# 1. INTRODUCTION AND OBJECTIVE OF THE RESEARCH

From the moment when the first biosensor for the determination of glucose was developed in 1962, the applications with biosensors have increased constantly, but not since the mid-90s there has been an increased recognition of the versatility and broad applicability of platforms based on piezoelectric acoustic biosensors (QCM- quartz crystal microbalance) for the analysis of molecular recognition and associated phenomena. Cooper, shows that during the period 2001-2005, there were a total of 1404 publications referenced as "quartz crystal microbalance" or QCM in the Web Science® database, of which 569 specifically involved molecular recognition studies *(Cooper 2007)*. The number of publications per year grew steadily in this period, from 241 in 2001, to 369 in 2005. The areas of application, as defined by the class of analyte were evenly distributed between life sciences research and diagnostic assay development, although interactions involving small molecules, immunoassays, lipids, oligonucleotides and polymer coatings were predominant *(Cooper 2007, Pavey 2002)*. Application to biological samples became possible when suitable oscillator circuits for operation in liquids were developed *(Nomura 1982)*. At the beginning, the relationship between mass adsorbed to the surface and resonant frequency of the crystal were only applicable in air or a vacumm *(Sauerbrey 1959)*. With apreciation in liquids, changes in density and viscosity, the sensor surface or withing the bulk solution have influence the frequency of the quartz.

A new category of QCM based biosensor application, is online observation of cell–substrate contacts or interactions with living cells growing on the quartz *(Heitmann 2007)*. Measurement of biochemical interactions or processes occurring in biological fluids is difficult in the traditional techniques. Accordingly, a lot of possible applications have been experimentally shown using QCM, amongst others, immunoassays *(Gerdon 2005, Halamek 2005, Michalzik 2005, Shen 2005)*, drugs screening *(Johnson 2001, Long 2001, Tan 2001)*, detection of viruses *(Amano 2005, Wu 2003)*, bacteriae *(Pohanka 2005, Su 2005)*, and eukaryotic cells *(Heitmann 2007)*.

The present work uses QCM technology to develop a groundbreaking technique for the real time analysis of the erythrocyte life cycle of *Plasmodium falciparum*, with special interest in the last six hours before the release of merozoites. Two main questions were asked: (I) Is it possible to measure changes in the frequency of the biosensor in association with merozoite release? (II) Once the merozoites have been released, will it be

possible to cause a reinfection of the healthy erythrocytes inside the biosensor system? Understanding these two processes allows several important statements about the mechanism of merozoite release and the invasion of new cells. Likewise, it also allows studying inhibitory effects on the release and reinfection of the erythrocytes of different inhibitory substances (protease inhibitors E64 and Leupeptin) as well as antimalarial drugs (Artesunate) and a lead compound of natural origin with possible biological activities on the cells (Chlorotonil).

In the year 2002, when the genome sequence of *P. falciparum* was completed, many of the barriers to perform state-of-the-art molecular biological research on malaria parasites were eliminated. Although new licensed therapies may not yet have resulted from genome-dependent experiments, they have produced a wealth of new observations about the basic biology of malaria parasites, and it is likely that these will eventually lead to new therapeutic approaches *(Winzeler 2007)*. These observations of the basic biology of *P. falciparum* (invasion of merozoites, liberation of merozoites and traffic of proteins from the parasites to erythrocyte) have led to the use of new microscopic and molecular techniques. Another possibility is opening up with the use of QCM for the study of malaria.

The real-time study of the erythrocyte cycle using QCM is first presented with the characterisation and optimisation of the biological layer (used for immobilisation of a cell on gold electrode) and the observation of the biosensor signal for 48 hours (Chapter sections 4.1 and 4.2). The observation of infected and non-infected erythrocytes inside the biosensor system is described in chapter sections 4.3 and 4.4. The comparison obtained in the signal once the merozoites are released is presented validating the method with external analyses through flow cytometry and Transmission Electron Microscopy (TEM). The reinfection of the healthy erythrocytes inside the equipment on second quartz inside the system containing healthy erythrocytes is presented, while the efficiency of this technique is evaluated (chapter section 4.5). In addition, the viability of the merozoites is presented, comparing them to other isolation techniques. Finally, the study of the merozoite inhibition using inhibitor proteases (E64 and Leupeptin), an antimalarial drug (Artesunate) and a recently isolated substance of natural origin (Chlorotonil) is described (chapter section 4.6.).

# 2. THEORY

This chapter describes the theoretical aspects, as a basis for the study of the erythrocyte cycle with QCM. The first section of the chapter will deal with the biosensor theory: mechanisms of biosensor, piezoelectric effect, piezoelectric crystal, relationship between added mass and frequency shift and biosensors in cell biology and pharmacy. The second section will deal with epidemiology of the malaria disease, the biology of the parasite, describing the characteristic features of the invasion, release of merozoites of the *P. falciparum*, chemotherapy and drugs resistance. The third and final part will deal with the biological layer for the use with Quartz Crystal Microbalance.

## 2.1. BIOSENSOR

A biosensor is an analytical tool consisting of biologically active material used in close conjunction with a device that will convert a biochemical signal into a quantifiable electrical signal *(Kumar 2000)*. Biosensors have many advantages, such as their simple and low-cost instrumentation, fast response times, minimum sample pre-treatment, and high sample throughput.

### 2.1.1 Mechanism of the biosensor

The receptor (biological part) is responsible for the selectivity of the sensor. The detector (physical transducer), translates the physical or chemical change by recognizing the analyte and relaying it through an electrical signal. The detector is not selective: the biological sensing element selectively recognizes a particular biological molecule through a physical or chemical process, specific adsorption, or a reaction, and the transducer converts the result of this recognition into a usable signal, which can be quantified *(Keusgen 2002, Kumar 2000)*.

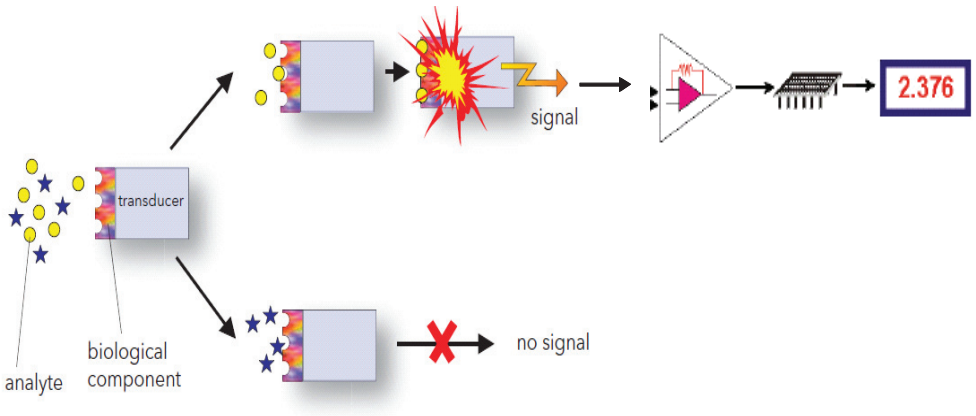


*Figure 2-1. Principle of the function of a biosensor. One compound (circles) of a mixture of substances specifically interacts with the biological part of the sensor. The resulting biological signal is converted into a physical signal (e.g. electric, optical or electrochemical) by a transducer. The signal is amplified, processed and displayed. Substances incapable of interacting with the biological component will not produce any signal. Modified from (Bergeret 2004, Keusgen 2002)*

As exemplified in Figure 2-1, a biosensor associates a bioactive sensing layer with any suitable transducer giving a usable output signal. Biomolecular recognition can be defined as the possibility of detecting analytes of biological interest, like metabolites, but also including drugs and toxins, using an affinity receptor which can be a natural system or an artificial one mimicking a natural one, able to recognize a target molecule in a complex medium among thousand of others *(Blum 1991)*. To obtain a quantified output signal (correlated with the amount or concentration of the analyte present in the medium) multiple events must take place sequentially. Briefly, a first chemical or physical signal consecutive to the molecular recognition by the bioactive layer is converted by the transducer into a second signal, generally electrical, with a transduction mode that can be electrochemical, thermal, optical or based on mass variation *(Blum 1991)*.

Table 2-1 summarizes a variety of biosystem-transducer combinations in terms of transducer, measurement mode and potential application.

*Table 2-1 Biosensor Components (Kumar 2000)*

| ***Transducer System*** | ***Measurement Mode*** | ***Typical Applications*** |
|---|---|---|
| Ion-selective electrode | Potentiometric | Ions in biological media, enzyme electrodes |
| Gas-sensing electrodes | Potentiometric | Gases, enzymes, organelle, cell or tissue electrodes |
| Field-effect transistor | Potentiometric | Ions, gases, enzyme subtrates, immunological analytes |
| Optoelectronic and Fiber OpticDevices | Optical | pH, enzymes, immunological analytes |
| Thermistors | Calorimetric | Enzymes, organelle, gases, pollutants, antibiotics, vitamins |
| Enzyme electrodes | Amperometric | Enzymes, immunological systems |
| Conductivity meter | Conductance | Enzyme substrates |
| Piezoelectric crystals | Acoustic (mass) | Volatile gases and vapors, antigen/antibody systems and other ligands/receptors |

2.1.2. Analytical Applications of Piezoelectric Crystal Microbalance

2.1.2.1 Piezoelectric Effect

During their work on the discovery of radium, the Curies employed what they called a *quartz crystal balance*, with the first application of a piezoelectric device as a chemical sensor. Nowadays, the so-termed *quartz crystal microbalance* technique is well established in non-biological applications. The term piezoelectric describes the generation of electrical charges on opposing surfaces of a solid material upon deformation (torsion, pressure, bending, etc.) along an appropriate direction *(Janshoff 2001)*.

Although a large number of crystals exhibit piezoelectricity, only quartz provides the unique combination for measuring mechanical, electrical, chemical, and thermal properties to make it usable *(Janshoff 2001)*.

The core component of the device is a thin quartz disc, which is sandwiched between two evaporated metal electrodes used in this thesis, and is commonly referred to as *thickness shear mode resonator* (TSM resonator) or *bulk acoustic wave sensor* (BAW). As this quartz crystal is piezoelectric in nature, an oscillating difference in the potential between the surface electrodes leads to corresponding shear displacements of the quartz disk. The mechanical oscillation responds very sensitively to any changes that occur at the crystal surfaces *(Janshoff 2001)*.

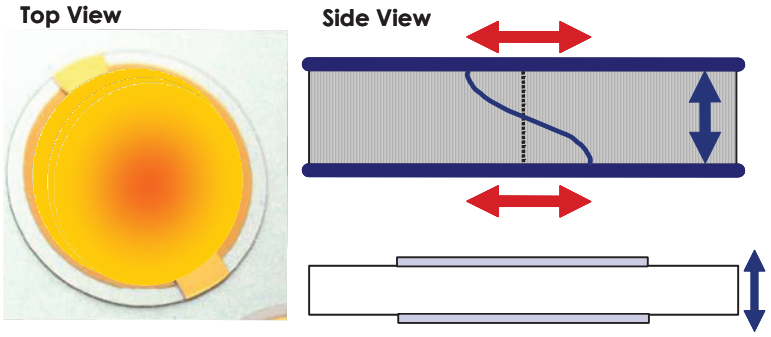


*Figure 2-2. Mass-sensitive devices and acoustic wave propagation modes. Black arrows indicate particle displacement, white arrows wave propagation Modified from (Kaspar 2000)*

Crystals have a natural vibration frequency, called resonant (or fundamental) frequency that depends upon their chemical nature, size, shape, and mass. Each vibration of a crystal involves its passing from a deformed configuration through its equilibrium configuration to an oppositely deformed configuration, and then back through the equilibrium configuration to the original configuration. This cycle occurs repeatedly as long as the crystal is vibrating *(Blum 1991)*. The most well-known piezoelectric material is quartz ($SiO_2$). When the vibrating crystal is piezoelectric, this cycle of oscillating deformity produces an oscillating electrical field; the frequency of the electrical oscillation is identical to the vibration frequency of the crystal *(Blum 1991)* .