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Comparative studies of Atomic Force Microscopy (AFM) and Quartz Crystal Microbalance with Dissipation (QCM-D) for real-time identification of signaling pathway

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Abstract- **Cell signaling is one of the fundamental processes that control the cell fate. It modulates the cell shape and mechanics. To identify the dynamic signaling pathway in situ, we need tools that are capable of monitor the real-time elasticity and viscosity changes as well as structural rearrangements. Atomic Force Microscopy (AFM) has been demonstrated to be an effective instrument to visualize membrane and cytoskeleton structures on live cells. It can also provide the mechanical stiffness information by recording force displacement curves. Meanwhile, the viscoelasticity change by signaling pathways can be measured as the change of dissipation of a monolayer of cells by means of a Quartz Crystal Microbalance with Dissipation (QCM-D). In the current study, we use the human epidermoid carcinoma A431 cell line as a model system which will be stimulated by epidermal growth factor (EGF). AFM was first used to image the structure of live A431 cells before and after stimulation; force measurement was also performed to analyze the dynamic elasticity change. The change of viscoelasticity of the A431 cell induced by EGF was monitored in real time on a QCM-D in terms of dissipation change and frequency shift. The mechanical property measurements from AFM and QCM-D experiment was analyzed and compared. Quantitative analysis can be performed to obtain the dynamic modulus of the material through theoretical modeling. This novel combination can be complementary to each other. A unified profile can therefore be generated as an effective indicator of signaling pathways such as cell proliferation and apoptosis.**

I. INTRODUCTION

Epidermal growth factor (EGF) is a ligand for epidermal growth factor receptor (EGFR), a cell surface receptor that has intrinsic tyrosine kinase activity [1]. Its activation by EGF can induce a variety of signaling events. The activation process itself is time-, dose- and context-dependent. Different activation can either stimulate or inhibit cell cycle progression, resulting in cell proliferation or cell apoptosis. The A431 human epidermoid carcinoma cell line has been widely used as a model for the study of signaling events after the interaction of EGF with its specific cell surface receptors [2].

The cellular response to the stimulation either proliferation or apoptosis can be significantly manifested in cell morphology and cellular mechanics change. Recent study [3] has shown that cytoskeleton elements (actin filaments and microtubule) will form a thick layer wrapping around the collapsed nucleus, and this result in a time-dependent stiffness change, first decrease and then increase. Therefore dynamic morphological and mechanical properties have been regarded as biomarkers, sometimes even regulators of the signaling and physiological processes. To characterize those properties requires special instrumentation and techniques.

AFM, due to the nature of its measurement process, its uniqueness of working in liquid with high resolution, is an ideal tool for visualization of the structural rearrangement in real time. We have demonstrated that nanometer scale cellular junction losses due to antibody binding to keratinocytes can be captured [4]. Detailed analysis of AFM deflection image also revealed significant surface roughness change in response to antibody interaction. Various other researchers have also showed reliable results from AFM imaging for cellular morphology observation [5]. Mechanical properties can be obtained by recording the force displacement curves via AFM nano indentation. Its effectiveness as biomarkers for physiological conditions has been reported previously [6].

QCM-D has been used for molecular recognition studies [7] to characterize protein adsorptions [8], cell spreading [9] and so on. It utilizes a quartz crystal chip that works in thickness-shear mode. The shift in resonance frequency of the quartz crystal indicates the mass change of the absorption layer; while the dissipation change will be used to construct the viscoelasticity property of the adsorbed surface film.

The combination of AFM and QCM-D for biological investigations has been realized in previous works. It can be categorized into two groups, functional and instrumentational. Functionally, the QCM-D can measurement the change of viscoelastic property change of the biological matter due to alteration of physiological conditions. This quantitative data can only be verified by probing the surface topography by AFM under the same physiological conditions accordingly. This functionality has been demonstrated by study the effect of different substrates on cell shape and cell mechanics [10]. The shape and mechanical properties are obtained by AFM imaging and force measurement while the QCM-D characterizes the adhesion between the cell and the substrates through the frequency and dissipation change. Instrumentation of AFM combined with QCM-D has been developed [11]. The work has achieved an instrument where the substrate for AFM experiment is an oscillating quartz crystal. Therefore the AFM imaging and measurement can be performed simultaneously with the QCM-D recording.

In this work, we combine AFM probing and the QCM-D observations on the same set of cellular signaling process. Both

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experiments monitor a monolayer of A431 cells that grow on substrate (glass substrate for AFM and quartz crystal surface for QCM-D) which will be stimulated by EGF. Whist QCM-D monitors the portions close to the quartz crystal surface; AFM will probe the apical surface away from the substrate. By analyzing and comparing the quantities data from both techniques, a unified profile can be generated as indicators of cell signaling pathways.

II. Materials and Methods

A431 cells have been cultured in the presence of CO_2 (5% v/v) at 37°C in Dulbecco's Modified Eagle's Medium (DMEM) (ATCC, Manassas, VA) supplemented with fetal bovine serum (10 % v/v) (Invitrogen.com) and 0.5% antibiotics (stock: 10000 U/mL Penicillin, 10000 µg/mL Streptomycin, Invitrogen). EGF is diluted to the concentration of 100 ng/ml using HBSS with 10% HEPES. The HBSS buffer solution is used as a control for the experiment.

All the experiments for AFM measurement and imaging were performed in aqueous environment at room temperature on Bioscope (Veeco Instruments, Inc. Santa Barbara, CA USA) using a Nanoscope IV controller. Before the stimulation, the cultured cells were incubated inside the HBSS buffer solution for around 2 hours to achieve equilibrium.

The electrochemical experiments were performed using a QCM recording instrument (Q-Sense E4, Go¨teborg, Sweden), contained of a Main Unit and an Oscillator, computer-controlled simultaneous measurement of resonant frequency (f) and energy dissipation (D) as a function of time. All electrochemical measurements were carried out in a polished gold-deposited circular (25 mm, QSX 301); AT-cut crystals with a fundamental resonant frequency of 5 MHz were used. The gold-coated QCM disks were oxidized for 10 minutes in an UV/ozone chamber (Bioforce nanosciences, Model: ProCleaner TM 110) to produce a hydrophilic surface suitable for cell adhesion.

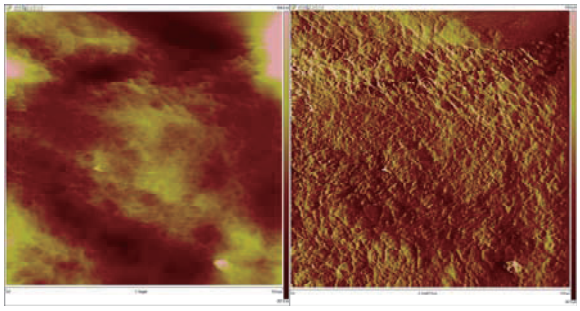

Fig. 1. A431 cells imaged live in HBSS buffer, the topography and deflection image with detailed structure of cytoskeleton; Scan size: 15µm

III. AFM for Energy Dissipation Measurement

As demonstrated by several researches, the structural information of cells is one of the important biomarkers to qualitatively identify cell physiological conditions. In fact, the reorganization of the cytoskeleton is the primary driving force behind the cellular migration and tissue growth. For AFM imaging and analysis, tapping mode AFM imaging was performed on cells in HBSS buffer solution. For live cell imaging, an individual cell will be monitored before and after the adding of specific reagents (EGF inhibitor or EGF ligand.) The topography and deflection image for A431 cell are shown in Fig. 1. The cytoskeleton structure underneath cell membrane is visualized in high magnification. Several parameters will be analyzed such as cell mean height and cell surface roughness to determine the cellular condition, i.e. cell growth or apoptosis. Since this is not the focus of the paper, detail for the imaging analysis is not presented.

Force displacement curves have been collected in HBSS on live cells. A silicon nitride probe with a spring constant of 0.30 N/m characterized by thermal tune method was used. Following Hertzian model analysis of force curves, quantitative Young's modulus was generated. Besides, by integration of the area between the loading and unloading portion of the force displacement curve, the energy dissipation for each indentation cycle was obtained.

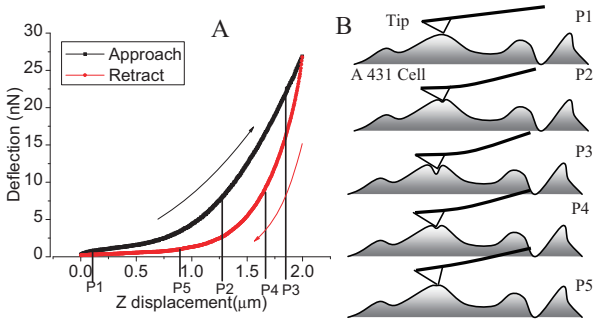

Fig. 2. Force measurement for energy dissipation calculation

The loading and unloading of the force measurement process is denoted by a series of positions, from P_1 to P_5 as shown in Fig. 2. During this process, the vertical direction piezo drives the cantilever moving towards the cell sample and deforms it. The position and the deformation can be depicted as the force-displacement curve. Besides, cantilever deflection picked up by position sensitive device (PSD) to generate the loading force, the deformation can be calculated by difference between the displacement and cantilever deflection. During each cycle, forces in approach curve is always larger than those in the retract curve meaning that there will be energy loss for each loading and unloading process, and this energy is calculated by integrate the area between the two curves. It is a measure of the viscosity behavior [12].

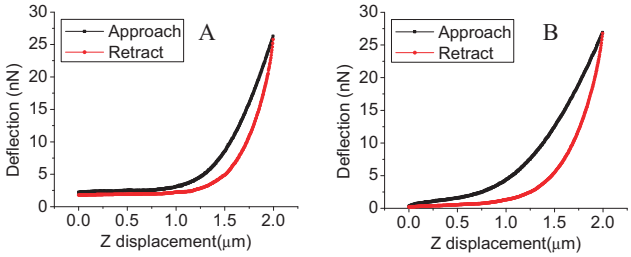

Fig. 3. Force displacement curve showing the dissipation (area) difference before and after the stimulation of EGF to A431 cells

The force measurement before and after the stimulation of the EGF are shown in Fig. 3A and Fig. 3B. As can be observed, the energy dissipation for each cycle, the area between the loading and unloading force curve, is increased from 3.0 to 6.8 femto-Joules after stimulation.

The whole stimulation process was also monitored dynamically in real time. After the EGF stimulation, force displacement curves were taken at a 0.8 Hz frequency. Each force curve was analyzed to obtain the energy dissipation using a Matlab routine. As shown in Fig. 4A, at minute 10, the stimulation started by adding 1 ml of the EGF containing buffer into 4 ml of the petri dish where the A431 cells sit inside HBSS buffer, to make the final concentration 20 ng/ml; all the force-displacement curves before that would be used as baseline. Right after the adding of EGF, there is an increase in energy dissipation; this sharp increase continues until around 10 minutes after stimulation. Then there is a slight drop of the stimulation effect as indicated by the decline of that discrepancy. Fig. 4B summarizes the dynamic change of the energy dissipation. Before stimulation, the energy dissipation for the normal A431 cells is 3.5 ± 0.85 femto-Joules; for the first 10 minutes after stimulation, the average dissipation is 6.5 ± 1.51 femto-Joules; while the continued observation for 30 minutes shows an average energy dissipation of 5.8 ± 0.80 femto-Joules.

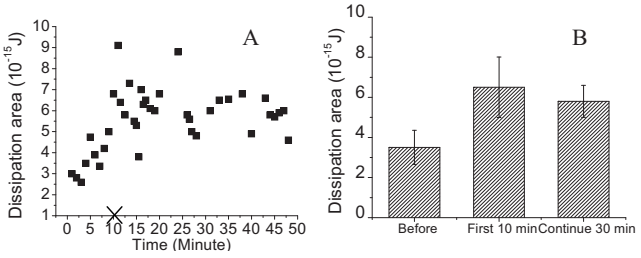

Fig. 4. Dynamic energy dissipation in real time (A), energy dissipation on different stages (B)

IV. QCM-D FOR ENERGY DISSIPATION MEASUREMENT

QCM uses a quartz crystal; as a result of its nature, the application of an alternating electrical field will generate a shear deformation [13], as shown in Fig. 5A and B. Both surfaces will move in parallel by in opposite direction working like an acoustic wave that propagate in the direction perpendicular to the crystal surface. The acoustic wave has a frequency that is inherent to the crystal: $f = n\frac{v}{2t_q}$ where f is the resonant frequency, n is the overtone number and t_q is the thickness of the crystal. If the sensor is immersed in liquid as shown in Fig. 5C, the acoustic wave will propagate into the liquid and there will be an exponential decay of the oscillation amplitude.

By switching on and off the driving power to the quartz crystal which is in thickness-shear-mode oscillation and recording the decay of the damped oscillation, the dissipation change ΔD and the resonance frequency shift Δf were measured in real time. ΔD is inversely proportional to the time constant of the decay and Δf is the period of the decaying oscillation.

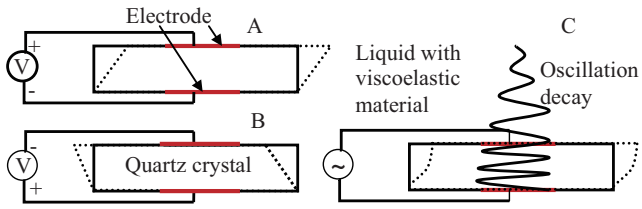

Fig. 5. Schematic of QCM-d dissipation in solid-liquid interface

For a single experiment, the measurements were carried out in a 400 µl static solution. The cells on the QCM disk were first bathed in close chamber with 400 µl of 20 mM HEPES HBSS buffer until a stable base line was obtained (around 2 hours). Following removal of 400 µl of HBSS buffer from the QCM chamber, 400 µl of pre-warmed HBSS buffer containing EGF inhibitor (VWR Scientific) or EGF ligand (Fisher Scientific) was added. During the process, automatic monitoring the change of dissipation and shift in frequency was recorded in 120 second interval.

The QCM-D experiment results for a continuous monitoring of 160 minutes after stimulation of EGF of dosage variation confirmed that the energy dissipation increased over time as observed by AFM. For the frequency response in Fig. 6B, overall we observe an increase of frequency and the increase is on a dosage dependent manner except for the 2mM stimulation. More importantly, as for the dissipation in Fig. 6A, after stimulation with EGF of different concentrations, the dissipation decrease is clearly dosage-dependent. Immediately after stimulation, the dissipation shows a sharp drop, then after about half hour it climbs up slightly. The initial sharp drop of dissipation indicates that more energy dissipation occurred after cells were stimulated with EGF. Qualitatively it indicates that cells become more viscous and more damping is presented in the cell-crystal oscillator. The change of mechanical property might be due to the reorganization of the cell cytoskeleton after EGF stimulated cell growth or even apoptosis.

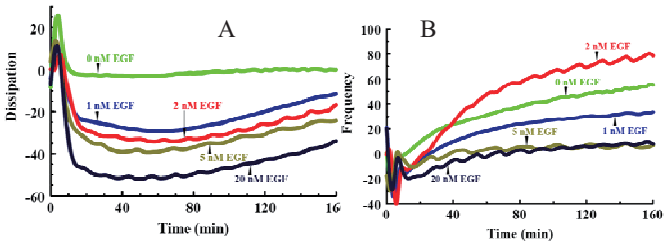

Fig. 6. QCM-D observation for EGF stimulation over 160 minutes, dissipation change (A) and frequency shift (B)

V. QUANTIFY THE VISCOELASTICITY BY MODELING

For pure elastic materials, viscosity is absent from them. There will be no energy dissipation. Accordingly, when performing AFM force measurement, the approach and retract curves would overlap with each other. However, for biological samples, viscosity is inherent to their material property; researches have shown that the cellular components are mostly

viscoelastic such as the cytoplasm fluid, even the cytoskeleton commonly regarded as elastic component is viscoelastic in nature [14]. Therefore, normal force curves on biological samples in liquid display large discrepancy between the approach and the retract curves.

However, integrating the discrepancy between the two curves is only a quasi-quantitative method to quantify the viscoelasticity of the material. More detailed study should resort to dynamic mechanical analysis where a small oscillatory stress is applied and the corresponding strain is measured. Complex dynamic modulus G^* is used to represent the relation between the stress and strain:

$$G^* = G' + iG'' \quad (1)$$

Where G' is the elastic modulus (storage modulus) and G'' is the viscous modulus (loss modulus). This dynamic modulus can be obtained by AFM based force modulation.

Force modulation imaging is an AFM technique that maps differences in surface stiffness [15]. It is developed on the basis of contact mode AFM imaging. The basic principle for force modulation imaging is to add a small vertical oscillation of the tip during the contact scanning. That means the contact force is modulated while the average of it is still the same with the contact mode. When the tip is modulated with a vertical displacement around 10 nm with frequency ranging from 0.5 Hz to 500 Hz [16], there will be a difference in the resistance of this oscillation from among areas even with the same contact force. This resistance can be measured as the viscoelasticity of the sample. The schematic drawing of the modulation process is shown in Fig. 7.

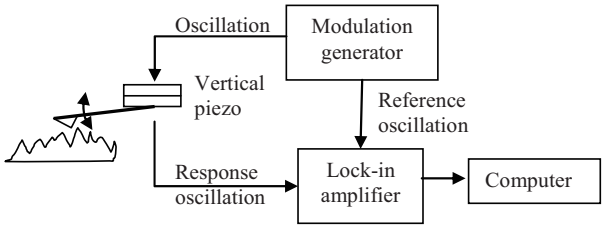

Fig. 7. Schematic of force modulation by AFM

For a spherical shaped tip, the applied normal force, a complex with different modulation frequency can be denoted as (for a spherical indenter):

$$F^* = \frac{4}{3}\frac{R^{\frac{1}{2}}}{1-\nu^2}(E_0\delta_0^{\frac{3}{2}} + \frac{3}{2}E_1^*\delta_0^{\frac{1}{2}}\delta_1^*) \quad (2)$$

Where ν is the Poisson ratio, R is the radius of the probe and E_0 is the elastic modulus at zero frequency. E_1^* is the frequency dependent elastic modulus. δ_1^* is the indentation displacement caused by the oscillation, and $\delta_0$is the main indentation caused by the applied force by contact AFM.

There are two components to the applied force: contact force and the oscillation force. The original Hertz model contact force is:

$$F = \frac{4}{3}\frac{E}{1-\nu^2}R^{\frac{1}{2}}\delta^{\frac{3}{2}} \quad (3)$$

The oscillation force is:

$$F_{osc}^* = \frac{2R^{\frac{1}{2}}}{1-\nu^2}E_1^*\delta_0^{\frac{1}{2}}\delta_1^* \quad (4)$$

The complex modulus obtained can be expressed as the relation between the applied force and the indentation as well as the indentation displacement:

$$G^* = G' + iG'' = \frac{f_{osc}^*}{2\delta_1^*(R\delta_0)^{\frac{1}{2}}} \quad (5)$$

From equation (5), we can calculate the dynamic modulus of the viscoelastic biological samples with G' and G''.

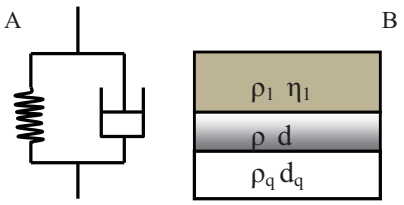

Fig. 8. The modeling of viscoelastic material (A) and the material configuration for QCM-D modeling (B)

Similarly for QCM-D, by proper modeling of the oscillation process and the viscoelastic property of the material, we can derive the same complex dynamic modulus G^*. The material absorbed onto the quartz crystal substrate was modeled as a Voigt viscoelastic element by a parallel configuration of a spring for elastic behavior and a dashpot for damping (Fig. 8A). The complex shear modulus is expressed as:

$$G^* = G' + iG'' = \mu + i2\pi f\eta \quad (6)$$

Where μ and η are shear modulus and shear viscosity. It is assumed that the quartz crystal of thickness d_q and density ρ_q is covered with this viscoelastic thin film with uniform thickness d and density ρ. The non-slip condition is applied to the Newtonian liquid with density ρ_1 and viscosity η_1 and the thin film surface (Fig. 8B). By solving the wave propagation equation, the change in frequency Δf and energy dissipation ΔD can be related to the viscoelastic modulus μ and η of the thin film by [17]:

$$\Delta f = \frac{Im(\beta)}{2\pi d_q\rho_q} \quad (7)$$

$$\Delta D = \frac{Re(\beta)}{\pi d_q\rho_q} \quad (8)$$

Where $\beta = \frac{\zeta_1(2\pi f\eta - i\mu)}{2\pi f}\frac{1-\alpha^2\zeta_1 d}{1+\alpha^2\zeta_1 d}$ and $\alpha = \frac{2\pi f\eta\zeta_1 - i\mu\zeta_1 + 2\pi f\eta_1\zeta_2}{2\pi f\eta\zeta_1 - i\mu\zeta_1 - 2\pi f\eta_1\zeta_2}$, with $\zeta_1 = i2\pi f\sqrt{\frac{\rho}{\mu + i2\pi\eta}}$ and $\zeta_2 = \sqrt{\frac{i2\pi f\rho_1}{\eta_1}}$.

Normally a few overtones will be tested $f = nf_0, (n = 1, 3, 5)$ to obtain the best fit for experimental data and the model.

VI. CONCLUSION

The results from both AFM and QCM-D measurements indicate that there will be cellular mechanical change after stimulation; and this change of viscoelasticity has the same manifestation in both the AFM and QCM-d measurement. The AFM force measurements showed the rapid increase of energy dissipation after EGF stimulation by integration of the approach and retract force curves. It followed by a slight drop of that increased amount. Meanwhile, the QCM-D measurements showed the same drop immediately after the EGF stimulation and small amount of climb afterwards. This quasi-quantitative analysis will be further improved by obtain

the dynamic modulus of the viscoelastic biological sample from both AFM and QCM-D modeling. The detailed modeling would provide the quantitative dynamic modulus change due to the physiological condition caused cellular response. We prove that dynamic cellular signaling events can be studied and identified by combination of AFM and QCM-D technique. This technique could facilitate the investigation of structural and mechanical biomarkers in cellular signaling processes.

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