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BIOTECHNOLOGY and BIOENGINEERING

An Alternative Quantitative Acoustical and Electrical Method for Detection of Cell Adhesion Process in Real-Time

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ABSTRACT: Sauerbrey [(1956), Z Phys 55:206–222] showed that the shift in resonance frequency of thickness shear mode (TSM) of a quartz crystal sensor is proportional to the mass, which is deposited on it. However, new powerful electrical circuits were developed that are capable of operating TSM quartz crystal sensors in fluids which enabled this method to be introduced into electrochemical and biological applications. These applications include the detection of virus capsids, bacteria, mammalian cells, the interaction of DNA and RNA with complementary strands, specific recognition of protein ligands by immobilized receptors, and last but not least the study of complete immunosensors. Piezoelectric quartz transducers allow a label-free identification of molecules; they are more than mass sensors since the biosensor response is also influenced by the surface charge of adsorbed proteins, interfacial phenomena, surface roughness and viscoelastic properties of the adhered biomaterial. These new characteristics have recently been used to investigate cell, liposome, and protein adhesion onto surfaces, thus permitting the rapid determination of morphological cell changes as a response to pharmacological substances, and changes in the water content of biopolymers avoiding of time-consuming methods. We validated an alternative quantitative acoustical engineering for cell adhesion process monitored by the TSM. Shear acoustical results (motional resistance) are further correlated to cell counting procedures and are sensitive of adhesion processes in real-time.

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Introduction

Cell adhesion plays a critical role in the formation of tissues and organs. Generally, adhesion behavior is a main indicator of viability for most mammalian cells (Hasmin et al., 2005; Toral et al., 2007). This phenomenon utilizes the cell adhesion to extracellular matrix (ECM), which influences many events of cell life, as division, growth, differentiation or apoptose.

Cell adhesion occurs when the adhesion receptor (integrins) expressed on the cell plasmic membrane interacts with the ECM molecule (fibronectin) or neighboring cells (Boulter and Van Obberghen-Schilling, 2006; Cuvelier et al., 2007; Evans and Calderwood, 2007; Nicolas and Safran, 2006; Reinhart-King et al., 2005; Tilghman and Parsons, 2008). The reversibility of this process, which is translated by adhesion and detachment cycles, allows the cells to move compared to cells or the ECM. The cell/cell and/or cell/ECM interactions induce the signal production during the activation of cell signal molecules, anchored in the adhesion area.

However, there are few quantitative data describing the adhesion and the mechanical interaction of cells with a substrate in real-time. Such information can be important to study the effect of specific ECM molecules, which can modulate the dynamics of cell adhesion.

Currently the majority of the methods used in biology to measure cell adhesion involve the use of mechanical forces

exerted against the cells or/and the quantification of the number of adherent cells by counting, using indirect colorimetric assays (Eibl et al., 2006). However these methods which use a centrifuge (Channavajjala et al., 1997; Park et al., 2006), do not explain why the cells adhere strongly or slightly to the surface and they do not measure cell adherence under the optimal conditions for cells in culture.

Other methods such as atomic force microscopy, micromanipulators or micropipette aspiration (patch clamp) (Karakecili et al., 2007; Levitan et al., 2000; Viigipuu and Kallio, 2004) allow to measure the forces necessary to detach the cells from a substrate. However, these methods allow to study a single cell and not many cells in culture.

The microscopic and/or ultrastructural techniques coupled to image analysis are also often used to observe and measure the molecules involved in cell adherence at given times. Such methods measure the adhesion phenomena in real-time. Consequently, the detection of the changes in the dynamics of the cellular adhesion and the cell viability under external pressure cannot be observed. Moreover, the majority of the methods quoted above involve special competence, instrumentation and man-power to produce reliable results.

In this study, we used and validated an alternative method using an acoustic sensor for the detection and measurement of cell adhering concentration in initial, spreading and growth phases in real-time monitoring motional resistance. The acoustic sensor consists of an AT-cut quartz crystal sandwiched between gold electrodes. An oscillating electric field applied across the electrodes propagates an acoustic wave through the quartz crystal (Sauerbrey, 1956). Authors such as, Fohlevova et al. (2007), Guo et al. (2008), Heitmann and Wegener (2007), Li et al. (2005), Thid et al. (2007), and Wei et al. (2007) successfully cultured different cell lines on the surface of shear sensors and recorded the cell adhesion by using the oscillator method monitoring frequency but not motional resistance as Marx et al. (2003), Haider et al. (2004), Le Guillou-Buffello et al. (2004, 2005, 2008). In this study, we examine in detail the feasibility of the use of acoustic sensors to monitor cell adhering concentration during the initial, the spreading and the growth phase. We chose to combine our experimental set-up to measure the motional resistance with the quartz crystal microbalance (QCM) technique in order to develop a biosensor allowing the measurement of cell adhering concentration. In order to reach this goal, we determined a temperature dependent, an intra- and extra-essays reproducibility, viscoelastic properties of cell monolayers, the influence of cell adhesion concentration, or cell number or cell surface. In addition, experiments of adherent cell detachment using two cell lines: Chinese hamster ovary (CHO) cells and McCoy fibroblastic cells were carried out.

Materials and Methods

Experimental Set-Up

The thickness shear mode (TSM) resonator set-up (Fig. 1A) is composed of a thin disk of AT-cut quartz (Mattel, Créteil, France) with two identical 5 mm diameter electrodes made of a 2,500 Å thick layer of gold deposited by evaporation on a thin chromium adhesion layer. The fundamental resonance frequency, and the quartz crystal diameter are respectively $f_0 = 9$ MHz and $d = 14$ mm. The upper face of the quartz disk in contact with the solution is limited by one of the O-ring joints. The experimental set-up including the quartz is placed inside an incubator under wet atmosphere (95% air +5% CO_2) where measurements were performed.

The application of an external electrical potential between the two electrodes produces an internal mechanical stress and thus induces a shear deformation of the crystal quartz due to the piezoelectric properties and crystalline orientation of the quartz. When applying a sinusoidal electric field perpendicular to the surface of the quartz crystal sensor, the deformation oscillates at the frequency of the induced field. The electronic instrumentation is further made up of a network analyzer (HP 4595A), which is electrically connected to the gold electrodes. Automatic measurements of the electrical admittance Y versus time at 30 s intervals were then carried out via an IEEE 488 bus to a PC computer with control software using HPVEE language. Finally, we measure the motional resistance which is directly proportional to viscosity and density at the cell–gold interface.

Figure 1B schematically depicts the signal transduction region of this biosensor, where the cells strongly adhere to the quartz crystal resonator. The cytoskeleton allows cells to stretch and spread, acquiring a greater area on the attachment surface, to which they subsequently attach themselves via focal adhesion complexes.

Analysis of the Impedance

Data analysis was performed using the lumped element Butterworth-Van Dyke (BVD) equivalent circuit. This circuit (Fig. 1C1) consists in a capacitor C_0 in parallel with the inductance L_1, a resistor R_1 and a capacitor C_1 in series (see Fig. 1C1). When the quartz crystal resonator is in contact with the cell suspension, this new element (Fig. 1C2) can be represented as a resistor R_2 due to the specific damping and an inductance L_2 due to the frequency shift. In this system, the R_2 value depends on the importance of the cell adhesion.

When cell suspension is deposited at the gold resonator surface, variations of the R_2 value are observed. These variations are related to modifications of the viscosity at the gold/cell interface.

R_2 resistance was directly calculated from the medium viscosity (Bandey et al., 1999; Martin et al., 1991):

$$R_2 = \left(\frac{N\pi}{4K^2 C_0 Z_q}\right)\sqrt{\frac{\omega\rho_m\eta_m}{2}} \quad (1)$$

where N is the harmonic resonance of the quartz ($N = 1$), K^2 is the effective electromechanical coupling factor for loaded

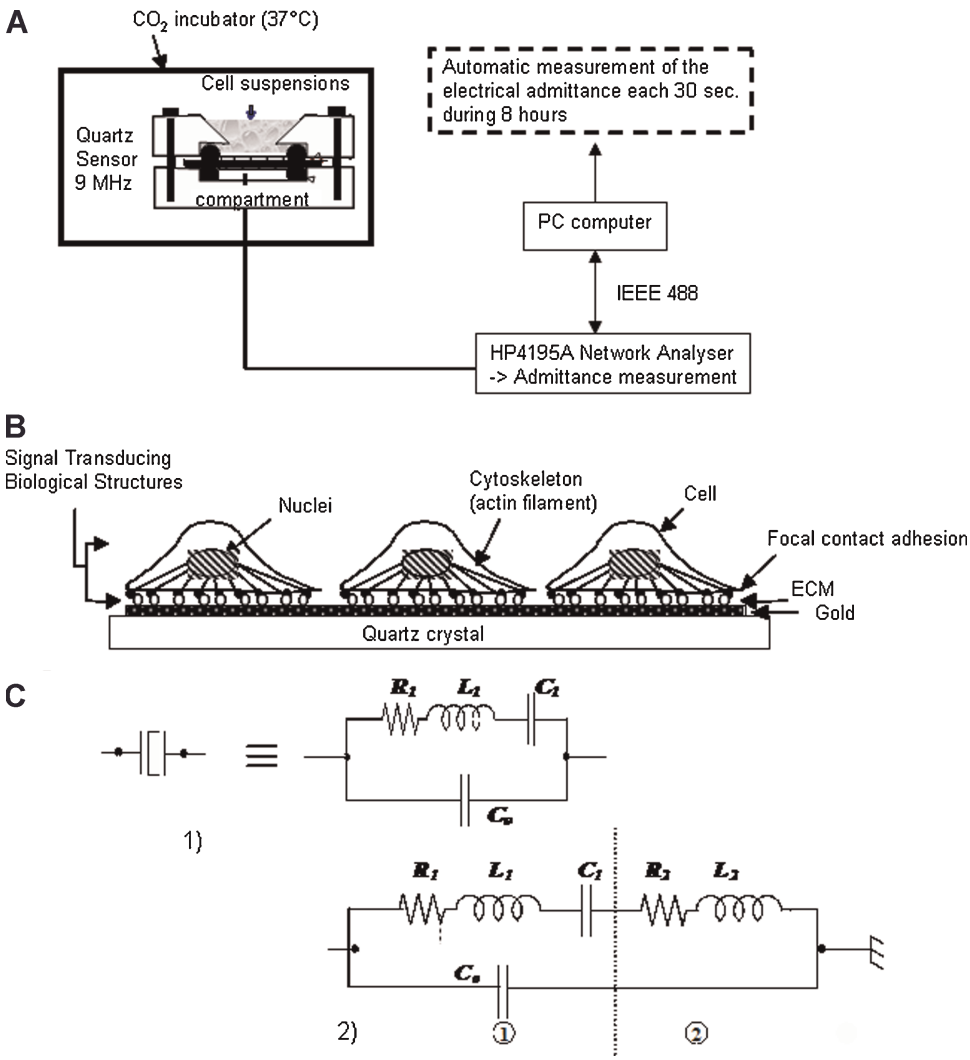

Figure 1. **A**: Scheme of the experimental device. **B**: Schematic picture of the signal transduction region of this biosensor, where the cells adhere to the quartz crystal resonator. **C**: BVD equivalent circuit an umperturbed AT-cut quartz resonator (1) or modified BVD equivalent circuit for a loaded AT-cut quartz crystal resonator (2).

quartz, C_0 the static capacitance arising from the presence of the dielectric quartz material between the electrode two surfaces, Z_q is the characteristic quartz impedance, ω is the angular series resonant frequency, ρ is the liquid viscosity and η is the liquid density.

The thickness δ of the oscillating boundary layer is involved in the measurements (shear waves at a frequency ω in a fluid of kinematic viscosity ν) (Bandey et al., 1999; Haider et al., 2004; Martin et al., 1991):

$$\delta = \left(\frac{\nu}{\omega}\right)^{1/2} \qquad (2)$$

A rough evaluation, taking into account the experimental conditions lead to a thickness of the order of $10^{-1}\,\mu m$ (Haider et al., 2004; Rajakovié et al., 1991; Wang et al., 2008).

Cell Culture

CHO cells line K_1 and McCoy fibroblasts are cells which adhere on a surface. CHO cells and McCoy human fibroblasts were purchased from ATCC (American Type Culture Collection, Manassas) (CRL-1696). Cells were cultured in 25 cm^2 tissue culture flasks with Dulbeco's Modified Eagle's Medium (DMEM, In Vitrogen, Cergy Pontoise, France) supplemented with 10% (v/v) fetal calf serum (FCS), L-glutamine (4 mM), penicillin (100 units/mL), streptomycin (100 μg/mL). Cultures were incubated at 37°C in a 5% CO_2 atmosphere humidified tissue culture incubator with twice weekly medium changes. Experiments were performed using cells in the 16th and 17th passage. T_4 Jurkat lymphocyte cells do not adhere on a surface and these cells are grown in suspension. Jurkat cells are maintained in long-term culture in RPMI medium. This culture medium was supplemented with 10% (v/v) FCS,

L-Glutamine (4 mM), penicillin (100 units/mL) and streptomycin (100 μg/mL). T4 Jurkat cells were grown in a 5% CO_2 humidified atmosphere at 37°C.

Temperature Variation

We measured the motional resistance after medium deposition (DMEM) at different temperatures (20°C, 25°C, 30°C, 35°C, and 40°C) on the gold resonator surface. The experiments were reproduced 10 times.

Reproducibility Test

For the extra-essay reproducibility, we measured the motional resistance after depositing the cell suspension or the DMEM medium on different gold surfaces at 37°C. For the intra-essai reproducibility test, we analyzed the motional resistance of the cell suspension (50,000 cells) or the DMEM on the same gold surface at 37°C. Experiments steps were 30 times with DMEM medium, and 10 times with cell suspension for extra- and intra-essays.

Cell Adhesion Analysis

Different experiments were carried out in order to study a cell adhesion on the gold surface over 8 h which are:

(1) to evaluate cell adhesion, we measured the motional resistance at different times of the medium culture, for 50,000 T_4 Jurkat lymphocyte cells and for 25 μg/mL of fibronectin matrix coated before the measurement during 30 min at 37°C;
(2) In order to study the cell adhesion on the different surface (gold surface or polystyrene surface), we measured the number of cells by a cell counter during 8 h on these two surfaces for CHO cells and McCoy fibroblasts cells. For that, we used a conventional method in biology, which is used in routine in the laboratory to count cells. First, after each time of analysis at 37°C, we wash the cells with the culture medium (removing the non adhering cells), then we wash with 200 μL of trypsin-EDTA solution (essential for the effectiveness of trypsin at maximum activity), then we add 200 μL of trypsin–EDTA solution. We leave it for 5 min at 37°C (temperature and time optimized for trypsin to split the integrin-fibronectin connections). After 5 min, we recover the cells in 5 mL of an isotonic medium. Then, we count the adhering cells using Coulting cells (Multisizer III—Coulter Counter from Beckman, Villepinte, France).
(3) In parallel, the motional resistance and cell number measured respectively during 8 h by a quartz crystal resonator and by a cell counter for the CHO cell or McCoy fibroblast. The percentage of cell increase calculated for cell counting and quartz crystal resonator were as following as:

Quartz crystal resonator technique

$$\frac{(R_{8h}-R_{5h})}{R_{8h}} \times 100 \quad (3)$$

where R is the motional resistance at time 8 h or 5 h.

Cell counting technique

$$\frac{(N_{8h}-N_0)}{N_{8h}} \times 100 \quad (4)$$

where N is the detached cell adhering number at time 8 h or initial (No; 50,000 cells)

(4) in order to study the cell adhesion versus cell (CHO or McCoy fibroblasts) concentration, we deposited on the gold surface the (5,000, 20,000, 50,000, 100,000, 500,000) cells and we analyzed the motional resistance ;
(5) in parallel, to measure the CHO cells or McCoy human fibroblasts cell adhesion on the gold or polystyrene surface, we observed by optical microscopy the cell adhesion every hour and for five concentrations (5,000, 20,000, 50,000, 100,000, 500,000 cells/quartz); we counted adhering cells every hour using a cell counter (Multisizer III—Coulter Counter from Beckman),
(6) in parallel, we measured the CHO cells or McCoy human fibroblast cell adhesion (50,000 cells/quartz) on the gold surface and at every hour we measured the fluorescence intensity by flow cytometry. For that, we have detached the cells with a trypsin–EDTA solution after each time of cell culture (see the protocol above) and place the adhering cells into a large volume (4 mL) of culture medium. The cells are then centrifuged at 1,500 rpm and we remove the supernatant. To the sedimented cells, we add 10 μL of antibody anti-integrin α5β1 marked with the FITC (Fluorescein isothiocyanate) (Invitrogen). We vortex then and leave the FITC solution during 30 min at room temperature protected from light. After 30 min, we wash with PBS 1×, we centrifuge and we recover the centrifugate in 400 μL of PBS 1×. We vortex and we pass the tube to the flow cytometry (Becton Dickinson LSRII, Le Pont-De-Claix, France). We obtain the result in intensity of fluororescence in arbitrary units (U.A.), which is specific of the number of integrins expressed on the cell surface. The integrins are implied in the link integrin–fibronectin during the cell adhesion.
(7) in parallel, we measured the CHO cells or McCoy human fibroblasts cell adhesion at 50,000 cells/quartz on the gold surface and we observed by optical microscopy the cell adhesion. The cell surface was calculated by a house method using to Matalb software.
(8) after cell adhesion on the quartz crystal surface during approximatively 10 h, we removed the cells by enzymatic digestion with trypsin–EDTA. In the second experiment, we added 50,000 CHO or McCoy fibroblasts cells on the gold surface after removal of the cell by trypsin digestion.

Statistical Analysis

Data obtained from 10 experiments were expressed as mean value ± standard deviation (SD) for each group of experiments (different cell concentration and type representation of cell adhesion) significant differences were calculated by ANOVA. Results of $P < 0.05$ were considered as significant (differences of $P < 0.001$ denoted by ***, $P < 0.01$ denoted by **, and $P < 0.05$ denoted by *). In addition, Student's *t*-test was used to compare the statistical significance of two different experiments. For optical microscopy, we analyzed 10 pictures by time and by cell type.

Results

Temperature Variation

In order to establish the shift between the motional resistance and the temperature of the medium deposited on the gold surface, we measured the motional resistance of culture medium from 20°C to 40°C. We observed in Figure 2A that the motional resistance decreased when the temperature increased. For this reason we chose to do all our experiments by quartz resonator technique at a fixed and stable temperature. For other results presented, we have chosen to work at 37°C, which is the physiologic temperature for cell culture.

Extra- and Intra-Essays Reproducibility

We have observed cell adhesion (Table IA and B) when we utilized the same quartz for different experiments, the motional resistance is more stable in time and the SD is weaker. For example, the mean values for CHO cells are respectively 242.7 Ω and 207.5 Ω at 8 h, and the SD is 10.7 ohms and 2.5 ohms for extra-essay reproducibility and for intra-assay reproducibility. These values give respectively as mean variation coefficient (CV) equal to 4.5% and 1.0%. These results were confirmed with the DMEM medium culture. The quartz crystal resonator is reusable and in future experiments we worked with the same gold surface.

Indirect Measurement of Density and Viscosity on the Quartz Resonator

Bandey et al. (1999) and Reed et al. (1990) showed that the motional resistance is sensitive to density and shear viscosity of Newtonian loading medium on the quartz crystal resonator. When we deposited the cell on the quartz surface, three elements which may influence the motional resistance (Fig. 2B). These elements are: (1) ECM, (2) cell contact, (3) medium culture. Figure 2C shows the results obtained with the fibronectin as ECM, with the T_4 Jurkat lymphocyte as a non-adherent cell, and with the medium culture (DMEM supplemented). In these three experiments, we observed that the motional resistance variation is not affected by the density variation due to the very thin layer of ECM and non-cell contact or medium culture. This signifies that the motional resistance measurement must be sensitive to all contacts when cells adhere on the gold surface.

Comparison of Cell Adhesion as a Function of the Support

Cells were grown during 8 h on the quartz crystal or on polystyrene surfaces. The results are illustrated in Figure 3A and B. We observed the cycle of duplication and division (the cell cycle is the essential mechanism by which all living things reproduce). The correlation coefficient between the cell cycle on the gold surface and on the polystyrene surface is respectively 0.997 (no significant difference: $P < 10^{-4}$, Student test) for CHO cells and 0.999 (no significant difference: $P < 10^{-4}$, Student test) for fibroblasts McCoy, meaning that the cell cycle is identical for two cell adhesion surfaces: gold and polystyrene. By optical microscopy, we observed (Fig. 3C) (1) in <2 h, cells are still rounded, already firmly attached (sedimentation phase), (2) between 2 h and 4 h, gradual cell flattening occurs spreading (adhesion phase) and (3) after 4 h, formation of a spread cell monolayer (multiplication phase). We show that there is no significant difference in response between the different supports. The morphological cell and the metabolism cell seem identical when cells adhere to a quartz crystal resonator.

Type Representation of Cell Adhesion

In Figure 4, we show a typical representation of the motional resistance versus time when living McCoy fibroblast cells or CHO cells adhere to the gold sensor surface. During the first 20 min only the cell sedimentation effects on the gold-quartz sensor is detected. Then, cells require to become attached to the ECM deposited on the quartz sensor surface to spread and grow. This induces a progressive increase of the motional resistance reaching a stationary level after a delay time of about 120 min. Next, the motional resistance increase with the multiplication and proliferation of the living cells process. This dependence of cell growth, proliferation, and survival on attachment to a substrate is known as anchorage dependence, and is mediated mainly by integrins and the extracellular signals that they generate (McClary et al., 1999). The last increase of the motional resistance observed is due to the increased cell number covering the quartz resonator. Indeed, we observed this response of cell adhesion for the two types of cells have a significant difference ($P < 0.01$, ANOVA). These results show that the response via resistance is dependent on the type of adherent cells on a gold-quartz resonator. They are further supported by optical microscopy observation

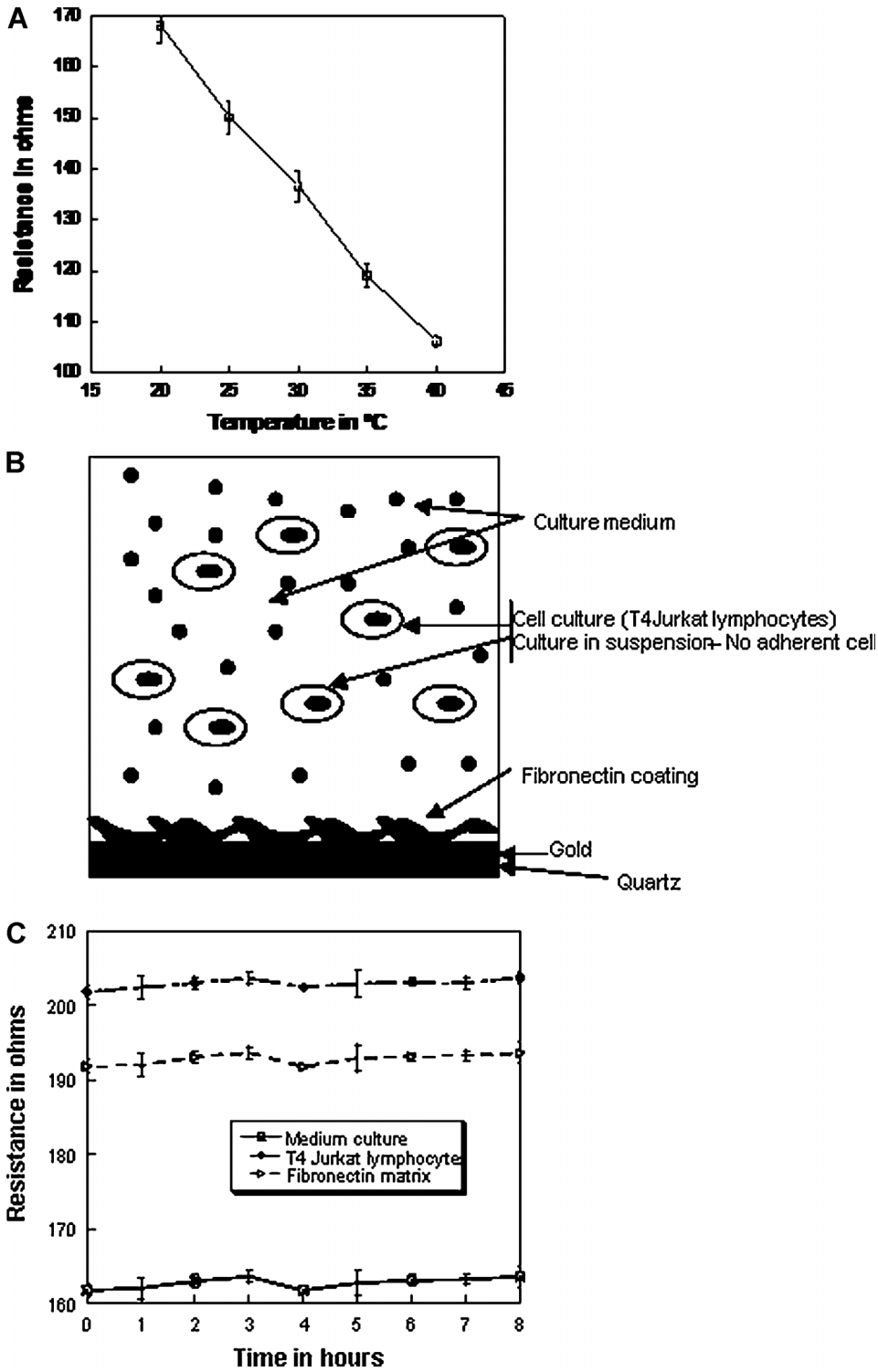

Figure 2. (**A**) Plot of the motional resistance versus the temperature for a loaded AT-cut quartz crystal resonator. Error bars represent the SD of 10 acoustic measurements. **B**: Scheme of experiments. Measurement by quartz crystal resonator technique:—culture medium (DMEM supplemented with 10% (*v/v*) FCS, L-Glutamin (4 mM), penicillin (100 units/mL), streptomycin (100 μg/mL), or—only T4 Jurkat lymphocytes (cells which are cultivated in suspension and are not adherent to cells), and or—fibronectin sole coating in order to observe the influence of matrix extracellular molecules. **C**: Time dependence of the motional resistance for a quartz crystal sensor loaded with a medium culture of CHO cells (☒), T4 Jurkat lymphocytes at 250,000 cells/mL (◆) or 25 μg/mL of fibronectin (ECM) (▷). Error bars represent the SD of the acoustic measurements.

Table I. Long time measurements of electrical motional resistance for a quartz crystal resonator loaded a monolayer of CHO or McCoy fibroblast and culture medium on the same gold surface of quartz (A) and on the different gold surface of quartz resonator (B). All experiments were made at 37°C. Error bars correspond to the SD of electrical measurements. The results obtained for a cells concentration for 25,000, 50,000, and 90,000 cells adhesion on gold surface for the two types of cellular lines recapitulates in (**C**).

	1 h	2 h	3 h	4 h	5 h	6 h	7 h	8 h
(A) Resistance on the same quartz								
CHO	160.2 ± 1.6	167.3 ± 2.0	170.3 ± 1.0	174.8 ± 2.3	175.2 ± 1.6	178.2 ± 0.7	190.3 ± 1.2	207.5 ± 2.5
McCoy fibroblast	160.2 ± 1.4	168.8 ± 1.7	178.9 ± 1.4	187.2 ± 2.3	194.3 ± 2.1	199.8 ± 1.0	207.3 ± 1.5	209.1 ± 1.9
Culture medium	160.2 ± 1.8	155.2 ± 1.9	155.8 ± 2.0	155.9 ± 1.2	155.8 ± 1.4	155.7 ± 1.7	155.8 ± 1.9	154.6 ± 1.6
	1 h	2 h	3 h	4 h	5 h	6 h	7 h	8 h
(B) Resistance on different quartz								
CHO	160.2 ± 6.7	89.6 ± 9.1	190.1 ± 9.7	195.5 ± 9.8	203.1 ± 10.0	211.2 ± 8.0	218.1 ± 8.7	242.7 ± 107
McCoy fibroblast	160.2 ± 7.5	92.2 ± 9.0	194.1 ± 9 3	195.5 ± 10.2	212.1 ± 10.2	250.1 ± 8.6	235.7 ± 9.9	243.2 ± 10.2
Culture medium	160.2 ± 7.7	59.8 ± 7.5	160.0 ± 7.5	160.7 ± 6.4	159.8 ± 6.7	159.7 ± 6.9	159.6 ± 7.7	159.1 ± 6.7

Adhesion phase	Cellular lines	Number of adhering	Resistance (ohms)	Total cell surface (ST) ($\times 10^4$ pixel)	Number of integrin expressed per cell (I) ($\times 10^3$ U.A.)	Ratio (ST/I) ($\times 10^4$ pixel/U.A.)
(C)						
Initial	CHO	25,000	358.5 ± 4	17.5 ± 2	335 ± 7	490 ± 40
Spreading	CHO	50,000	405.8 ± 3	35 ± 4	714 ± 6	490 ± 35
Growth	CHO	90,000	481.5 ± 2	58 ± 2	1288 ± 9	450 ± 60
Initial	McCoy	25,000	360.6 ± 3	9.5 ± 1	198 ± 4	480 ± 50
Spreading	McCoy	50,000	407.3 ± 2	22 ± 1	437 ± 5	500 ± 25
Growth	McCoy	90,000	481.9 ± 1	40 ± 5	828 ± 5	480 ± 40

reported in Figure 3C (for CHO cell, no data shown). We successfully observed three phases to the cell attachment on the gold-quartz resonator: (1) sedimentation phase, (2) spreading phase and (3) multiplication phase (growth phase). Moreover, we observe that the response of motional resistance is dependent on the cell type because motional resistances between CHO cell and McCoy fibroblast cells are significantly different at 8 h ($P < 0.01$, ANOVA). In Figure 4B, we show and confirm that the percentage of cell increases during 8 h. Indeed, the motional resistance increases proportionally with the cell number and we observed ≈50% of cell increase or increase of motional resistance. The CHO cell and McCoy fibroblast cell were duplicated thus during experiments.

Concentration Dependence of Cell Adhesion

When we studied the relationship between the motional resistance and the cell concentration, we show in Figure 5A and B that, the higher cell concentration, the higher the motional resistance. In Figure 7, we represent the motional resistance versus cell concentration at different times of the adhesion process. We observe that there is a significant difference at 8 h ($P < 0.01$, ANOVA) between 500,000 cells, 100,000 cells and groups (50,000, 20,000, and 50,000 cells) with CHO and McCoy fibroblasts cells. In Figure 5C and D, we observe two relationships. The first one for 5,000 cells to 50,000 cells on one gold-quartz sensor, and the second for 50,000 to 500,000 cells on the resonator. These results show that for a number of cells lower than 50,000, the cells in culture on the quartz resonator were only in contact with the ECM (Fig. 5C and D). For a number of cells higher than 50,000, the cells are in contact with the matrix and also with the neighboring cells. In Figure 6A, we have confirmed by optical microscopy that CHO and McCoy fibroblast cell number adhering increases with cell concentration and we show that the cell adhering number is proportional to motional resistance.

Cell Counting and Quartz Resonator Technique

We show that the shift in motional resistance of TSM resonator is correlated to the cell number in Figure 6B and C. The correlation coefficient between these two parameters (resistance and cell number) is respectively 0.976 for CHO cell (no significant difference: $P < 10^{-4}$, Student test) and 0.988 for McCoy fibroblasts (no significant difference: $P < 10^{-4}$, Student test).

Cells Surface and Quartz Resonator Technique

We show that the shift in motional resistance of TSM resonator is proportional to the cells surface in Figure 7A and B. The correlation coefficient between these two parameters (resistance and cells adhering surface) are respectively 0.968 for CHO cell (no significant difference: $P < 10^{-4}$, Student test) and 0.991 for McCoy fibroblasts (no significant difference: $P < 10^{-4}$, Student test).

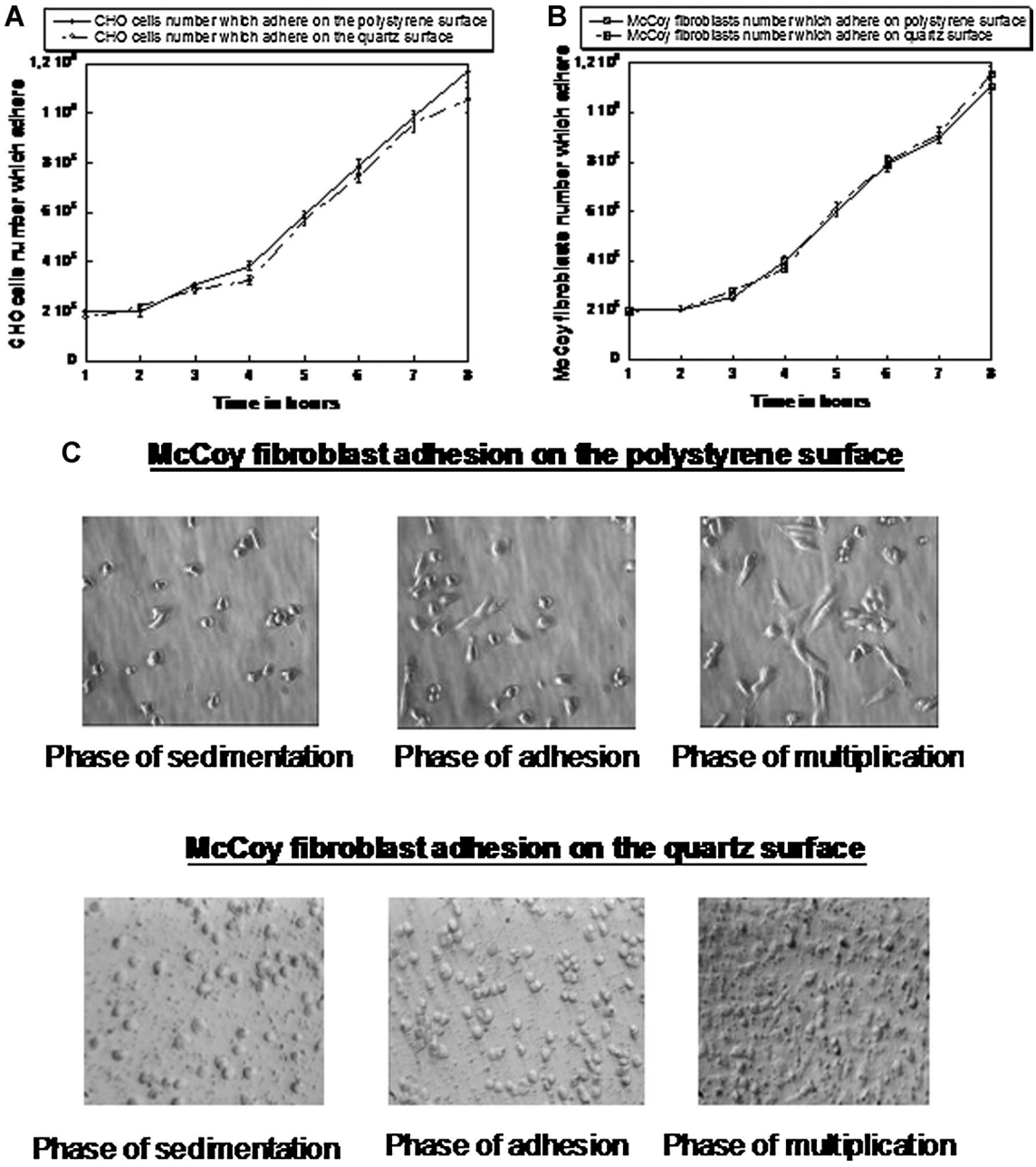

Figure 3. Correlation between respectively CHO cell number, which adhere to the polystyrene and to the gold surface (**A**), and McCoy fibroblast number which adhere to the polystyrene and to the gold surface (**B**). Experiments were repeated 10 times at 37°C, with the same gold surface. Error bars represent the SD of the motional resistance. **C**: Optical microscopy observation (400×) of McCoy fibroblast adhesion to gold sensor surface (for CHO cells, data not shown). At the sedimentation phase, the cells kept their spherical shape and did not spread actively on the gold surface. After a delay of 15 min the cells adhere and spread out progressively on the gold sensor surface. After a time of 300 min the cell multiplication process is starting.

The Influence of Integrin Molecules Measured by Quartz Crystal Resonator

The presence of integrin molecules measured by flow cytometry after having used anti-integrin $\alpha_5\beta_1$ antibodies. This marking is carried out with 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 7 h, and 8 h after measurement of motional resistance by quartz crystal resonator technique. We show that the shift in motional resistance of TSM resonator is proportional to the fluorescence intensity or integrin number per cell in Figure 7C and D. The correlation coefficient between these two parameters (resistance and fluorescence intensity) are respectively 0.992 for CHO cell (no significant difference: $P < 10^{-4}$, Student test) and 0.995 for McCoy fibroblasts (no significant difference: $P < 10^{-4}$, Student test).

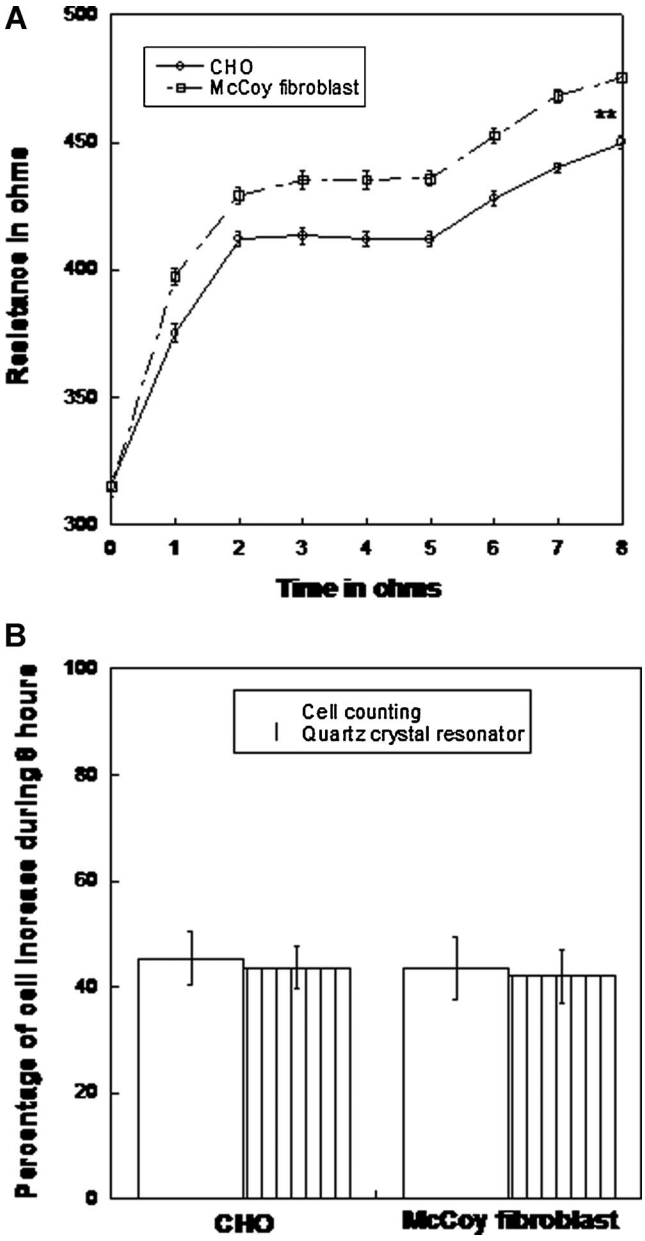

Figure 4. **A**: Typical representation of time relationship of the shear electrical motional resistance for a loaded quartz resonator with a monolayer of 50,000 CHO cells and McCoy fibroblasts. Acoustic experiments were repeated 10 times at 37°C, with the same gold surface. Error bars represent the SD of the motional resistance. **B**: Increase of cells during 8 h (%) with the CHO cells and McCoy fibroblasts by cell counting and quartz crystal resonator techniques. Experiments were repeated 10 times for each technique at 37°C, with the same quartz surface. Error bars represent the SD of the motional resistance and of the cell number, which adhere to the gold surface.

Cellular Removal by Enzymatic Digestion

When we add a trypsin solution, we observe an immediate cell removal process and consequently a drastic decrease of the motional resistance in Figure 8A and B since the trypsin property is to break the cell–matrix contact and to remove the cell from the substrate. Finally, this result confirms that the TSM resonator technique is valuable for all adhesion measurements while using inter-cell matrix contact. This measurement is affected by the intermediary matrix–cell contacts. However, to be effective, the quartz crystal resonator experiment series must be carried out with the same quartz, at a stable temperature. In Figure 8C, we have added the cell culture after trypsinization and we observe that the quartz crystal resonator is able to measure the new cell adhesion. The TSM quartz crystal sensor is therefore a technique to be introduced into a number of bioanalytic and biological applications such as measurements of cell adhesion processes via motional resistance.

Measure of Cell Adhering Concentration by a Quartz Crystal Resonator

Table IC shows the different results obtained for 25,000, 50,000, and 90,000 cells adhering on a gold surface for the two types of cellular lines. The values: R_2, the concentration of adhering cells, surface occupied by the cells (ST), number of the integrins per cell (I), and I/ST were measured or calculated. We observe that for each type of cell adhesion we have indeed an increase in the number of the integrins/cells (significant difference: $P < 10^{-4}$, Student test) and an increase in the surface occupied by the cells adhering (significant difference: $P < 10^{-4}$, Student test) when R_2 increases (significant difference: $P < 10^{-4}$, Student test). If we compare two types of cells at an increasing cellular concentration, we also observe an increase in the number of the integrins (significant difference: $P < 10^{-4}$, Student test) and surface occupied by the cell adhesion on the gold surface (significant difference: $P < 10^{-4}$, Student test). On the other hand, if we compare the two lines of cell with the same cell adhering concentration, we observe a resistance practically identical (no significant difference: $P < 10^{-4}$, Student test) but a significant difference ($P < 10^{-4}$, Student test) in the number of integrins and in the surface occupied by the cell adhesion ($P < 10^{-4}$, Student test). These results suggests that the cell adhering concentration seems to be a variable dependant on R_2 resistance but that the number of the integrins and the surface occupied by the cells adhesion on the gold surface are variables independent of the motional resistance. However, we notice that values of I/ST ratio are almost constant. These results suggest that there exists a relationship between the concentration of cell adhering, cell spreading, or cell growth on the gold surface measured by the quartz crystal resonator and the physical properties at the interface of the cell layer. From these results (Table IC), we write a function:

$$R_2 = f(\text{cell adhering number}) = 10^{-2}x\left(\frac{I}{\text{ST}}\right) \times \text{cell adhering number} + C \qquad (5)$$

Where ST is the total surface occupied by adhering cell (pixel), I is the number of integrins per cells (U.A.) and C is R_2 measured without cell adhering (ohm).

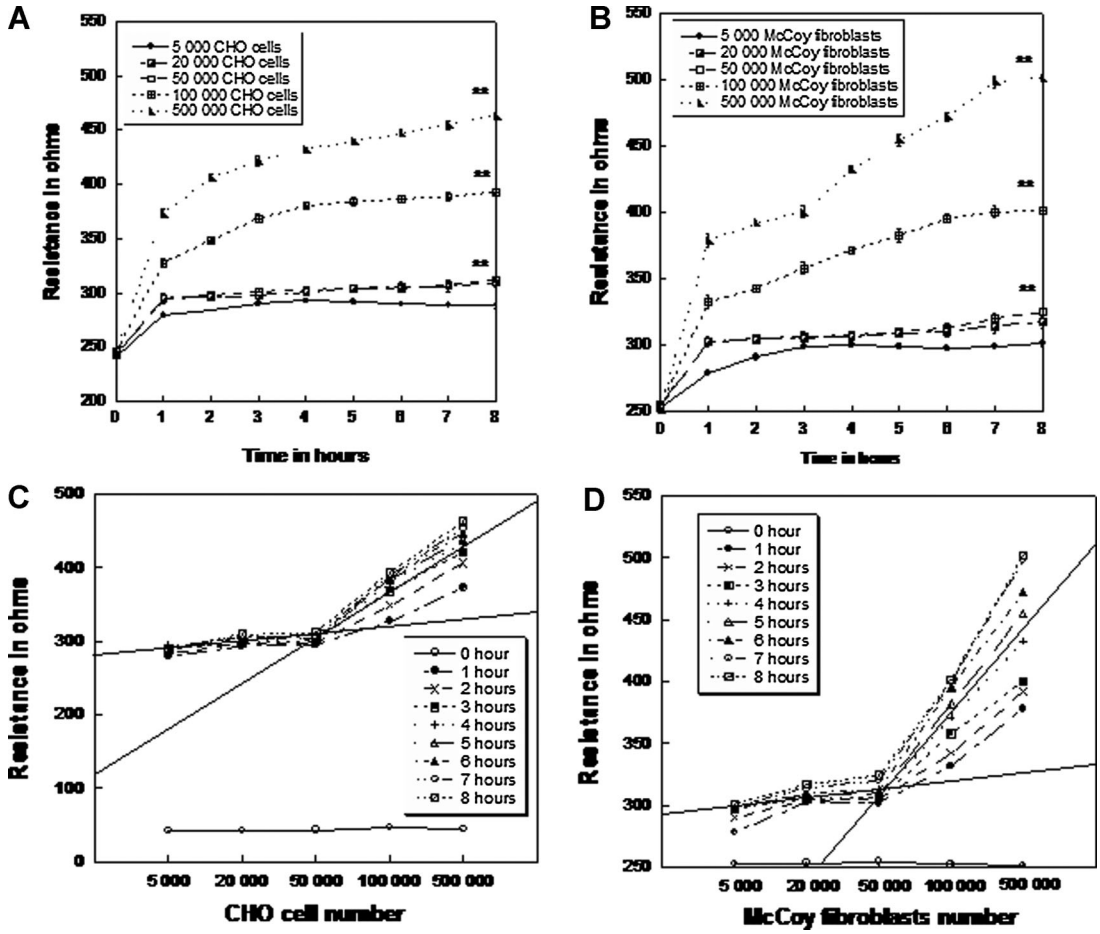

Figure 5. Time shift of the motional resistance for the CHO monolayer (**A**) and McCoy fibroblast-loaded quartz crystal sensor (**B**). The cell deposited at different density: 5,000 (●), 20,000 (◪), 50,000 (□), 100,000 (⊞), and 500,000 (◣) cell/gold sensor. Experiments were repeated eight times at 37°C. There is a significant difference ($P < 0.01$, ANOVA) between 500,000 cells/quartz, 100,000 cells/quartz, and group (50,000, 20,000, and 5,000 cells/quartz) for CHO and McCoy fibroblasts cells. CHO cells (**C**) and McCoy fibroblast (**D**) concentration dependence of the shear electrical resistance for different times of cell culture.

ST being dependant on adherent cells (ST $\approx$ cell adhering number $\times S$) and I/ST being practically constant (no significant difference: $P < 10^{-4}$, Student test), it will be possible to deduce I, ST or S (surface occupied by a cell) after having previously determined one of these three parameters by another biological technique (besides the quartz crystal resonator) allowing to measure the surface occupied by a cell or the number of the integrins per cell. Indeed, we observe for the two types of McCoy fibroblast and CHO cells at identical concentration of adhering cell, the factor as the resistance is close (no significant difference: $P < 10^{-4}$, Student test) whereas the number of integrins $\alpha_5\beta_1$ per cell which reflects the focal contact density is always very different (significant difference: $P < 10^{-4}$, Student test). These results suggest that the cell adhering concentration is the most factor involved in the resistance measurements.

Discussion

Characterization of the cell adhesion is of central importance in biological research, toxicology, and biotechnology (Kamarth et al., 1998; McGarry and McHugh, 2008; Morgan et al., 2008). Broad range responses toward multiple cell types and rapid analysis are often essential in these applications. For example, an easy determination of cell response to cell-affecting reagents is important in treatment of infectious diseases, or screening of new drugs and chemicals for their toxic effects.

Ebersole et al. (1991) suggest and anticipate that the piezoelectric sensor can be directly applied to the detection and characterization of eurokyotic cell growth. Previously reported piezoelectric methods for cell detection require selectivity, mass transduction, based on frequency-sensitive piezoelectric transduction and the quartz crystal resonator is

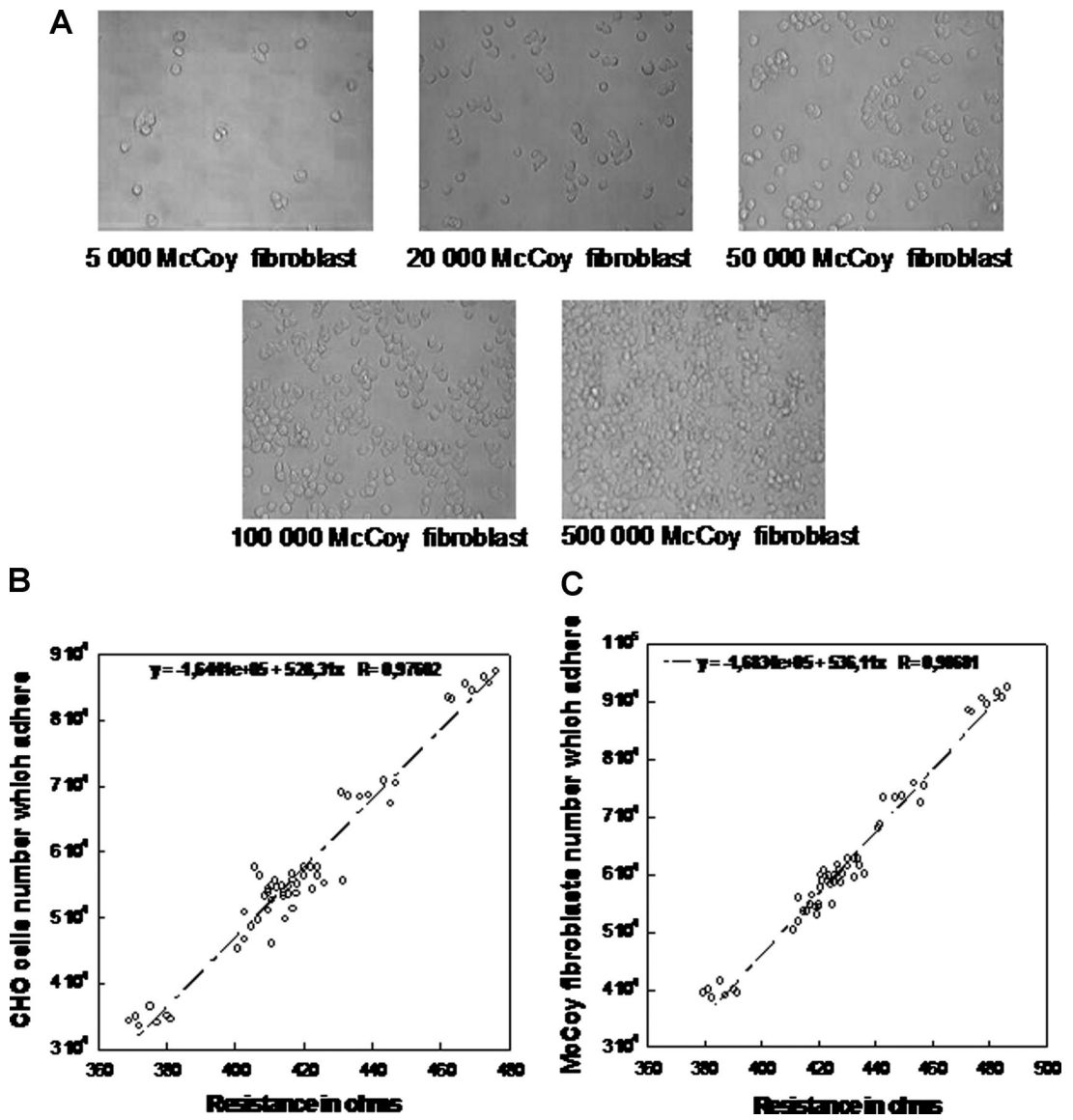

Figure 6. Optical microscopy observation (400×) at different density of McCoy fibroblast adhesion (**A**) (for CHO cell, data not shown) on gold sensor surface. At 50,000 McCoy fibroblasts, we have observed cell–cell and cell–matrix contacts in acceptable quantity. Correlation between CHO cells number which adhere and the motional resistance (**B**) and between McCoy fibroblast number, which adhere and the motional resistance (**C**). The variation coefficient is respectively 0.976 and 0.987 for CHO cells and McCoy fibroblasts.

not reusable (Fohlevova et al., 2007; Guo et al., 2008; Lord et al., 2008; Wegener et al., 1998, 2000; Welle et al., 2007). In our study, we have described and validated a simple, novel piezoelectric methodology based on resistance-sensitive piezoelectric transduction, to detect the presence of viable cells and to measure the rate of cell division.

Indeed, in this new method via the measurement of motional resistance the quartz sensor is reusable (better reproducibility of the acoustic response) and dependent on the temperature. Consequently, it will have to always be used at a constant temperature and in particular at 37°C, the universal temperature for eukaryotic growth cell.

Now it is clear by considering our results that the response of a quartz sensor is specific to cell contact because the cell environment such as the extracellular medium (culture medium), ECM (fibronectin) does not influence the acoustic response of the quartz crystal resonator. In addition, this assertion is confirmed by no cell adhesion such as immunizing cells (lymphocytes) which multiply in suspension and without contact with a surface do not modify motional resistance when they are deposited and cultivated in the TSM quartz crystal sensor. Moreover, other results implying the removal of adherent cells by tryspsin inducing a drastic decrease in motional resistance confirm the necessary of cells to adhere on gold surface in order to obtain an increase of the acoustical response.

In addition, Wegener et al. (1998, 2000) showed that the frequency measured by a QCM was proportional to the

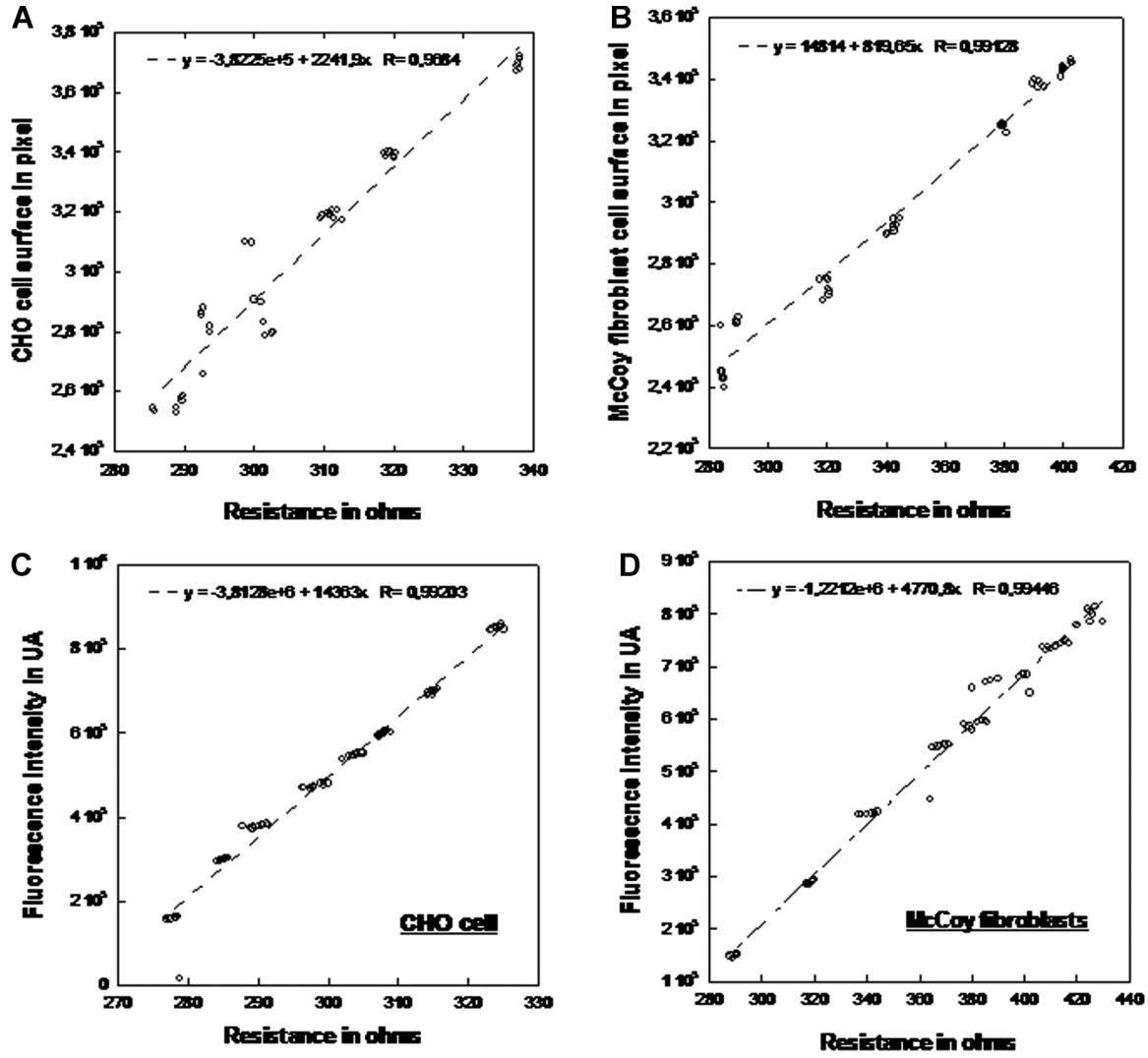

Figure 7. Correlation between the surface of CHO cells, which adhere and the motional resistance (**A**) and between the surface of McCoy fibroblast, which adhere on the quartz crystal resonator and the motional resistance (**B**). The variation coefficient is respectively 0.969 and 0.992 for CHO cells and McCoy fibroblasts. Correlation between the fluorescence intensity, which reflects the presence of integrin $\alpha_5\beta_1$ molecules and the motional resistance for CHO cells (**C**) and for McCoy fibroblast cells (**D**). The variation coefficient is respectively 0.992 and 0.995 for CHO cells and McCoy fibroblasts (no significant difference, $P < 10^{-4}$, Student test).

number of adherent cells on the gold surface but that this frequency was not dependent on the type of adherent cells. In this study, our device measuring motional resistance is proportional to the number of cells adhering to the gold surface but it is also dependent on the type of adherent cells. This fact is very important because it proves that our acoustic sensor can differentiate cell adhesion, spreading, and growth between different lines of cells. Moreover, the measurement of resistance is carried out every 30 s which makes it possible to follow the cell response in real time.

We also showed that motional resistance was dependant on the cell adhering concentration during the initial, spreading, and growth phases for the two types of different cell lines but not dependant on the surface of adhering cells on a gold surface and to the number of focal contacts (via the number of integrin molecules) between the cells and the ECM present at the gold surface. However, we show that the relation between the surface occupied by the cells on the number of integrins per cell is constant when the resistance increases or remains identical for two cellular types of lines. These results suggest that the motional resistance is a function of the cell adhering concentration with as a variable the surface occupied by the cells, on the number of the integrins per cell. Our assumption is that when the quartz crystal resonator vibrates at the frequency of 9 MHz, an acoustic wave is generated in the adherent cell whose rough penetration is 10^{-1} μm (Haider et al., 2004; Ladau and Lifschitz, 1991; Rajakovié et al., 1991; Wang et al., 2008) corresponding approximately to the thickness of the basal

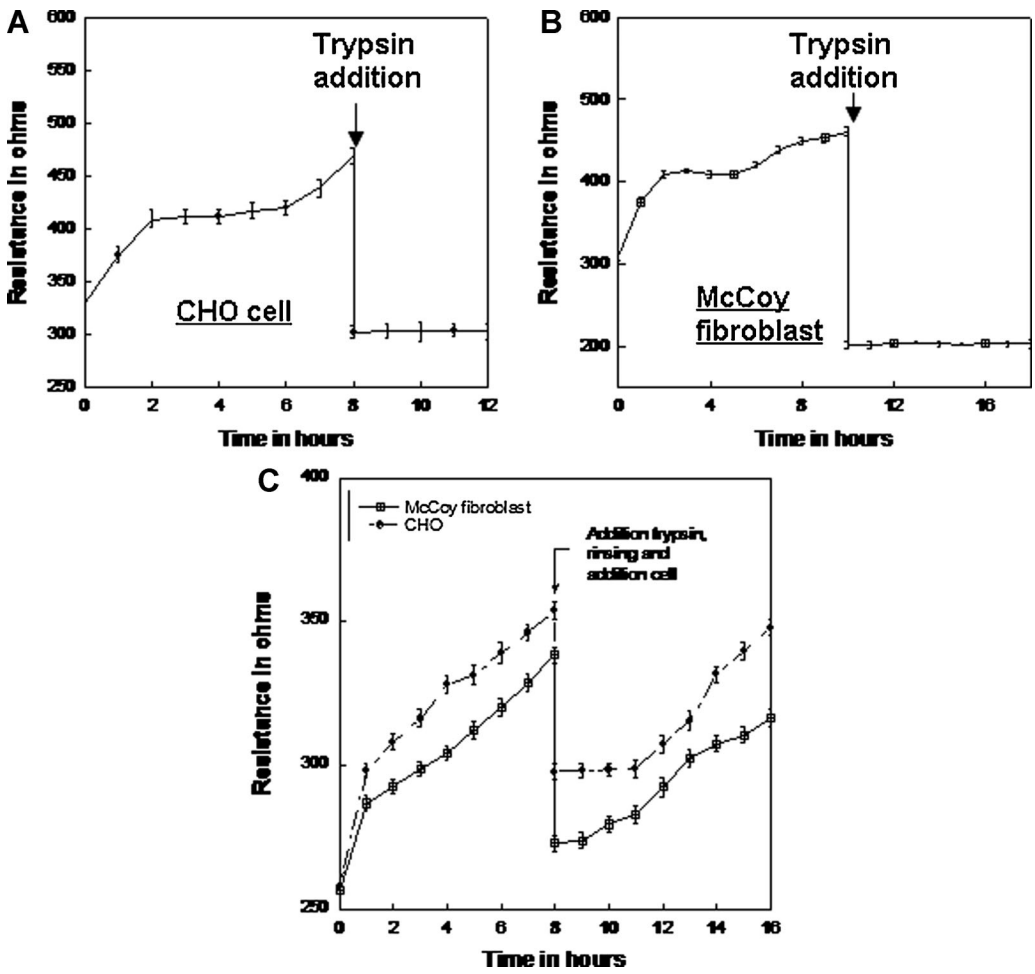

Figure 8. Time shift of the motional resistance for a loaded quartz crystal resonator with CHO cells (**A**) or McCoy fibroblasts (**B**) at 50,000 cells/quartz before and after addition of trypsin solution. Experiments were repeated 10 times at 37°C. Error bars represent the SD of the motional resistance. **C**: Time shift of the motional resistance for a loaded quartz crystal resonator with CHO cells and McCoy fibroblast at 50,000 cells/quartz. After 8 h, we added the trypsin solution, rinsed and added of new CHO cells or new McCoy fibroblasts at 50,000 cells/quartz. Acoustic measurements were repeated 10 times at 37°C. Error bars represent the SD of the motional resistance.

membrane of adhering cells (Fig. 9). The cells adhere in initial, spreading, and growth phases on the gold surface, the cells express their integrins as a function and dependence with the surface occupied by the cells using the cell–cell communication, integrin–fibronectin links with a several affinities providing the tensile strength (Li et al., 2007; Marx et al., 2003). Indeed, integrins play a crucial role in cell adhesion, migration, and signaling by providing transmembrane links between the ECM and the cytoskeleton, and integrins cluster in macromolecular complexes to generate

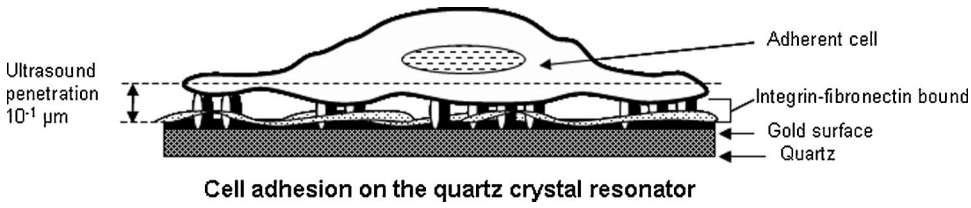

Figure 9. Scheme of cell adhesion to the quartz crystal resonator. When we measured the motional resistance for cells which adhere, the ultrasound penetration (Ladau and Lifschitz, 1991; Rajakovié et al., 1991; Haider et al., 2004; Wang et al., 2008) is 10^{-1} µm, corresponding to the thickness of the adherent cell. Basal membranes are detected. The presence of the integrin–fibronectin connection (ECM) and cell surface explain why the quartz crystal resonator measuring the cell adhering concentration.

cell–matrix adhesions such as focal points (Hynes, 1999; Wozniak et al., 2004). The space between integrins and the gold surface is 9–12 nm (Cavalcanti-Adam et al., 2006). The linear dimension for an integrin group within the basal membrane is 12–15 nm (Hynes, 1992, Cell), and the space needed for proteins such as vinculin that cross-links integrins to focal points is 60–75 nm (Zhao et al., 2009). Adding these numbers, the total space between integrins and the adhering surface is 82–102 nm. This estimate corresponds well to the penetration of acoustic waves ($10^{-1}\,\mu$m). Consequently, the TSM quartz crystal sensor quantifies the concentration of cell adhering, cell spreading or cell growth indirectly via two factors: the cells surface and the number of integrin molecules per cell. Moreover, the ratio (I/ST) is a constant and being much smaller than the number of adhering cell in the equation $R_2 = f(\text{cell adhering number}) = 10^{-2}x\left(\frac{I}{\text{ST}}\right) \times \text{cell adhering number} + C$, we can deduce that the resistance measurement by the QCM is a good measure of the cell adhering concentration and it will be possible to deduce is I or ST or S (surface occupied by a cell) after having previously determined one of these three parameters by another biological technique (besides the quartz crystal resonator) allowing to measure the surface occupied by a cell or the expression of the integrins per cell. The fundamental question raised by these results seems to be the following: Is it the cell concentration on the adhering surface or the focal contact density (these two items being possibly related in a non-trivial way), which is the most factor in the involved measurements? We believe that the concentration on the adhering surface is more strongly involved in the resistance measurements. Indeed, it is difficult to dissociate these two phenomena when we analyze the results of a cellular type but if we analyze the results of the two cellular types, this is more evident. We observe for the two types of Mc Coy fibroblast and CHO cells that when the cell concentration on the adhering surface is identical, the resistance is close whereas the number of integrins per cell which reflects the focal contact density is very different.

On the other hand, it was crucial to check whether the morphology and the number of adherent cell in different phase measured by the quartz crystal sensor were or not modified by their adhesion to the gold-quartz surface compared to a standard system of biological cell culture made up of polystyrene (standard box of cell culture). Indeed, if there is a difference, questioning the advantage of the quartz crystal resonator for the biological and biochemical research. However, our results showed that the morphology and the number of adhering cell on gold surface are identical to those obtained on a more conventional cell culture surface. Consequently, the acoustic response via motional resistance is quite specific induced by the adhesion or/and growth of different cell lines, when the acoustical measurement is taken at constant temperature at 37°C in a CO_2 incubator.

The applications of this piezoelectric eukaryotic growth cell sensor which measures the concentration of cell adhesion, spreading, or/and growth are numerous. This possibility should be useful in screening new therapeutic drugs, cytotoxic chemicals, and growth regulators on a real time basis. Previously our work showed that the sensor technology can be used with a polymer (Le Guillou-Buffello et al., 2004, 2005) and the bioactive biomaterial (Le Guillou-Buffello et al., 2008) coated on the gold surface in order to characterize the adhesion, the multiplication and the metabolism of specific cell types. Indeed, previously work demonstrated that bioactive PMMA (Polymethyl Methacrylate), bearing sulfonate and carboxylate groups present, inhibit properties of McCoy fibroblastic cells adhesion when compared to non-functionalized polymer PMMA and polymers bearing only one of these functional groups by a quartz crystal resonator technique (Le Guillou-Buffello et al., 2004, 2005). These polymers are excellent candidates for biomedical application where inhibition of cell adhesion and proliferation is needed: they can be used in the intra-ocular lens application to prevent the secondary cataract. Also, we showed previously that functionalization of titanium alloys with hydroxyapatite with and without RGD (Arg-Gly-Asp) peptides could increase cell adhesion (HOP cell: Human OsteoProgenitor cell) by a quartz crystal resonator (Le Guillou-Buffello et al., 2008). Such functionalization approaches could be used for bone prosthese applications to induce osteointegration and osteoconduction. It is anticipated that miniaturization of piezoelectric sensors will extend the usefulness of this technology for clinical microbiology, industrial quality control (fermentation, food processing) and research. The sensors eventually can be constructed as an array, or single probe devices, and can be used in either in disposable or reusable formats.

The piezoelectric method described here is quantitative in real-time and has several advantages over existing techniques. Currently quantifying cell adhesion methods involve exerting mechanical forces and quantifying the cells resisting to such a force (indirect spectrometric assays or by counting). These methods do not account for cells that are weakly adhering to the surface and they also fail to measure adhesion under environmental conditions ideal for cells. More sophisticated methods measure the forces necessary to remove single cells from a given substrate using atomic force microscopy, micromanipulators or micropipette aspiration. These methods lack the ability to study several cells in a culture. These adhesion events can be quantified by microscopic or ultra structural techniques often coupled with image analysis. Such methods, however, lack the ability to study the adhesion phenomena in real-time. Consequently, they are unable to detect changes in external conditions that may alter the dynamics and viability of adherent cell. In addition, most of the above methods involve a lot of skill, instrumentation and man-power to produce reliable results.

In conclusion, the development and the use of quartz crystal resonators have a great potential for future studies of adhering cells in initial, spreading and growth phases. This technique should be particularly suitable for the comparison of several cellular lines and for the assessment of adherence

when cell are exposed to therapeutic molecules, biomaterials or other materials.

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