Cell adhesion and spreading processes studied by the thickness shear mode resonator technique

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ABSTRACT

The Thickness Shear Mode (TSM) quartz crystal resonator has been extensively used as a sensitive sensor in a variety of research such as electrochemical or biological field. This technique based on the transverse propagation of an acoustic shear wave generated by a sinusoidal electric field through a piezoelectric quartz resonator provides a non destructive and a powerful means for characterizing physical properties of materials. Electrical impedance analysis is further better suited for modeling the loaded resonator systems near sensor mechanical resonance and then gives quantitative information on the interfacial processes. In the present study, kinetic of attachment and spreading of adherent living cells is investigated using the thickness shear mode quartz crystal sensor technique. Within the framework of the Butterworth-Van Dyke equivalent circuit (BVD), experimental results of the electrical motional resistance in the vicinity of the sensor resonant frequency have shown that the increase of this parameter is strongly correlated with the evolving surface coverage during attachment and spreading of the adherent living cells on the quartz sensor surface. Both the dependence of the electrical motional resistance on the cell concentration and the contribution of the extracellular matrix on the acoustical response of the thickness shear mode quartz resonator are further analyzed near mechanical resonant frequency. Finally the ability of the thickness shear mode quartz resonator technique for monitoring specific cell-substrate interactions is discussed.

Keywords : Thickness shear mode (TSM), quartz crystal resonator, impedance analysis, cell adhesion.

INTRODUCTION

There is a growing interest in the use of the thickness shear mode quartz crystal sensor technique in a variety of electrochemical [1-3] and biological research [4-8] either for monitoring *in situ* adsorption processes or studying cell-substrate interactions on quartz sensing surface. Because of its high sensitivity, this technique based on the transverse shear mode oscillations provides a noninvasive and a powerful means for probing changes at solid-solid or solid-liquid interfaces. Indeed, various media in contact with the vibrating quartz sensor create mechanical perturbations that affect the system resonance characteristic, resulting in a frequency shift and an increase in resonance damping [9,10]. The general goal in the former is to identify the relationship between the quartz sensor electrical response and its surface loading. Several mechanical properties of the foreign materials such as accumulated mass, liquid density-viscosity or shear complex modulus then can be extracted and studied [9,10].

Electrical acoustic impedance analysis is widely used for modeling the shear acoustic sensor device response and thus gives quantitative information on interfacial processes [8-10]. The well established Butterworth-Van Dyke equivalent circuit (BVD) provides a powerful tool for relating the electrical properties of the quartz to mechanical properties of the material loading quartz surface in the vicinity of the sensor mechanical resonance [11]. With some approximations, this model can be further translated into the lumped element model (LEM) generally used in electrical engineering which represents mechanical interactions by their equivalent electrical circuit components. The real part of the measured impedance gives the motional resistance or resonance damping and the imaginary part is then representative of the frequency shift [11].

On the other hand cell-cell interactions play a prominent role in various physiological processes such as cell differentiation during development, tissue regeneration or cell migration in tumor metastasis [12]. Cell-substrate interactions further constitute a great interest for studying the compatibility of biomaterials surfaces with living cells [13]. In the past few years considerable efforts have been made for a better understanding of the complex mechanisms governing the adhesion and spreading of living cells on surfaces [4,8]. However little is known about the exact relationship between these underlying processes and the response of the acoustic quartz sensor device.

In the present study, kinetic of attachment and spreading processes of adherent living cells is investigated using the thickness shear mode (TSM) quartz crystal sensor technique. In a first part we present experimental results of the electrical motional resistance derived from the BVD equivalent circuit during adhesion and spreading of adherent living cells on the quartz resonator. Both the dependence of the motional resistance on the cell concentration and the contribution of the extracellular matrix are further analyzed in the vicinity of the sensor resonant frequency. Finally the ability of the thickness shear mode quartz crystal resonator technique for studying specific cell-substrate interactions is discussed.

MATERIAL AND METHOD

CELL CULTURE

Chinese Hamster Ovary cells (CHO) type K1 were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen or Gibco, France). The culture medium was supplemented with 10% (v/v) fetal calf serum medium (FCS, Invitrogen), 4 mM L-glutamine (Gibco), 100 units/ml penicillin and 100 µg/ml streptomycin (Invitrogen). Cells were grown to confluence in a humidified incubator with 5% CO_2 atmosphere at 37°C. The confluent CHO-K1 cell monolayers were washed twice with DMEM and were then removed from the culture substrate by 0.25% (w/v) trypsin supplemented with 1mM EDTA (5 min at 37°C). Trypsin digestion was terminated by the addition of an excess of complete culture medium. Cells were spun down at 110 x g for 10 min. The pellet was resuspended in culture medium, and aliquots of the cell suspension were transferred onto the quartz disk after the cell number had been determined with a malassez cell. Before inoculation, the quartz plates were treated with ethanol 100% for 3-5 min, which provides both an intense cleaning of the gold surfaces and sterilization of the entire chamber.

THICKNESS SHEAR MODE SENSOR DEVICE

The thickness shear mode resonator device (figure 1) consists of a thin disk of AT-cut quartz (Mattel, Creteil, France) with two identical 5 mm diameter electrodes made of a 2500 Ã thick layer of gold deposited by evaporation on a thin chromium adhesion layer. The quartz crystal diameter is $d = 14\,\text{mm}$ and the fundamental resonance frequency $f_o = 9\,\text{MHz}$. The electrode contacting the solution is limited by one of the O-ring joints. On the opposite side in contact with air, a silica gel is used to maintain the degree of humidity constant. The test cell with the quartz is placed inside an incubator under wet atmosphere (95% air + 5% CO_2) were measurements were performed.

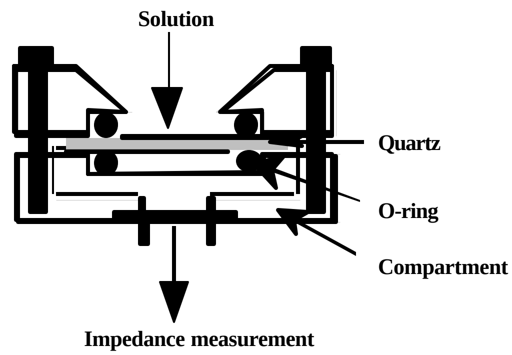

Figure 1. Shear acoustic sensor device

The application of an external electrical potential between the two electrodes produces an internal mechanical stress and induces a shear deformation of the crystal because of the piezoelectric properties and crystalline orientation of the quartz [11]. When applying a sinusoidal electric field perpendicular to the surface of the crystal, the deformation oscillates at the frequency of the induced field. The quartz crystal resonates at a frequency determined by the thickness of the crystal. This corresponds to a crystal thickness that is an odd multiple of half the acoustic wavelength l [11]. At the natural mechanical resonance a standing waves pattern is generated across the crystal thickness. The displacement occurring is maximum at the free surfaces and vary sinusoidally across the quartz thickness.

METHOD OF IMPEDANCE ANALYSIS

The electroacoustical response of the sensor device was first investigated for an unperturbed resonator. Data analysis was performed using the lumped element Butterworth-Van Dyke (BVD) equivalent circuit which consists of a capacitor C_o paralleled by a series combination of an inductance L_1, a resistor R_1 and a capacitor C_1 (figure 2.a). The combination of L_1, R_1 and C_1 is referred to as the motional branch of the BVD equivalent circuit because only these impedance elements are associated with the shear motion of the quartz [11]. The parallel capacitance C_o arises from the presence of the dielectric quartz material between the two surface electrodes but it also contains parasitic contributions of the set up [11]. The components of the BVD equivalent circuit were then deduced from the Nyquist plot which describes the resonator admittance Y at different frequencies (figure 2.b). The Nyquist plot satisfies the following equation [14, 15] :

$$\left(G-\frac{1}{2R_1}\right)^2+\left(B-wC_o\right)^2=\left(\frac{1}{2R_1}\right)^2 \qquad (1)$$

where G represents the conductance (real part of the admittance) given by :

$$G=\frac{R_1}{R_1{}^2+\left(L_1w-1/C_1w\right)^2} \qquad (2)$$

and B, the susceptance (imaginary part of the admittance) scaling as :

$$B=wC_o-\frac{\left[L_1w-\left(1/C_1w\right)\right]}{R_1{}^2+\left[L_1w-\left(1/C_1w\right)\right]^2} \qquad (3)$$

Equation (1) is representative of a circle whose radius is inversely proportional to the resistance thus scaling as $1/2R_1$ while the center is located at $\left(1/2R_1, wC_o\right)$. The frequency f increases in the clockwise direction of the admittance circle of the Nyquist plot. The static capacitance C_o has the effect to raising the center of the circle by an amount wC_o in the vertical direction because of the dielectric capacitance C_o of the quartz crystal. The capacitance C_1 of the motional branch was fixed at a constant value during all fitting procedures because it is determined by the material properties of the quartz and the dimensions of the resonator [14, 15].

In a second step measurements of the shear complex electrical admittance Y were performed with the network analyzer as the cell suspensions were placed on one side of the acoustic resonator device. The shear admittance Y as a function of the conductance G and susceptance B for the modified Butterworth-Van-Dyke equivalent circuit then may be expressed as [14, 15] :

$$Y=G+jB=\frac{R}{R^2+U^2}+j\left(wC_o-\frac{U}{R^2+U^2}\right) \qquad (4)$$

where $R=R_1+R_2$, $U=\left(wL-1/wC_1\right)$ and $L=L_1+L_2$.

The electrical components of the modified lumped element Butterworth-Van-Dyke equivalent circuit (figure 2.c) was fitted to experimental measurements of the electrical admittance Y using nonlinear least-square methods thus determining both the inductance L_2 and resistor R_2. The series resonant frequency in this case may be defined as the frequency at which the motional reactance vanishes [14, 15]:

$$w_S=2pf_S=1/\sqrt{LC_1}=1/\sqrt{\left[\left(L_1+L_2\right)C_1\right]} \qquad (5)$$

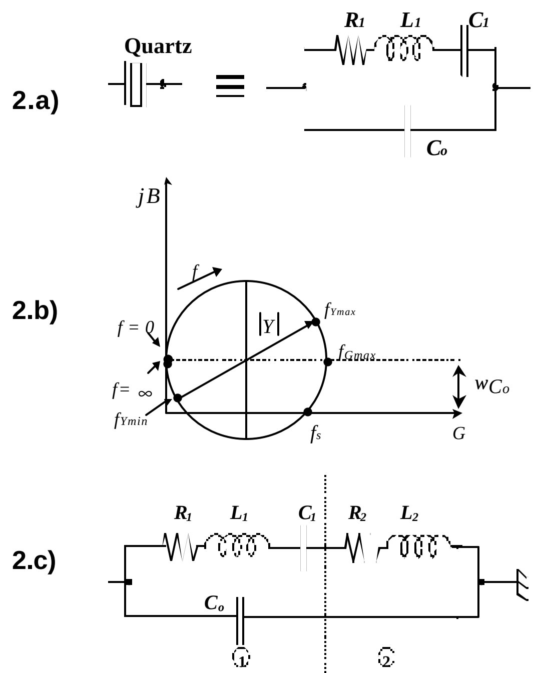

Figure 2. Butterworth-Van Dyke (BVD) equivalent circuit for an unperturbed AT-cut quartz resonator (2.a) , Nyquist representation of the electroacoustic impedance (2.b) or modified Butterworth-Van Dyke (BVD) equivalent circuit for a loaded AT-cut quartz resonator (2.c).

EXPERIMENTAL RESULTS AND DISCUSSION

CALIBRATION OF THE ACOUSTIC QUARTZ SENSOR DEVICE

The first experiment consisted of calibrating the response of the acoustic quartz sensor system with different Newtonian liquid loading conditions. For this purpose, electrical admittance measurements over a frequency range near the fundamental resonance were performed with various solutions of water mixed with concentrations in glycerol ranging from 0 to 72%. Solutions density and viscosity were derived from literature values. Both the frequency shift Δf_s and changes in motional resistance $\Delta R = R_2|_{w=w_s}$ are plotted on figures 3 and 4. The frequency shift Δf_s is linearly related to the liquid loading parameter $(r_m h_m)^{1/2}$ and decreases when the product $(r_m h_m)^{1/2}$ increases. Conversely, the motional resistance $\Delta R = R_2|_{w=w_s}$ increases linearly as the product $(r_m h_m)^{1/2}$ increases (figure 4). These results are in agreement with both theoretical predictions of Martin *et* al. reported in [9, 10]. Indeed, the motional resistance due to Newtonian liquid loading in the vicinity the resonant frequency scales as :

$$R_2 = \left(\frac{Np}{4K^2 w_s C_o Z_q}\right)\left(\frac{wrh}{2}\right)^{1/2} \tag{6}$$

where w is the angular frequency ($w = 2pf_o$), K^2 is the effective electromechanical coupling factor, $(N = 1,3,5\ldots)$ is the resonator harmonic number, w_s is the angular resonant frequency, Z_q is the characteristic quartz impedance, r_m and h_m represent respectively the density and shear viscosity of the Newtonian loading medium.

The frequency shift Δf_s produced by the liquid load is given by the following expression [9,10] :

$$\Delta f_s = -f_o^{3/2}\left(p r_q h_q\right)^{-1/2}\sqrt{h_m r_m} \tag{7}$$

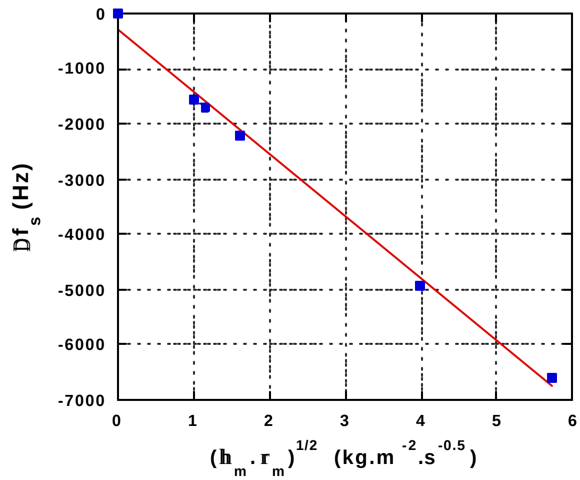

Figure 3. Frequency shift Δf_s *versus* the loading parameter $(h_m r_m)^{1/2}$. The solid curve was obtained from linear regression $\Delta f_s = -1126\sqrt{h_m r_m} - 299.55$ in good agreement with equation (7).

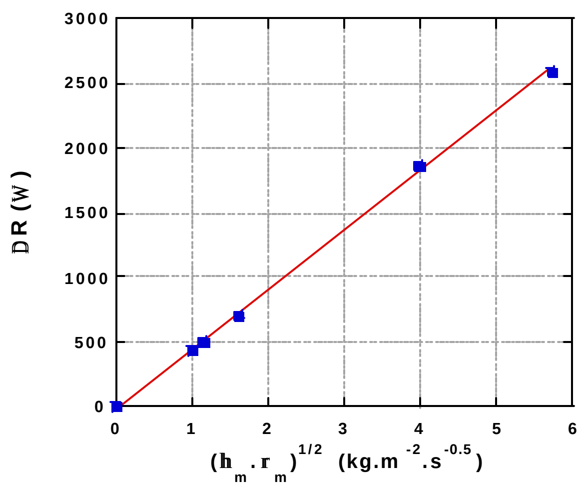

Figure 4. Motional resistance $\Delta R = R_2|_{w=w_S}$ *versus* the loading parameter $(h_m r_m)^{1/2}$. The solid line was obtained from linear regression $\Delta R = 460.67\sqrt{h_m r_m} - 10.038$ in good agreement with equation (6) .

KINETIC OF ATTACHMENT AND SPREADING OF LIVING CELLS

Attachment and spreading of living cells on surfaces is a complex process initiated by non-specific cell interactions with the surface, followed by the establishment of specific molecular contacts of the receptor-ligand type. The non specific interactions mainly involve van der Walls forces, repulsive electrostatic interactions and steric stabilization. The net contribution of these non specific forces is slightly pro-adhesive but generally not of sufficient magnitude to hold the particle in place. In contrast the stronger non specific interactions are mediated by transmenbrane proteins called integrins. These α,β-heterodimeric proteins connect the intracellular cytoskeleton to the extracellular matrix and thus provides the mechanical stability of cell-substrate adhesion.

We report in this section an experimental study of the kinetic of attachment and spreading of adherent CHO living cells on the quartz sensor surface. For this purpose, the motional resistance $\Delta R = R_2\big|_{w=w_S}$ was deduced from the shear impedance measurements in the vicinity of the resonant frequency and was reported as a function of time as the quartz sensor was loaded with the suspension of Chinese Hamster Ovary (CHO) cells. As shown on figure 5, during the first twenty minutes only the cell sedimentation effects were detected, the cells kept their spherical shape and did not actively spread on the quartz sensor surface. Adhesion and spreading of Chinese Hamster Ovary cells on the quartz resonator surface induces a progressive increase of the motional resistance $\Delta R = R_2\big|_{w=w_S}$ which reaches a stationary level after a delay time of about 120 minutes. Multiplication of the living cells process further starts after a time of about 300 minutes and thus results in an increase of the electrical motional resistance $\Delta R = R_2\big|_{w=w_S}$ because the cell number covering the quartz sensor surface increases. In contrast as the quartz sensor device was loaded with suspension of spherical latex particles with density close to those of the CHO cells, at the same concentration, the motional resistance $\Delta R = R_2\big|_{w=w_S}$ increases slightly only during the first twenty minutes and remains constant during time (figure 5).

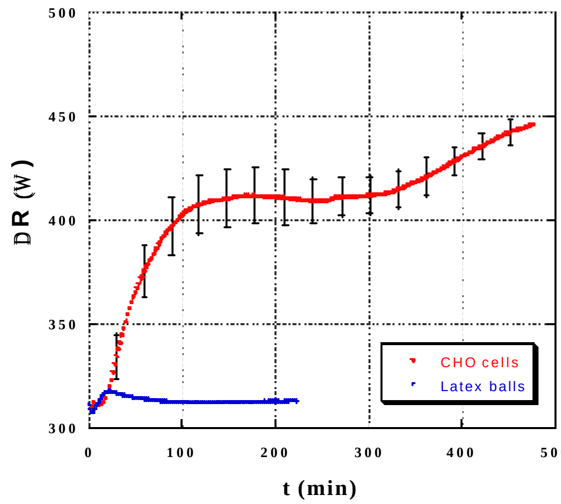

Figure 5. Time dependence of the motional resistance $\Delta R = R_2$ in the vicinity of the resonant frequency f_S for a quartz crystal sensor loaded with a monolayer of Chinese Hamster Ovary cells (CHO) ( ) or spherical latex particles (-) . The experiments were repeated ten times in the same conditions with 50 000 cells or 50 000 latex particles dispersed on a area of about 50 mm^2.

Furthermore, the time variation of the motional resistance $\Delta R = R_2\big|_{w=w_S}$ is strongly related to the evolving surface coverage during attachment and spreading of the cells on the vibrating resonator surface because of the cell integrin-mediated contacts established with the proteins covering the resonator surface. These results have been found in good agreement with optical microscopic observation reported in [4].

DEPENDENCE OF THE ELECTRICAL MOTIONAL RESISTANCE ON THE CELL CONCENTATION

The dependence of the motional resistance $\Delta R = R_2\big|_{w=w_S}$ on the cell concentration near resonant frequency was also analyzed. When plotting the motional resistance $\Delta R = R_2\big|_{w=w_S}$ as a function of time for different cell concentration (figure 6), the motional resistance increases because the cell number interacting with the sensing surface increases. This experiment well confirms the time variation of the motional resistance $\Delta R = R_2\big|_{w=w_S}$ during the cell multiplication phase observed on figure 5.

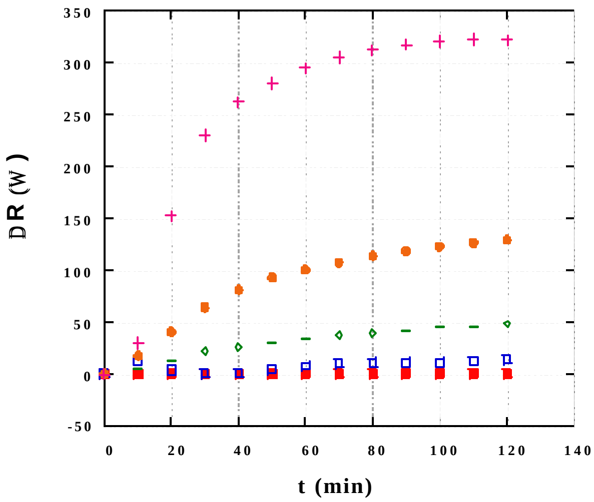

Figure 6. Time dependence of the motional resistance $\mathbf{D}R = R_2$ in the vicinity of the resonant frequency f_s for a loaded quartz crystal sensor with a monolayer of Chinese Hamster Ovary cells (CHO) at different cell concentration ((, Culture medium), (, 20 000 CHO), (, 50 000 CHO), (, 100 000 CHO), (+, 500 000 CHO)).

CONTRIBUTION OF THE EXTRACELLULAR MATRIX 0N THE ACOUSTICAL RESPONSE OF THE LOADED THICKNESS SHEAR MODE QUARTZ RESONATOR

An immobilized layer of adhesion promoting proteins is required to allow cells to form specific molecular bonds. Extracellular matrix proteins can originate from precoating the quartz sensor surface, spontaneous adsorption of serum proteins or secretion of endogenous proteins by the cells. The contribution of the extracellular matrix proteins during the adhesion processes was finally investigated. The variation of the motional resistance $\Delta R = R_2|_{w=w_S}$ *versus* time was then studied as the quartz sensor surface was coated with fibronectin. As shown on figure 7, the presence of this protein favors the living cells to make specific integrin-mediated contacts on the quartz sensor surface and thus results in an increase of the electrical motional resistance $\Delta R = R_2|_{w=w_S}$.

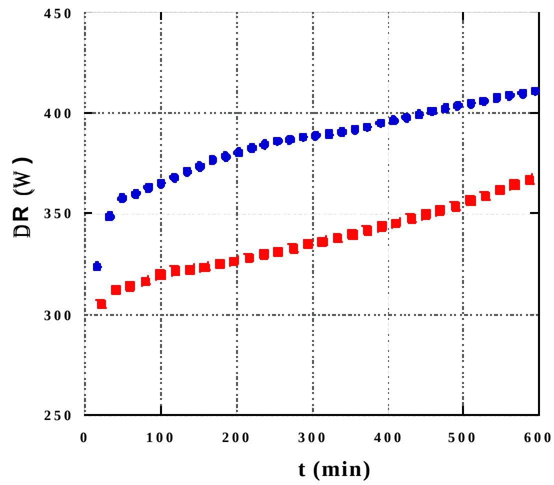

Figure 7. Time dependence of the motional resistance $\mathbf{D}R = R_2$ in the vicinity of the resonant frequency f_s for a loaded quartz crystal sensor with a monolayer of 50 000 cells of Chinese Hamster Ovary cells (CHO) dispersed onto non coated quartz surface ( ) or fibronectin coated surface (·).

CONCLUSION

The present study demonstrates that the thickness shear mode resonator technique is better suited for studying attachment and spreading of adherent living cells on vibrating quartz sensor surfaces. The measured electrical motional resistance derived from the Butterworth-Van Dyke equivalent circuit (BVD) is very sensitive to specific receptor mediated interactions of the cells with the extracellular matrix proteins adsorbed or secreted on the quartz surface and thus varies as the evolving surface coverage during adhesion and spreading processes. Because of its noninvasive nature and high sensitivity, this technique gives quantitative information and thus provides a powerful means for a better understanding of specific cell-substrate interactions.

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