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Electrochemical lab on a chip for high-throughput analysis of anticancer drugs efficiency

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Abstract We describe a new method for rapid, sensitive, and high-throughput detection of colon cancer cells' response to differentiation therapy, using a novel electrochemical lab-on-a-chip system. Differentiation-inducing agents such as butyric acid and its derivatives were introduced to miniature colon cancer samples within the nanovolume chip chambers. The efficacy of each of the differentiation-inducing agents was evaluated by electrochemical detection of the cellular enzymatic activity level, whereas reappearance of normal enzymatic activity denotes effective therapy. The results demonstrate the ability to evaluate simultaneously multiplex drug effects on miniature tumor samples (~15 cells) rapidly (5 minutes) and sensitively, with quantitative correlation between cancer cells' number and the induced current. The use of miniature analytical devices is of special interest in clinically relevant samples, in that it requires less tissue for diagnosis, and enables high-throughput analysis and comparison of various drug effects on one small tumor sample, while maintaining uniform biological and environmental conditions.

Key words: Bio-MEMS; Lab-on-a-chip; Nanochip; Colon cancer; Differentiation therapy

High-throughput detection of nanovolume tissue and cell samples offers great potential for increasing the amount and quality of biomedical data and has a wide range of applications. In cancer research this technology can greatly improve diagnosis and therapy, including basic research, detection of cancer markers, and testing of patients' response to different treatment modalities and thereby tailoring the treatment to an individual patient. Currently it is known that one cannot predict the response of a tumor to a particular treatment only from its type and anatomical location, but must consider the tumor's overall individual characteristics.[1,2] Examination of the particular tumor responses to several drug types simultaneously and under exactly the same conditions will be invaluable in providing the optimal treatment to each patient.

In this study we present a unique high-throughput electrochemical system for the detection of colon cancer cells' response to various differentiation-inducing agents. Current cancer therapeutic strategies focus predominantly on achieving the removal or death of cancer cells within the patient, through three basic approaches: surgery, chemotherapy,[3] and γ-irradiation[4] These methods are aggressive, highly toxic, and often nonspecific.[5] "Differentiation therapy" is an alternative less toxic approach for cancer treatment, which makes use of agents that modify cancer cell differentiation.[6] Upon appropriate treatment, cancer cells

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restrain their own growth and return to their normal growth rate. [7,8] In addition, the differentiation therapy can serve as a complement to conventional surgical treatment, in that the latter can eliminate most, but not all the patient's cancer cells, leading to remission of the disease.

Butyric acid (BA) is a potent differentiation agent in a wide variety of cancer cells in vivo and in vitro. [9,10] Butyrate has been shown to specifically affect gene regulation by transcriptional and post-transcriptional modifications. It induces dose-dependent differentiation and inhibits proliferation of various malignant cell types including erythroleukemia, embryonal carcinoma, and colon carcinoma. [11,12] BA induced the expression of specific differentiation-associated genes when used at concentrations between 0.5 and 10 mM. Differentiated cells are characterized by the appearance of regulatory enzymes such as alkaline phosphatase. [13]

Butyric acid is potentially useful in differentiation therapy but is limited by the requirement for millimolar concentrations and short metabolic half-life for efficacy. To overcome these problems BA derivatives (prodrugs) have been synthesized and screened. Among them pivaloyloxymethyl butyrate (named AN-9) demonstrated impressive anticancer activity in preclinical and clinical studies. [9,10,14] AN-9, metabolized intracellularly to acids and aldehyde, affects and penetrates cancer cells about 100 times faster than BA. [11]

A second generation of BA prodrugs possessing similar biological activities to AN-9 but having greater aqueous solubility, greater selectivity, and increased activity was developed. [15] The lead compound of this family, butyroyloxymethyl-diethyl phosphate (AN-7), was shown to effectively mediate apoptosis and inhibit tumor growth and metastases. In 22Rv1 human prostate cancer xenografts, AN-7 treatment resulted in tumor regression in greater than 25% of the animals and significantly increased their survival. [16]

In this work we present a proof-of-principle experiment for high-throughput screening of human cancer cells' response to differentiation therapy. HT-29 human colon cancer cells (ATCC, Petach-Tikva, Israel) were previously proved to be sensitive to differentiation therapy agents such as BA and its derivatives (AN-7 and AN-9) by enhancement of the alkaline phosphatase activity. [11,17] According to the enzymatic activity level of the treated cancer cells, the efficiency of the particular drug treatment was evaluated. In general, normal enzymatic activity denotes that the cells differentiate properly as a consequence of the particular drug treatment, whereas lack of enzymatic activity denotes ineffectual drug treatment for the particular cancer tumor and for the particular patient.

Methods

Device design and characteristics

The electrochemical chip was fabricated using the same method that was described earlier by our group. [18] Briefly, the chip was produced on silicon wafers and includes arrays of eight independent temperature-controlled electrochemical cells. Each cell can hold 100 nL of solution and consists of three embedded electrodes: (1) a gold working electrode, (2) a gold counter electrode, and (3) an Ag-AgCl reference electrode. The wall of the chambers is constructed from photopolymerized polyimide (SU-8, Tel Aviv, Israel). The device was manufactured as two parts. The first part consists of a disposable silicon chip, with the electrochemical cell arrays wire bonded to a special printed circuit board (PCB) platform that directly connects to the data processing units (Figure 1). The second part of the device is reusable and includes a multiplexer, potentiostat, temperature monitor, and a pocket personal computer for sensing and data analysis. The signal sensing is performed by the handheld palm-potentiostat with an interface to electronic circuitry for electrode signal regulation and detection (BV-2004; Palm Instruments, By 2004, Houten, The Netherlands). The electronics consists of eight independent duplicate circuits of electrochemical cells. A potential is applied between a working and a reference electrode in each electrochemical cell, and the output current is measured. This design enables simultaneous performance of multiple experiments wherein each electrochemical cell can be measured independently.

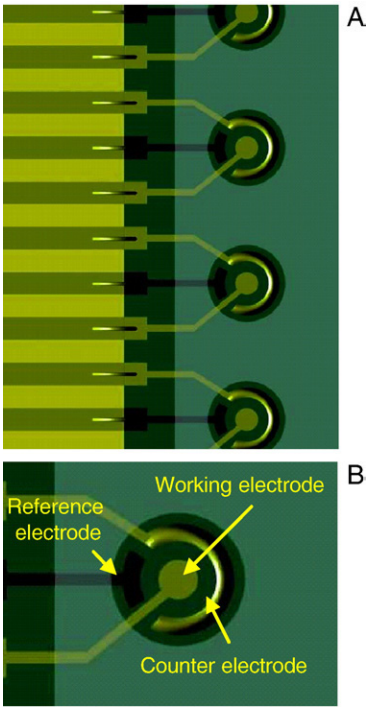

Figure 1. Image of the electrochemical silicon chip wire bonded to the printed circuit board (PCB) platform. **A,** The silicon chip that contains an array of electrochemical cells is glued to the tailored PCB platform, and the chip's gold pads are wire-bonded to the gold PCB's electrodes. The PCB board enters directly to the socket of an external sensing circuit. **B,** The 100-nL electrochemical cells consist of three embedded electrodes: gold working electrode, gold counter electrode, and Ag-AgCl reference electrodes.

Cell lines and cultures

HT-29 human colon cancer cells (ATCC) were grown in Dulbecco's Minimal Essential Medium in the presence of fetal bovine serum at 37°C in 95% air, 5% CO_2 for 3 days before drug treatment. BA at 0, 0.078, 0.156, 0.312, 0.625, 1.25, 2.5, 5, and 10 mM concentrations was added to the HT-29 cell cultures. The optimal BA concentration, 50% lethal concentration (LC_{50}), and viability were calculated accordingly. The measurements were performed in phosphate-buffered saline (PBS) with the intact cells and without additional treatment of the cancer cells such as lysis.

Butyric acid (Sigma, Tel Aviv, Israel) at concentrations ranging between 0.08 and 10 mM was introduced to HT-29 colon cancer cells and incubated for 72 hours for optimization. BA at a constant concentration of 2.5 mM was applied in all electrochemical experiments. The BA derivatives, AN-7 and AN-9, were synthesized. AN-7 was solubilized in PBS and AN-9 in dimethyl sulfoxide followed by dilution with medium to a final dimethyl sulfoxide concentration of 0.1% or less. The prodrugs were then introduced to the cells at a constant concentration of 50 μM and incubated for 96 hours at 37°C in 95% air, 5% CO_2 conditions. After incubation with the agents (BA, AN-7, and AN-9) the viable cells were counted by trypan blue exclusion. Before all electrochemical measurement the cells were centrifuged and diluted in PBS. All measurements were performed in PBS. The influence of BA, AN-9, and AN-7 was examined for the appearance of alkaline phosphatase enzymatic activity.

Results

Multiplex detection of differentiation-induced agents

The activity of the enzyme alkaline phosphatase is determined by using *p*-aminophenylphosphate (PAPP) as a substrate. The product of the enzymatic reaction, *p*-aminophenol (PAP), is oxidized on the working electrode at 220 mV. This oxidation current is monitored.

An array of eight-channeled 100-nL electrochemical chambers were loaded with HT-29 human colon cancer cells treated with BA or its derivatives. The treated cells were placed in the electrochemical chambers, and PAPP was added to a final concentration of 1 mg/mL at a total volume of 100 nL. Alkaline phosphatase activity was measured by monitoring the PAP oxidation current. The disposable electrochemical chips were replaced after every experiment.

After measuring various concentrations we found that the optimal BA concentration was 2.5 mM. In addition, the effect of BA, AN-9, and AN-7 on the HT-29 colon cancer cells was examined after incubation for 96 hours by measuring the induced alkaline phosphatase activity. Each electrochemical chamber on the array was loaded with cells exposed to different agents. The results are shown in Figure 2.

Normal enzymatic activity denotes that the cells differentiate normally as a consequence of the particular drug treatment. As shown in Figure 2, BA and AN-7 induced

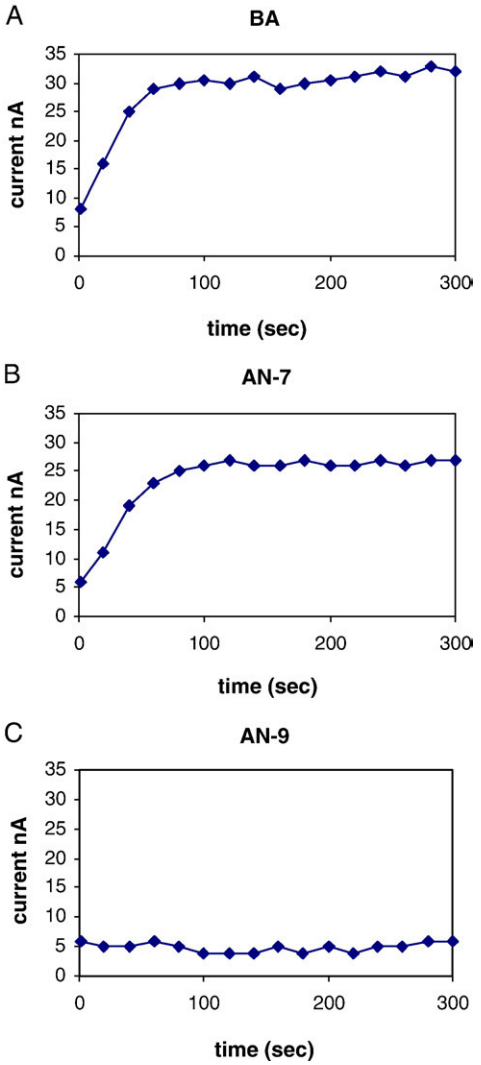

Figure 2. HT-29 colon cancer cells' response to **A,** butyric acid (BA), **B,** butyroyloxymethyl-diethyl phosphate (AN-7), and **C,** pivaloyloxymethyl butyrate (AN-9). Amperometric response curves for monitoring of alkaline phosphatase activity using the electrochemical array chip. The HT-29 colon cancer cells were exposed to the differentiation agents: BA (2.5 mM), AN-7 (50 μM), and AN-9 (50 μM). The HT-29 cells with the substrate *p*-aminophenylphosphate (PAPP) were placed into the 100-nL volume electrochemical chambers on the chip. The current was measured using the amperometric technique at 220 mV.

enzymatic activity of alkaline phosphatase, whereas AN-9 did not induce any enzyme activity. AN-9 showed no induction as compared with AN-7 at 50 μM concentration, which may be related to its reduced potency regarding HT-29 cancer cells. [19,20] It should be noted that AN-7 at a 50 μM concentration exerted a similar effect on differentiation to that of BA at a 2.5 mM concentration, indicating that AN-7 was 1.5 orders of magnitude more potent than BA.

To exclude the possibility of false positive and false negative signals, all arrays included controls chambers (data not shown); For false positive control each one of the components (the untreated HT-29 cells, the treated HT-29 cells, and the drugs)

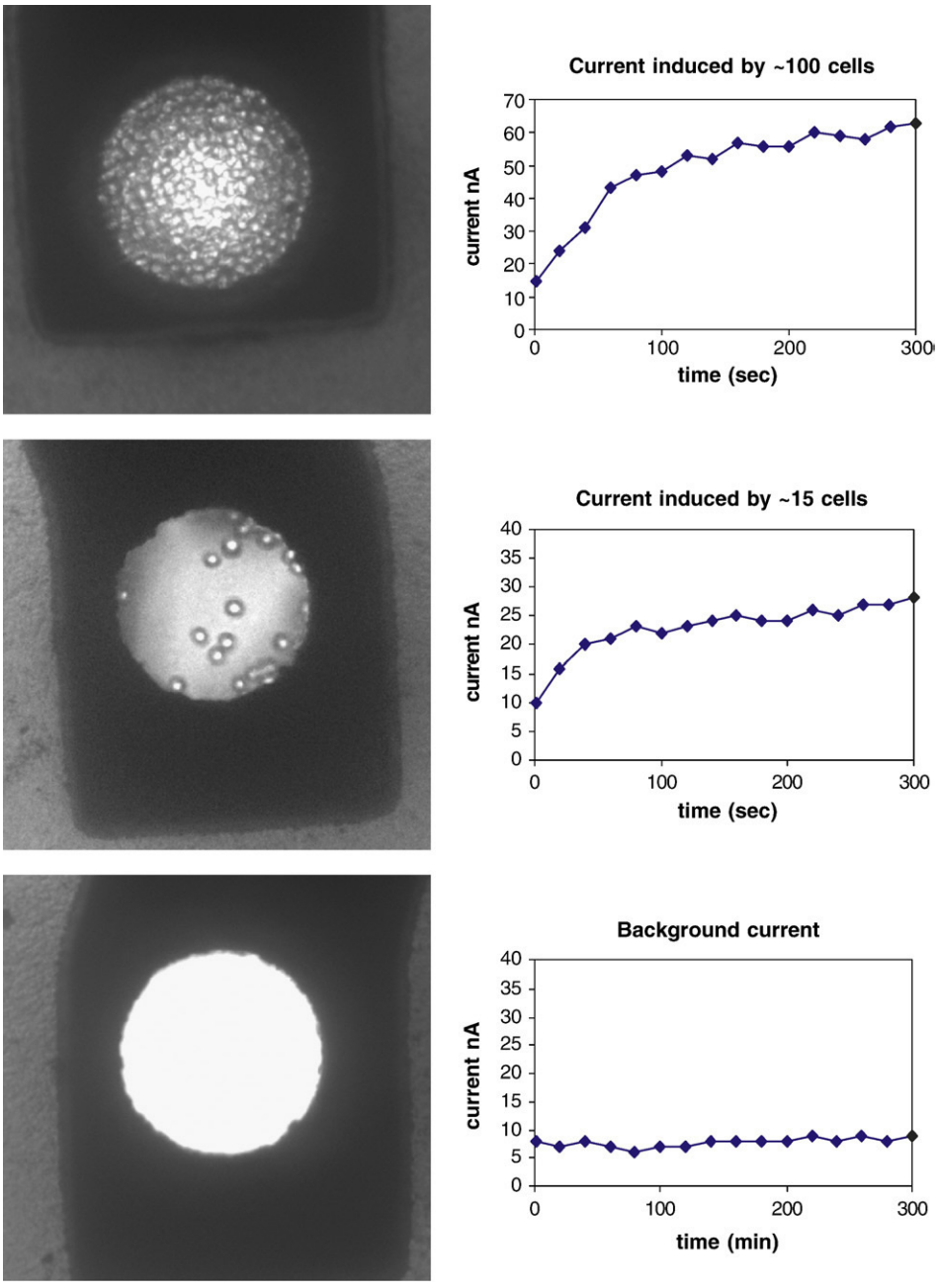

Figure 3. Enzymatic activities versus cancer cell numbers. Right column: Amperometric response curves of alkaline phosphatase activity. The HT-29 colon cancer cells were exposed to butyric acid (2.5 mM). The current was measured at 220 mV. Left column: microscopic imaging of the HT-29 cells inside the 100-nL chip chambers. Upper, middle, and lower curves represent the current response of about 100 cells, 15 cells, and 0 cells counted inside the chamber, respectively.

was amperometrically measured alone. Next, the combinations of untreated HT-29 cells with and without PAPP and the different drugs with PAPP, were measured. In all false positive control experiments the background signal was steady, and below 8 nA. For a false negative control, a chamber was loaded with purified alkaline phosphatase and PAPP; thus, in any case current was generated.

Quantification of cancer cells by the biochip

We further examined the correlation between the current density and the number of cells present in the chip chamber. The HT-29 cells were counted under the microscope, and the induced current was measured as shown in Figure 3.

Multiple measurements demonstrated a high correlation between the number of cells counted inside the chamber and alkaline phosphatase activity (Figure 4).

Noise characterization

In addition to the desired signal we can observe two types of unwanted noise signals: (1) electrical noise, and (2) biological and biochemical noise. The electrical noise may stem from the electrodes' thermal noise (Johnson noise) and

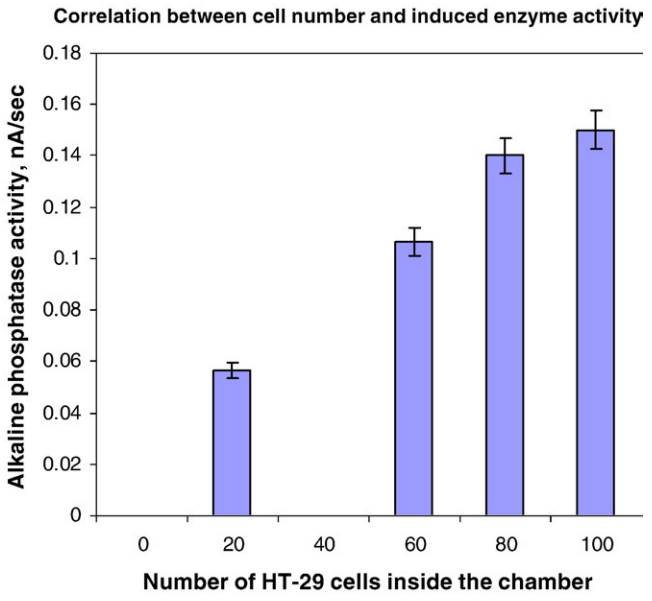

Figure 4. Correlation between HT-29 colon cancer cell number and the induced alkaline phosphatase enzymatic activity. Activity is presented by Δ current/Δ time. Each result represents the mean of three measurements. The current was measured using the amperometric technique at 220 mV.

the corpuscular nature of the electrochemically induced current (shot noise). There is also measurement-induced noise, which, in our case, is dominated by quantization noise caused by the analog-to-digital conversion. The biological and biochemical noise is a fixed or slowly variable background signal that is the result of unwanted biochemical processes and other unidentified oxidation-reduction reactions in the electrochemical cell, whereas the electrical noise stems from thermal noise and shot noise. In the present experiments the biological and biochemical background noise signal was measured without the desired oxidation reaction. For example, amperometric measurement was performed for each of the following components: the untreated HT-29 cells, the treated HT-29 cells and the drugs, the combinations of untreated HT-29 cells with and without PAPP, and the different drugs with PAPP. This background signal was measured and varied from 0.5 nA to 8 nA, which is much larger than the electronic noise. Therefore, the electronic noise has a negligible influence on the system. As demonstrated in Figure 3, the signal-to-noise ratio is always larger than 1.

Discussion

A significant quantitative correlation between the induced current signals and the number of cancer cells counted inside the nanovolume electrochemical chambers was found. The ability to quantify the enzymatic reaction of a few living cells is due to the new geometry, in which the electrochemical cell dimensions were reduced to nanoscale. The construction of an array of nanochambers on one silicon chip leads to high throughput in addition to the capability of performing multiple experiments simultaneously and independently.

The miniaturization of the electrochemical cells and the entire device toward lab-on-a-chip format is highly important because of portability, convenient handling, and reduced amount of reagents required. Moreover, there are several additional fundamental advantages. The favorable area-to-volume ratio leads to a short diffusion distance for the PAP molecules toward the electrode surface, providing improved signal-to-noise ratio, faster response time, enhanced analytical performance, and increased sensitivity. These characteristics result in more sensitive and rapid detection of the biochemical and physiological processes of the cancer cells.

In conclusion, we have demonstrated a proof-of-principle experiment of multiplex electrical detection of colon cancer cells' response to differentiation therapy, using an array of nanovolume electrochemical cells. As a proof of principle we measured simultaneously eight independent electrochemical cells; however, by using the same fabrication process hundreds of electrochemical cells can be integrated on a single silicon chip, providing the ability to test online the effect of multiple drug agents on the same miniature biopsy sample. This technology enables one to tailor effective cancer therapy to the individual patient, thus having allowing substantial progress toward the personalized medicine era.

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