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CELL-BASED ACOUSTIC SENSORS FOR BIOMEDICAL APPLICATIONS

M. Cole[1], J.W. Gardner[1], S. Pathak[1], Z. Rácz[1], R.A.J. Challiss[2], D. Markovic[2]
[1]University of Warwick, Coventry, United Kingdom
[2]University of Leicester, Leicester, United Kingdom
E-mail: M.Cole@warwick.ac.uk

ABSTRACT
The development of a novel surface acoustic wave biosensor for liquid phase ligand detection is described in this paper. The functional layer of the biosensor comprises human embryonic kidney cells that can be efficiently used for the expression of specific ligand receptors and is coupled to the acousto-electric transducer. We outline the successful design and development of a low loss shear horizontal surface acoustic wave device for detection of receptor-ligand interactions in heterologous systems. The proof of concept implementation of a protocol to immobilize cells expressing either muscarinic acetylcholine or insect olfactory receptors on the device surface has been successful. In addition, the background effect of the cell growth medium has been examined and results presented. We believe that this biological sensor also can be used more generally to monitor cell viability when challenged with toxins, drugs or other substances.

KEY WORDS
Biosensor, Surface acoustic wave, Human embryonic kidney (HEK) cells, Ligand detection

1. Background and Introduction

We have reported on the use of chemical sensors to detect infections from the headspace of cultured bacterial pathogens [1], clinical eye swabs [2] and clinical blood/urine samples [3]. However, the use of solid-state gas sensors or even mass spectrometers has only had a limited success because of their limited chemical specificity. In a related EU project called iCHEM [4] we are exploring the technique of inserting specific odorant receptors (ORs) into cells that can subsequently be made to change their biochemical/physiological properties, such as cytoplasmic Ca^{2+} concentration.

Eusocial insects have extremely sophisticated systems to detect and identify the complex and diverse semiochemicals that they rely upon to find food, locate conspecific mates, organize colonies and divide labour. Insects are able to detect these volatile chemicals with the help of olfactory receptor neurons (ORNs), which are embedded in the hair-like chemosensory sensilla located on the antennae [5, 6]. Recognition of a broad range of chemicals requires the synchronous activity of receptors such that the chemical diversity of the odorants can be matched [7]. The key specificity in the response to extracellular molecules (e.g. odours, pheromones or toxins) is considered to be due to the specificity of the ligand binding olfactory receptors (ORs). Due to the diversity of ligands, molecular recognition can be achieved through a large family of proteins encoded by olfactory receptor genes.

The exact molecular mechanisms of insect olfactory receptor signal transduction remain unclear despite a long and prolific history of research [8]. Figure 1 shows a recently proposed model of OR signal transduction in insects in which a ligand responsive variable ORx subunit is a G-protein coupled receptor and OR83b is a cyclic nucleotide-gated ion channel [8, 9]. When stimulated by an odorant, the ORx activates the channel protein OR83b by an ionotropic and a metabotropic pathway [9]. The direct activation of OR83b leads to a fast and transient ion conductance. The metabotropic pathway includes activation of G_s proteins. The $G\alpha_s$ subunit stimulates adenylyl cyclase (AC), thereby increasing cyclic adenosine monophosphate (cAMP) production which in turn slowly activates a long-lasting non-selective cation conductance.

The goal of the work presented here is to develop a surface acoustic wave (SAW)-based sensor that is functionalized with a biological layer and enables the detection of chemicals at very low concentrations – not only for semiochemistry, but also biomedical application.

A heterologous expression system, human embryonic kidney 293 (HEK293) cells, were employed for the development of an olfactory receptor-based sensor for

detecting pheromone signaling, in which ORs can be efficiently expressed and then coupled to the artificial acousto-electric system for ligand detection.

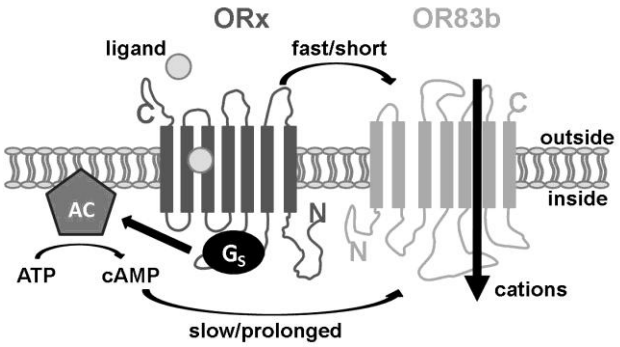

Figure 1. Schematic model of insect olfactory receptor signal transduction that has been successfully expressed in human embryonic kidney cells.

Changes inside and on the surface of the HEK293 cells induced by the ligand-receptor interaction are detected by surface acoustic waves that – depending on the frequency – penetrate into different regions of the cells, such as the nucleus, the cytoplasm and the bilipid layer. Figure 2 shows a schematic 'fried-egg' representation of a HEK293 cell adhering to a flat piezoelectric substrate with the desired SAW penetration depths.

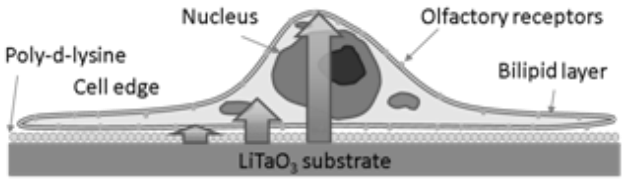

Figure 2. Schematic 'fried-egg' representation of a human embryonic kidney 293 cell on a lithium tantalate surface acoustic wave device showing the different wave penetration depths required to monitor ligand binding-induced changes inside and on the surface of the cells.

Lithium tantalate-based custom SAW devices have been designed and fabricated to facilitate the characterization of HEK293 cells. A microfluidic chamber and a cell immobilization protocol have also been developed for the deposition and growth of HEK293 cells on the device surface [10]. This biosensor is an integral part of a novel biosynthetic chemoreceiver module developed for infochemical detection and can be applied to monitoring the affect of toxins, drugs, etc.

2. Experimental Design

2.1 Background Theory

Acoustic sensing is based on the change of the propagation characteristics of the surface acoustic wave travelling along the surface of the SAW device because of either electrical or mechanical changes in the adjacent medium. For high sensitivity liquid phase sensors, substrate materials that generate coupled shear horizontal surface acoustic waves (SH-SAWs) are required to avoid excessive damping of the SAW by the liquid medium. For such applications, a rotated Y-cut (36°) X-propagating $LiTaO_3$ crystal was chosen as the substrate material for the biosensors in this work due its efficient electromechanical coupling constant (K^2=0.047) and comparatively low temperature coefficient of delay of approximately 32 ppm/°C [11].

The acousto-electric potential associated with the shear horizontal wave on the surface of the lithium tantalate extends into the adjacent liquid; the extent of the penetration of this potential determines how far from the crystal/liquid interface a change in property can be detected by the sensor. The potential skin depth was calculated to be approximately $1/7^{th}$ of the wavelength of a SH-SAW for an unmetallised surface [12]. Consequently, the distance of detection is directly proportional to the SAW frequency as shown in Figure 3. For metallised surfaces, the potential penetration is practically zero. Such devices are only sensitive to the mechanical changes in the adjacent liquid.

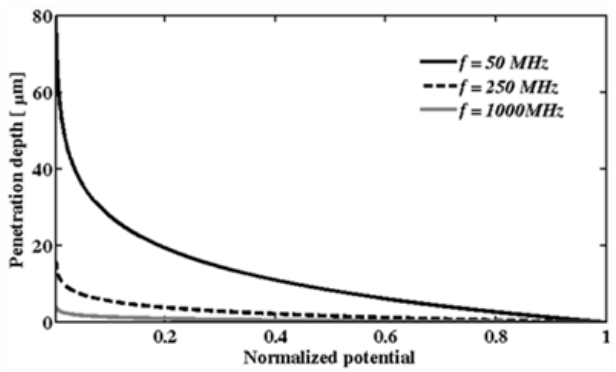

Figure 3. Penetration profile of the normalised electric field potential for shear horizontal surface acoustic waves with a frequency of 50, 250 and 1000 MHz in $LiTaO_3$ and with water as the adjacent liquid.

In order to enable the characterization of entire HEK293 cells, the potential penetration depth was set to 9.7 μm

which gives the corresponding SAW frequency of 60.56 MHz.

2.2 SH-SAW Sensor

The SH-SAW biosensors were designed in dual delay-line configuration to allow differential measurements in which only one delay-line of the pair is coated with functionalized HEK293 cells expressing olfactory receptors while the other is coated with non-functionalized (i.e. wild type) HEK293 cells, Figure 4(a) [13]. Measuring the difference between the signals of the two delay-lines ameliorates environmental and other common mode variations and ensures that the measured responses are produced purely by the functionalized cells. The changes in the velocity, $\Delta v/v$, and the attenuation, $\Delta\alpha/k$, of the propagating SH-SAW due to the response of the HEK293 cells to ligands can be approximated by the following equations [14]:

$$\frac{\Delta v}{v} = -\frac{K^2}{2}\frac{\left(\frac{\sigma'}{\omega}\right)^2 + \varepsilon_0\left(\varepsilon_r' - \varepsilon_r\right)\left(\varepsilon_r'\varepsilon_0 + \varepsilon_P^T\right)}{\left(\frac{\sigma'}{\omega}\right)^2 + \left(\varepsilon_r'\varepsilon_0 + \varepsilon_P^T\right)^2} \quad (1)$$

$$\frac{\Delta \alpha}{k} = -\frac{K^2}{2}\frac{\left(\frac{\sigma'}{\omega}\right)^2\left(\varepsilon_r\varepsilon_0 + \varepsilon_P^T\right)}{\left(\frac{\sigma'}{\omega}\right)^2 + \left(\varepsilon_r'\varepsilon_0 + \varepsilon_P^T\right)^2} \quad (2)$$

Here, k is the wave number, ω is the angular frequency, K is the electromechanical coupling constant, ε_0 is the electrical permittivity of free space, ε^T_P is the effective permittivity of the SAW crystal, ε_r is the relative permittivity of the unperturbed biological layer, and ε'_r and σ' are the relative permittivity and conductivity of the perturbed biological layer, respectively.

Split finger interdigital transducers (IDTs) were chosen instead of single finger IDT transducers in order to suppress Bragg reflection and edge effects, see Figure 4(b). The IDTs were fabricated using a 150 nm thick layer comprising of 30 nm of chromium followed by 120 nm of gold. Both the input and the output IDTs consist of 14.5 finger pairs with a periodicity of 8.5 μm. The acoustic aperture and the delay path lengths were set to 40λ and 90λ, respectively, where λ is the acoustic wavelength.

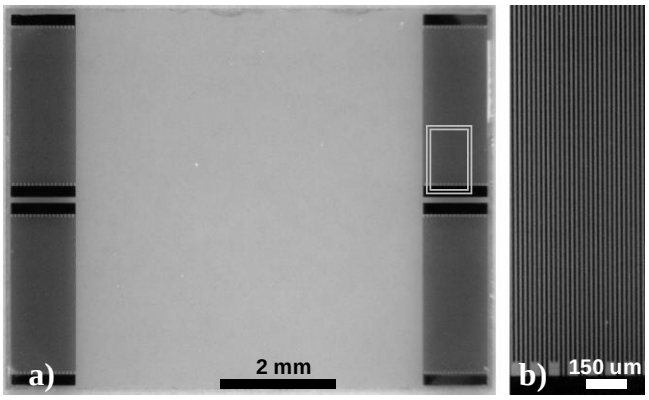

Figure 4. (a) Optical microscope image of a 60.56 MHz unmetallised dual delay line SH-SAW sensor fabricated using Au/Cr electrodes and a $LiTaO_3$ substrate. (b) Higher magnification image of a split-finger interdigitated transducer electrode.

2.3 Microfluidic Chamber

A novel microfluidic chamber was designed and built using micro-stereolithography (MSL) to enable the in-situ culturing of HEK293 cells on the SH-SAW microsensor. The chamber consists of a 500 μl inner reservoir, as shown in Figure 5, which can store growth medium for up to 2 days in an incubated environment providing stable growth conditions for the cells. The inner sidewalls of the chamber are angled to promote the easy spreading and uniform distribution of liquids with high surface tension. Replacement media, antibiotics and odour carrying liquid mixtures can also be circulated through the reservoirs via the microchannels on both sides of the chamber. The whole setup is designed to fit under high magnification microscope lenses to enable real-time optical imaging of cell growth on the device surface.

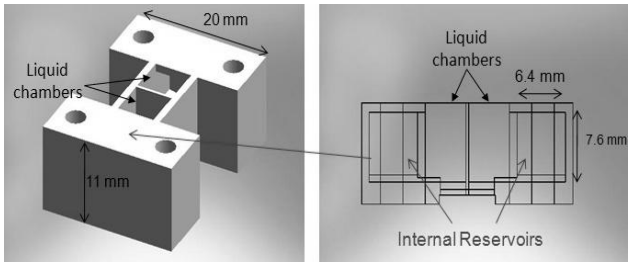

Figure 5. Microfluidic chamber to hold the cell growth medium (top and side view) and test liquids.

2.4 Cell Growth and Immobilization Protocol

A protocol has been developed by which HEK293 cells can be coated onto the surface of the SH-SAW microsensor. Initially, cells were cultured in 75 cm^2 tissue

culture flasks with Dulbecco's modified Eagle's medium (DMEM, L-Glutamax) which was supplemented with 10% fetal calf serum (FCS), penicillin/streptomycin and were incubated at 37°C with 5% CO_2 in humidified air [10]. The medium was replaced after two days while the cells were sub-cultured at 80% confluency. The sub-culturing of cells was performed by adding trypsin/EDTA to the cells and then briefly incubating the flask until all the cells detached from the bottom surface. After adding fresh medium to the trypsinised cells the entire content of the flask was spun down at 2000 r.p.m. for 4 min. A pre-sterilized SH-SAW biosensor chip was placed in a sterile 24 well plate and the chip was drip-coated with a layer of poly-D-lysine to promote cell adhesion. Next, the chip was incubated for 1 min and then fresh medium containing serum was pipetted onto the chip followed by a further 10 min incubation. Finally, 50 µl of the cell-medium mixture was pipetted onto the device surface and the entire setup was covered and incubated in a sterile environment at 37°C with 5% CO_2 in humidified air. A similar procedure was performed with only the incubation times having been modified when printed circuit board (PCB) mounted chips were used.

2.5 System Setup

The 60.56 MHz SH-SAW biosensor chip was initially dip-washed in acetone, isopropanol and deionized water, then mounted onto a PCB and ultrasonically wire-bonded. In order to sterilize the sensing area of the biosensor, the PCB (along with the chip) with the MSL chamber was submerged in an ethanol bath for 20 min followed by a phosphate-buffered saline (PBS) rinse and 15 min incubation. The MSL chamber was then tightly mounted on top of biosensor chip using nylon washers and screws.

In order to characterize the SH-SAW sensors, an Agilent E50718 network analyzer (Agilent Technologies, Santa Clara, CA, United States) was used. During the measurements, the sensor setup had to be taken out of the incubator; therefore, a dry block heater (Techne Inc., Burlington, NJ, United States) was employed to maintain the ambient temperature at 37°C. An optical microscope and a desktop computer used to obtain real-time digital images of cells growth completed the measurement setup that is illustrated in Figure 6.

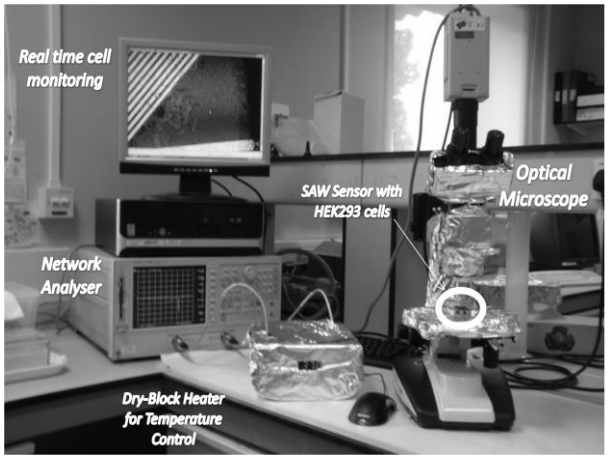

Figure 6. Measurement setup to optically observe and acousto-electronically characterize the behaviour of HEK293 cells on SH-SAW sensors.

3. Results

3.1 SAW Device Characterization

The insertion loss for the 60.56MHz SH-SAW microsensor was obtained from the transmission coefficients using an Agilent E50718 network analyzer. The measured frequency was found to be very close to the design frequency and the insertion loss obtained for both devices was −4dB, as shown in Figure 7.

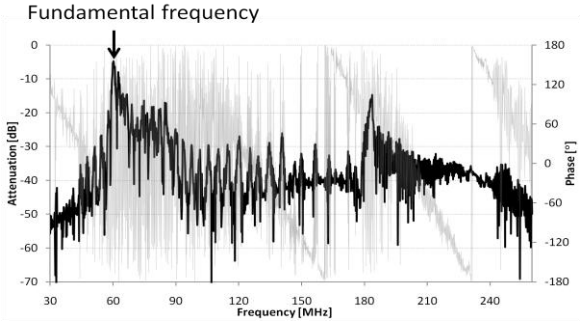

Figure 7. Frequency vs. attenuation and phase plot of the 60.56 MHz SAW sensor.

3.2 Characterisation of Biological Media

In order to examine the effect of cell growth media on SAW measurement, the response of the SAW biosensors was measured when loaded by three different biological media (DMEM, DMEM+, KHB) and deionized water (for reference). Each liquid was tested five times with all the tests performed in a random order to mitigate possible memory effects of the system.

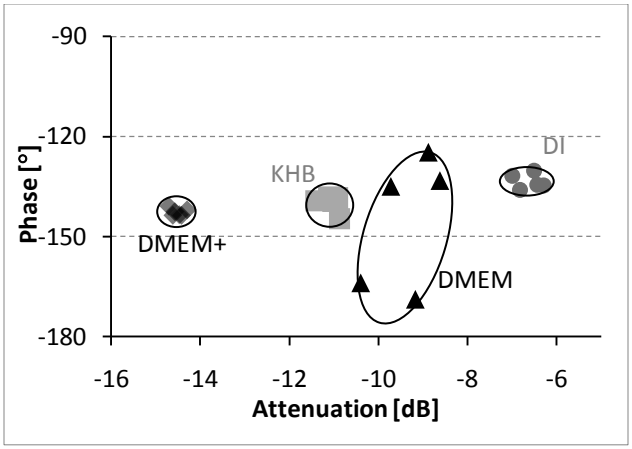

Figure 8. Attenuation vs. phase diagram for three cell culture media and deionized water.

The attenuation vs. phase diagram for all 20 measurements, shown in Figure 8, shows that the cell culture media can be separated from each other despite being 99% water based. It is also visible that cell culture liquids depending on their concentration will produce a 2-8 dB increase in wave attenuation without significantly changing the phase characteristics.

3.3 Scanning Electron Microscopy of Cells

In order to confirm HEK293 attachment and viability on LiTaO3 and Au/Cr/$LiTaO_3$ surface, HEK293 cells (10,000-150,000 cells/well) were immobilized onto pre-sterilized SAW sensor chips using the immobilization protocol outlined earlier. The cells were allowed to grow in an incubator environment for a period of 2 days and to confirm this the cell morphology on the sensors was examined under a scanning electron microscope, Figure 9. An MTT cell viability assay was also performed and the results are shown in Figure 10. Both the electron micrographs and the MTT assay confirmed that HEK293 cells had grow on both metallised and unmetallised sensing areas on $LiTaO_3$, and they do not appear to have a preferred growth region.

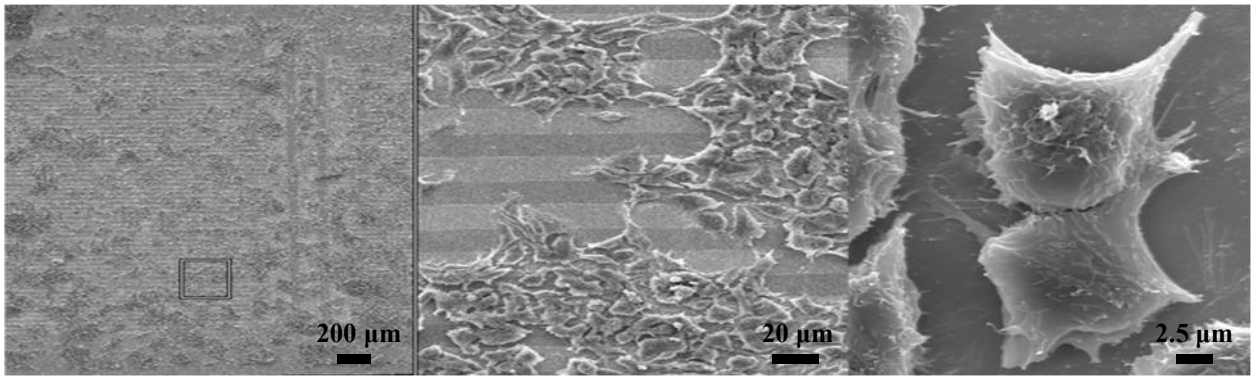

Figure 9. Scanning electron micrographs (increasing magnification, left to right) of HEK293 cells grown on a $LiTaO_3$ SAW device with Au/Cr electrodes.

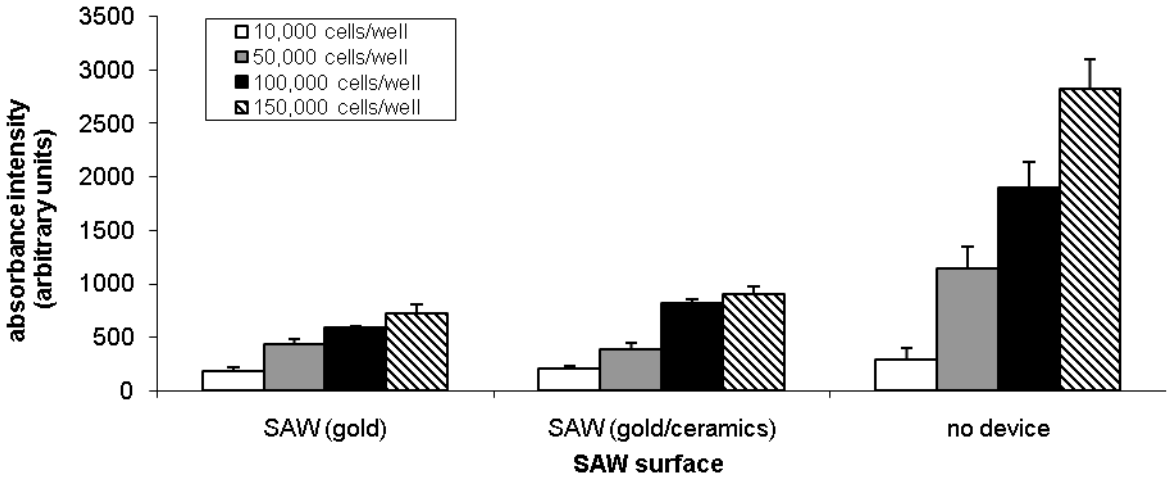

Figure 10. MTT cell viability assay results.

HEK293 cells were grown on the biosensors integrated with the PCB to verify that the developed immobilization protocol can be used to deposit and grow HEK293 cells on an assembled SAW/microfluidic chamber setup. After the cells were deposited on the biosensor chip, the device setup was covered with a glass cover slip and kept in an incubated environment for 2 days.

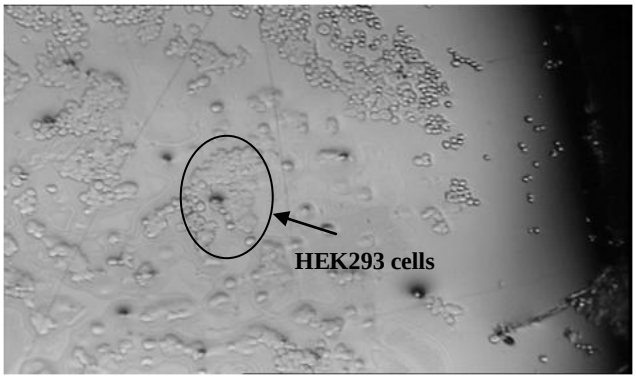

Figure 11. Optical microscope images of HEK293 cells grown on a SAW biosensor.

The cell culture medium in the MSL chamber was refilled every 18 h to provide cells with fresh growth medium. The cells on the sensor chip were observed under an optical microscope after a period of 48 h and as Figure 11 shows, HEK293 cells were successfully deposited and grown on the fully assembled device setup.

4. Conclusion and Further Work

In this paper we have described the development of a biosensor consisting of a low-loss SH-SAW biosensor, a biological functional layer of HEK293 cells that was deposited and grown on the SAW chips using a customized immobilization protocol and a designated microfluidic chamber. A cell monitoring and measurement system was designed and built that allows the simultaneous real-time optical imaging and electrical characterization of the biosensor.

We have shown that cells can be grown on acoustic sensors and that the signals are related to both cell growth and density. The basic principle means that the sensors probe inside the cells and are thus sensitive to its physical properties such as ionic conductivity and viscoelasticity. The use of a pair of acoustic biosensors and differential measurements should allow high common mode rejection of interfering signals and higher sensitivity to transfected cells.

Further work is underway to probe acoustically the growth of HEK293 cells on SH-SAW biosensors and establish the relationship between the acousto-electric signal and the growth phase and condition of the cells. Cells with ligand binding sites will also be immobilized on the biosensors and their response to various chemicals (ligand inhibitors and activators) be acoustically monitored. In this way we can monitor in real-time the viability of cells and in particular their response to specific ligands including biological molecules such as proteins and hormones as well as toxins and drugs.

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