperometry. The influence of the hypersolutes on the detection system within the biosensor could thus be more fully investigated.

The system studied was first characterized, as a reference, before the stabilizing agents were added. The stability of the systems was assessed, comparing the biosensors response, whilst facing stress conditions, with the hypersolutes as opposed to without. It would have been interesting to compare these results to those if the biosensor were to have been stabilized using other stabilizing agents, already commercially available, such as trehalose. This was done later on, for some of the immunosensor study; it wasn't, however, for the enzyme-based biosensor and the study is lacking because of this.

It is to be noted that the manner in which this study was conducted influenced in a way the results obtained. The tests were designed with an utopian objective firmly in mind: that these hypersolutes were, in the long run, to be industrially produced and commercialized. If indeed they were interesting, they would be used to stabilize biosensors and thus added as part of the biosensor fabrication process. The stabilizing agents, in the tests performed for this thesis, were therefore added along with the enzymes rather than added to the biosensor along with the enzyme substrate. This proved to influence the results obtained. Indeed, a study was realized by a partner within the HotSolutes project on the influence the hypersolutes might have on enzyme-based biosensors against pH variations. The conclusion that was reached, whilst testing this system in the light presented just above, was that there was no significant stabilizing influence. However, the partner had, with his study, reached just the

opposite conclusion. The way their study was conducted was to prepare a biosensor and then test it with a substrate solution at different pH, with or without stabilizing agents. Also, their test was significantly longer, time-wise, as those realized for this thesis. This emphasizes the importance of the light in which these tests were designed and realized.

The study on operational stability could not be pursued to fruition, due to the fairly tight schedule this study was on, being part of a European project, as well as to the instability of the system used. Yet, it was an important point to study and this test would have to be further optimized and then realized. The pump that was made available wasn't powerful or reliable enough to be used with the system that had been designed. Either another pump would have to be tried, or the system would have to be changed, maybe by lowering the number of cells tested simultaneously.

Again, the hypersolutes made available for this thesis aren't characterized very much. While testing their influence on storage, one of them, firoin, created, after 4 months, a white deposit that completely disabled every biosensor. The nature of this deposit wasn't investigated, but it would have been an interesting study to realize, using X-ray diffractometry for example. It might have allowed a foray into understanding the way these stabilizing agents function.

The second part of this thesis concentrated on immunosensors. A model system was designed using ELISA tests and then was studied on the BIAcore and Akubio systems.

Although the ELISA tests were more time-consuming than the other systems used, it might have been interesting to test the influence of the stabilizing agents with these as well, especially using the electrochemical detection. This would have allowed a nice comparative study between enzyme-based biosensors and immunosensors systems.

As with enzyme-based biosensors, it would also have been interesting to compare the hypersolutes available to trehalose at least and maybe a couple other

stabilizing agents, right from the beginning of this study. Although in this case, it was done later on, on some of the characterization that was done.

Another comment must be made on the immunosensor study done: The binding of the stabilizing agent on the chip surface wasn't studied. It is to be expected that there would be some non-specific binding occurring and that hasn't been assessed. It can be extrapolated that the results obtained regarding the stabilizing effect of the hypersolutes was therefore slightly overestimated. This, however, shouldn't impact the results obtained too much and the conclusions drawn at the end of this study remain valid.

Finally, as several systems were studied for enzyme-based biosensors, it might have been interesting to test several immunosensors. An existing immunosensor system, for example, could be investigated. Several systems were considered but again, lack of time prevented the realization of this study. [Padeste *et al* (1998)]

The last part of this thesis was centered on DNA-based biosensors. These are notoriously tricky systems to exploit. One of the partners of the HotSolutes project was to characterize a system, but went bankrupt. Thus, the design of a model system wasn't realized until this part of the study was started. Optimization of the system that was to be studied took enough time that the study of the stabilizing effect of the hypersolutes was sketchy at best. This is but a preliminary study and a full study of the influence of the stabilizing agents on a DNA-based biosensor still remains to be more thoroughly done.

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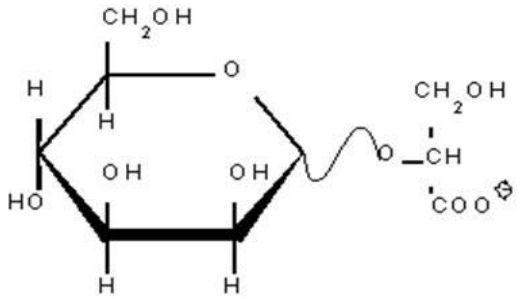
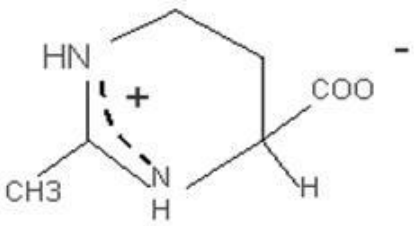
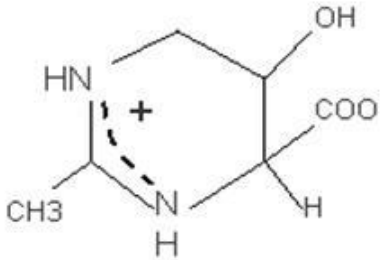
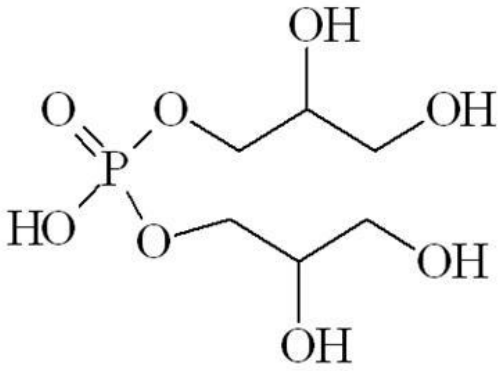
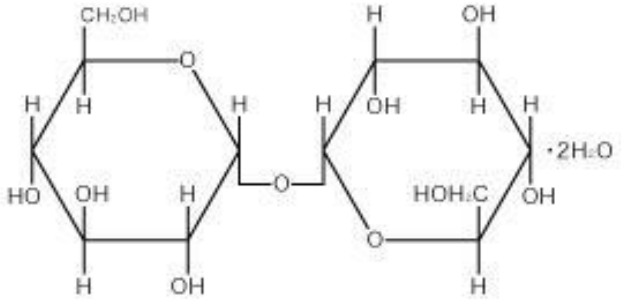
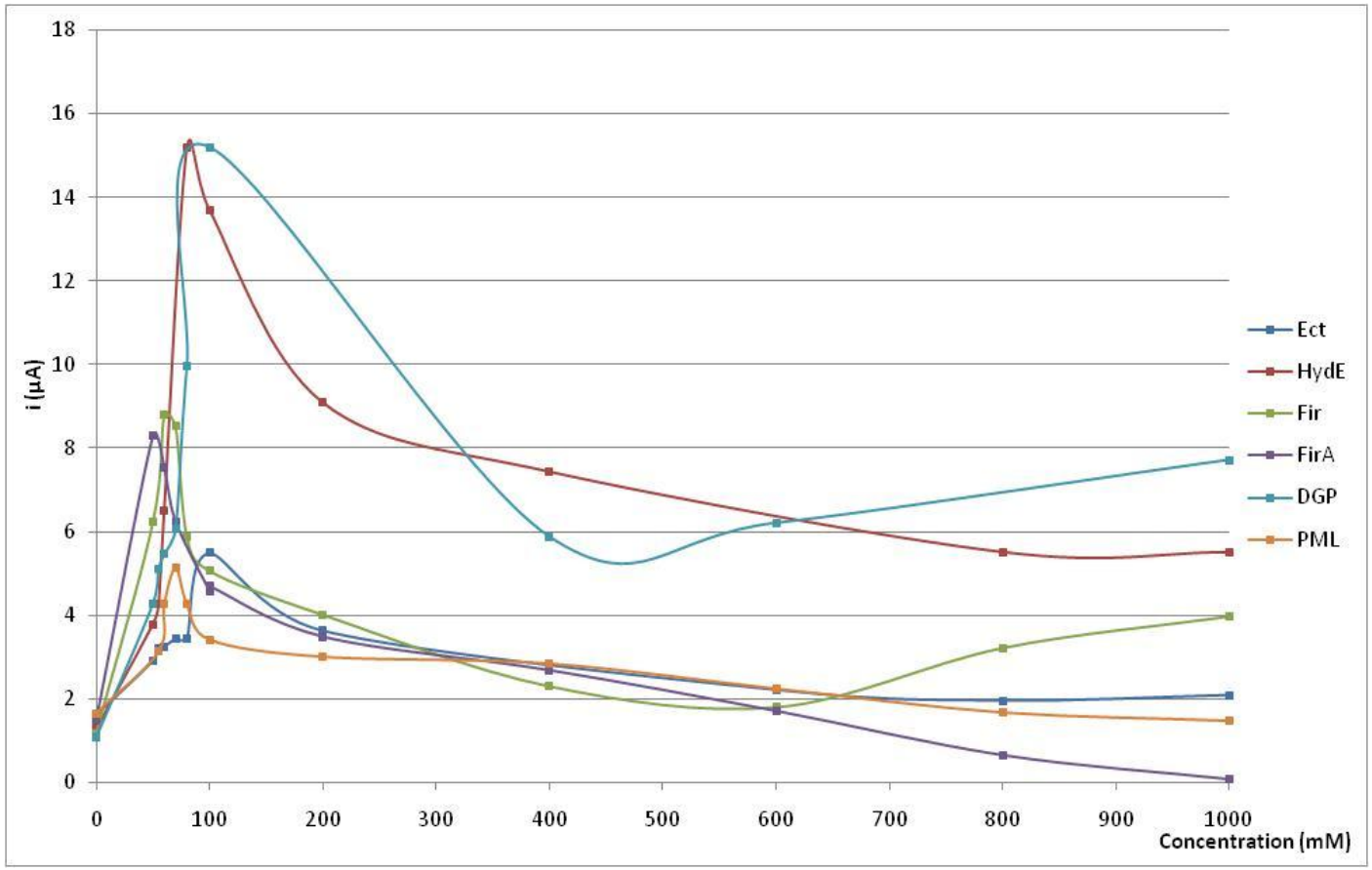
7. APPENDIX

7.1. Hypersolutes structures (cf. p.105)

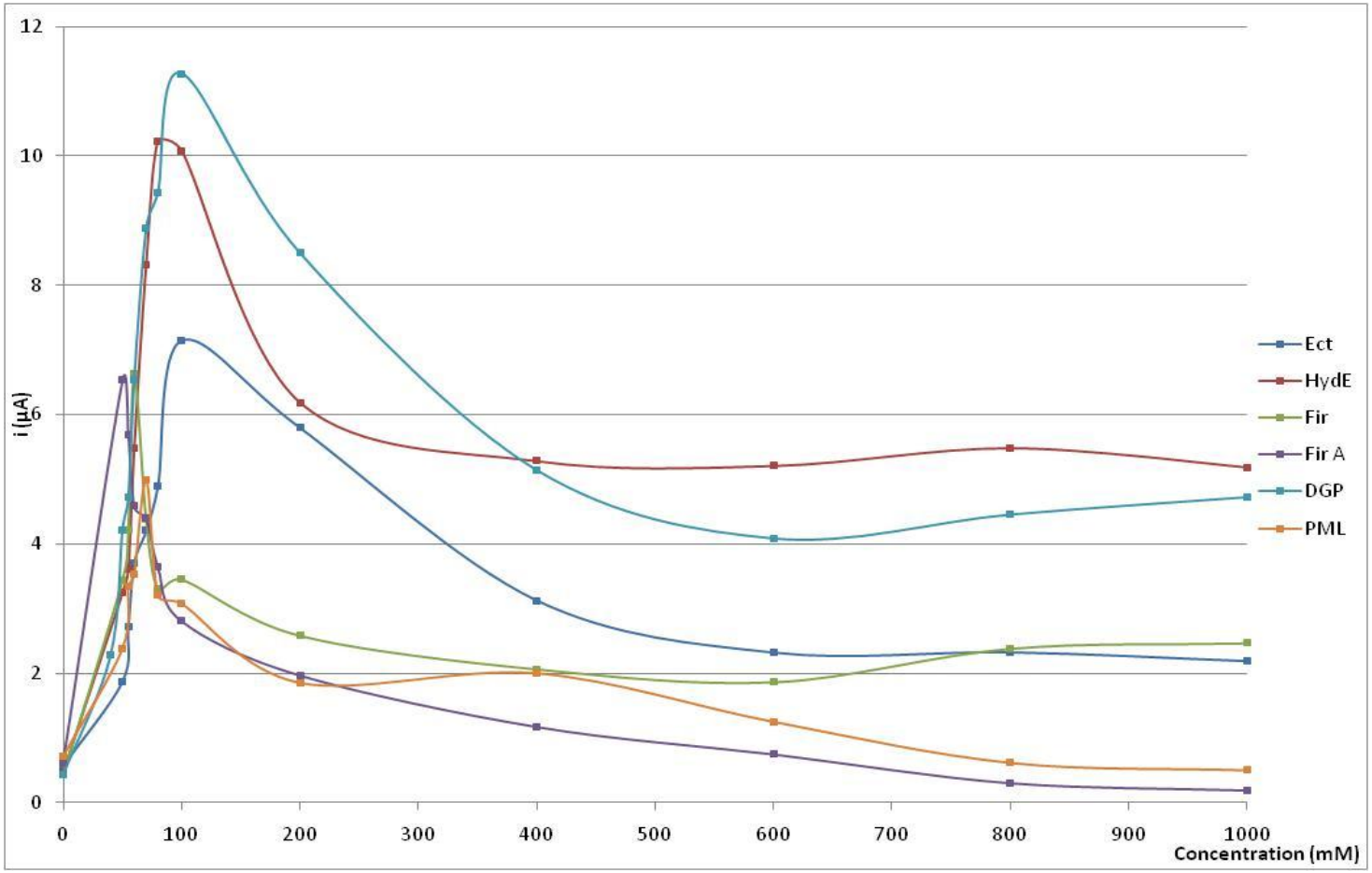
Firoin	Ectoine	Hydroxyectoine
		
Diglycerol Phosphate	**Trehalose**	
		

7.2. Optimisation of hypersolutes loading on glucose oxidase biosensors after 14 days of storing at 37°C against glucose 5mM (cf. p.152)

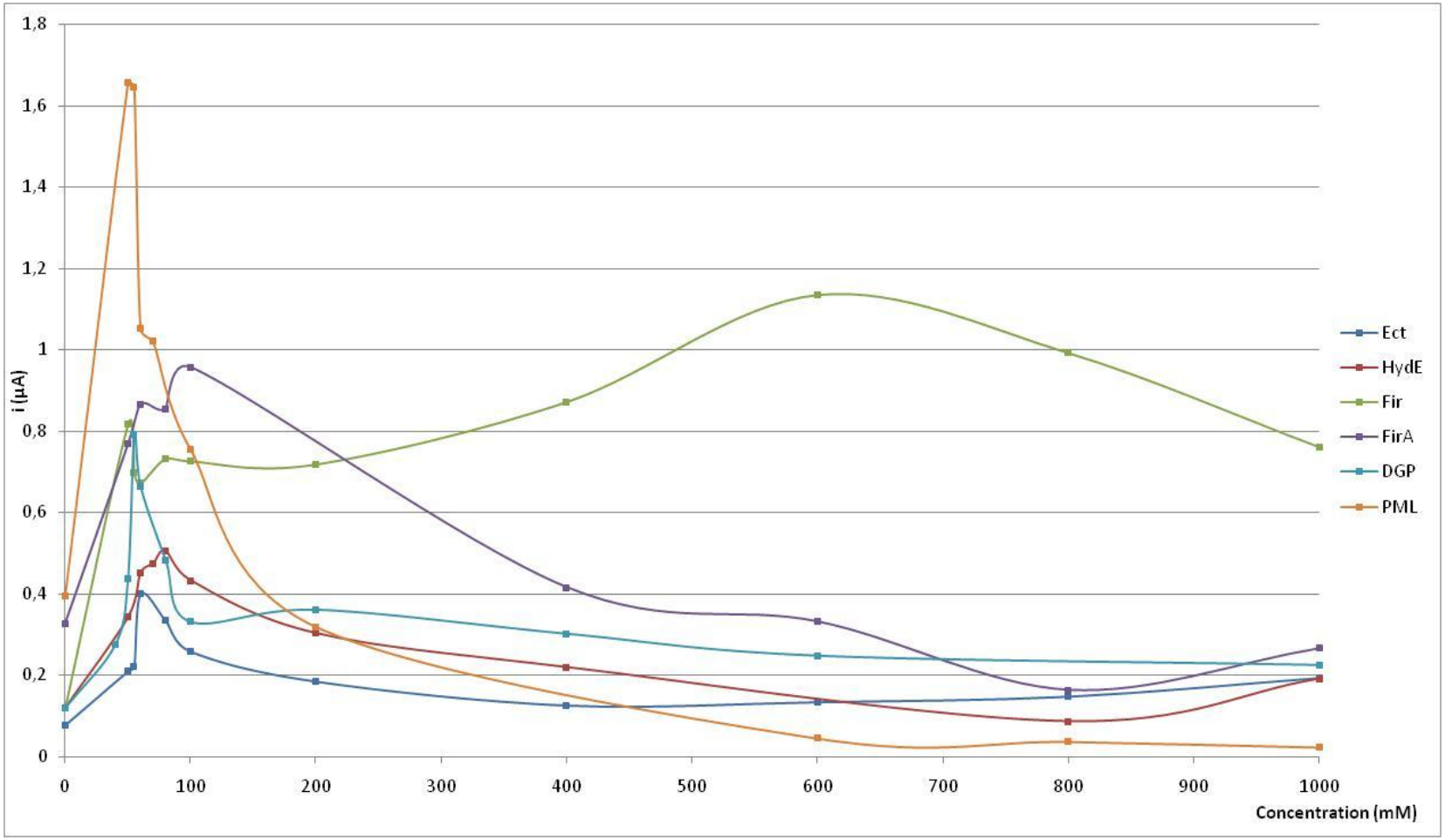


7.3. Optimisation of hypersolutes loading on enzyme-based biosensors after 2 months of storing at room temperature (cf. p.153)

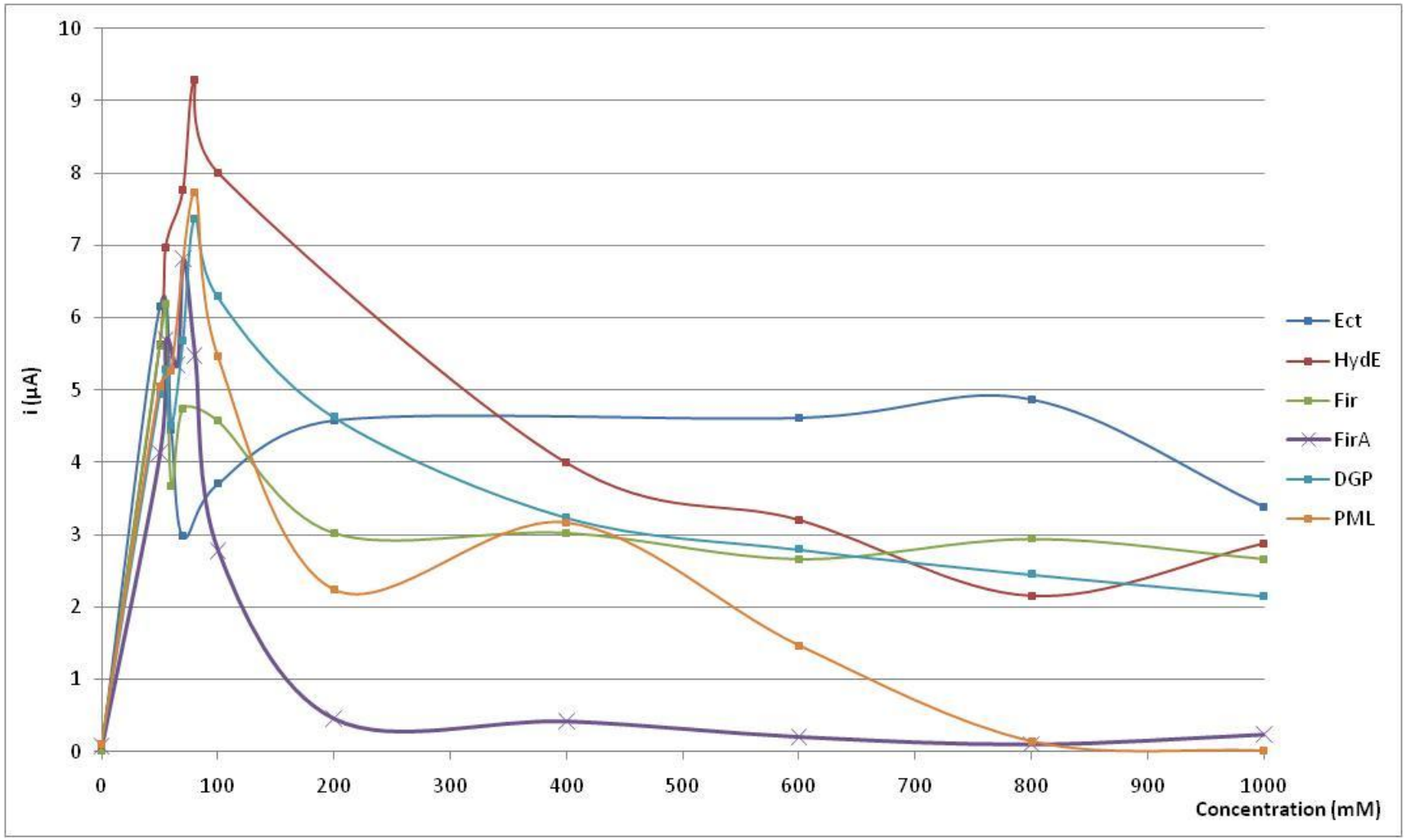
7.3.1. Glucose oxidase biosensor



7.3.2. Alcohol oxidase biosensor

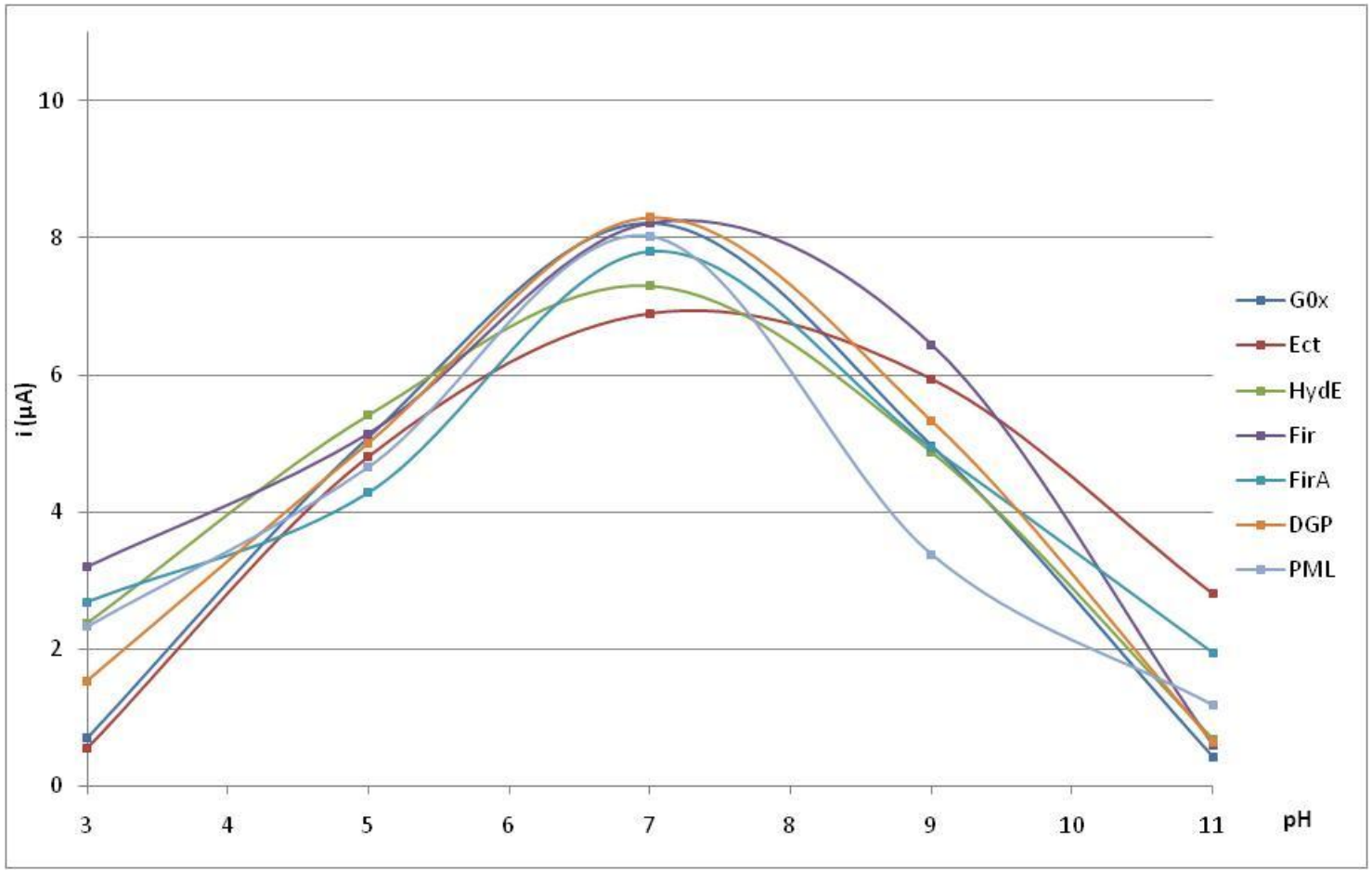


7.3.3. Acetylcholine esterase biosensor

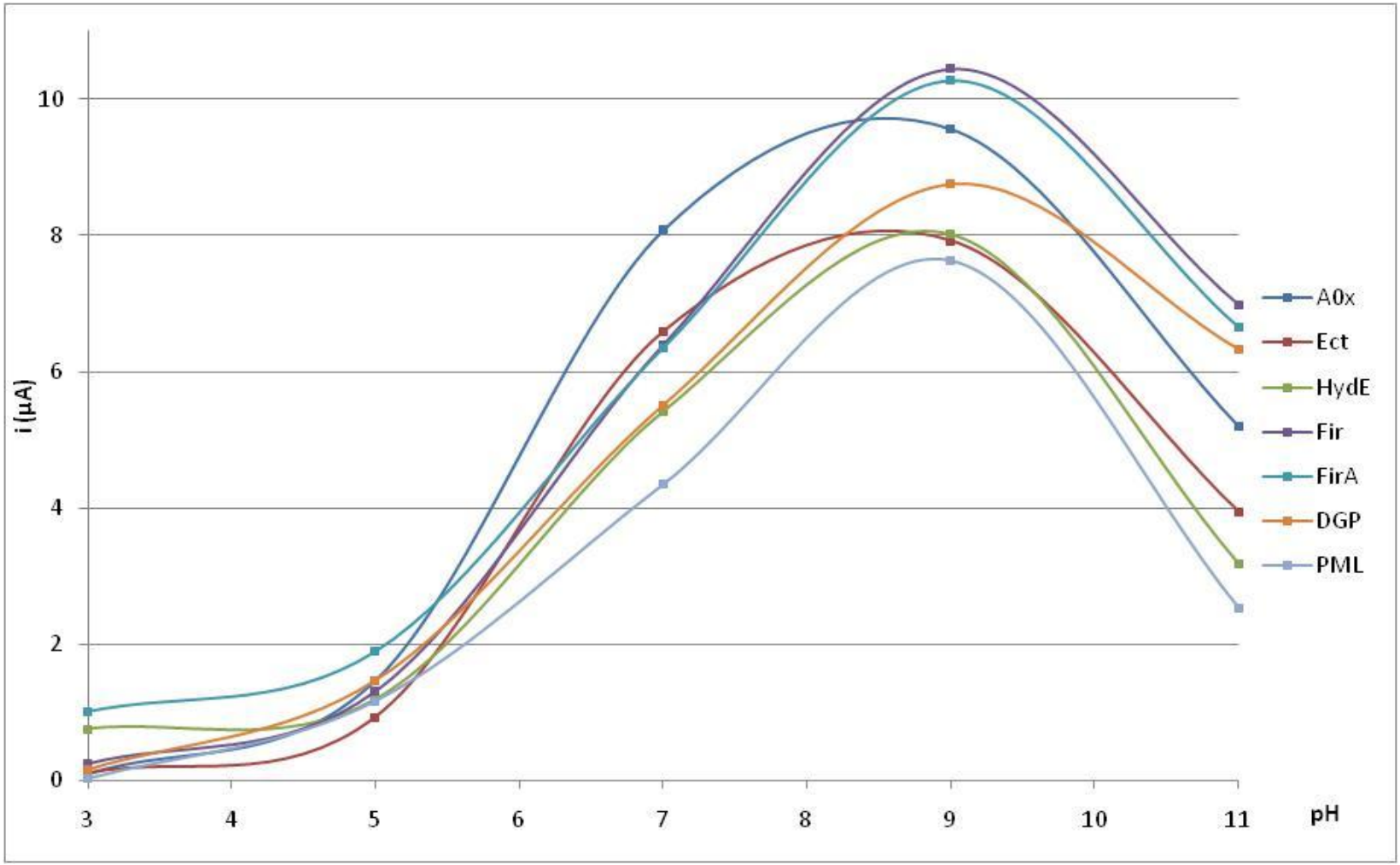


7.4. Influence of the hypersolutes on enzyme-based biosensors against pH variations (cf. p.157)

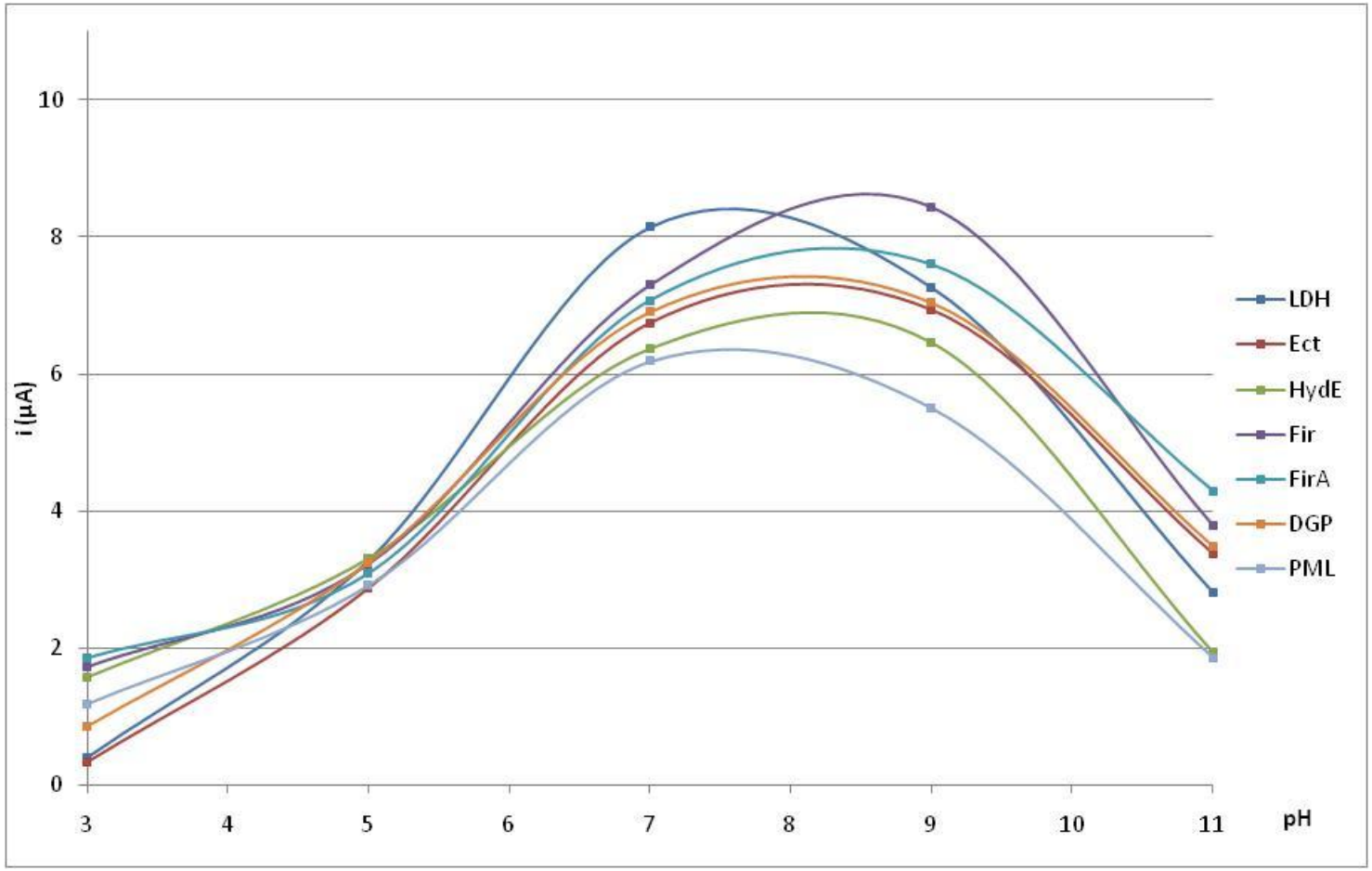
7.4.1. Glucose oxidase biosensor



7.4.2. Alcohol oxidase biosensor

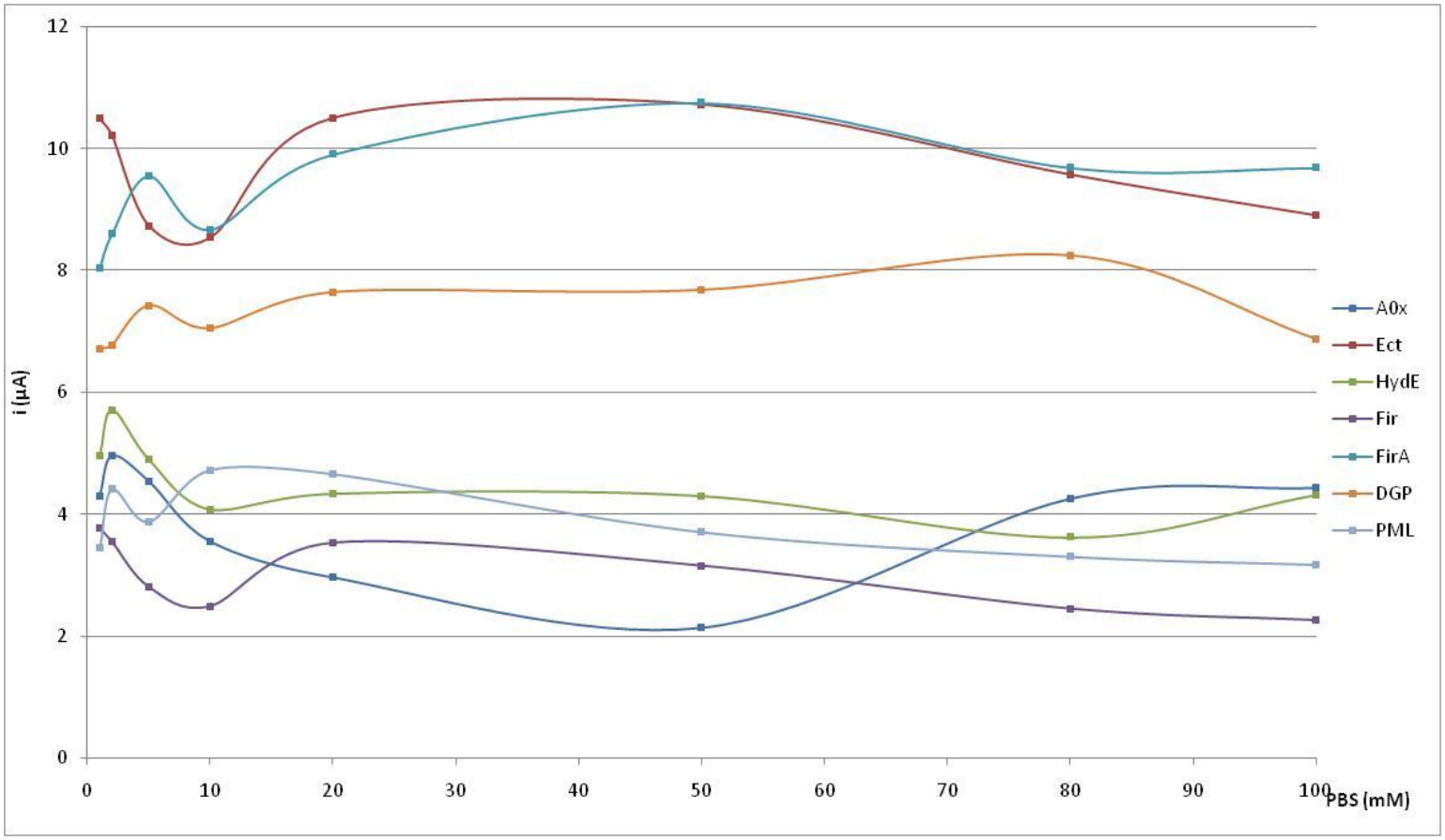


7.4.3. Lactate dehydrogenase biosensor

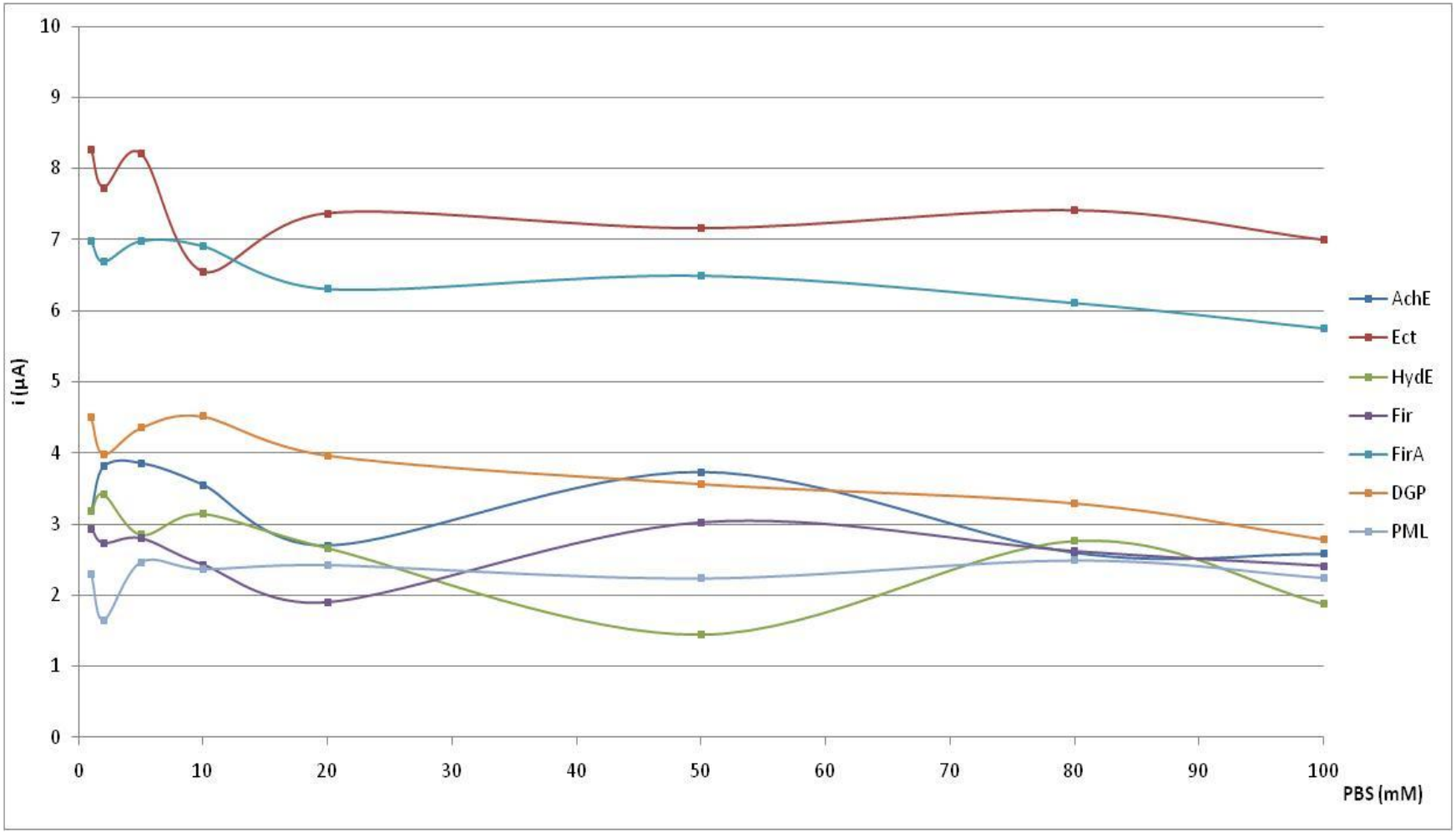


7.5. Influence of the hypersolutes on enzyme-based biosensors against ionic strength variations – PBS over the range 0 to 100mM (cf. p.157)

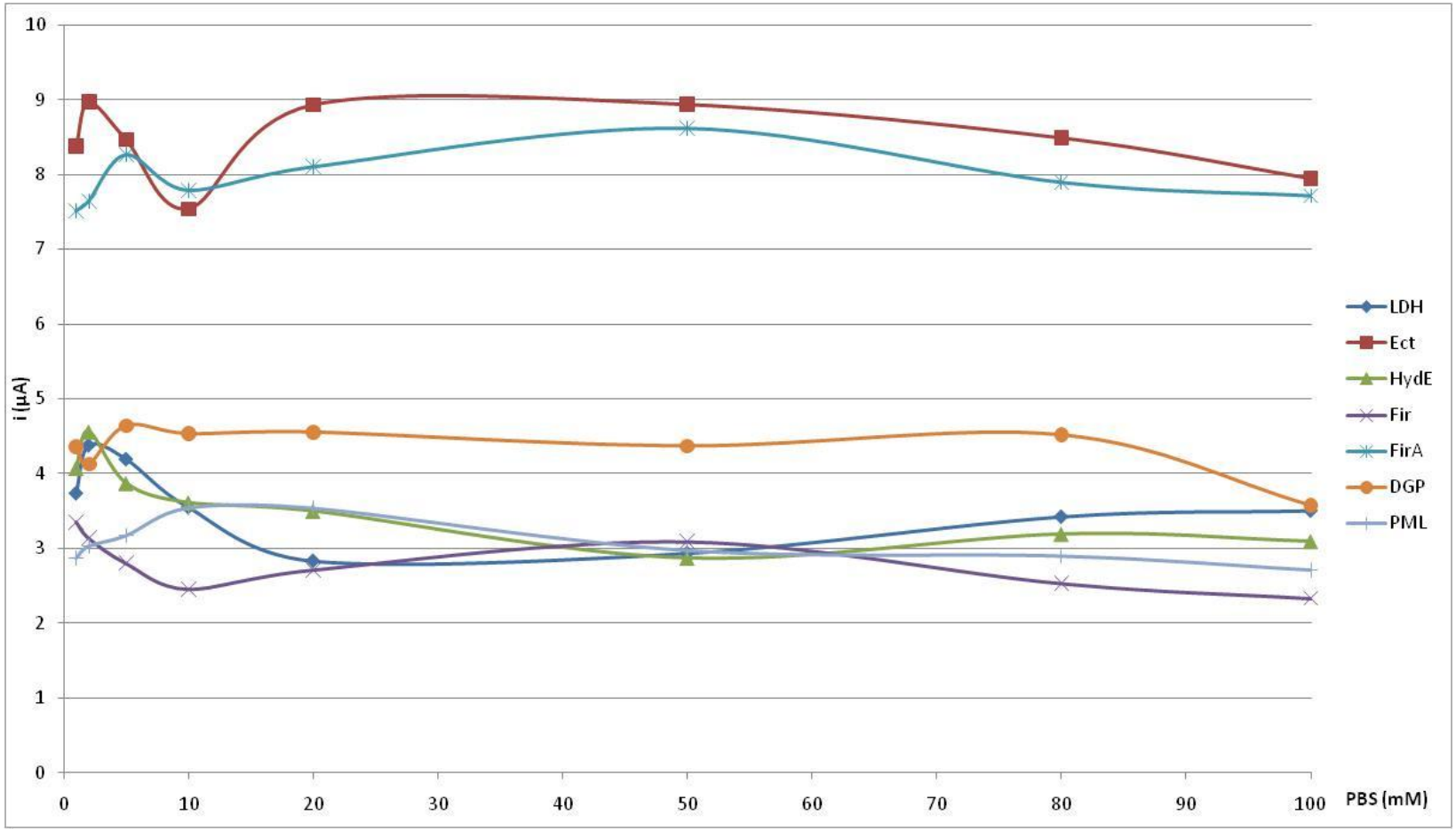
7.5.1. Alcohol oxidase biosensor



7.5.2. Acetylcholine esterase biosensor



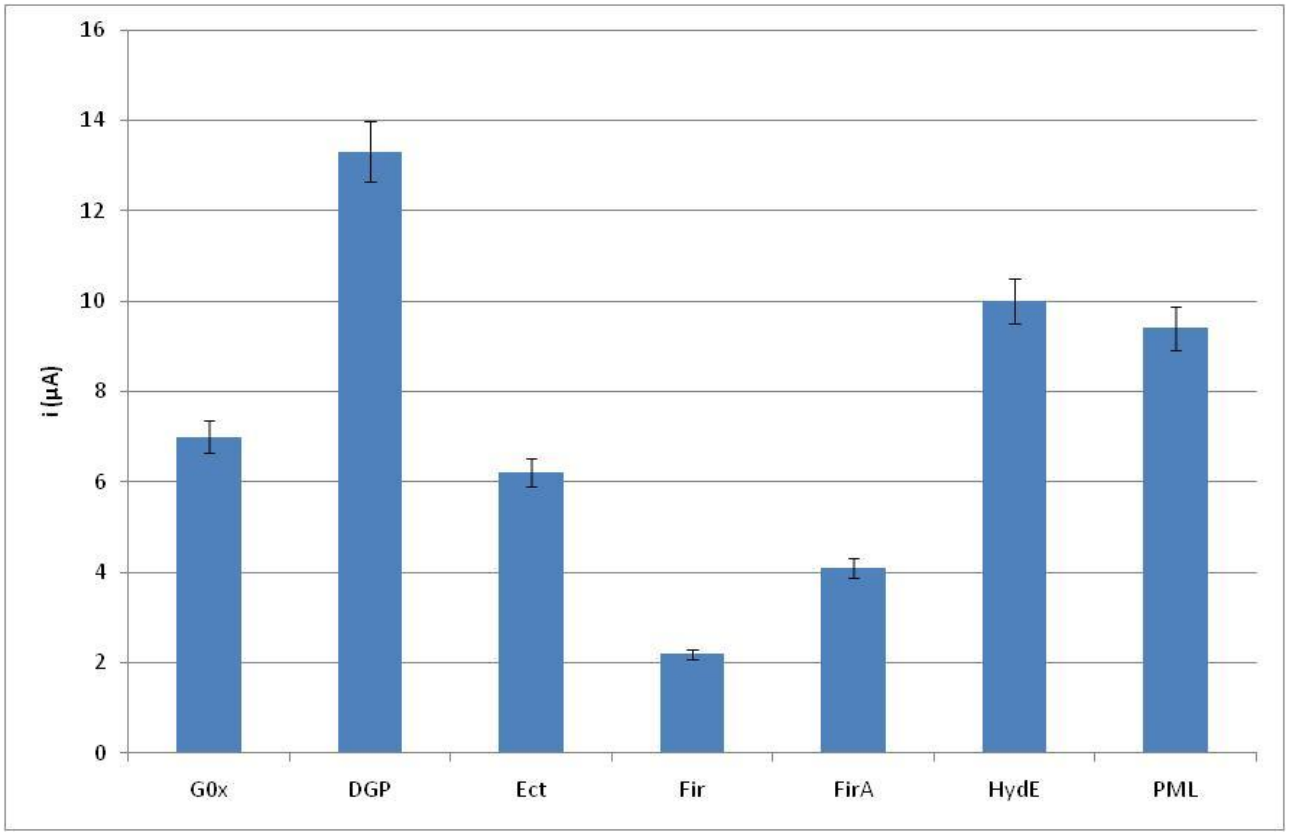
7.5.3. Lactate dehydrogenase biosensor



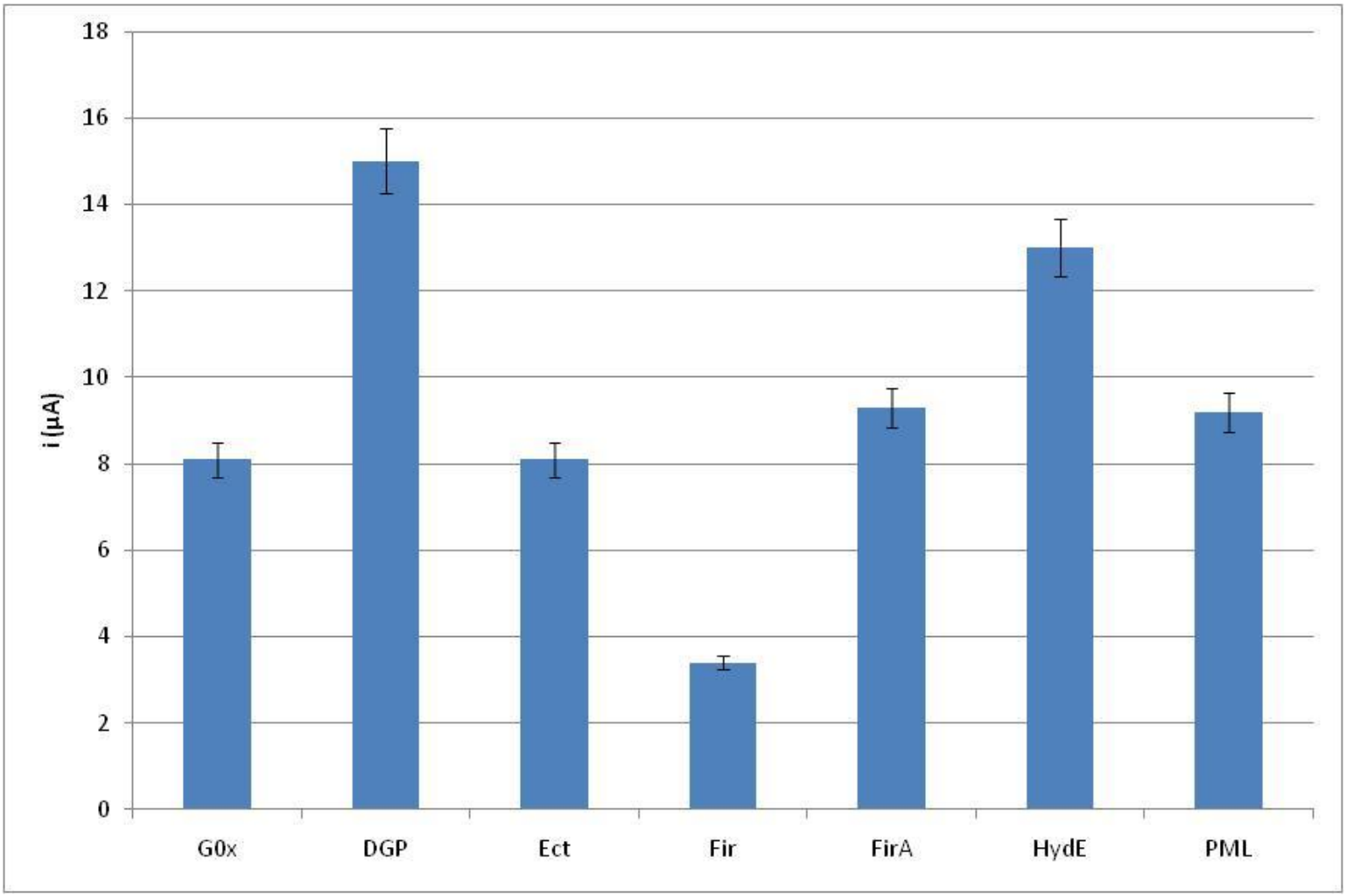
7.6. Influence of the hypersolutes on enzyme-based biosensors against storage (cf. p.161)

7.6.1. Glucose oxidase biosensor

7.6.1.1. Storing 14 weeks at 4°C

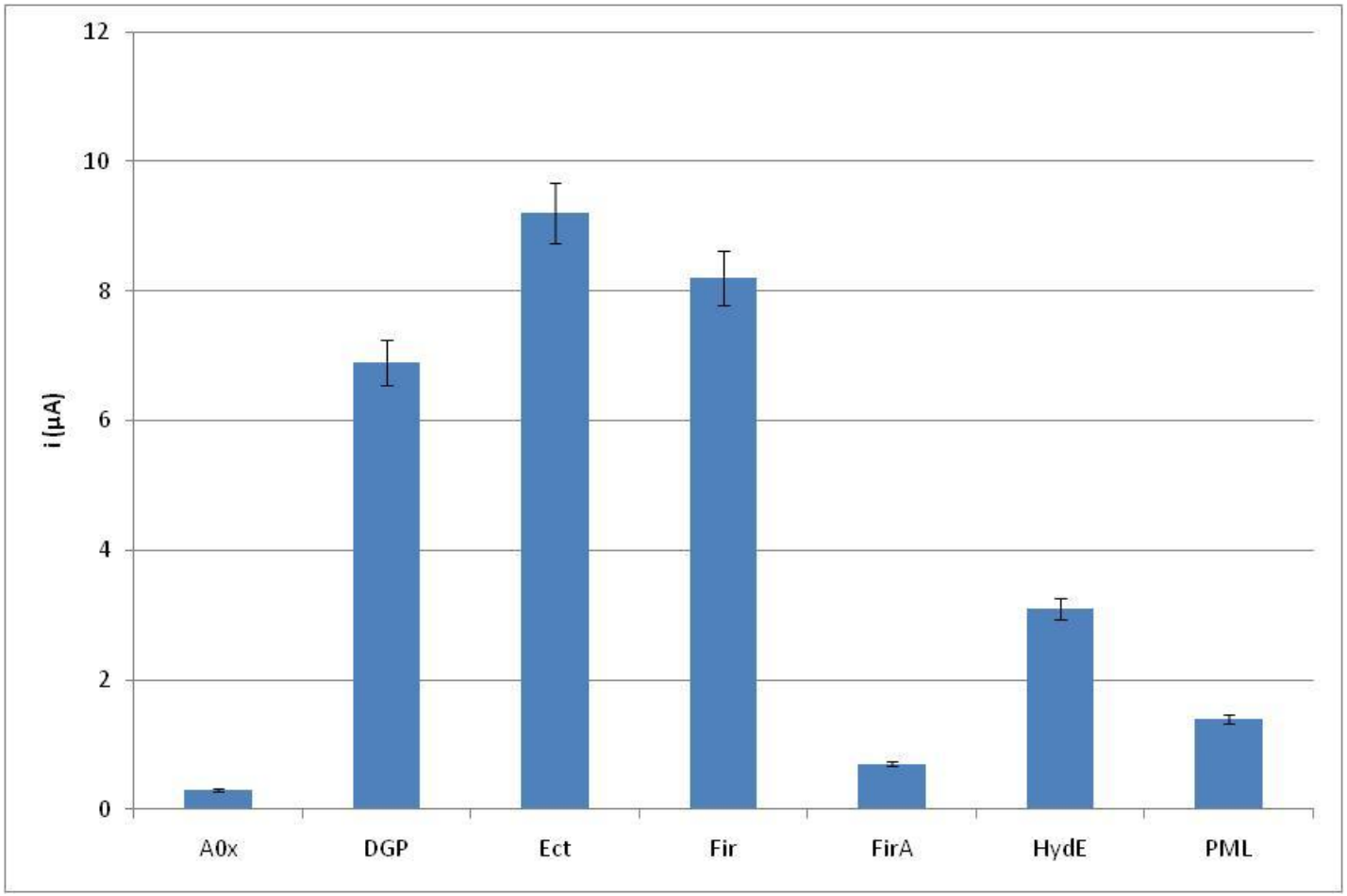


7.6.1.2. *Storing 2 months at room temperature*

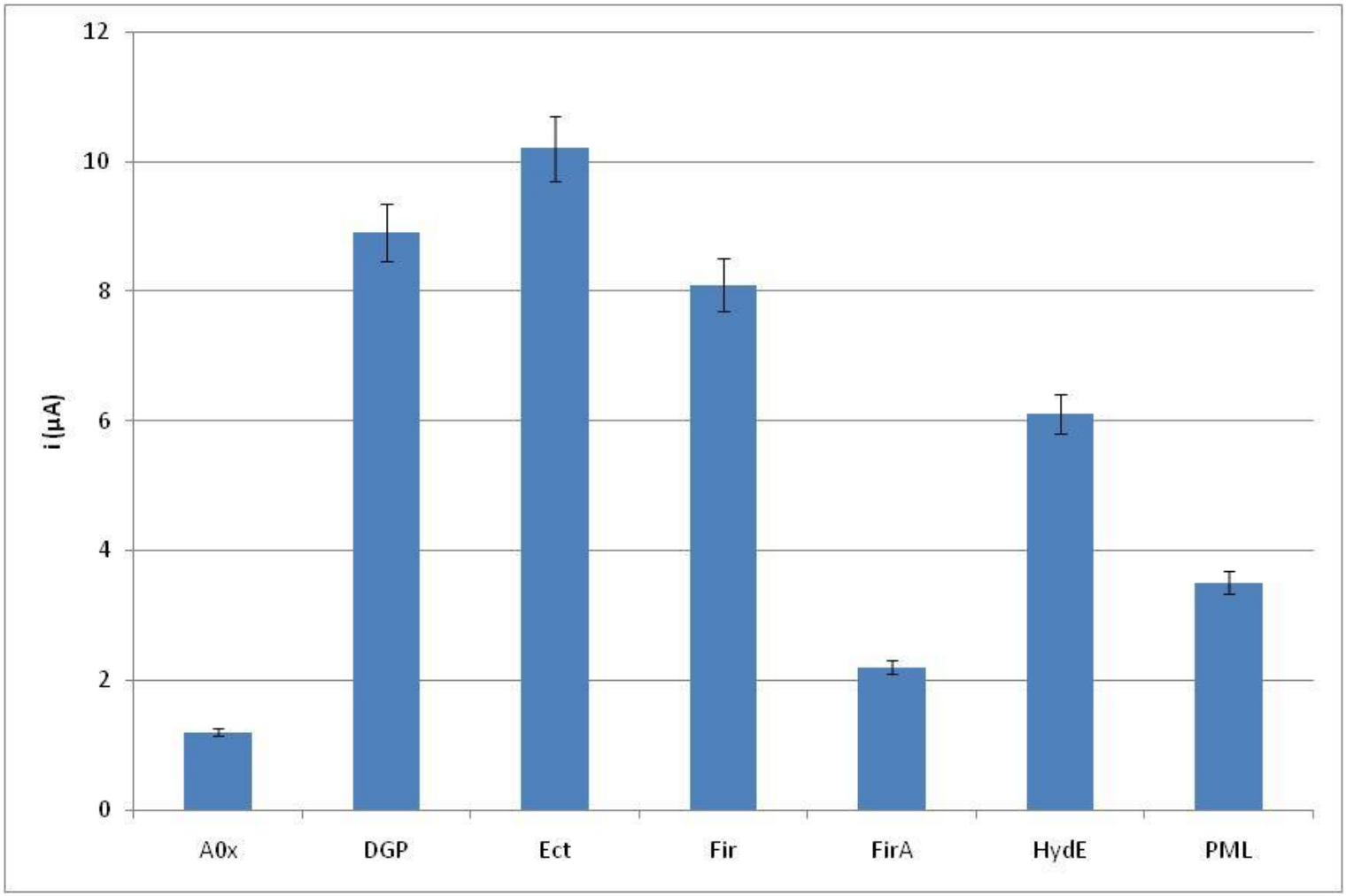


7.6.2. Alcohol oxidase biosensor

7.6.2.1. Storing 14 weeks at 4°C

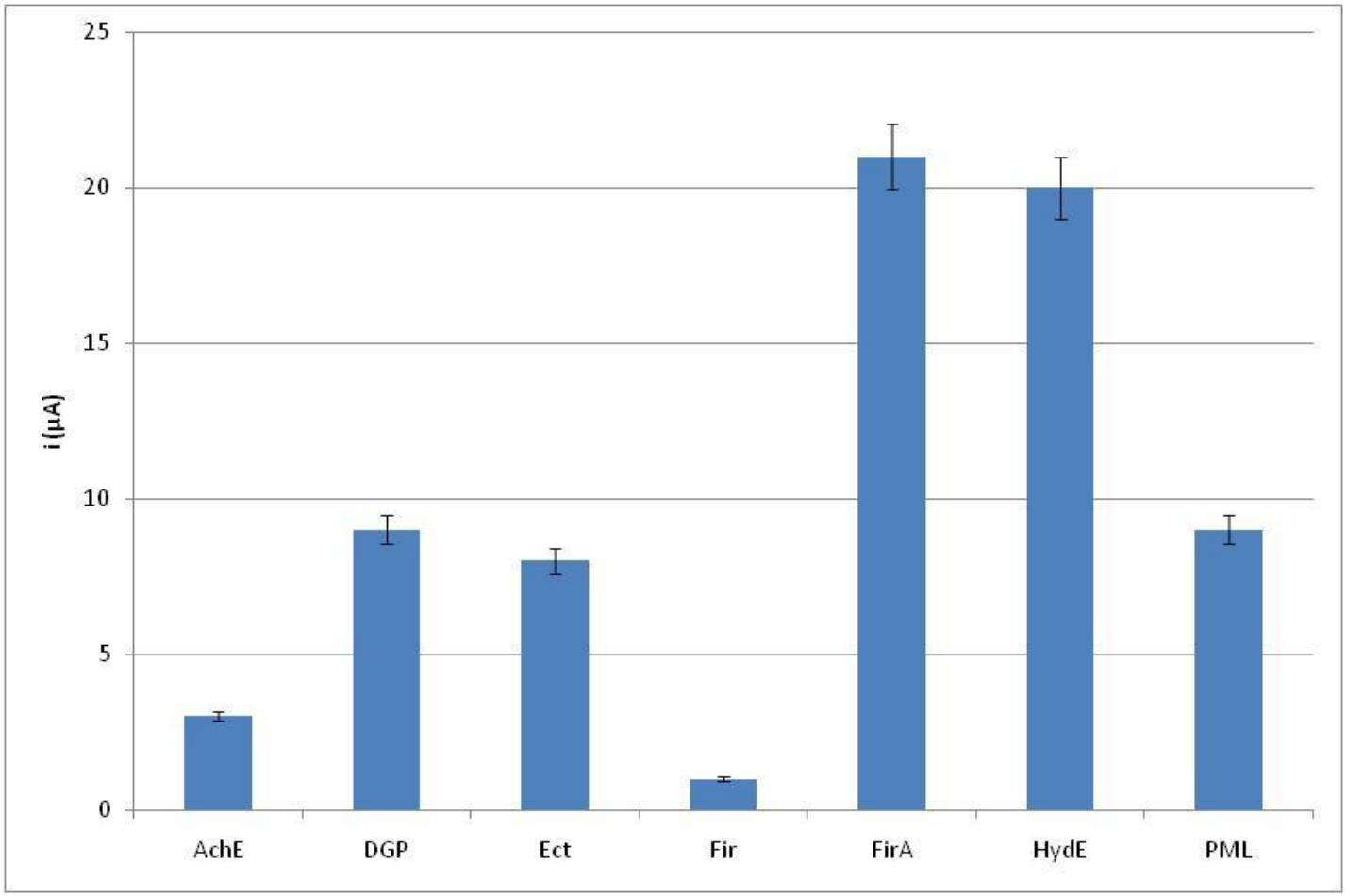


7.6.2.2. *Storing 2 months at room temperature*

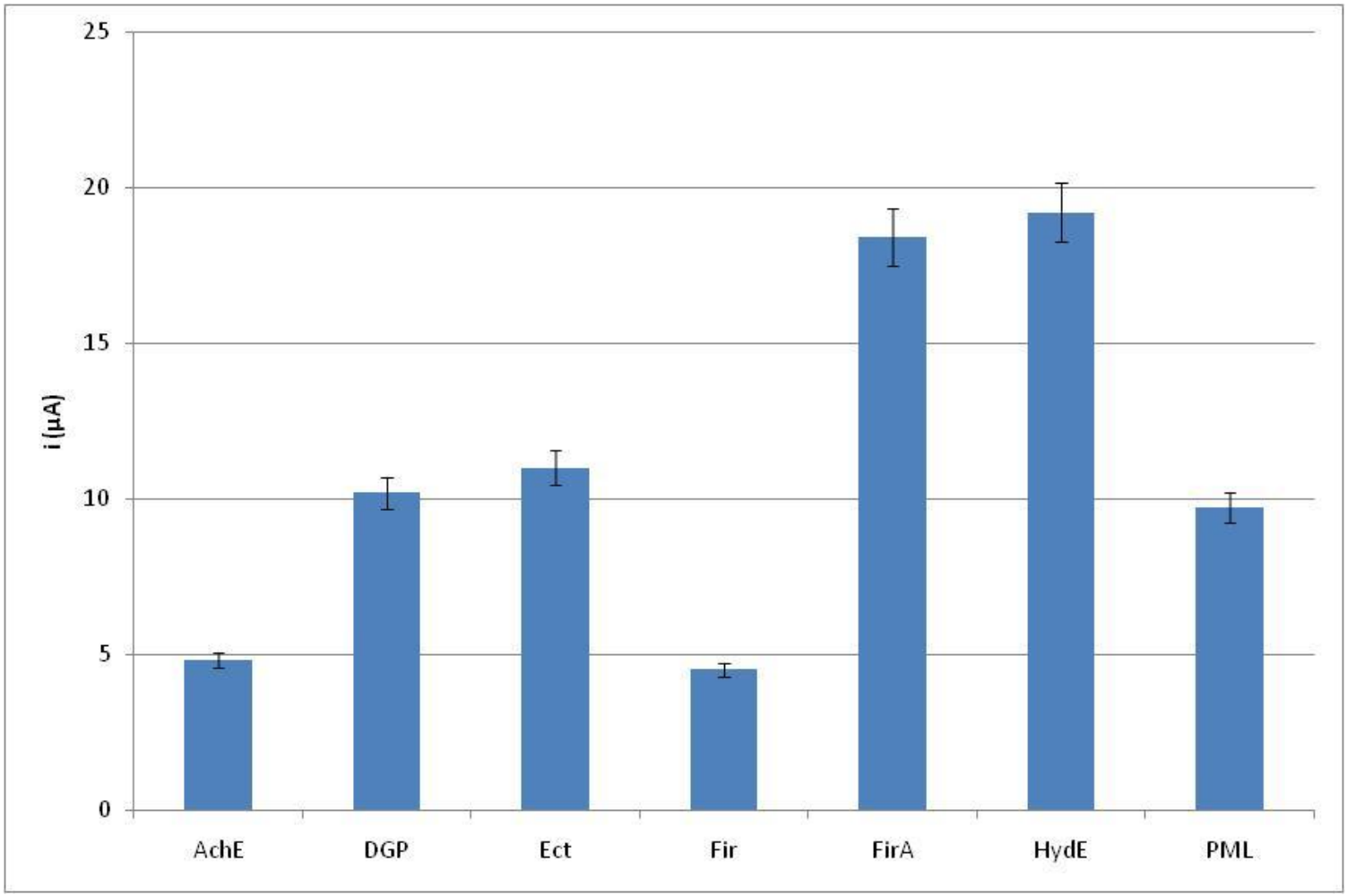


7.6.3. Acetylcholine esterase biosensor

7.6.3.1. Storing 14 weeks at 4°C

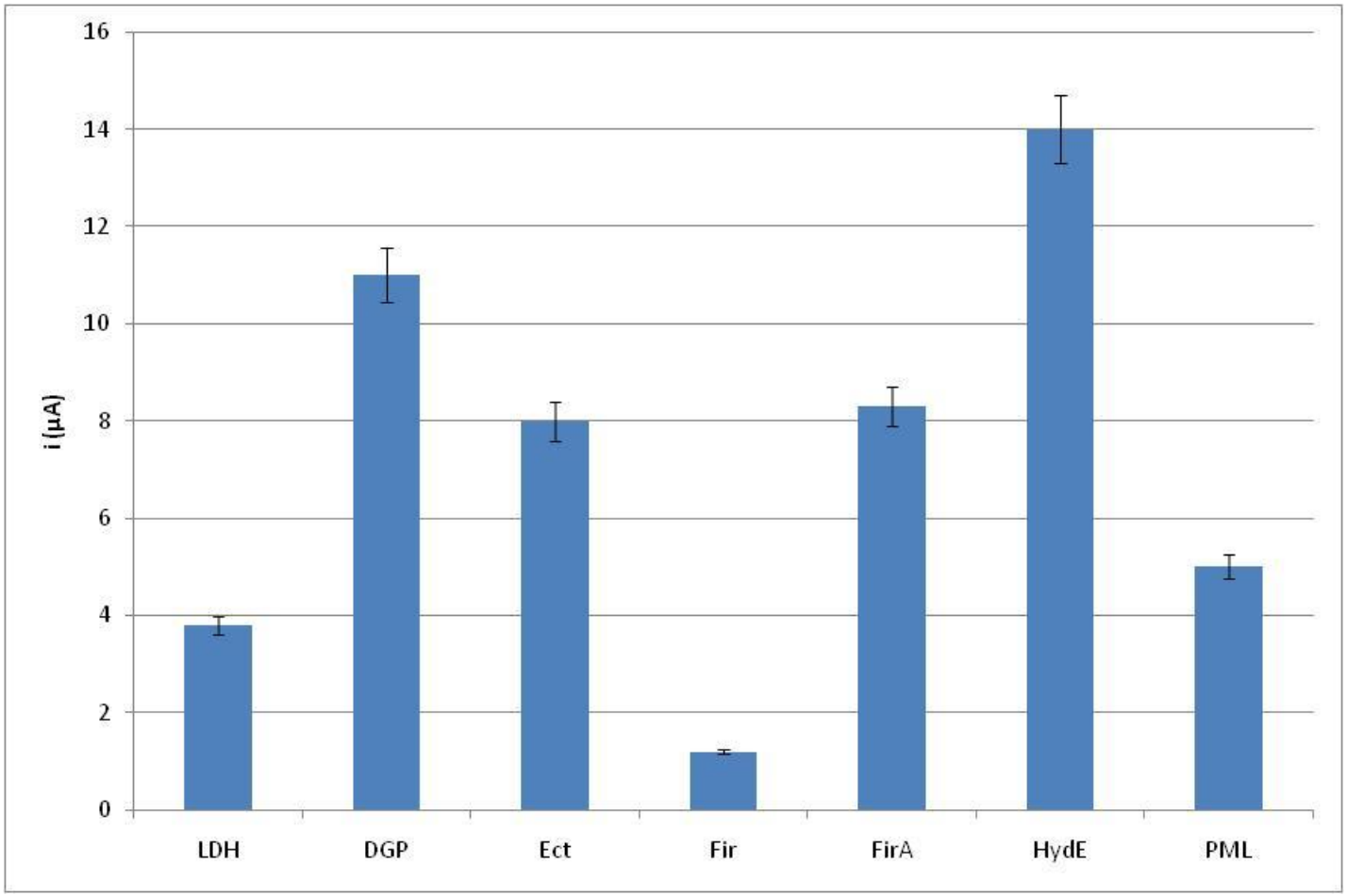


7.6.3.2. *Storing 2 months at room temperature*



7.6.4. Lactate dehydrogenase biosensor

7.6.4.1. Storing 14 weeks at 4°C



7.6.4.2. *Storing 2 months at room temperature*