Detection of vital bacteria and protein ligand binding using the QCM/HCC

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ABSTRACT

Quartz Crystal Microbalance/ Heat Conduction Calorimetry (QCM/HCC) is a new sensor platform that enable simultaneous measurement of mass accumulation, rheological changes and heat flow due to a surface process. In this work we examine the applicability of the QCM/HCC for biological systems. We studied the binding of warfarin to Human Serum Albumin (HSA) and the growing of *Escherichia Coli* on the QCM surface coated with agar. A significant changes in all three parameters are observed, in both cases. We demonstrate that the QCM/HCC is a powerful platform for detection and studying biological systems.

INTRODUCTION

The QCM/HCC is a new technique for studying surface phenomena occurring at the solid/gas and solid/liquid interfaces. The technique combines two complementary methods – *Q*uartz *C*rystal *M*icrobalance and *H*eat *C*onduction *C*alorimetry. The first provides data on the mass change and changes in the viscoelastic properties, while the latter measures the heat generated / consumed by the surface process. Direct measurement of heat, mass change, and changes in mechanical properties provides thermodynamic and kinetic information, as well as rheological properties in some cases. The QCM/HCC can be used both as a technique for characterizing interfacial processes and as a sensor.

Over the last decade there has been an increasing need for the analysis and detection of biomolecular interaction. Better characterizing of protein-protein interaction and protein-drug interaction results in understanding the driving force for biological processes and in the development of new drugs. In this work we show that the QCM/HCC is very sensitive for the analysis of small amount and concentration of biologically interacting species. Moreover, the technique does not require labeling of the protein/drug studied, so no chemical modification are required. Thermodynamic information of interfacial processes is a unique feature of the system. The data can also suggest conformational changes on the surface.

The QCM/HCC can also serve as an advanced biosensor in which the detection of the analyte is based not only on one signal, but on the response of the three measurable parameters. That fact is important in real environments when other surrounding materials can interfere one or more signals in different ways.

Measurement in the gas phase for the determination of the rheological properties of polymers were published elsewhere (1-3). Our aim in this work is to validate the QCM/HCC technology to biological application by demonstrating that it could provide thermodynamic and other information for the introduction of human serum albumin (HSA) with an organic ligand. The drug tested in this work, warfarin, is an anticoagulant used for treatment of increased risk for blood clots. The system of HSA/warfarin is well studied (4-9 and references within) and therefore we can compare the results obtained with the results of other techniques. Cysteamine was used as a linker to attach the HSA covalently to the gold electrode on the quartz crystal (6).

Although warfarin is a small molecule, pronounced mass, motional resistance and heat changes were observed. Significant signals were obtained even at very low warfarin concentration, at the micro-molar range. Frequency change is found to depend on the bulk warfarin concentration. Motional

resistance data shows a significant variation as the warfarin was added, suggesting a possible conformational change. Heat generated by interfacial process was also obtained.

We propose that the QCM/HCC is applicable for studying protein/drug interaction. This high precision technique is sensitive for characterizing the binding properties.

A second biological application is the study of vital bacteria on surfaces. The heat produced by the bacterial metabolism can be used as a indicator of the growth. The QCM/HCC can give simultaneous information on the mass change on the surfaces due to the bacterial growth and the the heat generated by the growth process. Measurements of the QCM/HCC are fast and direct, in contrast to conventional measurements (such as pour plate count) that are time consuming, and less informative.

We studied the growth of *Escherichia Coli* on agar media. This is a challenging systems due to the small amount of the heat expected to be generated. A thin agar film was applied to the crystal surface and then inoculated. Heat and mass change were measured for at least 24 hours. The QCM/HCC is shown to be very sensitive tool, measuring heat generated in the range of micro-Watts and mass change in the range of nano-grams. Heat is found to be in phase with the mass change at the initial stages of the growth.

The data provided from this technique can yield information on the growth process, as well as the effectiveness of antimicrobial agents.

EXPERIMENTAL

The QCM

As was mentioned in the introduction, the QCM/HCC is a combination of the techniques quartz crystal micro-balance and Heat conduction Calorimetry.

QCM is based on a piezoelectric crystal, that oscillates in the thickness shear mode. The response of the crystals to external perturbations, such as absorption, follows the Sauerbrey equation (10), in which the change in frequency is inversely proportional to the mass change:

$$\Delta f / f_0 = -2 f_0 \Delta m / (\rho\mu)^{0.5} \qquad [1]$$

where Δf is the change in frequency, f_0 corresponds to the fundamental frequency of the crystal, Δm is the change in mass per unit area and ρ and μ the density and the shear modulus of the quartz.

The Sauerbrey equation holds for piezoelectric sensors such as the QCM when the thickness of the adsorbed film is small compared to the crystal thickness (i.e.~1%), and when the crystal is in contact with a gas.

The frequency may also shifted due to variation in the viscoelastic properties of the layer on the surface, the temperature and the pressure. Whereas the temperature and the pressure could be controlled easily, the changes in the viscoelastic properties are inherently related to the process occurred on the surface. Less rigid layer on the QCM surface, increases the dependency of the frequency shift on the viscoelastic properties of the layer (11,12).

Indication of the changes in the viscoelastic properties are measured through the motional resistance. A non rigid layer results in energy losses and therefore high motional resistant value.

The Heat Flow Sensor

The heat flow calorimetry is base on the measurement of thermal power, flowing from the sample to a heat sink as a function of the time. The total heat associated with the process is just the time integral of the thermal power.

The Thermal power, P, is measured by a thermoelectric module which follow the Tian equation :

$$P=1/S[U+\tau(dU/dt)] \qquad [2]$$

where S is the thermopile sensitivity ($V \cdot W^{-1}$), U is the thermopile voltage and τ is the time constant of the calorimeter. At steady state, $U=S \cdot P$, and the output voltage is proportional to the thermal power dissipated on its surface. The time constant is the ratio of vessel heat capacity and the thermal conductance of the thermopile. In our case τ=55sec.

The Apparatus

A sketch of the QCM/HCC is shown in figure 1. The apparatus contain two identical sensors – reference and sample. The gases / solution introduced to the system through a flow cell. A further description of the system is given elsewhere (3).

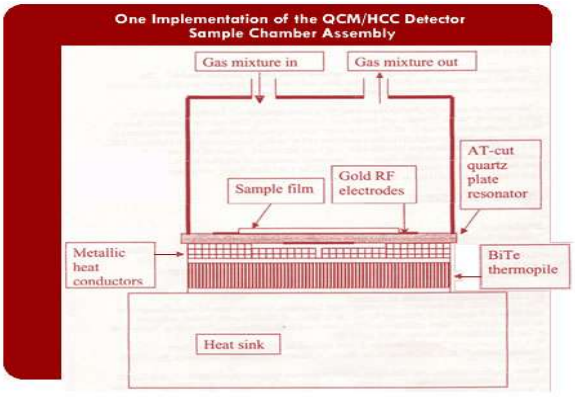

Figure 1

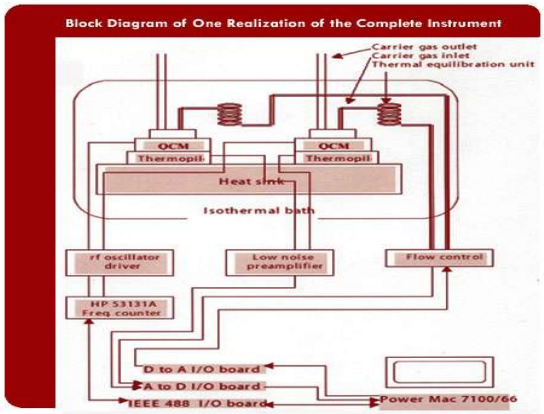

Figure 2

Figure 1. Sketch of the QCM/HCC apparatus (taken from Masscal Co. website)

Crystal Preparation

AT – cut, polished 5MHz piezoelectric quartz crystals microbalance (Maxtek Inc.) were incorporated to the HCC/QCM flow system. The crystals are disks 2.54 cm in diameter and 0.332 mm thick,with gold electrodes evaporated on the top and bottom.
Only one side of the crystal was exposed to liquid/gas flow in the flow cell.

HSA Immobilization Procedure

The electrode exposed to the liquid flow was cleaned with acetone, ethanol and water then coated according to the following procedure (6):

1. The electrode was immersed in a 0.1% solution of cysteamine (Sigma) in water overnight prior to being treated with 2.5% GA(Sigma) in PBS(Sigma) buffer for 1 h.
2. The device was washed with water and dried and then placed into HSA(Sigma) solution for about 1h. The protein solution was composed of 1 mg/ml.
3. The device was then dried and placed into the HCC/QCM.
4. The buffer solution used was 5%DMSO in DPBS. Warfarin(Sigma) in 5% DMSO in DPBS was

injected to the system several times with different warfarin concentrations (the binding process is reversible and the warfarin can be dissociate from the HSA with buffer flowing).
The flow rate was 50µL/min. The stabilized temperature was 25ºC.

Preparation of Crystals Coated with Agar

The crystals were cleaned in Piranha solution for 5 min and then washed many times in distilled water. In order to make the gold surface hydrophilic to obtain a uniform agar coating, one face of the crystal was immersed in 1mM 3-MPA in ethanol solution for about 16 hours and then rinsed with water and ethanol. The QCM's were sterilized and coated in a sterile environment at 60°C with solutions of Luria broth agar and growth medium of appropriate viscosity to produce uniform coatings. The agar was prepared from agar powder, with sterile distilled water at powder concentration of 15 grams per liter of water. The crystal was heated on a small Petri dish, containing hot water,to about 60 ºC when the agar drop was applied to the surface. The crystal and the dish were allowed then to cool naturally in order to minimize internal stress in the agar film. Coating thicknesses were determined by mass change of the QCM. Since viscous media such as agar will damp the QCM resonators, the optimum thickness was determined by ascertaining that cells will grow for the duration of the experiment without using up the medium. The mass of the agar on the surface,determined both with the QCM and with a balance, was found to be 305 µg, with good agreement between the methods. A small change (1 ohm)in the motional resistance accompanied the agar addition to the surface.

The cells studied were *Escherichia co*li. The behavior of the agar itself, without *E.col*i, was measured in the QCM/HCC for 24 hour in order to establish baseline signals. The measurements were taken at 25 ºC. Air (aerobic condition) or nitrogen (anaerobic condition) with 50% relative humidity was flown above the crystals exposed surfaces at flow rate of 10ml/min. Then the crystal was removed from its chamber and inoculated with *E.coli* from a stock in a sterile environment,and returned immediately to the chamber. A second 24 hour measurement at the same conditions was taken.

RESULTS AND DISCUSSIONS

Detection of Warfarin Binding to an Immobilized Film of Human Serum Albumen

The results obtained for the adsorption/desorption of three warfarin concentrations are shown in figures 2 and 3.

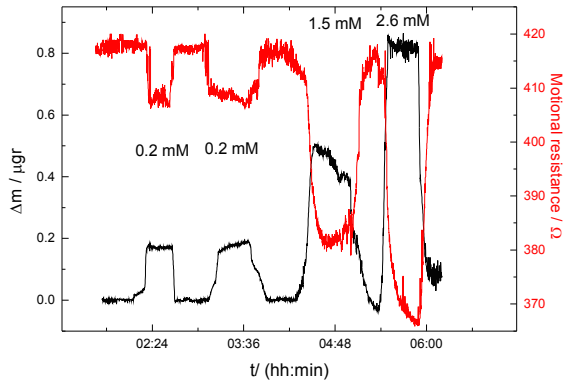

Figure 2. Changes in mass and motional resistance due to three concentrations of warfarin adsorption/desorption.

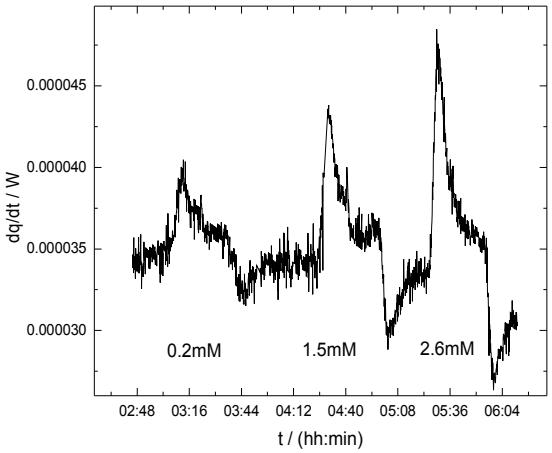

Figure 3. Changes in thermal power due to three concentrations of warfarin adsorption/desorption.

Graphs 2 and 3 show the pronounced signals obtain in the three parameters measured – thermal power,mass change and motional resistance. The Thermal power signal was calculated from the Tian equation from the thermopile voltage signal. All the signals are in phase with each other: changes in the thermal power followed the changes in mass and motional resistance. The thermal power and mass signals increase with the increase of warfarin concentration while the motional resistance decreases as the concentration increases. The big changes in the motional resistance suggest that the binding process is followed by conformational changes in the HAS film on the crystal surface.

There are two sources for the obtained thermal power. The binding of warfarin to the HSA and the heat generated by the QCM itself. The later should be subtracted from the thermal power signal in order to have the heat generated by the reaction. Using the motional resistance measured and the equations 3 and 4 (given by the crystals manufacturer) the QCM contribution was calculated as can be shown in figure. 4. The signal after the subtraction is given in Figure 5.

$$dq/dt_{crystal}=(V_{soc})^2(V_{cond}/100)(1- V_{cond}/5) \quad [3]$$

$$V_{cond}=100/(R+20) \quad [4]$$

Where V_{soc} is the open circuit crystal drive voltage, for the this system V_{soc}=0.125V. R is the motional resistance value.

Figure 6 shows the integrated heat for each adsorption/desorption step. The fact that the integrated heat increases as the warfarin concentration increase means that the surface sites for warfarin adsorption are not being saturated at these concentrations.

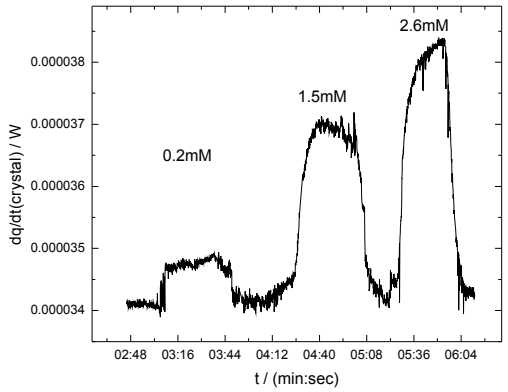

Figure 4. The calculated thermal power generated by the crystal

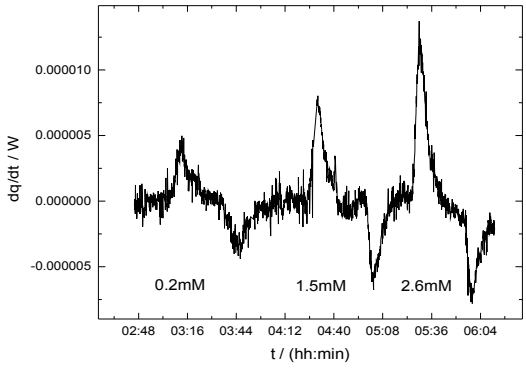

Figure 5. The net thermal power for the binding process

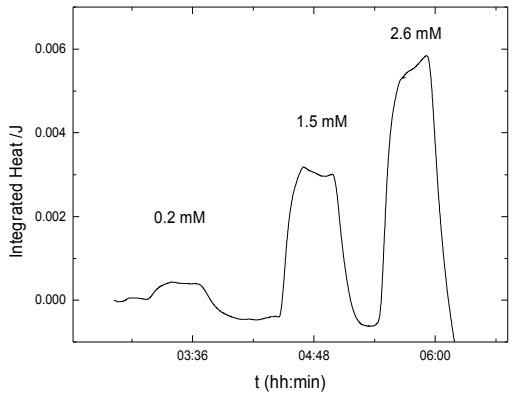

Figure 6. The integrated heat change for the adsorption/desorption of three concentrations of warfarin.

Although the integrated heat show linear dependence with the measured “mass”increase (Figure 7), the attempt to calculated the enthalpy results in higher value (by 10) than expected (5,7). In other words, in order to obtain the literature enthalpy data the mass change should be a factor of ten higher than the measured mass measurement.

Two main reasons contribute to this “missing”mass effect, which are well discussed in the literature:

1.The Sauerbrey equation is not consistent with the large viscoelastic damping caused by the immersion of the crystal in solution. For pure water, any film thicker than 0.25 mm produces an identical frequency shift because the acoustic wave generated by the crystal does not penetrate further into the solution. The Sauerbrey equation assumes that the film on the surface is rigid. However,the HSA may be non rigid to some extend. All those factors and their contribution to the missing mass effects are discussed in (11,12).

2.When warfarin adsorbs to the HSA its adsorption is not the only process taking place. Water molecules are rearranged and desorbed from the protein film. This explanations supported by the decrease in the motional resistance upon adsorption. Many other works report similar phenomena in which water desorbed from surface due to adsorption or on the effect of trapped water in the adsorption process (13-15).

A better quantitative analysis of these results will be discussed elsewhere.

These results with a previously characterized protein-ligand binding system are the first measurements to be taken of the energetics of ligand binding to thin protein films. It is clear that the QCM/HCC is sensitive enough to detect and to characterize the
adsorption of a small molecule such as warfarin on a protein.

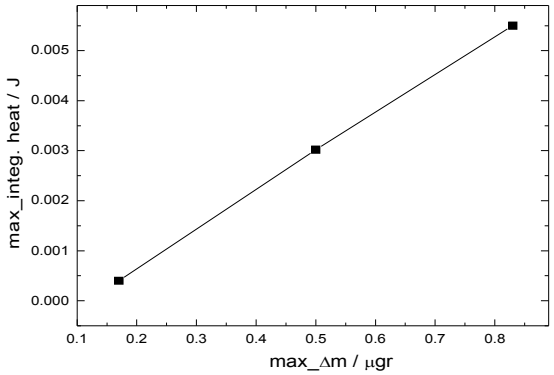

Figure 7. The dependence of the maximum integrated heat measured with the maximum “mass”change measured.

Detection of Vital Bacteria

The first attempt to detect *E.Coli* on the crystal coated with agar was in aerobic environment. Figure 8a. shows the mass change on the QCM surface in two cases:(a)only agar is on the crystal,and (b) the agar film was inoculated with *E.Coli*. While almost no mass change is observed during the 24-hour measurement with agar alone,a mass increase of 3.7 μg (1.2%of the agar mass)is observed in the case where *E.Coli* was added to the agar film.

The increase of mass is clearly not exponential at early times,and eventually the mass stops changing.

Figure 8 b shows the change in motional resistance upon inoculation. This variable is a measure of the

viscoelastic damping of the QCM by the film. The inoculation process causes an initial step increase in damping as the soft viable bacteria contact the growth medium,and as the bacteria replicate there is an increase in damping.

The thermal power generated during this process is shown in figure.8c. Inoculation of *E.coli* produces immediate heat generation from metabolic activity. As the mass on the surface increases the thermal power decreases, but the rate of change of mass is not proportional to the thermal power,as can be seen in figure 9.

In another experiment,the same procedure was repeated,but in an anaerobic environment (pure nitrogen, 50%RH). As expected no change was observed during this period in all three signals. After 24 hours in this environment we change the atmosphere to air at 50% RH. Approximately two hour later a similar response to the previous experiment was observed :mass increase, small increase in motional resistance and similar exothermic change in the thermal power (figure 10).

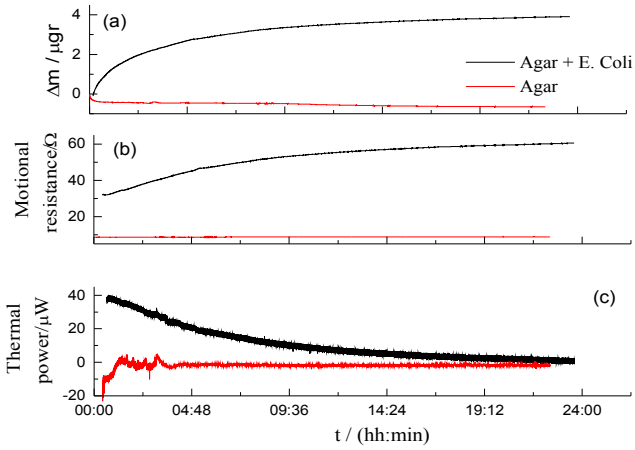

Figure 8. Changes in mass,motional resistance and thermal power when an agar film is inoculated and not inoculated with *E.coli in* air at 25°C,50%RH.

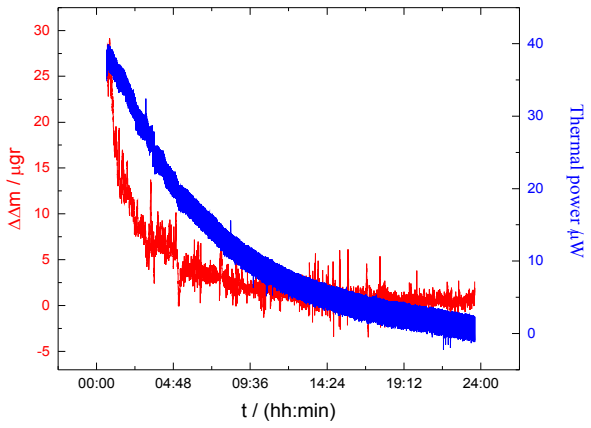

Figure 9. The rate of change of mass and thermal power when an agar film is inoculated with *E.coli* in air at 25°C,50%RH.

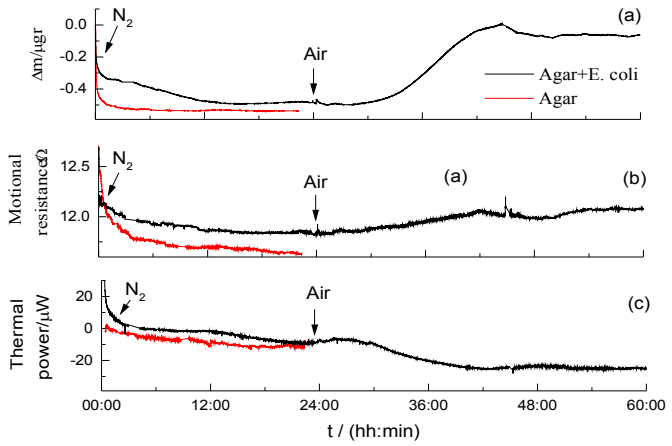

Figure 10. Changes in mass,motional resistance and thermal power when an agar film is inoculated and not inoculated with *E.coli*,anaerobically and then aerobically.

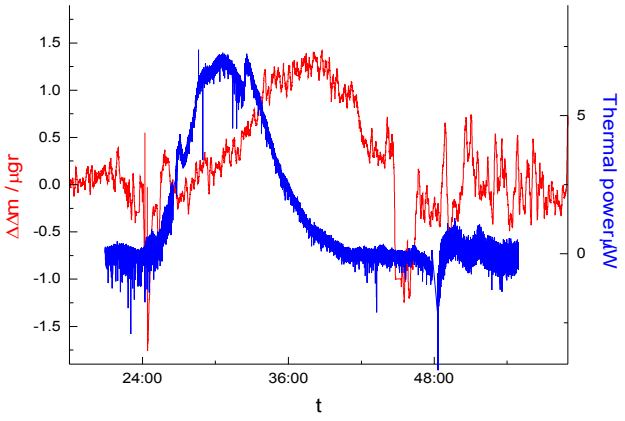

Figure 11. The rate of change of mass and thermal power when an agar film is inoculated with *E.coli*,anaerobically and then aerobically.

It is very clear from the results that we are observing a bacterial growth processes on the surface of the agar. There is a noticeable difference in all three signal measured with inoculated and not-inoculated agar.

The thermal power signal increases upon inoculation and then slowed down with time. However we observed that the thermal power began to decline earlier than the mass. Similar behavior was observed before by Kemp (16) who explained that after a certain time substrate depletion occurs and/or toxic byproducts accumulate. The bacteria at that stage can still divide for a limited period of time under a stressed metabolism but cannot eventually complete the growth process.

The small increase in the motional resistance is also reasonable if we consider that part of the water generated in the growth process may remain on the surface,making it less rigid.

Another explanation is that the agar/*E.coli* layer has different viscoelastic properties than the agar itself. We observe that the top of agar film with *E.coli* inoculated on the surface become much softer after one

day.
Most of the models of bacterial growth assume a homogeneous environment for all the bacteria in aqueous growth media. In those cases,the initial phase of bacterial growth is exponential. In the case of bacteria growing at the interface,the situation might be different. Not all the bacteria see the same water environment,some see water from the agar itself and some from the air. Under these conditions it is possible that not all the bacteria show the same stoichiometry during their growth process.
Furthermore,with the current method of inoculation we do not control the amount of bacteria deposited on the surface. When we inoculate the agar with a large number of bacteria we might see three-dimensional growth instead of surface growth,and the growth in this case may be other than exponential. There is a need to develop a model to predict this growth process.
We believe that the positive mass change can be explained by different growth stoichiometry,different behavior of three dimensional growth process or by the fact that the *E.coli* are hygroscopic and adsorb water from the environment.

CONCLUSIONS

In this work we show that the QCM/HCC can be a powerful tool for studying biological application. Two different example were examine – the interaction of HSA with the anticoagulant drug, warfarin, and the growing of *E. Coli* on agar substrate. Significant signals were obtained in both case, in the solid / liquid interface (first example) and solid / gas interface (second example). From the results, details about the nature of both process can be learned. In this paper we present mainly a qualitative analysis but more quantitative analysis can be done.

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