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Acoustical sensing of cardiomyocyte cluster beating

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ABSTRACT

Spontaneously beating human pluripotent stem cell-derived cardiomyocytes clusters (CMCs) represent an excellent *in vitro* tool for studies of human cardiomyocyte function and for pharmacological cardiac safety assessment. Such testing typically requires highly trained operators, precision plating, or large cell quantities, and there is a demand for real-time, label-free monitoring of small cell quantities, especially rare cells and tissue-like structures. Array formats based on sensing of electrical or optical properties of cells are being developed and in use by the pharmaceutical industry. A potential alternative to these techniques is represented by the quartz crystal microbalance with dissipation monitoring (QCM-D) technique, which is an acoustic surface sensitive technique that measures changes in mass and viscoelastic properties close to the sensor surface (from nm to μm). There is an increasing number of studies where QCM-D has successfully been applied to monitor properties of cells and cellular processes. In the present study, we show that spontaneous beating of CMCs on QCM-D sensors can be clearly detected, both in the frequency and the dissipation signals. Beating rates in the range of 66–168 bpm for CMCs were detected and confirmed by simultaneous light microscopy. The QCM-D beating profile was found to provide individual fingerprints of the hPS-CMCs. The presented results point towards acoustical assays for evaluation cardiotoxicity.

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1. Introduction

There is increasing demand to access human material for evaluating existing and new chemical entities. In cardiotoxicity research, cells should be species relevant, have high reproducibility, and exhibit specific markers and functional similarities to adult human cardiac myocytes. Human pluripotent stem cells (hPS) have the potential to provide derivatives such as cardiomyocyte cells in large volumes [1,2]. Specifically, spontaneously beating hPS-derived cardiomyocytes in a cluster format (CMC) have become interesting for toxicity research. These cell clusters range in size from 200 to 300 μm in diameter and exhibit specific markers and functional similarities to adult human cardiac myocytes [3]. They are considered to be an excellent *in vitro* tool for studies of human cardiomyocyte function and are applied for cardiac safety pharmacology assays [4–8]. Cardiotoxicity assay development towards real-time, label-free monitoring of rare cell function using array formats, and monitoring of changes in optical or electrochemical properties of cells, is in progress [4,9], whereas techniques directly measuring changes in the mechanical properties of cells are largely lacking. Atomic force microscopy assays are under development, and forces exerted by individual CMCs have recently been measured [10].

The tight link between mechanical properties of cells and important cell processes (e.g. chronotropic events) suggests that acoustical sensing methods might have potential, alone or in combination with other techniques, in cell-based drug screening platforms, as well as to increase the fundamental understanding of cell properties. One such technique, the quartz crystal microbalance (QCM) technique, has already been successfully applied to studies of cells [11–13]; e.g., attachment and subsequent spreading of cells to the sensor surface [14,15], changes in cells exposed to cytomorphic agents [15,16], exocytotic events in neural cells on the sensor surface [17], pigment redistribution in melanophores [18], and cell responses when exposed to nanoparticles and nanotubes [19]. Furthermore, QCM has been applied to detect beating of cardiac myocytes, grown in a monolayer on the sensor surface [20]. This finding shows the potential of the QCM technology as a platform for non-invasive monitoring of chronotropic characteristics in a label free and real-time manner, aiming not only for the detection of, e.g., arrhythmias, but likely also properties of the cardiomyocyte contractile machinery.

This study addresses the monitoring of mechanical (viscoelastic) properties of single cell clusters by acoustical sensing using QCM with dissipation monitoring (QCM-D). A windowed QCM-D module was used for the detection of spontaneous hPS CMCs beating to allow for simultaneous live imaging by light microscopy (Fig. 1). Based on these results, we support the idea of QCM-D alone

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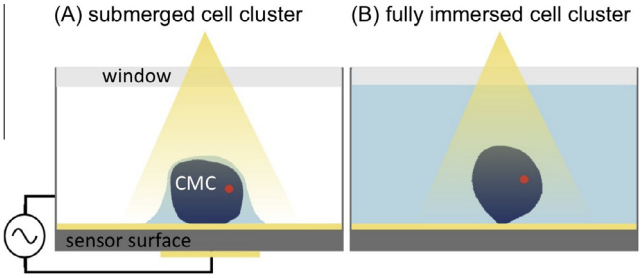

Fig. 1. Illustration of the experimental design. The CMCs are seeded *ex-situ* on either gelatin coated, or uncoated QCM-D sensors, and cultured for several days prior to measurement. Two measurement modes are shown, (A) under a liquid film where the CMC rests at the surface, and (B) in a chamber, filled with liquid, where the same CMC is tethered to the surface. The dot to the right in the cluster indicates the pacemaker cell.

or in combination with another technique (e.g., microscopy or impedance spectroscopy) as an attractive alternative to existing cardiotoxicity platforms.

2. Materials and methods

Unless otherwise stated chemicals were from commercial suppliers and used as received. Water was purified (filtered and deionized until a resistivity of 18 MΩ cm) using a MilliQ unit (Millipore, France).

2.1. Surface preparation

Surface preparation steps of SiO_2-coated QCM-D sensors (Q-Sense AB, Sweden) were as follows. QCM-D sensors were cleaned by a UV-O_3 treatment for 15 min followed by sterilization in 70% ethanol and rinsing with sterile water. For experiments using gelatin coatings, the cleaned and sterilized sensors were soaked for 30 min in aqueous gelatin solution (0.1%) at room temperature. Excess gelatin was removed followed by addition of a droplet of medium onto the sensor surface.

2.2. Cell culture

hPS-CMCs were obtained from Cellectis Stem Cells (Cellectis AB, Göteborg, Sweden). Briefly, CMCs were routinely cultured in knockout Dulbecco's Modified Eagle Medium (DMEM) medium supplemented with 20% fetal bovine serum (FBS), 1 mM GlutaMAX, 0.1 mM β-mercaptoethanol, 1% Minimal Essential medium (MEM) non essential amino acids, and 1% penicillin as described previously [3]. For QCM-D experiments, the sensors were placed in the central well of a humidified IVF culture dish, to keep the underside as clean and dry as possible during the cell attachment phase. Single clusters (~300 μm in diameter) were seeded centrally on sensors (gelatin pre-coated or plain) in a droplet of medium and incubated without further interference for 4–5 days in standard cell culture conditions (5% CO_2 and 95% humidity).

2.3. QCM-D experiments

The QCM-D experiments were performed using a Q-Sense E1 window module and QCM-D sensors with a fundamental frequency, f_0, of 5 MHz (Q-Sense AB, Sweden). Prior to mounting, cluster beating was verified by microscopic evaluation. The sensors were carefully taken from the culture dishes, excess medium was removed from the upper surface, and the under surface (and electrode) were gently blotted and dried with tissue. Care was taken not to perturb the attached cluster during the sensor mounting steps. All experiments were performed at 37 °C, and CMCs were either covered by a film of liquid only, or fully immersed in liquid carefully added on top (Fig. 1). Added solutions were pre-heated to 37 °C (to avoid formation of gas bubbles in the measurement chamber) and flowed at a slow rate (~25 μl/min) to fill the chamber without dislodging the cluster. Measurements were recorded at a single overtone (3rd) with the highest rate of sampling in order to ensure detection of rhythmic cell signals occurring in the range of 60–200 bpm. Frequency shifts were normalized to the fundamental frequency by dividing the values by three. Cluster beating (and continued attachment) was verified periodically throughout the QCM-D measurement.

2.4. Fourier transformation

The QCM-D signals were analyzed by fast Fourier transformation (FFT). FFT is particularly useful to look for periodicities of a signal. FFT of a periodic time series provides the corresponding power spectral density (PSD) function (or power spectrum), where any periodicity within the experimental data is identified as a peak at the corresponding frequency (here beating rate of the cell cluster). The absence of peaks in the PSD plot is indicative for a time series without any periodicity. In other words, FFT analysis of the recorded QCM-D data provides a frequency-resolved view on the composition of the measured time series. Note that, in order to avoid confusion with the frequency shifts recorded during the QCM-D measurement (Δf), the term beating rate will be used when the PSD and the clusters are described.

3. Results and discussion

The overall aim of the present study is to investigate the applicability of QCM-D for the detection of changes in spontaneously beating single cell clusters, hPS-CMCs, as a platform for studies on human cardiomyocytes based on changes in viscoelastic properties near the sensor surface. We will first describe a typical experiment where CMC beating is detected and analyzed, followed by discussing the origin of these signals and potential application areas of this new platform.

3.1. Analysis of QCM-D signals recorded during CMC beating

Cell clusters were added to the surface of QCM-D sensors *ex situ*, where the CMCs readily attached and grew under common cell culture conditions. Immediately after mounting of the sensor in the QCM-D instrument, spontaneous beating of single CMCs submerged in a thin liquid film on the sensor surface was observed in the registered signals, as verified by light microscopy using a windowed module. Typical frequency and dissipation signals (3rd overtone) as a function of time under such conditions are shown in Fig. 2A. The QCM-D signals (typical shifts from peak to peak were $\Delta f \sim 3$ Hz and $\Delta D \sim 1{\cdot}10^{-6}$) exhibit a clear periodicity in agreement with the visually observed beating. Direct information about the periodicity of the signal is obtained by transforming the measured time-dependent QCM-D signal to a power spectrum in the frequency domain using fast Fourier transformation (FFT). The FFT analysis of the QCM-D frequency and dissipation signals shows clear peaks corresponding to a beating rate of 168 bpm (Fig. 3A, and Table 1 "CMC 04"). Peaks appearing at higher beating rates are integers of the beating rate, so called harmonics, that occur for signals which differ from a sinusoidal shape (at an extreme, a rectangular signal would give an infinite number of harmonics which rates are an integer of the main peak). The observed beating rate is in agreement with previously reported rates, such as 94 ± 33 bpm [21], 12–120 bpm [6], 81 ± 31 bpm, 151 ± 40 bpm,

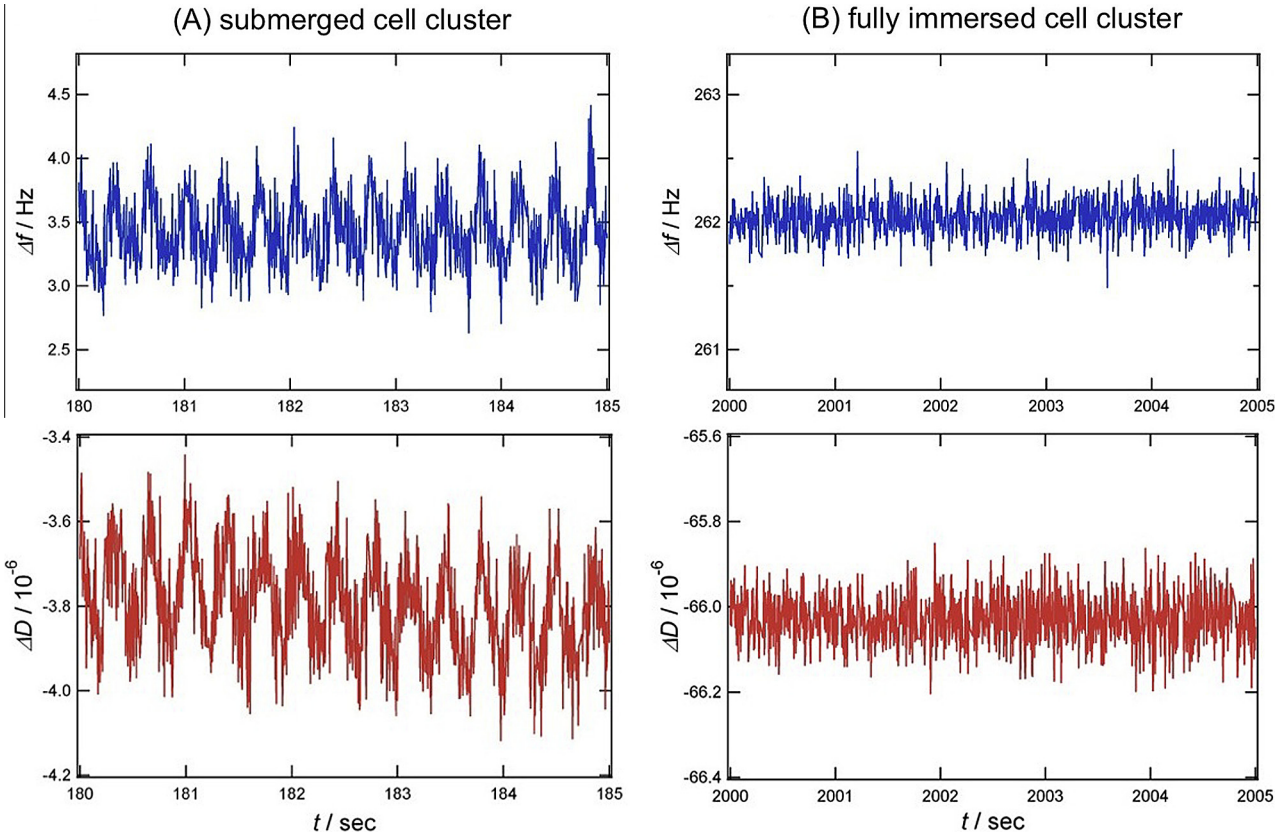

Fig. 2. QCM-D detection of CMC beating. (A) Frequency (top, blue) and dissipation plots (bottom, red) for a single, spontaneously beating CMC cultured on a QCM-D sensor (uncoated) (A) under a liquid film and (B) fully immersed in media, leading to ceasing of the beating.

and 130 ± 47 bpm for ventricular, atrial and nodal clusters, respectively [1], and 60 bpm [22]. A movie of spontaneous CMC contraction can be found in the Supplementary section. Upon filling the chamber with medium, CMC contractions and thus the QCM-D signal ceased (Figs. 2B and 3B).

Interestingly, we observed each CMC to have a unique beating profile, which may be dependent on the characteristics of the cluster, such as the position of the pacemaker cell and its location relative to the sensor surface (see Fig. 1). Variations in beating rate for four individual clusters can be seen in Table 1. In the following, we discuss the profile of one particular cell cluster, described in Fig. 4A, which shows a short time frame of the QCM-D signal displaying three beating cycles. The signal is observed to be essentially sinusoidal for both the frequency and the dissipation (verified in the power spectrum, Fig. 3A and Table 1, where only one or two harmonics are observed; see above). We observed that the number of harmonics varies for each cell cluster, indicating each CMC to have an individual signal deviating differently from a sinusoidal shape. Taking a closer look at the signal in Fig. 4A, we observe a phase shift between the frequency and the dissipation signals (fittings of the signals to sinusoidal functions were included to help the eye and to estimate the phase shift more easily). Plotting of the dissipation shift against the frequency shift for the curves in Fig. 4A results in an ellipse where the angle between the semi-major-axis and the *x*-axis (frequency) is the phase shift between the dissipation and the frequency shift. A linear relation between the dissipation and the frequency would indicate that the frequency and dissipation curves are in phase or shifted by 180°, whereas a circle in the Df-plot indicates that the phase shift between the frequency and dissipation is 90° or 270°. The observed phase shift between the frequency and the dissipation signal is, like the shape of the signal, individual for each cell cluster and varies between 23° and 143° (Table 1).

3.2. Origin of the QCM-D responses

Conventionally, QCM-D has been used to measure adsorption processes and interactions with biomimic layers, for example the interaction of peptides, proteins or virus like particles with functionalized lipid bilayers. Interpretation of the QCM-D signal obtained in these kinds of processes is "simple" in the sense that the signals probe deposition of material which is directly bound to the sensor surface, although models of increasing complexity are required to model soft layers formed on the surface [23,24]. Furthermore, in some situations, the surface adlayer can be acoustically and mechanically "decoupled" from the sensor surface, through slip between the deposited material and the sensor surface [25]. In the case of cell studies (involving non-homogeneous surface layers), it is obvious that interpretation of the QCM-D signal will not be straight-forward. Thus, studies of cells by QCM-D being described in the literature have often been phenomenological in the sense that signals are recorded and correlated to cellular processes without a mechanical model correlating the signals to the processes. In several studies it has been concluded that the observed QCM-D signals correlate to rearrangements of the cytoskeleton. For example, the effect of cytomorphic agents is typically sensed by QCM-D [12,15]. An issue that has to be taken into consideration for the interpretation of the QCM-D signal is what kind of mechanical link is formed between the cells and the sensor surface. The cells might adhere either (I) directly via focal adhesion points to the sensor surface (Fig. 1B) or (II) simply reside on the surface and a layer (of proteins or media) between the basal

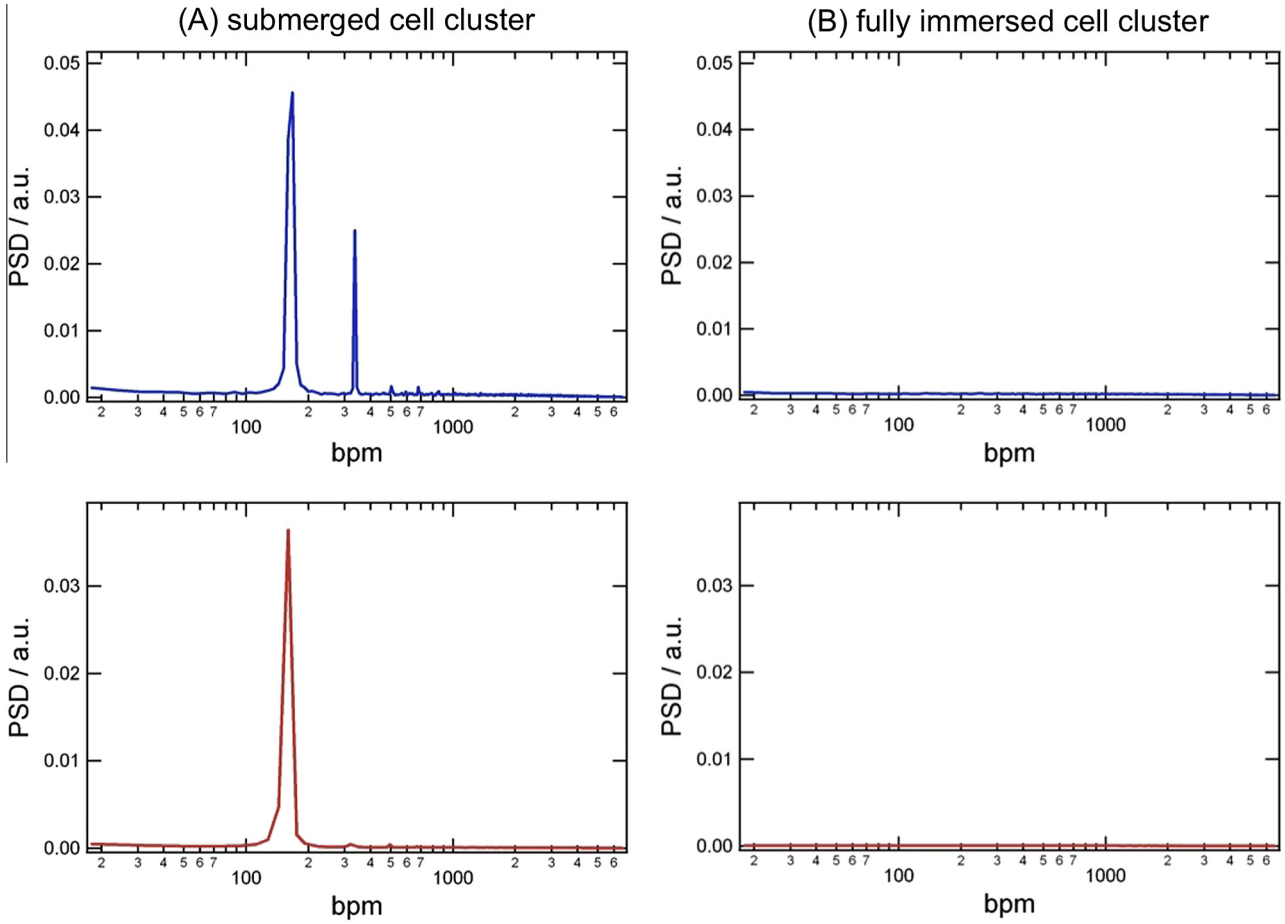

Fig. 3. Power density spectra of the QCM-D signal. (A) PSD of the QCM-D frequency (top, blue) and dissipation (bottom, red) signal of a submerged CMC and (B) a fully immersed cell cluster. .

Table 1
Beating rates determined by FFT of the QCM-D frequency and dissipation signals of four different clusters.

CMC name	bpm	Phase shift	Number of harmonics	
			Based on QCM-D frequency signal	Based on QCM-D dissipation signal
01[a]	66	143°	2	2
02	84	23°	4	2
03[a]	156	92°	1	3
04	168	112°	2	1

[a] CMCs cultured on gelatin coated sensor surfaces.

laminar of the cell and the sensor surface, while potentially still trying to establish for firm links directly to the support (Fig. 1A).

In the former case (I), the cells have developed strong adhesion to the sensor surface, meaning that changes occurring in the cell within the QCM-D sensing depth can be transduced directly to the recorded signals. It is also likely that changes occurring primarily in the bulk of the cell (e.g., cytoskeletal rearrangements) can be sensed also in the membrane region through associated effects in the focal adhesion region (e.g., less or more strained protein complexes, or changes in the number of adhesion sites [26]). This is in

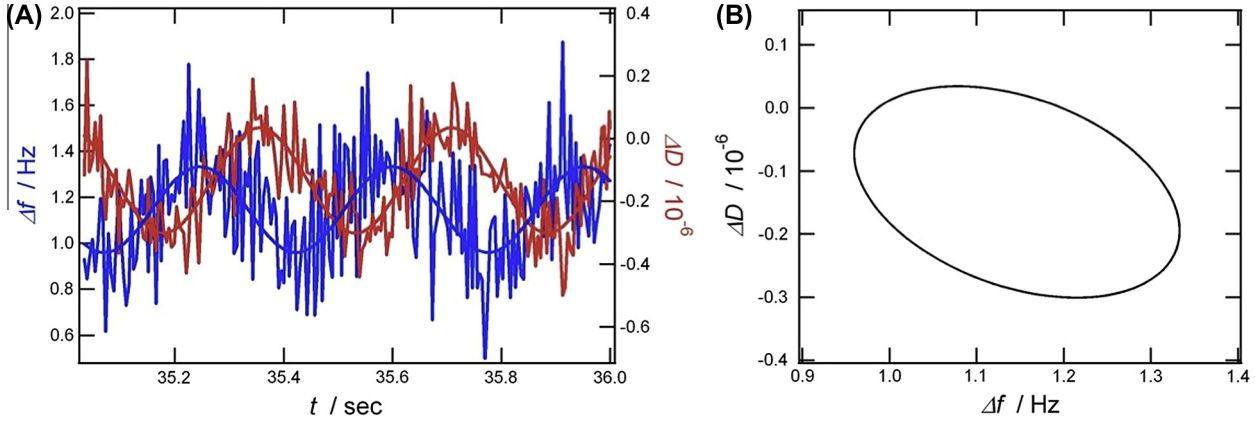

Fig. 4. Phase behavior of QCM-D signal. (A) Frequency (blue) and dissipation (red) shift versus time, and (B) dissipation versus frequency shift of the fit. (For interpretation of color in this Figure, the reader is referred to the web version of this article).

agreement with our previous measurements on fibroblasts [15] and melanophores [18], where induced cytoskeleton changes led to changes in the QCM-D signal (see also e.g. [12,16]). In analogy, the observed signals from the beating CMCs can be interpreted as changes in the bulk viscoelastic properties of the cells themselves as they synchronously cycle through contraction (the cytoskeleton becomes more rigid) and relaxation (the cytoskeleton becomes softer) phases. However, the signals are diminished or lost upon liquid immersion while the CMC is still attached, which is rather speaking in favor of a model as in case (II), where cells are indirectly coupled to the oscillatory motion of the QCM-D sensor via a transducer layer, in our case a thin liquid film. With such a model of the CMC – QCM-D sensor interface, it is likely that the contraction of the CMC is sensed as a pressure wave near the sensor surface, rather than as the results of direct mechanical links (being stretched or relaxed) between the surface and the cluster. Since the QCM-D is sensitive to pressure [27,28], a CMC contraction-induced pressure wave could cause changes in the resonance frequency of the oscillating quartz crystal sensor. It is difficult to say what effects a pressure wave inside a closed chamber would have on the cells and this might explain why the CMCs do not beat when the windowed measurement cell is filled with liquid (Fig. 1B). The incompressibility of the liquid in the filled volume is likely perceived by the cluster, maybe causing the beating to stop (as opposed to the thin liquid film in contact with air where volume changes can occur readily).

In addition to mechanical changes in the beating CMCs, electrochemical variations might be detectable by QCM-D. During a beating cycle, the ion concentration between the cell cluster and the QCM-D sensor is fluctuating. This fluctuation could lead to changes in the QCM-D signal. Furthermore, the beating of the hPS CMC gives rise to an electromagnetic field. However, this cannot affect the QCM-D responses due to the grounding of the top electrode.

Taken together, the most likely model for how the changes in the beating CMCs are transmitted to the QCM-D signals is via secondary effects in the layer the closest to the sensor surface, rather than via forces exerted on the sensor via direct mechanical bonds through focal adhesion sites.

3.3. QCM-D beating rate measurements in relation to other techniques

Typical assays for the study of CMC beating include transmembrane action potential (TAP) recording, microelectrode arrays, voltage-clamp studies, hERG channel inhibition, ion channel trafficking, and proarrhythmic potential measurements. The golden standard of these assays for evaluating the cardiotoxic risk of new chemical entities is TAP, although being labor intensive. Platforms that incorporate multielectrode arrays are more amenable for general use, but the attachment of the cells can sometimes be challenging. Furthermore, precision plating is required when working with advanced three-dimensional models to ensure near contact between the cells and electrode. More recently there have been developments in real time cellular analyzers (RTCA). For example, the xCELLigence RTCA Cardio System (which can measure the contractility of cardiomyocytes in a 96 well plate impedance-based system) relies on the complex connections between cardiomyocytes growing in a monolayer, which inherently demands larger cell quantities [9].

All of the assays mentioned above have in common that they detect electrochemical changes during a cardiac beating cycle. To our knowledge there are no established assays for the detection of the mechanical properties of beating cardiomyocytes. As demonstrated in our study, QCM-D provides the possibility to directly detect changes in the mechanical and viscoelastic properties of cell clusters attached to the sensor surface. At present, it is not possible to compare QCM-D curves that reflect changes in the viscoelastic properties during a beating cycle to standard contraction profiles derived from, e.g., patch clamp measurements on individual cells. However, we observe a signal that provides an individual fingerprint of hPS-CMCs, which we assume, is related to the dimensions of the cluster and the positioning of the pacemaker cell within it.

Further benefits of QCM-D are its convenience and robustness. Common assays mentioned above are typically performed using labor-intensive techniques that require highly trained operators and precision plating. We did not experience difficulties in plating cell clusters on QCM-D sensor surfaces, neither did the performance of the experiment require extraordinary skills. In addition, it is of great advantage that only relatively small volumes of tissue-like material are needed to perform QCM-D experiments. This is highly valuable for investigations of more rare cell types, such as patient derived induced pluripotent stem (IPS) cells [29].

In summary we have demonstrated a combined QCM-D and hPS-CMC platform to be a versatile tool to detect chronotropic events in a human cardiac cell model. Particularly the ease of preparation and the low quantities of cells required, combined with the straight-forward QCM-D detection in real time is a potential advantage compared with established sensor assays. Most interestingly, the detected QCM-D signal gives rise to an individual fingerprint of the hPS-CMC containing information which is potentially unique to the QCM-D technique. Studies along these lines will contribute to the development of real-time cardiotoxicity assays for rares cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.04.070.

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