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# *In vitro* real-time characterization of cell attachment and spreading

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A method based on the piezoelectric quartz crystal microbalance (QCM) technique for *in vitro* real-time characterization of cell attachment and spreading on surfaces has been developed. The method simultaneously measures the resonant frequency, *f*, and the dissipation energy, *D*, of the oscillating system. The QCM responses are sensitive to very small amounts (a few hundreds) of cells and highly specific to surface chemical properties. The first results from deposition of cells on two polystyrene surfaces of different wettability in serum-containing medium are reported. It has previously been shown that a decrease in *f* is related to the degree of cell spreading. In our data it appears that the extent or quality of cell attachment is reflected in an increase in *D* caused by adhering cells. The combined information from *f* and *D* measured by this technique might therefore be useful to probe cell–surface interactions for biomaterials. © *1998 Kluwer Academic Publishers*

## 1. Introduction

One of the major shortcomings in biomaterials research and development is the lack of suitable tools for *in vitro* evaluation of real-time cell–surface interactions. Such techniques would be useful, for instance, to optimize chemical aspects or topography of the biosurface. This is of great interest both to find materials which favor cell adhesion, which is desirable for many medical implants, and to develop materials which prevent cell adhesion, e.g. to stop biofouling.

Important disadvantages with some conventional methods for characterization of cell adhesion are that they are interruptive/destructive, such as flow-chambers for quantifying the strength of cell adhesion to surfaces and fixing/staining techniques. Other methods are indirect, such as analysis of signal substances from the cells, or subjective, such as optical microscopy techniques to study the physical appearance of cytoskeletal features in adhered cells. Some techniques are also restricted to certain surface materials.

The quartz crystal microbalance (QCM) technique has previously been used to characterize various types of biofilms and offers the opportunity to probe directly the cell–surface interactions at the interface (more specifically, the evolution of the attachment and spreading, secretion of adhesion proteins and cytoskeleton modifications in the cell) in real-time. Examples of recent studies include protein adsorption [1, 2] and adsorption of lipid vesicles or bilayers [3]. The high sensitivity of the QCM, concentrated to a fraction of micrometre from the surface, and the possibility to deposit biomaterial overlayers on the QCM electrode (e.g. titanium films or polymer coatings), makes the technique a promising method to study cell–surface interactions. A few studies have reported measurements of the resonant frequency while cells adhere to the QCM surface. Attempts to quantify the total weight of the cell layer have failed due to the non-rigid (viscoelastic) properties of the cells, which could not be accounted for in these measurements [4, 5]. It was, however, found that the change in resonant frequency was proportional to the contact area between a cell and the QCM [4, 5].

In this paper we describe a method to characterize cell adhesion in liquid environments by dynamically monitoring the QCM response to adhering cells. The method is based on a novel technique [6, 7] of simultaneously extracting the resonant frequency (which is affected by the degree of spreading of the adhering cells) and the dissipation factor (which is related to the viscoelastic properties and attachment of the adsorbate) from the QCM. This technique has recently been applied to study the adhesion of monkey kidney epithelial cells and Chinese hamster ovary (CHO) cells to polystyrene surfaces in a serum-free environment [8].

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Here, our first studies of CHO cells adhering to surfaces in a serum-containing environment are reported. The aim is to provide further insights into the importance of the dissipation factor in QCM measurements on cells.

## 2. Materials and methods

### 2.1. QCM sensors

The QCM consists of a 25.4 mm diameter disc made from an AT-cut 5 MHz quartz crystal with gold electrodes on both sides (Maxtek, Inc, USA). One of the electrodes of the quartz crystal was coated by a thin film of polystyrene, acting as the substrate for the cells to be deposited (any thin film can be applied). The polystyrene was spin-coated from a solution of approximately 0.5% (wt/vol) polymer in toluene at 1000 r.p.m. This produced surfaces of continuous polystyrene films [9], approximately 20–40 nm thick (measured by profilometry), of reproducible quality. After experiments, these polymer films can be dissolved in toluene, using an ultrasonic technique.

The as-prepared polystyrene surfaces were hydrophobic (advancing water contact angles in excess of 90°) in their pristine state. The polystyrene-coated discs were, in some experiments, modified by a UV/ozone treatment [10], which provides successively more hydrophilic surfaces as the exposure time is increased (Table I). To mimic polystyrene of tissue culture quality, 3 min exposure time was used, resulting in hydrophilic properties of the surface (receding water contact angle less than 5°).

The polystyrene coated QCM discs were exposed to foetal calf serum in the measurement chamber for 2 h prior to the deposition of cells. Both hydrophobic and hydrophilic polystyrene surfaces (identical to those utilized in previous experiments in serum-free medium [8]) were used. During the incubation, the chamber was heated from room temperature to 37 °C. Over this time, serum proteins compete and deposit on the surface and the temperature of the liquid stabilizes at 37 °C. QCM measuremets during incubation confirm that proteins adsorb differently on the two different surfaces. Owing to space-limitations, and because the study was focused on the qualitative comparison between the two surfaces (hydrophobic and hydrophilic), the serum deposition will not be further discussed here. The cell measurement chamber for the QCM sensor [8] and the technique simultaneously to extract the resonant frequency and dissipation factor [6, 7] have been described previously.

TABLE I Contact angle measurements of water on various substrate surfaces

| Average Θ | Gold electrode | Polystyrene | | |
|---|---|---|---|---|
| | | UV: 0 min | UV: 1 min | UV: 3 min |
| $\Theta_{adv}$ | 47° | 94° | 56° | 29° |
| $\Theta_{rec}$ | 22° | 86° | 25° | 5° or less |

### 2.2. Cell preparation

Chinese hamster ovary (CHO) epithelial cells were grown on plastic tissue culture flasks and maintained in Iscove's medium supplemented with 10% foetal calf serum, 1% penicillin and streptomycin (all from Gibco, Paisley, UK). The medium was replaced every 2–3 d. Prior to the experiments, the cells were dissociated with trypsin-EDTA and centrifuged at 5000 r.p.m. for 10 min in fresh medium. Cells were subsequently suspended in serum-free medium, centrifuged again, and then re-suspended in serum-free medium for the cell deposition. The cells were deposited on the QCM by injection of cell suspension via a 50 μm diameter capillary.

### 2.3. The *Df* plots

Most previous applications of the QCM technique have implicitly or explicitly assumed that the adsorbed films are rigid and adsorb evenly on the surface with no slip. Sauerbrey has shown that the change in frequency under such conditions is proportional to the adsorbed mass, and thus to the thickness of the overlayer [11]. For cell adhesion, however, the Sauerbrey equation is not valid. This is due to two major reasons: (i) the cells have viscoelastic properties, which means that only a fraction of the true cell mass is registered by the QCM, and (ii) the lateral sensitivity varies over the QCM surface, which makes the result dependent of the spatial distribution of the cells. The viscoelasticity of the cells gives rise to energy losses on the oscillating QCM and contribute to the dissipation factor, which is the sum of all energy losses in the system ($D = 1/Q$). These issues have been discussed elsewhere [8, 12] amounting to an advantageous way of representing and qualitatively comparing data in terms of *Df* plots.

The *Df* plots ($\Delta f[t]$ versus $\Delta D[t]$) are alternative representations of the raw QCM data, where the time is implicit. The magnitude of the QCM signals is not uniquely determined by the type of cell or even the number of cells deposited on the QCM, for the two reasons mentioned above, but the slopes of the *Df* plots at each phase of the cell–surface interaction appear to give unique values describing that phase, reproducible and comparable to other systems. Because the shape of the lateral sensitivity variation for $\Delta f$ and $\Delta D$ are the same [13], a plot of $\Delta f$ versus $\Delta D$ is qualitatively independent of the number and spatial distribution of cells on the surface.

In the present case, the proportion of the surface that is covered by the cells is much smaller than the uncovered surface, so that the cells can be considered as small perturbations to the general interfacial properties of the QCM immersed in the liquid. The area covered by cells after a typical deposition (a few hundred cells) is estimated to be less than 1% of the active area of the electrode and the cells are not forming water-trapping clusters, but are predominantly isolated single cells dispersed over the surface. For the area not covered by the cells, the influence of the liquid on the QCM, described by the Kanazawa–Gordon equation [14], is kept constant during the measurements.

## 3. Results and discussion

The QCM response to adhesion of CHO cells in serum-containing medium for the hydrophobic and hydrophilic polystyrene surfaces is shown in Fig. 1. In the QCM response from CHO cells on serum-exposed, hydrophobic polystyrene surfaces, a distinct increase in the dissipation factor is observed while no frequency shift due to cell deposition can be detected. CHO cells deposited according to the same procedure on serum-exposed hydrophilic polystyrene surfaces initially give rise to a clear but limited decrease in resonant frequency, while a substantial and sustained increase in energy dissipation is observed. The slope $\partial D/\partial f$ in the initial regime of the $Df$ plot is approximately $4 \times 10^{-6}/2.5(\mathrm{Hz}) = 1.6 \times 10^{-6}(\mathrm{s})$ while the second regime of the $Df$ plot is vertical.

In the microscope, no significant cell spreading was observed on the serum-coated hydrophobic surface, while considerable cell spreading was observed on the serum-coated hydrophilic surface in parallel with the QCM signal. These observations correlate well with studies of CHO cell adhesion on polystyrene reported in the literature [15].

The large dissipation shift (Fig. 1), with no accompanying frequency shift is extremely interesting. In order to explain the apparent lack of mass (as indicated by the small or non-existing frequency shift) for cells deposited on serum-exposed hydrophobic polystyrene, it must be remembered that $\Delta f$ is proportional to the cell–surface contact area, which might be very small for non-spread, spherical cells. Moreover, the average density of a single cell is only a few per cent larger than the surrounding liquid, already loading the QCM. A receptor-mediated attachment or focal contact, even if it is not extensive enough to stimulate cell spreading, might well lead to significant dissipative processes in the acting part of the cytoskeleton. We therefore tentatively suggest that cell attachment can cause energy dissipation detectable by the QCM measurement, before cell spreading occurs.

How does this hypothesis fit with previous results? In Fig. 2, QCM responses to deposition of CHO cells on comparable hydrophobic and hydrophilic polystyrene surfaces in serum-free medium are shown, for comparison. Similar results have been discussed in general term previously [8].

In the present context, two issues need to be addressed: (i) why are the responses for the two hydrophobic surfaces so different with and without serum, and (ii) are the responses for the two hydrophilic surfaces as similar as they appear?

Both questions might be answered if one assumes that the initial response in the two cell adhesion measurements under serum-free conditions are due mainly to adhesive agents (adhesion proteins) secreted by the cells. The slopes $\partial D/\partial f$ in the initial regimes of the $Df$ plots are around $2.5 \times 10^{-6}/15(\mathrm{Hz}) = 1.7 \times 10^{-7}(\mathrm{s})$ and $3 \times 10^{-6}/30(\mathrm{Hz}) = 1 \times 10^{-7}(\mathrm{s})$, respectively. This is, indeed, around one order of magnitude smaller than the initial slope in the $Df$ plot for the cell adhesion on the serum-coated hydrophilic surface ($1.6 \times 10^{-6}$) and closer to typical values for adsorption

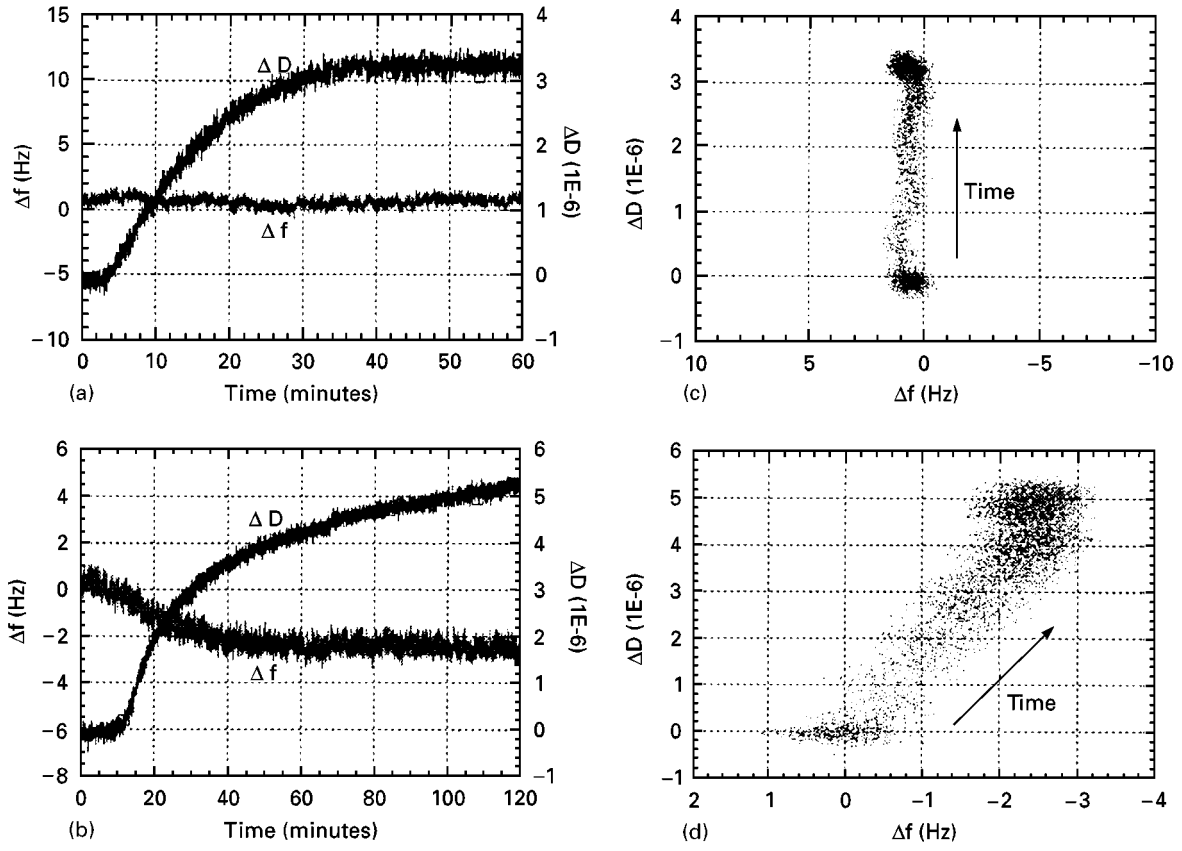


*Figure 1* (a, b) Resonant frequency and energy dissipation shifts as functions of time, and (c, d) energy dissipation shift, $\Delta D$, versus resonant frequency shift, $\Delta f$, for (a, c) around 400 CHO cells deposited on hydrophobic polystyrene and (b, d) around 200 CHO cells deposited on hydrophilic polystyrene in serum-containing medium. Serum incubation time was approximately 140 min.

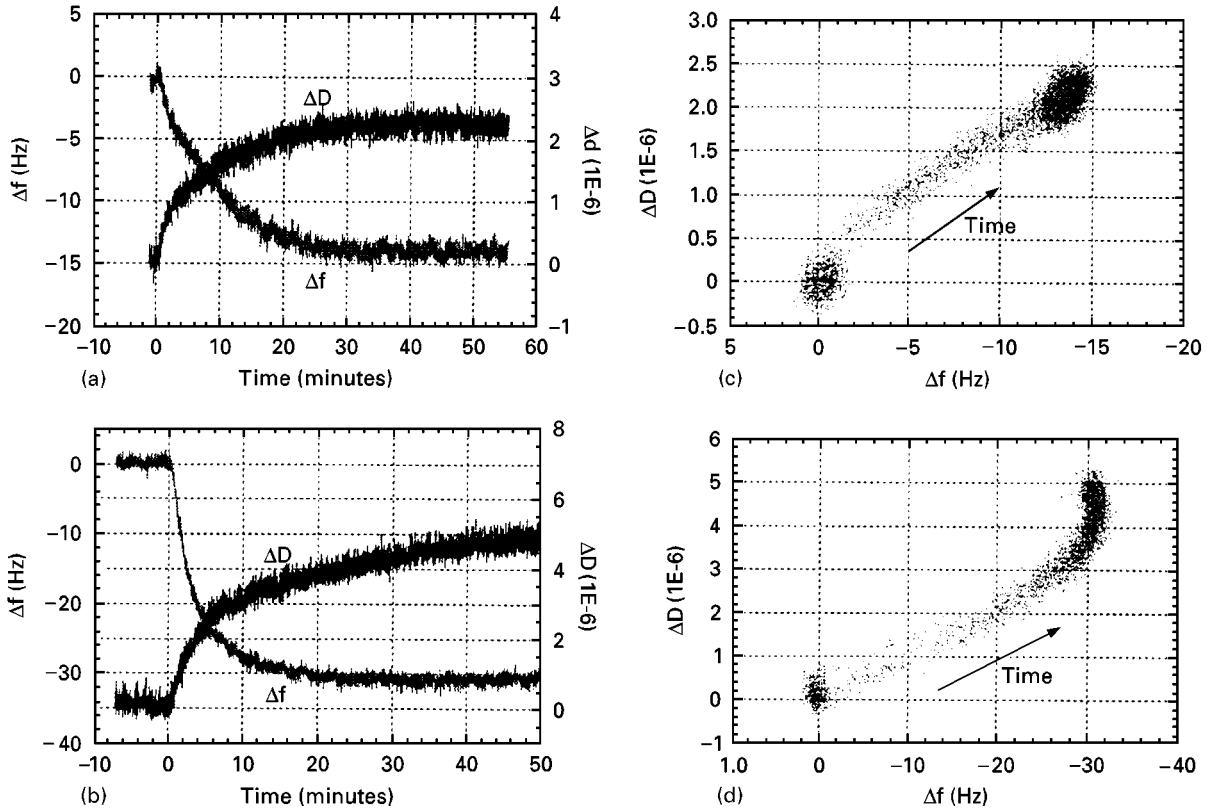


*Figure 2* (a, b) Frequency and dissipation shifts as a function of time after cell injection and (c, d) dissipation versus frequency shift for adhesion of 600–700 CHO cells to (a, c) hydrophobic and (b, d) hydrophilic polystyrene under serum-free conditions.

for adhesion proteins [1]. In the presence of serum, a possible interpretation of the vertical *Df* plot is dissipative energy losses caused by cell attachment in the absence of secretion (or perhaps failure of secretion to replace serum proteins) and with no significant associated cell spreading. The relatively steep slope for the hydrophilic surface then corresponds to attachment in combination with cell spreading. Note also that the responses in the presence of serum, presumably receptor mediated, are delayed 3–4 min after cell deposition. The vertical regimes in the *Df* plots found for the hydrophilic surfaces, with or without serum present, might be attributed to increasing attachment of the type (independent of spreading) seen for the deposition of cells on hydrophobic surfaces in serum-containing medium.

## 4. Conclusion

The results obtained support the QCM technique, as used in this study, as a powerful method independently to monitor cell attachment and spreading in both serum free and serum-containing environments, which may constitute a new screening method for biomaterials research.


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