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Screening between normal and cancer human thyroid cells through comparative adhesion studies using the Quartz Crystal Microbalance

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ABSTRACT

In this work, the Quartz Crystal Microbalance with Dissipation monitoring (QCM-D) was used to distinguish the dynamic cell adhesion behavior of human normal (Nthy) thyroid epithelial cells from poorly differentiated anaplastic carcinoma cells (ARO). The surfaces used to facilitate cell adhesion were bare titanium (Ti), gold (Au) and fibrinogen-coated gold (Fg-Au). The pattern of cell adhesion for both cell lines was that the largest acoustic signals were observed on Ti, followed by Au and last by Fg-Au; in addition, ARO cells always produced smaller acoustic signals than Nthy cells on the same surface and for the same number of cells in suspension. Moreover, the calculated acoustic ratio of energy dissipation over frequency change suggests a higher ability of Nthy cells to spread and potentially form more attachment points on the surface than the ARO cells, something observed in SEM images. Finally, we demonstrated that the application of two surfaces for cell adhesion experiments, one of which is Au and the other either Ti or Fg-Au, can discriminate with accuracy between the two particular cell types and potentially form a platform for differentiation between normal and cancer thyroid cell types.

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1. Introduction

Cell adhesion is a vital process in biology. Cells adhere to their surrounding environment in order to migrate, differentiate and proliferate. The interaction of cells with the extracellular matrix (ECM) is mediated by adhesion receptors of the integrin superfamily which recognize peptide motifs such as the Arg-Gly-Asp (RGD) found in ECM proteins [1]. Adhesion complexes are formed and located at specific sites termed focal adhesion points (FAs) that secure the anchorage of the cell to the substrate. Cell adhesion studies are of interest to cellular biophysics and find applications in nanotechnology and biomaterials; for example in orthopedic research understanding osteoblast adhesion to putative biomaterial surfaces is crucial to optimize the bone-biomaterial interface [2]. For cell-substrate interaction tracking, many techniques are employed such as optical, mechanical, electrochemical and acoustic [3,4].

Acoustic sensors have been extensively employed in cellular studies at the solid-liquid interface in real time and without the need of labels. The acoustic sensing procedure is non-invasive so that the adhered cells remain intact and can be further analyzed. For this reason, acoustic devices such as the Quartz Crystal Microbalance (QCM) or Thickness Shear Mode Resonator (TSM-R) and the Love wave device have been used to monitor cell-cell interactions, cell response to drug administration, cell mechanics, cellular signaling and cell-substrate adhesion [5–7]. Cell-cell interaction studies employing sigmoidal-shaped cell adhesion curves have been used to quantify cell cooperativity [8,9]. The cell response to external stimuli and subsequent modulation of its morphology has been reported with the QCM-D and Love wave device [10–13] during the addition of cytochalasin D that acts as actin filament inhibitor or jasplakinolide which acts as a stabilizer. Also, drugs such as nocodazole [14,15] and taxol [15] have been used to study the effect of modulation of the microtubule network. Mechanical (viscoelastic) properties of cells during cell attachment to a QCM sensor surface have been assessed by using multiple parameters like resonant frequency, maximum oscillation amplitude and decay time constant [13,16]. Finally, cell signaling pathways at surfaces have been reported [17], for example, changes in energy dissipation of a MCF-10A cell monolayer and the re-modeling of focal adhesions induced upon exposure to an epidermal growth factor [18].

Cell-substrate interaction studies have also received extensive attention and are studied with acoustic sensors; cell adhesion response can vary with the cell type and surface coating as well as surface characteristics like topography, composition, energy and mechanical properties [19–23]. Up to now, the QCM-D (or TSM-R) has been used to study the attachment and spreading of various mammalian cell lines such as NIH 3T3 [24,25], MCF-7 [9], MDCK [26] and LG2 [11,27]. Cell surface-adhesion studies were performed on uncoated metal surfaces like gold [14], tantalum [28], titanium [29], hydroxyapatite [30] and silica [31].

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Functionalization of metal surfaces includes adsorption of ECM proteins [32,33], modification by RGD peptides [34,35], formation of supported lipid bilayers [36,37] and multilayer buildup [38]. Among all the metal substrates, gold is used as a standard reference material while titanium finds applications in the orthopedic and dental implant industry. Fibrinogen, an extracellular protein found in blood in concentrations of 3 mg mL^{-1}, is also used as a surface coating [39,40]. Finally, the QCM-D has also been utilized in tumor disease studies. Frequency fluctuations have been monitored as an indicator of cell micromotility to assess the invasiveness of colon and pharynx derived malignant cells [41]. In other studies, the dynamic viscoelastic properties of normal (HMEC) and malignant (MCF-7) cells were evaluated upon adhesion and spreading on gold [9] while lectin-induced cell agglutination was employed to discriminate between normal (*L*-02) and cancer (Bel7402) hepatic cells [42].

Anaplastic Thyroid Cancer (ATC) is a fatal tumor malignancy of the human endocrine system; average survival rate is estimated in months and one-year survival is 15% [43]. ATC accounts for a minority (<2%) of all the thyroid malignancies and its rarity is counterbalanced by its aggressive phenotype. If the primary tumor is treated with surgery long term survival is possible [44]; nevertheless at later stages, the tumor is resistant to all methods of treatment like surgery, radiotherapy and chemotherapy [43,45]. Until now, there is little advance in disease-specific biomarker research, hence the disease is characterized by low prognosis [46,47]. In this study, we examined the potential of QCM-D as a diagnostic tool of ATC, by trying to elucidate differences between the adhesion pattern of normal thyroid and anaplastic thyroid cancer cells. Starting from the hypothesis that cancer cells may exhibit a different adhesion ability we decided to exploit possible differences in cell adhesion patterns on three surfaces, namely, titanium, gold and fibrinogen-coated gold. In parallel, scanning electron microscopy images were used to possibly correlate the morphology of cells on these surfaces with acoustic measurements. Results indicate that, indeed, screening of the two types of cells can be achieved by combining adhesion patterns of each cell line on the above three surfaces. This result points to the potential use of the QCM sensor as a diagnostic platform, complementary to other existing ones, for cell malignancy detection.

2. Materials and methods

2.1. Preparation of surfaces

In this work, gold (Au) and titanium (Ti) surfaces were used as substrates for cell adhesion. For the acoustic experiments Au and Ti-coated AT-cut crystals (QSX-301 and QSX-310, respectively, from Biolin, Q-Sense, Sweden) with a diameter of 13.7 mm and a fundamental frequency of 5 MHz were employed. For the microscopy scanning metal-coated coverslips were produced in-house (Microelectronics Facility, University of Crete). Specifically, glass coverslips (Agar Scientific), with a diameter of 13 mm and a thickness of 0.19–0.23 mm, were cleaned with acetone, propanol and milli-Q water and then inserted in a vacuum chamber (10^{-7} Torr) of an electron beam evaporator (Temescal BJD-1800). The evaporation process resulted in two model surfaces: glass coated with 15 nm Cr and a subsequent top coat of 15 nm Au and glass coated with 15 nm Ti. Surface cleaning methods included rinsing of all surfaces with acetone and milli-Q water, drying with nitrogen and plasma etching (Harrick PDC-002) in air prior to experiments.

2.2. Protein solution preparation

Fibrinogen from bovine plasma (>95% clottable proteins) (Merck Millipore) was dissolved in sterile milli-Q water and solubilized at 37 °C. Before QCM-D and static experiments, fresh dilutions of fibrinogen were prepared in phosphate buffered saline PBS (Sigma) with a final concentration of 10 μg mL^{-1}.

2.3. Cell preparation

Two thyroid cell lines, Nthy-ori 3–1 (Normal human primary thyroid follicular epithelial cells) and ARO (poorly differentiated human anaplastic carcinoma) were grown in RPMI-1640 medium (Gibco by Life Technologies) supplemented with 10% fetal bovine serum (Biowest) (inactivated at 56 °C for 30 min), 2 mM L-glutamine (Gibco by Life Technologies) and 1% penicillin-streptomycin (Gibco by Life Technologies). Cell cultures were maintained at 37 °C in a humidity-controlled incubator with 5% CO_2.

For QCM-D and static experiments cells were washed twice with sterile PBS and detached from culture plates using 1× trypsin-EDTA (Gibco by Life Technologies). The cell suspension was centrifuged for 5 min at 1,200 rpm and the cell pellet was resuspended in PBS. Cells were stained with trypan blue (Biosera) and cell number was counted on a Neubauer slide. Prior to cell adhesion experiments, various cell dilutions in the range of 0.5 to 20 × 10^4 cells mL^{-1} were prepared and left at room temperature.

2.4. QCM-D measurements

The QCM-D experiments were performed using the Q-Sense E4 (Biolin, Q-Sense, Sweden) instrument and AT-cut quartz disks (5 MHz). Fibrinogen (10 μg mL^{-1}) adsorption experiments were conducted at 25 °C under a constant flow rate of 50 μL min^{-1}. Before and after the addition of fibrinogen solution, the crystal was equilibrated with PBS. Cell adhesion experiments on Ti, Au and Fg-Au crystals were conducted at 25 °C under a constant flow rate of 10 μL min^{-1}. Crystals were equilibrated with PBS and cell dilutions were continuously inserted in the system until equilibrium was achieved. The cell adhesion experiments were completed with a PBS rinse. All data are reported as the average of three to six measurements along with their standard deviations.

2.5. Static experiments

Cell adhesion experiments were performed in a static mode on glass surfaces coated with Au or Ti at 25 °C. For Fg-Au surfaces, fibrinogen (10 μg mL^{-1}) solution was allowed to equilibrate for 1 h and was then rinsed away. Cell adhesion on Ti, Au and Fg-Au surfaces occurred when seeding a cell suspension of 20 × 10^4 cells mL^{-1} on each surface for 4 h. At the end of the static experiments cells were fixed for scanning electron microscopy imaging.

2.6. Scanning electron microscopy (SEM)

The morphology of adsorbed cells on each surface was analyzed by SEM. After static cell adhesion experiments, surfaces were rinsed with 0.1 M sodium cacodylate buffer (SCB) for 5 min and then cells were incubated in a fixative solution of 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M SCB for 30 min. Then, surfaces were washed with 0.1 M SCB for 5 min and stored in the same solution at 4 °C overnight. The following day, the sample was washed with 0.1 M SCB for 5 min, dehydrated with an ethanol gradient (30%, 50%, 70%, 90% and 100%) and the solvents were replaced with liquid CO_2 by critical point drying. Dry samples were mounted on a glass slide and sputter-coated (BAL-TEC SCD 050) with Au (thickness 12.5 - 15 nm) prior to observation with the microscope (JEOL JSM-6390LV).

2.7. Data analysis

QCM acoustic data were collected by QSoft 401 (Biolin, Q-sense, Sweden) software and exported (in MS Office Excel format) by Q-tools (Biolin, Q-sense, Sweden) software. All data reported here were obtained at the 35 MHz frequency (7th overtone, harmonic) and are used as raw numbers, i.e., without dividing them by the overtone. Scanning electron

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