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Cellular Microarrays for Chemical Sensing

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The broad-spectrum sensitivity of cell-based biosensors offers the capability for detecting known and unknown chemical/biological agents. One cellular parameter that is often measured is the extracellular potential of electrically active cells. Membrane excitability in osteoblasts plays a key role in modulating the electrical activity in the presence of chemical agents. However, the complexity of this signal makes interpretation of the cellular response to a chemical agent difficult to determine. By analyzing shifts in the signal's power spectrum, it is possible to determine a frequency spectrum also known as the signature pattern vector (SPV) which is specific to a chemical. It is also essential to characterize single cell sensitivity and response time for specific chemical agents for developing detect-to-warn biosensors. To determine the real time sensing capability of single osteoblast sensors, multichemical sensing, also termed "cascaded sensing," is performed and the performance of the sensor is evaluated. A system is described for the measurement of extracellular potentials from cells isolated onto planar microelectrode arrays. We used a 4×4 multiple microelectrode array system to spatially position osteoblast cells, by using a gradient AC field. Fast fourier transformation (FFT) and wavelet transformation (WT) analyses were used to extract information pertaining to the frequency of firing from the extracellular potential. Quantitative dose response curves and response times were also obtained with the cultured single cell systems using local time domain characterization techniques. Future applications of this technique are also discussed.

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1. Introduction

Biosensor technology is the driving force in the development of biochips for the detection of gaseous pollutants,[1] biological and chemical pollutants,[2] pesticides[3] and microorganisms.[4] A novel challenge is the development of effective biosensors based on fundamental research in biotechnology, genetics and information technology, which will change the existing axiom of "detect-to-treat" to "detect-to-warn."

Conventional methods for detecting environmental threats are primarily based on enzyme,[5] antibody[6, 7] or nucleic acid-based assays,[8–12] which rely on chemical properties or molecular recognition to identify a particular agent.[13–15] The current methods involved in risk assessment for humans fail in field situations due to their inability to detect large numbers of chemical agents, characterize the functionality of agents and determine human performance decrements.[16]

Cell based sensing[17] is the most promising alternative to the existing biosensing techniques as cells have the capability of identifying very minute concentrations of environmental agents. Mammalian cells with excitable cell membranes are used as cell-based biosensors. The cell membranes of mammalian cells with excitable cell membranes are comprised of ion channels. These ion channels open or close based on changes in the internal and external local environments of the cells. This results in the development of ionic gradients that are responsible for the modification of electrical conductivity. The modification of electrical conductivity is directly associated with a change in the extracellular membrane potential. This extracellular measurement can be considered to be a reliable indicator for the determination of the presence of a specific agent in the cell's local environment. The cell membrane potential will indicate any change associated with the local environment in a noninvasive manner that will not interfere with the existing cell. These minute concentrations can be measured in parts per million (ppm) and in the case of certain agents in parts per billion (ppb). Cell based sensing is the means for developing highly sensitive and accurate biosensors as they yield physiologically relevant information that can be used to characterize the specific nature of the chemical agent. Most mammalian cells have excitable cell membranes that can be activated and deactivated based on external agents. Moreover, extracellular microelectrode arrays offer a noninvasive and long-term approach to the measurement of extracellular potentials.

Cells express and sustain an array of potential molecular sensors. Receptors, channels and enzymes that are sensitive to an analyte are maintained in a physiologically relevant manner native to the cellular machinery. In contrast with the antibody-based approaches, cell-based sensors should optimally only respond to functional biologically active analytes. Cell-based biosensors have been implemented using microorganisms in particular forthe purpose of environmental monitoring of pollutants. Biosensors incorporating mammalian cells have the distinct advantage of responding in a manner that can identify the physiological effect of the analyte. Several approaches to cell signal transduction are available. These include measurement of cell fluorescence, cell metabolism, impedance, intracellular potentials, and extracellular potentials. Some analytes, such as pollutants,[18] can activate microorganism pathways involved in the metabolism or nonspecific cell stress, resulting in the expression of one or more genes. However, pollutants are often mixtures of chemicals

and the presence of other inhibiting/stimulating agents in a sample can affect sample integrity. Fluorescence imaging has proven to be an invaluable tool for monitoring changes in concentration of ions and protein expression related to cellular signaling.[19] There are three disadvantages associated with this technique: the ability to readily transfect reporter genes in mammalian cells is limited to tumor or tumor derived cells, cell loading with fluorescent dyes is a potentially invasive treatment, and finally, the analytes under study need to be tested for auto fluorescence. Another category of cellular biosensors[20] relies on the measurement of the energy metabolism, which is a common feature in all of the living cells. Proper interpretation of data acquired by this method requires parallel experiments in the presence of known receptor antagonists.

The membranes of cells exhibit dielectric properties. By culturing cells over electrode contacts changes in the effective electrode impedance can be measured. Impedance measurements are based on the fact that intact living cells are excellent electrical insulators at low signal frequencies, hence a noninvasive assay of cultured cell motility, adhesion, and spreading.[21–24] In terms of its use in a biosensor, the impedance technique relies on changes in the cell migration or morphology which are rather slow.[25]

An important aspect of the information that can be derived from cell-based biosensors relates to the functional or physiologic significance of the analyte to the organism.[26] To this end, the bioelectric phenomena of excitable cells have been used to relay functional characteristic information concerning the cell status. Thus analytes that affect the excitability of the cell membrane are expected to have profound effects on an organism. Furthermore, the nature of changes in the excitability can yield physiologic information on the organism. The associated disadvantage of the technique is its invasiveness.

The current cell based biosensing technology is based on combining these concepts; however, to evaluate the degree of threat from a specific chemical agent it is essential to detect the sensitivity limit and the responsiveness of a single cell through reliable extracellular measurements of their electrical activity modulation. Until now, no such measurements have been quantified. This lack of information is due to the inability to isolate a single cell over a single electrode. In this paper, we present a novel single cell osteoblast sensor, which can detect specific chemicals based on modification of their extracellular electrical activity. We have developed a novel technique for isolating and positioning a single osteoblast cell over an individual electrode, utilizing gradient alternating current (AC) fields based on the variance in the cell's dielectric properties. We have investigated rat osteoblast cells because they a have highly permeable and excitable cell membrane. Combining the microelectrode array technology with our new technique of cell isolation, we are able to determine the electrical response and sensitivity of a single cell to different chemical agents. Finally, we are able to analyze the signal modulation in the frequency domain to generate a SPV, using FFT analysis for a given cell type depending on the chemical agent. Each SPV comprises the frequencies of maximum relative amplitudes that correspond to the stable burst rates of firing. These are termed as "eigen vectors." The associated amplitudes are termed as "Eigen Values." Hence, a collection of response patterns for each cell type for a variety of agents is developed. Local signal characterization is performed using WT analysis to determine the detection sensitivity and the response time for each specific chemical agent. Thus, the chemical agent acting upon a single

osteoblast is determined by synthesizing the time domain and frequency domain response to yield a unique "Signature Pattern" (SP). Finally, to evaluate the ability of an individual cell to recognize different chemical agents, we introduced two chemical agents in cascaded mode and determined the SP.

2. Materials and Methods

2.1 *Biosensing system*

The biosensing system comprises a chip assembly and an environmental chamber for maintaining a stable local environment for accurate data acquisition.

2.1.1 *Chip assembly*

The microelectrode array fabrication and cell patterning have been achieved using a previously developed procedure[27] and are now briefly described. A 4×4 microelectrode array comprising platinum electrodes (diameter: 80 μm, center-to-center spacing: 200 μm) spanning a surface area of 0.88×0.88 mm^2 on a silicon/silicon nitride substrate with electrode leads (6 μm thick) terminating at electrode pads (100 μm×120 μm) is fabricated using standard lithography techniques (Fig. 1). To achieve a stable local microenvironment the microelectrode array is integrated with a silicone chamber (16×16×2.5 mm^3) with a microfluidic channel (50 μm wide) for pumping in the testing agent and pumping out the test buffer once the sensing process has been completed. The flow rate of the buffer is 40

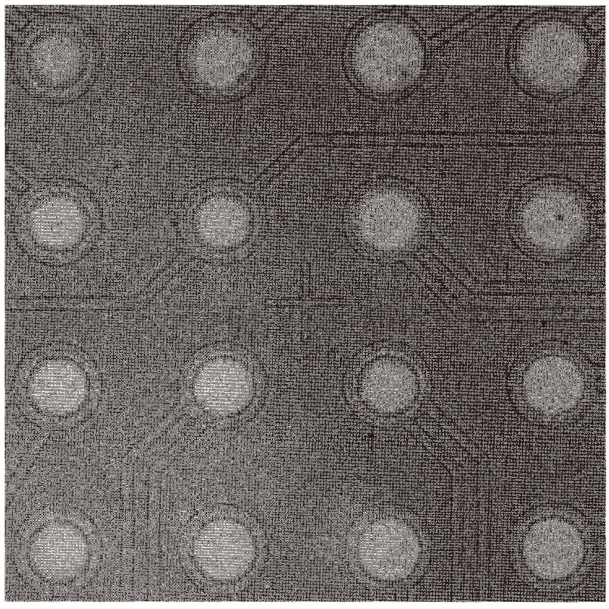

Fig. 1. 4×4 microelectrode array comprising platinum electrodes, with 80 μm diameter and 200 μm center-to-center spacing.

μL/min. The silicone chamber is provided with an opening (8×8×2.5 mm^3) and is covered by a glass cover slip for in situ monitoring. Simultaneous electrical and optical monitoring is achieved by using a Microzoom™ (Nyoptics Inc., Danville, CA) optical probe station under 8× and 25× magnification. The electrical stimulation and measurements are achieved utilizing micromanipulators (Signatone, Gilroy, CA).

2.1.2 *Environmental chamber*

The optical probe station along with the chip assembly is enclosed by an acrylic chamber (S&W Plastics, Riverside, CA). The environment in the chamber is controlled so as to maintain a constant temperature of 37°C. A heat gun (McMaster, Santa Fe Springs, CA) inside the chamber heats the air in the chamber and this is linked to a temperature controller (Cole Parmer, Vernon Hills, Illinois) that stops the heat gun from functioning above the desired temperature. A 6" fan (McMaster, Santa Fe Springs, CA) inside the chamber circulates the hot air to maintain temperature uniformity throughout the chamber and is monitored by a J-type thermocouple probe attached to the temperature controller. The carbon dioxide concentration inside the chamber is maintained at 5% and is humidified to prevent excessive evaporation of the medium. This chamber with all of its components will ensure cell viability over long periods of time and stable cell physiology in the absence of chemical agents.

2.2 *Cell culture*

Primary rat osteoblast cells were cultured to a concentration of 10,000 cells in 1 mL for sensing experiments. To achieve the patterning of a single cell over a single electrode, 10 μL of cell culture solution was mixed with 500 μL Dulbeco modified eagle medium (DMEM; Gibco, Grand Island NY) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island NY), 100 μg/mL penicillin, and 100 μg/mL streptomycin (P/S; Gibco, Grand Island NY). The cells were centrifuged and re-suspended in 1 mL of separation buffer consisting of 1:9 dilutions of Phosphate Buffer Saline 250 mM Sucrose (Sigma, St Louis) and de-ionized water (weight/volume). The conductivity of the separation buffer was 4.09 mS/cm and it had a pH of 8.69. The separation buffer was replaced with a test buffer ((DMEM)/ Fetal Bovine Serum (FBS)/Phosphate Buffer Saline (PBS)) with conductivity of 2.5 mS/cm and a pH of 8.06.

2.3 *Experimental measurement system*

Figure 2 shows a schematic representation of the measurement system. It comprises of extracellular positioning, stimulating and recording units. The osteoblast cells were isolated and positioned over single electrodes by setting up a gradient AC field using an extracellular positioning system comprising a pulse generator (HP 33120A) and micromanipulators (Signatone, Gilroy, CA). The signal from the pulse generator was fed to the electrode pads of the selected electrodes using the micromanipulators. The extracellular recordings from the individual osteoblasts obtained from the electrode pads were amplified and recorded on an oscilloscope (HP 54600B, 100 MHz). The supply and measurement systems were integrated using a general purpose interface bus (GPIB).

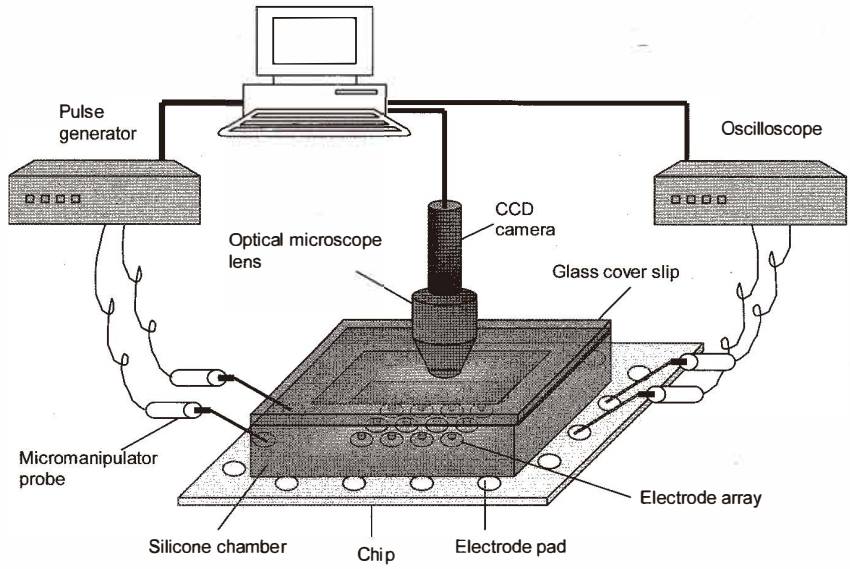

Fig. 2. Schematic representation of the measurement system. It simultaneously provides electrical and optical monitoring capabilities.

2.4 *Cell isolation and positioning*

A gradient AC field is set up among the electrodes by adjusting the parameters of the applied frequency, peak-to-peak voltage and conductivity of the separation buffer. Osteoblasts under the absence of an electric field have a uniformly distributed negative charge along the membrane surface. On applying the gradient AC field, a dipole is induced based on the cell's dielectric properties. Due to the interaction between the dipole and the nonuniform electric field, the osteoblasts experience a positive dielectrophoretic force that causes their migration to the electrodes, which are the regions of high electric fields.[28] In this manner, osteoblasts are isolated and positioned over the electrodes.

2.5 *Signal processing*

Changes in the extracellular potential shape have been used to monitor the cellular response to the action of environmental agents and toxins. The extracellular electrical activities of a single osteoblast cell are recorded in both the presence and absence of chemical agents and the modulation in the electrical activity is determined. However, the complexity of this signal makes interpretation of the cellular response to a specific chemical agent difficult to interpret. It is essential to characterize the signal in both the time and frequency domains for extracting the relevant functional information. The use of power spectral density analysis as a tool for classifying the action of a chemically active agent was investigated and found to be a suitable technique for data analysis. The power spectrum of the extracellular potential is a better indicator of the cell response than the monitored peak-to-peak amplitude.

Additionally, by examining the root mean square (RMS) power in different frequency bands, it is possible to approximate the power spectral density analysis performed numerically herein. Using the FFT analysis, we analyze the shifts in the signal's power spectrum. The FFT analysis extracts the modulation in the frequency of the extracellular potential burst rate and hence is termed as "frequency modulation" and generates the SPV. The "eigen vectors" corresponding to the modulated firing rate of the osteoblast cell are determined from the SPV. However, the FFT analysis is a transformation based on the whole scale, i.e., either absolutely in the time domain, or absolutely in the frequency domain. Therefore, it is impossible to extract the local information in the time domain. Thus, we use WT analysis to extract the information from the local time domain. WT is a time-scale (time-frequency) analysis method whereby multiresolution analysis of the parameters is achieved. This can express the local characterization of signals in both the time and frequency domains, hence, functional information extracted from the extracellular potential, such as the response time and the limits of detection. As this analysis relies on the determination of the modulation of the amplitude of the signal due to the effect of the chemical agents, it is termed as "Amplitude Modulation."

2.6 *Selection of chemical agents*

It is essential to obtain the effect of a broad spectrum of chemical agents, ranging from highly toxic and physiologically damaging to relatively less toxic, to determine and evaluate the time window of response of a particular cell type for a specific known agent based on varying concentrations, and finally determine the limit of detection for a specific chemical agent. All the experiments were conducted based on the hypothesis that a unique SPV would be generated for each cell type for a specific chemical. This was hypothesized as it has been scientifically proven that different chemicals bind to different ion channel receptors and thus modify the electrical response of a cell in a unique manner.[29,30] We present here the responses of single osteoblast cells to the effect of the following chemical agents: Ethanol, Hydrogen peroxide, Ethylene diamene tetra acetic acid (EDTA), and Pyrethroids, for n=15.

2.6.1 *Ethanol*

Ethanol produces anesthetic effects but in a milder form as compared to pentobarbitone and ketamine, though the mechanism of action is essentially assumed to be the same.[31] We hypothesized that determination of single cell ethanol sensitivity would help us to identify the lowest threshold concentration, for the family of chemicals whose physiological response mechanism would mimic that of ethanol. The concentration ranges tested for ethanol were from 5000 ppm to 15 ppm. The detection limit for ethanol using this technique was determined to be 19 ppm.

2.6.2 *Hydrogen peroxide*

Hydrogen peroxide is one of the major metabolically active oxidants present in the body and leads to apoptosis. Hydrogen peroxide also leads to the degradation of cells. As the behavior of hydrogen peroxide *in vivo* is similar to the behavioral responses obtained from exposure to carcinogenic chemicals such as rotenone, we estimated that hydrogen peroxide would make an ideal candidate for sensing studies.[32] The range of concentration

of hydrogen peroxide varied from 5000 ppm to 20 ppm. The sensitivity limit for a single osteoblast due to the action of hydrogen peroxide was determined to be 25 ppm.

2.6.3 *Pyrethroids*

Pyrethroids are active ingredients in the commercially used pesticides. Pyrethroids share similar modes of action, which resemble that of DDT. Pyrethroids are expected to produce a "knock down" effect *in vivo*; the exact *in vitro* response at a cellular level has not yet been clarified. Hence, they are ideal candidates for the analysis of this genre of chemicals. The concentration range of pyrethroids varied from 5000 ppm to 850 ppb. The detection limit for pyrethroids was determined to be 890 ppb.

2.6.4 *Ethylene diamene tetra acetic acid (EDTA)*

EDTA belongs to a class of synthetic, phosphate-alternative compounds that are not readily biodegradable and once introduced into the general environment can re-dissolve toxic heavy metals. The target specificity of EDTA in a single osteoblast cell has not been electrically analyzed to date. The concentration ranges for EDTA varied from 500 ppm to 175 ppm. The limit of detection in this case for a single osteoblast was determined to be 180 ppm.

In all the experiment cycles, we obtained a unique response of the osteoblast cells to a specific chemical. We determined the detection limit of a single osteoblast cell for every chemical agent. The firing rate of a single osteoblast in the absence of a chemical agent was determined to be 668 Hz after the FFT analysis of the recorded extracellular electrical activity of the individual osteoblast cells. On performing FFT analyses on the recorded modified extracellular electrical activity in the presence of the specific chemical agents at varying concentrations, specific burst frequencies were obtained that can be used as identification tags for recognizing the chemical agents. These are known as eigen vectors.

To simulate real time sensing conditions, we performed cascaded sensing using the same osteoblast cell to detect the response to two chemical agents induced in a cascaded form. The results obtained for cascaded ethanol-hydrogen peroxide sensing are presented below.

3. Results

3.1 *Osteoblast sensing*

3.1.1 *Control experiment*

In order to determine the SPV corresponding to a specific chemical, the initial activity pattern vector for each cell type was determined. Using the process of dielectrophoresis, a single cell was positioned over a single electrode and its initial electrical activity was recorded.

3.1.2 *Ethanol sensing*

3.1.2.1 *Determination of signature pattern vector*

Single osteoblast cells were positioned over individual electrodes. The sensing agent was then introduced onto the microelectrode array using the microfluidic inlet channel.

The initial concentration of ethanol used was 5000 ppm and the modified electrical activity was recorded. The concentration of ethanol was decremented in a stepwise manner and in each case, the modified electrical activity was recorded. The lowest concentration of ethanol sensed by a single osteoblast was 19 ppm. The lowest concentration of ethanol (19 ppm) detectable by this technique is far more sensitive than the detectable limits obtained with the optical waveguide technique[33] (35 ppm: 0.4×10^{-6} M) that is considered to be one of the most sensitive detection techniques to date.[34]

The analysis was performed on the acquired data pertaining to the modified extracellular potential to yield the SPV. The instant at which the chemical is added to the chip system is denoted by t=0 s. Figure 3(a) represents the SPV for a single osteoblast due to the action of ethanol at 19 ppm. Osteoblasts have the unmodulated firing rate of 668Hz. This corresponds to the frequency of firing of osteoblasts in the absence of a chemical agent. There are two eigen vectors (514 Hz and 722 Hz) in the SPV corresponding to the modulated firing rate of the osteoblast. During the first phase of the sensing cycle (t= (0, 60) s) the modulated firing rate is focused at 722 Hz. During the second phase of the sensing cycle (t=(60,120) s) the modulated firing rate shifts towards the lower frequency value (514 Hz). During the third phase of the sensing cycle (t=(120, 180) s), the modulated frequency shifts back to the original higher frequency bursting (668 Hz and 722 Hz) as observed in the first phase. As the concentration of ethanol is very low, the cell quickly recovers and on reintroduction of the chemical at t=180 s, the SPV starts to repeat itself (t= (180, 240) s).

The WT analysis is performed on the acquired data to yield the local time domain characteristics in order to extract the first modulated maxima corresponding to the first eigen vector of the response. Figure 3(b) indicates the extraction of the first eigen vector using WT at an ethanol concentration of 19 ppm. The response time for an osteoblast is also determined using WT analysis. The response time is defined as the time taken for the functional sensing element-osteoblast to respond to the specific input-chemical agent, and reach its first extreme value. Figure 3(c) indicates the response time for the osteoblast at an ethanol concentration of 19 ppm. We determined that the response time of the osteoblast to a specific chemical until the detection limit remained constant irrespective of the concentrations of the chemical agent. The response time of a single osteoblast to ethanol was determined to be 0.41 s and is denoted by T_R.

3.1.3 *Hydrogen peroxide sensing*

The single osteoblast cells were isolated and positioned over individual electrodes in the manner previously described. The initial concentration of hydrogen peroxide used was 5000 ppm and the modified electrical activity was recorded. The concentration of hydrogen peroxide was decremented in a stepwise manner and in each case, the modified electrical activity was recorded. The lowest concentration of hydrogen peroxide sensed by a single osteoblast was 25 ppm. FFT analysis was performed on the acquired data pertaining to the modified extracellular potential to yield the SPV. The instant at which the chemical is added to the chip system is denoted by t=0 s. Figure 4(a) represents the SVP.

There are three eigen vectors (257 Hz, 565 Hz and 873 Hz) in the SPV corresponding to the modulated firing rate of the osteoblast. The frequency of 668 Hz corresponds to the

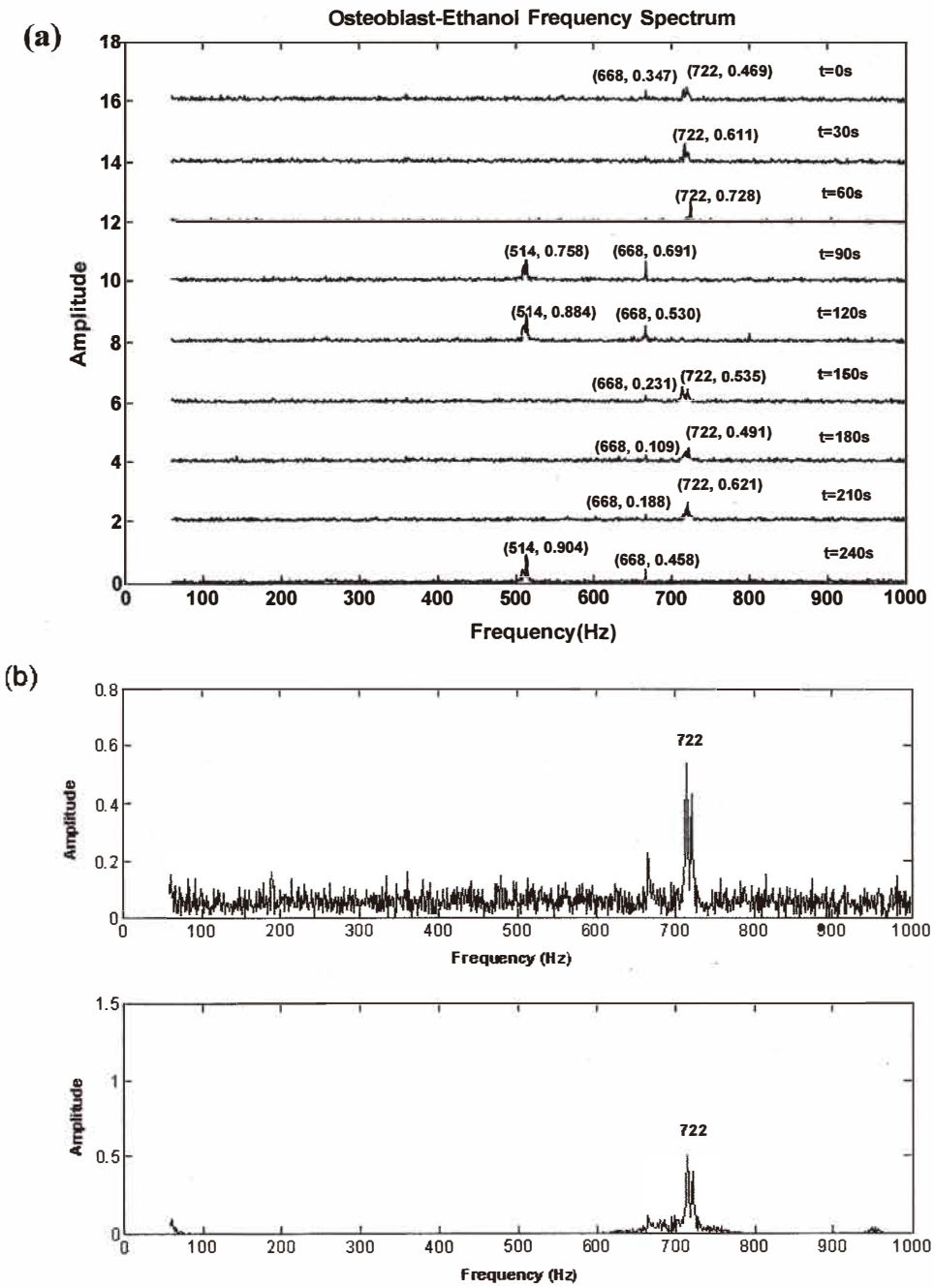

Fig. 3. (a) Signature pattern vector of single osteoblast due to the action of ethanol at 19 ppm. (b) Wavelet transformation analysis to determine the first eigen vector of a single osteoblast due to the action of ethanol at 19 ppm.

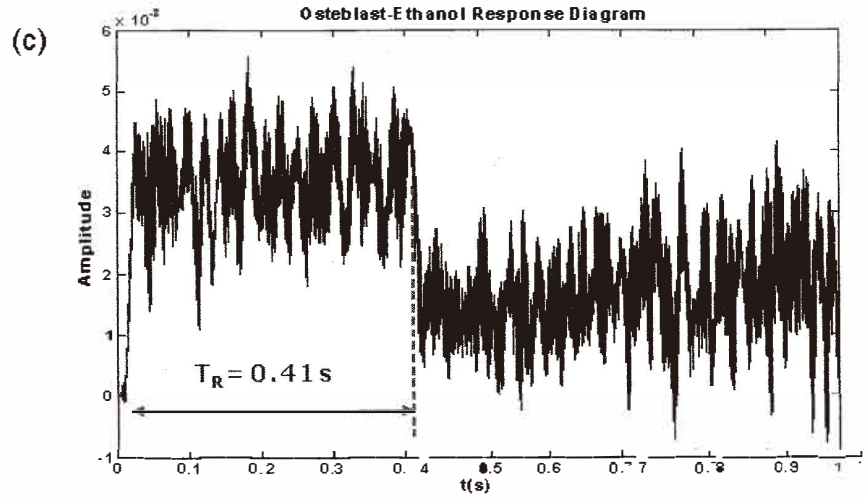

Fig. 3. (continued from the previous page) (c) Response time of a single osteoblast due to the action of ethanol.

osteoblast firing rate in the absence of a chemical agent. During the sensing cycle, low-frequency subsidiary peaks (129 Hz, 334 Hz, 257 Hz and 437 Hz) are expressed. We hypothesize that these occur due to possible nonspecific interactions between the chemical agent and the sensing system. The hypothesis is based on the fact that the control burst frequency for a single osteoblast is 668 Hz and the eigen vector range for the eigen vectors due to the interaction of the chemical agents has been observed to vary by ± 30% of the control value.

The WT analysis is performed on the acquired data to yield the local time domain characteristics in order to extract the first modulated maxima corresponding to the first eigen vector of the response (spectrum not shown). The response time for an osteoblast is determined using WT analysis by evaluating the time required to achieve the first maximum after the application of hydrogen peroxide. Figure 4(b) indicates the response time for the osteoblast at a hydrogen peroxide concentration of 25 ppm. This technique produces a sensitivity of 2.94×10^{-8} M (25 ppm) in comparison to the sensitivity of 1.2×10^{-6} M: 42 ppm produced by the optical waveguide technique.[35]

We determined that the response time remained constant for various concentrations of hydrogen peroxide. The response time of a single osteoblast due to hydrogen peroxide was determined to be 0.71 s for various concentrations.

3.1.4 *Pyrethroid sensing*

The initial concentration of pyrethroid used was 5000 ppm and the modified electrical activity was recorded. The concentration of pyrethroid was decremented in a stepwise manner and in each case, the modified electrical activity was recorded. The lowest

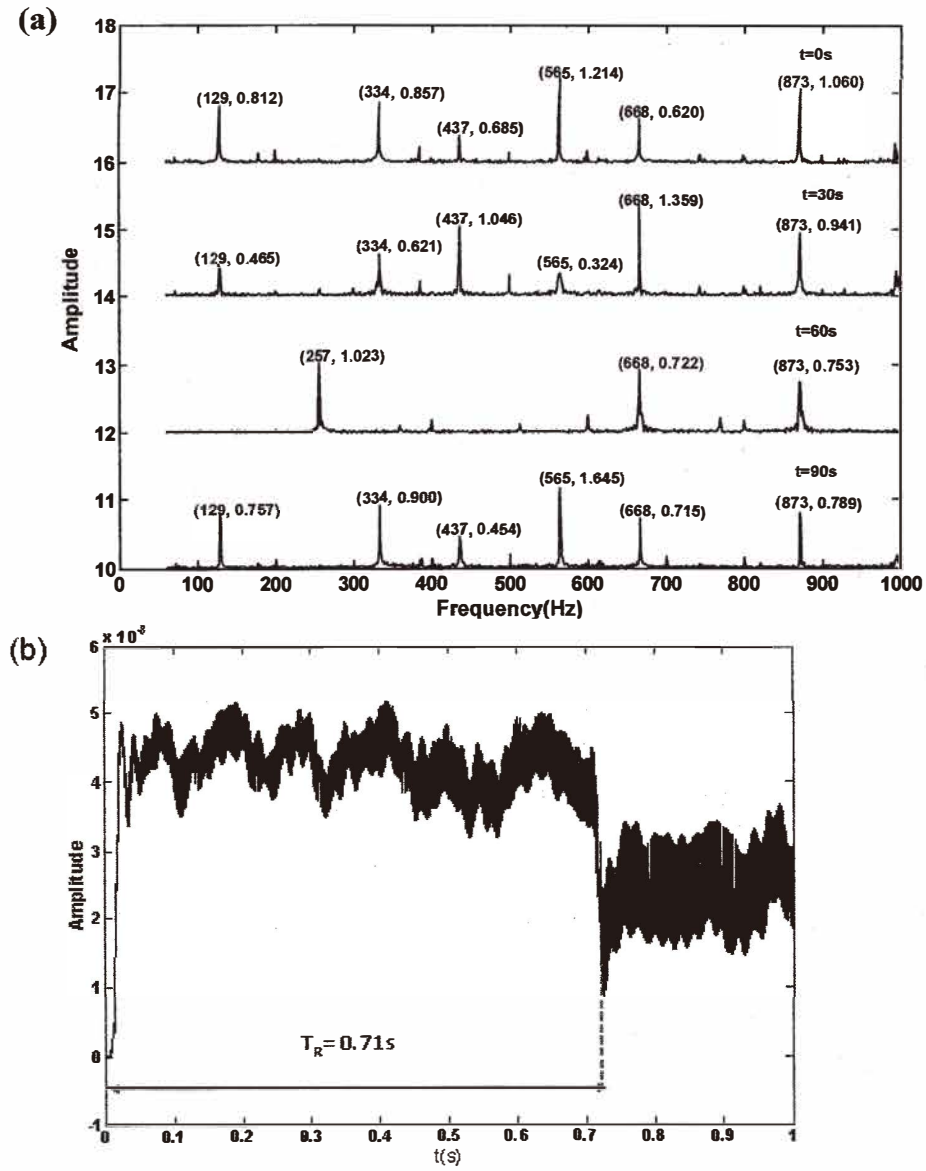

Fig. 4. (a) Signature pattern vector of single osteoblast due to the action of hydrogen peroxide at 25 ppm. (b) Response time of a single osteoblast due to the action of hydrogen peroxide.

concentration of pyrethroid sensed by a single osteoblast was 890 ppb. The sensitivity limit obtained via this technique is far more sensitive than that obtained through the waveguide detector technique (≈ 950 ppb: 0.1×10^{-6} M).[36] FFT analysis was performed on

the acquired data pertaining to the modified extracellular potential to yield the SPV. The instant at which the chemical is added to the chip system is denoted by t=0 s. Figure 5(a) represents the SVP.

There are two eigen vectors (257 Hz, and 873 Hz) in the SPV corresponding to the modulated firing rate of the osteoblast. The frequency of 668 Hz corresponds to the osteoblast firing rate in the absence of a chemical agent. During the first half of the cycle, there are subsidiary peaks corresponding to 129 Hz, and 565 Hz corresponding to the non-specific interactions of the chemical agent within the sensing system.

The local time domain characteristics are obtained by performing WT analysis on the acquired data. The first modulated maximum is extracted and this corresponds to the first eigen vector of the response (spectrum not shown). The response time for an osteoblast is determined using WT analysis by evaluating the time required to achieve the first maximum after the application of pyrethroid. Figure 5(b) indicates the response time for the osteoblast at a pyrethroid concentration of 890 ppb. We determined that the response time remained constant for various concentrations of pyrethroid. The response time of a single osteoblast to pyrethroid was determined to be 0.23 s and this value remained constant irrespective of the concentrations of pyrethroid.

3.1.5 *EDTA sensing*

The initial concentration of EDTA used was 5000 ppm and the modified electrical activity was recorded. The concentration of EDTA was decremented in a stepwise manner and in each case, the modified electrical activity was recorded. The lowest concentration of EDTA sensed by a single osteoblast was 180 ppm. The sensitivity of this technique is far superior to that obtained from previous studies which resulted in a detection limit of 4.6×10^{-6} M: 210 ppm.[37]

FFT analysis was performed on the acquired data pertaining to the modified extracellular potential to yield the SPV. The instant at which EDTA is added to the chip system is denoted by t=0 s. Figure 6(a) represents the SPV.

The initial peak in the frequency spectrum is observed at 514 Hz corresponding to the first eigen vector. This is obtained at t=0 s, after the immediate application of EDTA. Osteoblast cells then regain their control of the firing rate, corresponding to 667 Hz. The next two eigen vectors of 258 Hz and 872 Hz are obtained in the time interval (t= (60, 90) s). Subsidiary low frequency peaks are observed at 129 Hz, 334 Hz, and 437 Hz and high-frequency peaks are observed at 514 Hz and 565 Hz due to the nonspecific interactions.

The local time domain characteristics and functional information are obtained by performing WT analysis on the acquired data. The first modulated maximum is extracted and this corresponds to the first eigen vector of the response (spectrum not shown). The response time for an osteoblast is determined using WT analysis by evaluating the time required to achieve the first maximum after the application of EDTA. Figure 6(b) indicates the response time for the osteoblast at a pyrethroid concentration of 180 ppm. We determined that the response time remained constant for various concentrations of EDTA. The response time of a single osteoblast to EDTA was determined to be 0.14 s and this value remained constant irrespective of the concentration of EDTA.

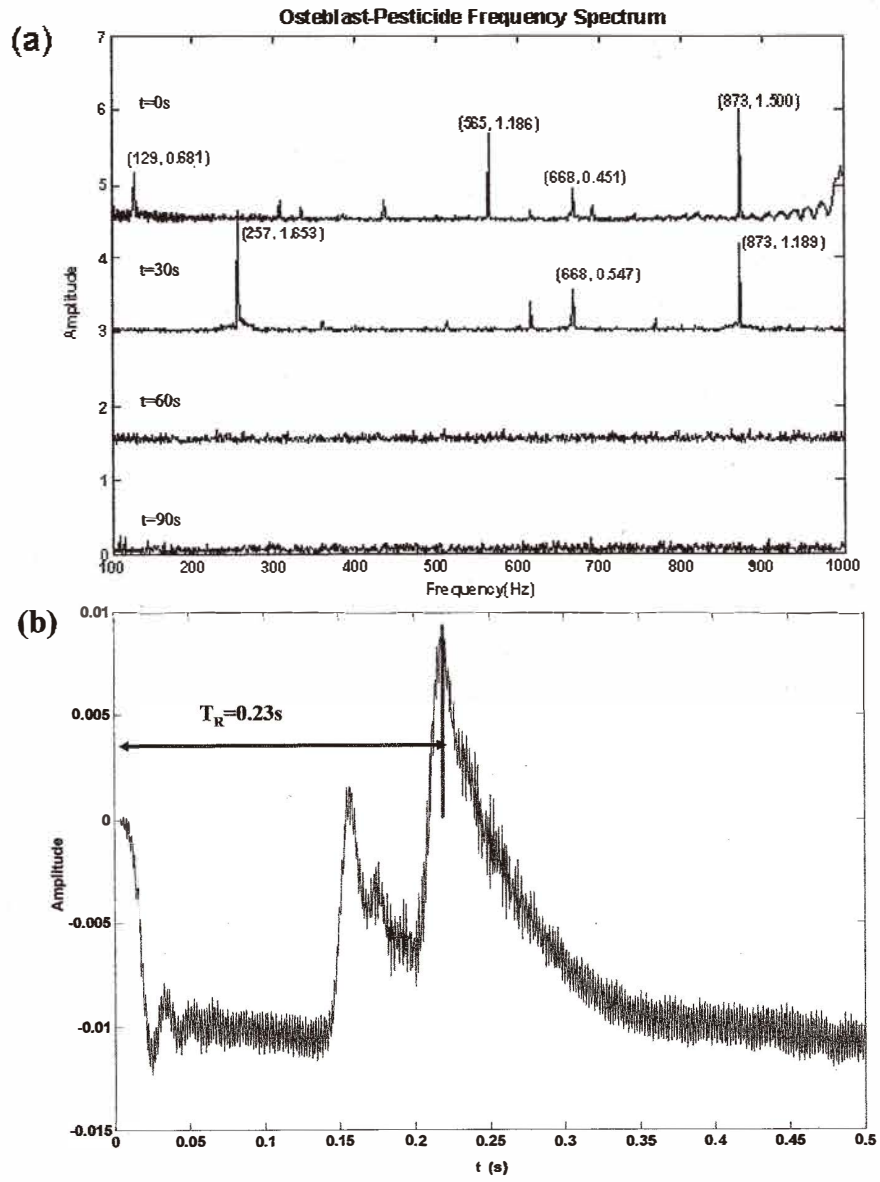

Fig. 5. (a) Signature pattern vector of single osteoblast due to the action of pyrethroids at 890 ppb. (b) Response time of a single osteoblast due to the action of pyrethroids.

3.2 *Comparison of detection limits and response time*

We determined that a single osteoblast cell was the most sensitive to ethanol (19 ppm) whereas it was the least sensitive to pyrethroid (890 ppb). Also, single osteoblast cells

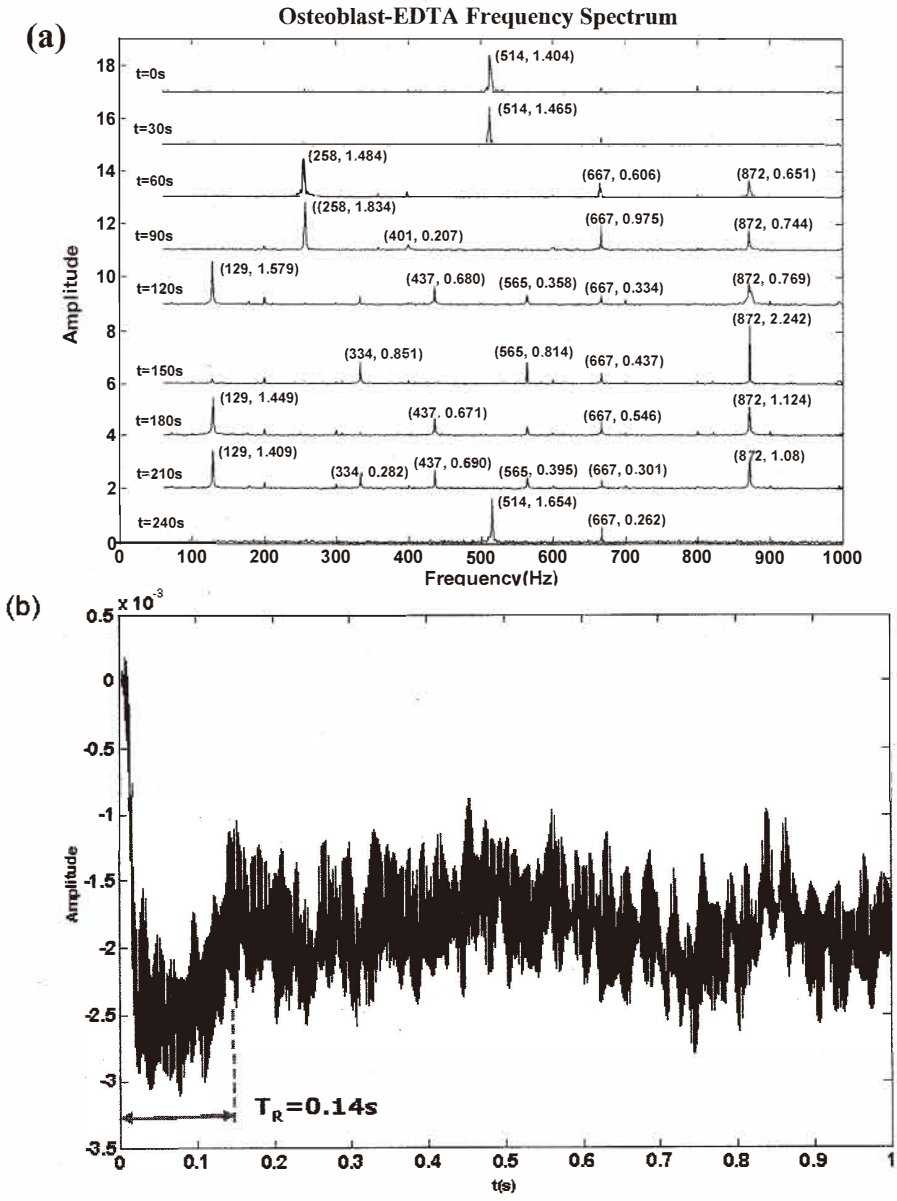

Fig. 6. (a) Signature Pattern Vector of single osteoblast due to the action of EDTA at 180 ppm. (b) Response time of a single osteoblast due to the action of EDTA.

respond the quickest to EDTA (0.14 s) whereas they take the maximum time to respond to hydrogen peroxide (0.71 s). This data has been summarized in Table 1.

Figure 7 is a graphical representation of the response times obtained for each specific

Table 1
Comparison of chemical concentrations and response times.

	Ethanol	Peroxide	EDTA	Pyrethroids
Response time(s)	0.41	0.71	0.14	0.23
Concentration	19 ppm	25 ppm	180 ppm	890 ppb

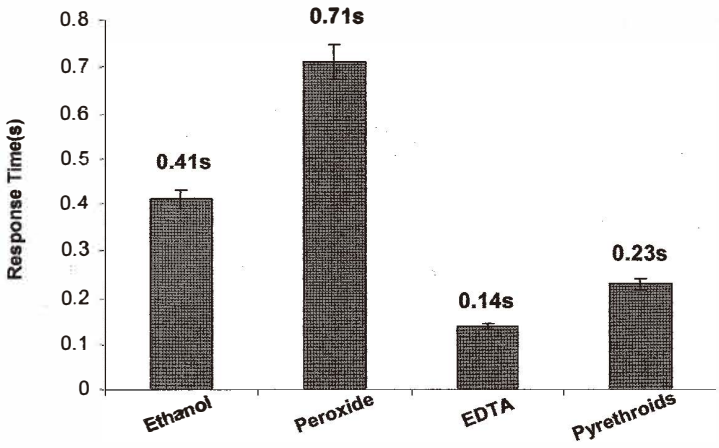

Fig. 7. Representation of response times for specific chemical agents.

chemical agent. The graph shows the repeatability of the response. The response time for each chemical was determined by testing a specific agent in three cycles and each cycle comprised of three runs.

3.3 *Effect of varying concentration of chemical agents*

We observed for all the chemical agents that the amplitude of response decreased as the concentration of the chemical agent in the local microenvironment increased. We performed a WT analysis, where the local time domain characterization of the amplitude was performed as a function of concentration. This analysis identified the amplitude shifts corresponding to the varying concentration. WT analysis indicated that at a higher concentration (1000 ppm), there was a large decrement in the amplitude of the time domain characterization of the extracellular potential. For low concentrations around the detection limit the decrement of the amplitude was much smaller, being by a factor of about 80%. Figures 8(a) and 8(b) represent the variation in amplitude due to low (180 ppm) and high (1000 ppm) concentrations of EDTA. It was also observed that there is no noticeable difference in the response times due to various concentrations for a specific chemical.

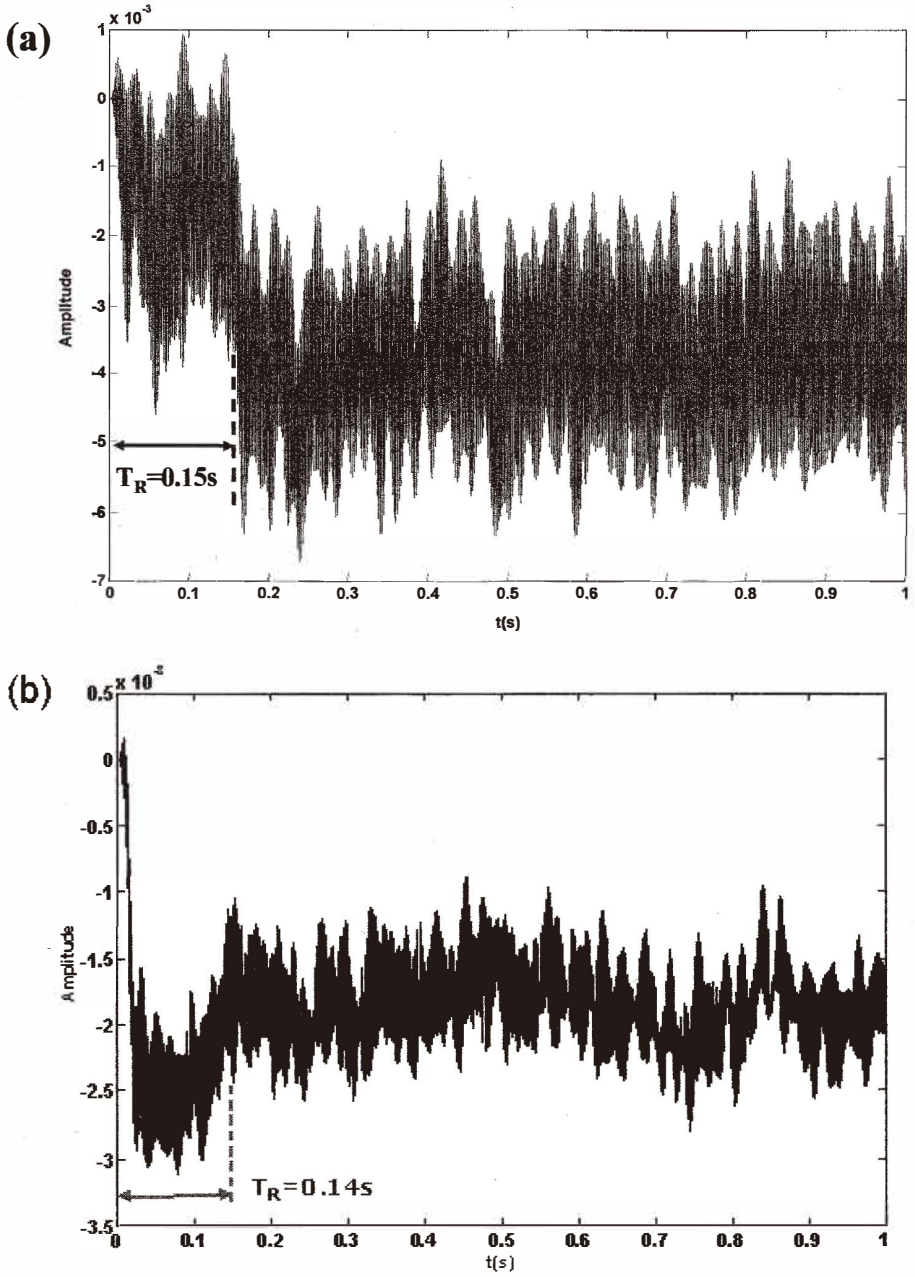

Fig. 8. (a) Variation in amplitude of single osteoblast due to the action of EDTA at 180 ppm. (b) Variation in amplitude of single osteoblast due to the action of EDTA at 1000 ppm.

3.4 *Cascaded sensing*

To simulate real time conditions we tested the selectivity of the single osteoblast sensor. We determined the ability of the sensor to identify specific chemical agents when introduced in cascade by exhibiting the SPV corresponding to each agent. Here, we describe the cascaded sensing of ethanol-hydrogen peroxide by single osteoblast cells. After determining the detection limits for both of the chemical agents, we first introduced ethanol at 19 ppm into the chip sensor and recorded the modified extracellular potential. As observed previously the osteoblast cell then regains its initial spectrum after undergoing modulation. Hydrogen peroxide at 25 ppm was then introduced into the chip sensor and the modulated response was recorded. FFT analysis of the acquired data indicates that the SPV obtained in the cascaded sensing exactly correlated with the SPVs obtained from the individual sensing of ethanol and hydrogen peroxide. The eigen vectors corresponding to ethanol (514 Hz and 722 Hz) and those corresponding to hydrogen peroxide (257 Hz, 576 Hz and 852 Hz) can be correlated to those obtained during individual chemical sensing. There is a slight shift in two of the eigen vectors of hydrogen peroxide from 565 Hz to 576 Hz and from 873 Hz to 852 Hz which can be accounted for by the interaction between ethanol and hydrogen peroxide. Figures 9(a) and (b) represent the SPVs of a single osteoblast cell due to the cascaded action of ethanol and hydrogen peroxide, respectively (for n=15).

4. Discussion

It is well known that membrane excitability plays a key physiological role in osteoblasts for the control of ion flow, which eventually determines the extracellular potential, measured from the cell membrane. The presence of specific chemical agents modulates the transmembrane flow of ions and thus modifies the extracellular potential. Hence, measurement and analysis of extracellular potential due to the effect of a specific chemical agents is a noninvasive and accurate method for determining the effect of specific chemical on osteoblasts. In order to develop detection methods for developing detect-to-warn biosensors in real time situations, it is essential to determine the detection limit and response time of single osteoblast cells for various chemical agents. Moreover, it is also necessary to determine the modulation pattern of the electrical activity in the presence of a specific chemical to identify the agent in field conditions from the analyzed data obtained from experimental analysis. All the experiments were conducted based on the hypothesis that a unique SPV would be generated for a specific chemical. This was hypothesized as it has been proven that different chemicals bind to different ion channel receptors for different periods on the cell membrane and thus modify the electrical response of the cell in a unique manner. SPV integrates the frequency domain analysis, obtained from FFT and the local time domain characterization, obtained from WT. As WT analysis is a multiresolution analysis, functional data pertaining to a broad spectrum of parameters can be obtained from a single analysis technique. The detection limit and the response time of single osteoblasts to various specific chemical agents are determined in this manner. It was established that the response time of the osteoblast is virtually independent of the concentration of the chemical agents and is dependent only on the nature of the agent.

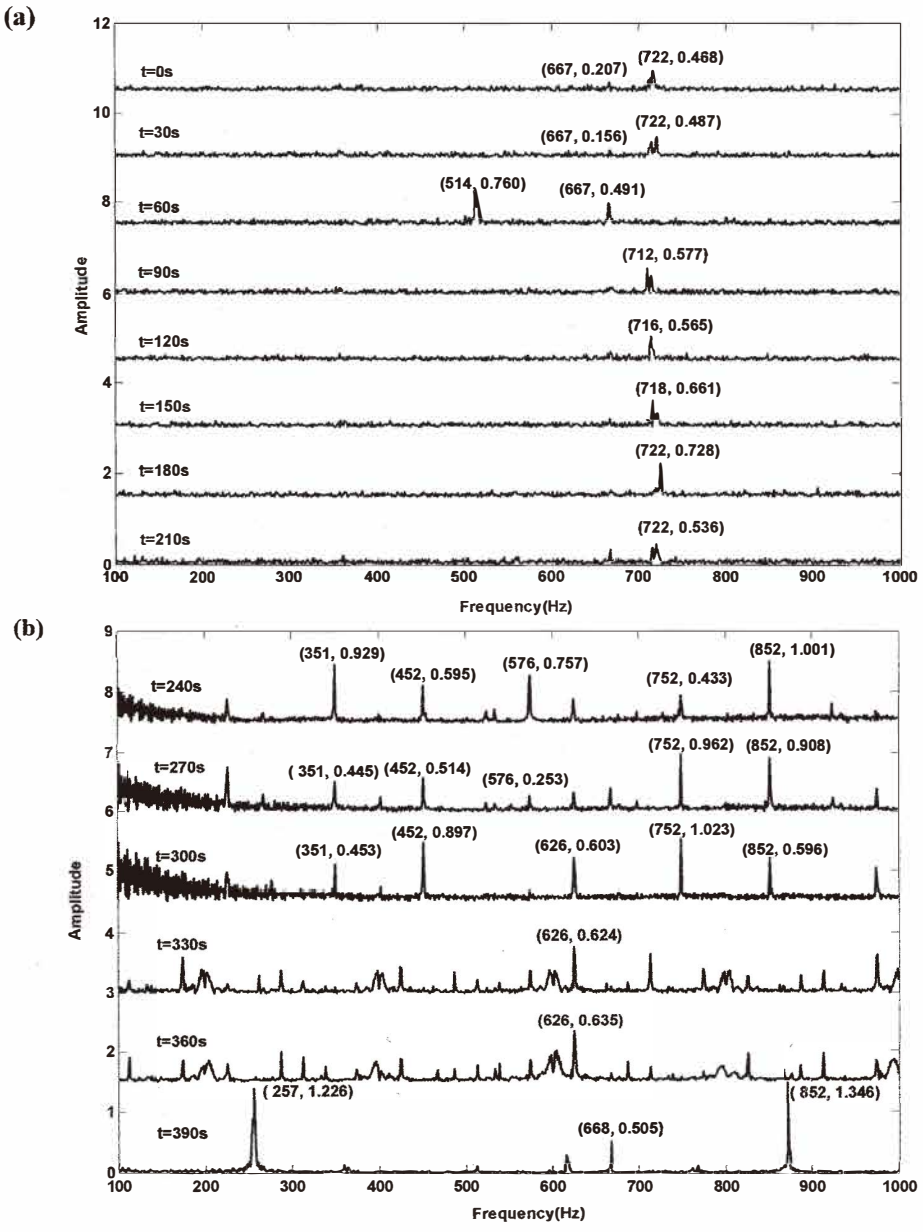

Fig. 9. (a) Signature pattern vector of single osteoblast due to the action of ethanol at 19 ppm. (b) Signature pattern vector of single osteoblast due to the cascaded action of ethanol-hydrogen peroxide at 19 ppm and 25 ppm, respectively.

To determine the effect of a broad spectrum of agents, it is essential to determine the specific SPV associated with each chemical as it provides an easy method for identification. It is possible to create a lookup table consisting of chemical agents and their corresponding SPVs, and this would provide us with a means of identifying the genre of an unknown agent based on comparing the SPV generated by the unknown agent to those generated by known agents listed in the lookup table.

Cascaded sensing established the ability of single osteoblast sensors to function effectively in real time situations. The generation of SPVs associated with specific chemicals under cascading conditions close to detection limits indicates the ability of osteoblasts to physiologically recover, which provides us with the possibility of reusing single osteoblast sensors.

Sensing performed on a mixture of chemical agents results in the generation of SPVs that are identical to the various components of chemical agents in the mixture. Some subsidiary eigen vectors are also generated, which do not correspond to the eigen vectors of the individual chemicals. We hypothesize that the generation of these eigen vectors is related to the interaction of the individual chemicals in the mixture, resulting in the generation of by-products which act upon the cell membrane and produce a modification in the extracellular activity that on FFT analysis yields the new eigen vectors.

In the future, we would like to develop a library of SPVs corresponding to chemical/biological agents of various genres and devise a lookup table of SPVs that would aid in the identification of unknown agents.

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