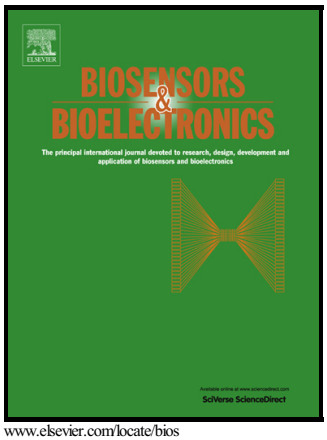

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Monitoring the spreading stage of lung cells by Silicon nanaowire electrical cell impedance sensor for cancer detection purposes

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Abstract

We developed a silicon nanowire based electrical cell impedance sensor (SiNW-ECIS) as an instrument that detects cancerous cultured living lung cells by monitoring their spreading state at which the cells stretched and become extended on nanowires. Further current penetration into the extended membrane of malignant cells in respect to normal ones (In the first 6h after cells interaction with surface) are the key mechanism in our diagnosis procedure. The developed device applied to monitor the spreading-induced electrical differences between cancerous and normal lung cells in an integral fashion. Detection was performed so faster than the time required to complete cells mitosis. Morphology and architecture of doped Si nanowires covered microelectrodes observably enhance the contact area between cells and electrodes which support accurate signal recording from stretched cells as indicated by SEM and florescent images.

Keywords: SiNW-ECIS, Cell spreading, Current penetration, Impedance, Biosensor, Cancer diagnosis.

1. Introduction

Cancer cells are different from healthy cells in reproduction, adhesion (Ertel et al., 2006), proliferation rate (Meadows et al., 2008), maturation (Sell, 2010) and function (specialization) (Shay and Wright, 2010) which all might affect the electrical (Abdolahad et al., 2013) and chemical (Rehm, 2006) (M. Abdolahad et al., 2012) signals recorded from the cell. Impact of proliferation among such differences make an individual definition from cancer as biologists introduce the cancer as a disease characterized by the autonomous aimless and excessive proliferation of cells (Victor R. Preedy, 2012). Therefore, cell signaling specially that regulating growth and proliferation, is intimately associated with oncogenesis. Also, the proliferation of cancer cells is so faster than that of healthy cells which replicate many times (Skeel, 2007). Cell proliferation assays are extensively applied in clinical research, such as the chemically induced mutagenicity and carcinogenicity assays (Cunningham and Matthews, 1995), the screening of novel growth and differentiation factors (Antczak et al., 2012; Theus et al., 2012) and the detection of oestrogenic compounds (Baker, 2001). In recent years, designing new biosensors for cell proliferation and/or viability assays was considered by many research groups. Now days, In vitro cell proliferation investigations are carried on by colony formation assays (Kintzios et al., 2006) . The requirement to enzymatic detachment of cells from the surface of the culture plate is a critical restriction of such method. Other conventional manners for assaying cell proliferation such as flowcytometry (Nguyen et al., 2003) and use of selective stains for DNA and RNA determination (Li et al., 2006) are so expensive which need biomarkers and complicated technical and practical considerations. Remarkable improvement in microfabrication technology

has opened many opportunities for miniaturizing these analytical devices and offered high potential for scientific research and clinical diagnostics. Among various microfabricated biosensors, electric cell-substrate impedance sensing (ECIS) method has been applied as a valuable tool for real time monitoring of cell proliferation (Wang et al., 2008). Many reports were published on investigation of cancer cells proliferations based on impedimetric sensors (Hong et al., 2011; Pradhan et al., 2014b; Yang, 2013). Both real impedance and the phase angle (of the corresponding impedances) in highly and poorly metastatic cells were measured by such devices (Frazier et al., 2009). The spite of wide applications of cell proliferation studies in medical research, time consuming procedure of the investigation (At least 24hrs are required to complete proliferation.) and the problems of maintaining system stability in such period of time, limited their usage especially in diagnosis purposes. Recently, the migration of single metastatic and primary cancer cells were investigated by microfluidic based electrochemical biosensors (Nguyen et al., 2013). The method was so faster but involved to complicated micro fluidic systems in which the cells motion in fluidic channels and their entrapment on Au working microelectrodes limited their applications in clinical samples (biopsy or pup smear). In addition using self-assembled monolayers (SAMs) to promote the cell adhesion on the Au layer would affect the electrical signal extraction from the cells by the working electrodes. So, there is still a paucity of specific, efficient methods to assay cancerous state of the cells in faster approach by ECIS systems. On the other hand, spreading stage as one of the important pre-proliferation subsequences (occurred 10h before proliferation stage) would contain many distinguishable parameters between normal and malignant cells. However, the impedimetric monitoring of spreading stage in normal and cancer cells has not been carried out for diagnosis applications till date. Fabrication of cancer cells ECIS biosensors with the ability to diagnose their spreading

differences could lead to much faster responses and be a helpful alternative for conventional impedance sensor. Recently nanosturctured materials, have been interested due to their suitable Bioelectrical properties as nanoscale interactors and new generation of nanostructured based ECIS has been developed by our group (Abdolahad et al., 2013; Mohammad Abdolahad et al., 2012). Electrically active nanomaterials (CNT, SINW, metallic nanoparticles,…) could apply well-directed electrical interaction with cell outer-wall to penetrate the electric field into the membrane for signal recording purposes (Mohammad Abdolahad et al., 2014; Xu et al., 2013). Among various nanomaterials applied in biosensing processes, silicon nanowires (SiNWs) have found a wide range of applications in the field of bioelectronics, owing to their unique chemical and physical properties as well as their compatibility with electronic device fabrication processes (Duan et al., 2013). The biocompatibility of such structure and some of its derivatives (like silica NWs) in interaction with many kind of cells were investigated (Alexander et al., 2012; Coffer, 2014; Garipcan et al., 2011). SiNWs can be considered as a cancer bio field effect transistor (bioFET) for cancer detection based on charge sensing (M. Abdolahad et al., 2014b) or cancer protein markers (such as PSA) detecting (Zheng et al., 2005). Recently, Silicon Nanowire Immunosensor has been developed for Detection of 8-Hydroxy-2'-Deoxyguanosine Oxidative Stress Cancer Biomarker (Thomas et al., 2014). Using complicated functionalization procedure limited the application and increased the probability of nonspecific binding between weakly functionalized nanowires and non-targeted markers. Finding label free cancer detection methods based on SiNW electrical biosensor (with simple fabrication and testing processes) would be so helpful especially when large amount of the cells required to be checked (in the cases such as biopsy, fine needle aspiration and etc.). The present report we demonstrate a novel, miniaturized SiNW-ECIS as a new label-free chip capable to diagnose the lung metastatic cells (QUDB) from

normal ones (MRC-5) much faster than a conventional proliferation assay. The mechanism based on the differences in spreading stages, as a pre proliferation subsequence, between normal and malignant cells occurred in the first 7h after their initial interaction with NWs which induce different electrical impedance in the response of biosensor. Extracting the electrical signals from the seeded cells on doped silicon nanowire covered microelectrodes, allows for the simultaneous investigation of cells dielectric parameters and prediction of their cancerous state. Electrical current flow into the cell would cause local depolarization in its membrane depend on electrical permeability which determines the current blocking by the cell. Cancerous transformation would cause degradations and disruptions in cell membrane phospholipids (the main dielectric part of the membrane) (Mohammad Abdolahad et al., 2014)]. Extension of the cell during spreading stage would affect such blocking ability in cancer cells. This might increase the current penetration toward them in comparison with that in normal cells and lead to a diagnostic response of SiNW-ECIS biosensor in first hours of culturing process. Morphology and nano architecture of doped Si nanowires observably enhance the contact area between cells and electrodes which strongly reflect the increased current penetration into malignant cells during spreading stage as indicated by SEM and florescent images. In addition, the great compatibility of SiNW with electronics fabrication technology makes SiNW-ECIS a powerful tool in transducing the variations in dielectric parameters of seeded cells (without need to any functionalization protocols) to electronic signals. Its sensing mechanism is straightforward and is predominated by dielectric and beta dispersion changes due to the direct attachment and stretch of cells on silicon nanowires.

2. Materials and methods

2.1. Sensor fabrication process

The schematic of the fabrication process has been presented in Fig. 1. Silicon wafers used as substrates in the present experiment were cleaned through standard RCA #1 cleaning method ($NH_4OH:H_2O_2:H_2O$ solution and volume ratio of 1:1:5 respectively). Subsequently, a thin layer (~ 300 nm) of SiO_2 was grown on the substrate by wet oxidation furnace (Fig.1A). A 10 nm gold thin layer has been coated on SiO_2 by sputtering system (Veeco Co.) as catalyst layer (Fig. 1B). The gold has been patterned and the design of the sensor transferred on the substrate (Fig. 1C). Then the sample was placed in LPCVD chamber and SiNW was grown by the assistance of H_2 and SiH_4 gases in 450^oC as we reported previously (Taghinejad et al., 2013) (Fig. 1D).

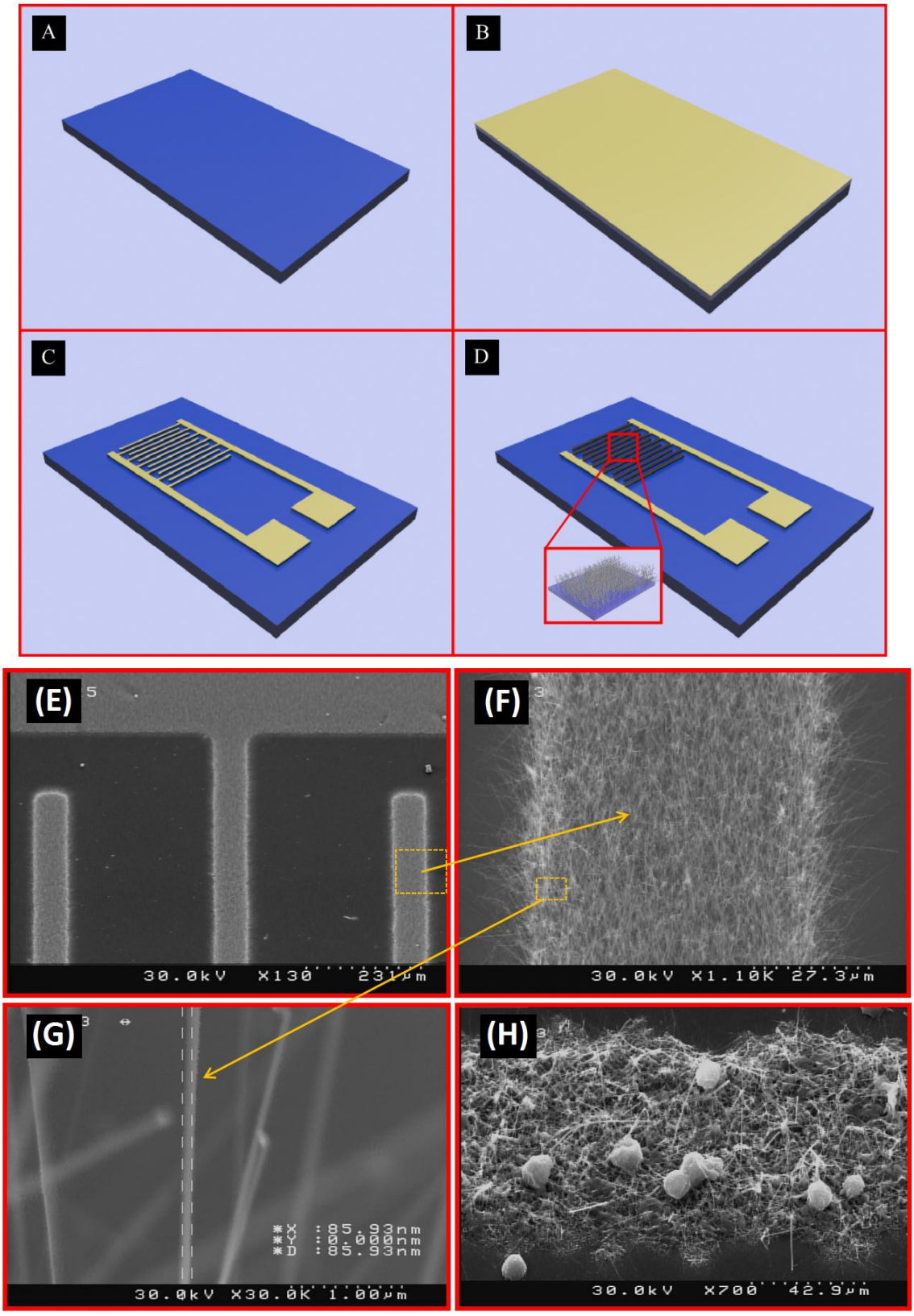
A
B
C
D
(E)
30.0kV X130 231µm
(F)
30.0kV X1.10K 27.3µm
(G)
*X :85.93nm
*Y :60.00nm
*D :85.93nm
30.0kV X30.0K 1.00µm
(H)
30.0kV X700 42.9µm

Fig. 1

Finally, the sensors have been transferred into phosphorous doping furnace to enhance the conductivity of NWs. SEM images of Fig. 1 presented the geometry and architecture of SiNW-ECIS before (Fig. 1E-G) and after (Fig. 1H) the cells interactions with the surface.

2.2 Cell culture

MRC-5 and QUDB cell lines were isolated from normal and malignant human lung tissue, respectively. These cells were obtained from the standard cell Banks of Iran (Pasteur Institute) and they were maintained at 37^{o}C (5% CO_2, 95% clean air) in RPMI-1640 medium (Sigma 8758) supplemented with 5% fetal bovine serum (Gibco), and 1% penicillin/streptomycin (Gibco). The fresh medium was replaced every other day.

2.3 Cells seeding on SiNW arrays

The sensor was packed in a plaxy glass cover sealed with biograde silicon rubber tube (Fig. 2). Same concentration of MRC-5 and QUDB cells (10^4#/ml) were dropped on the surface of the sensor with final volume of 300µl. The sensors were held in incubator (new brunswik Co.) and the electrical measurements were experimented after the desired period of times.

2.4 Impedance measurement

The electrical impedances of normal and malignant cells were carried out using NI DAQ USB 6323 connected to the sensors with coaxial wires. We designed and fabricated a readout electronic board with similar signal extracting mechanism for further investigations (Fig. 2). Measurements were performed with applied voltage of 400 mV. The real time monitoring of the cells activities was performed by measuring the impedance at the frequencies ranged from 200Hz to 150kHz in the time interval between 3.5h to 9.5h after the cells were dropped on the

NWs. A suitable response of the cells membrane to stimulating signals were reported in such range of frequencies (Abdolahad et al., 2013; M. Abdolahad et al., 2014a; Mohammad Abdolahad et al., 2014). Final impedance value for each frequency was the mean value of 10 subsequent measurements. To eliminate the effect of medium, the differentiated impedance value has been calculated by comparing the response of the device in various stages of cell culturing.

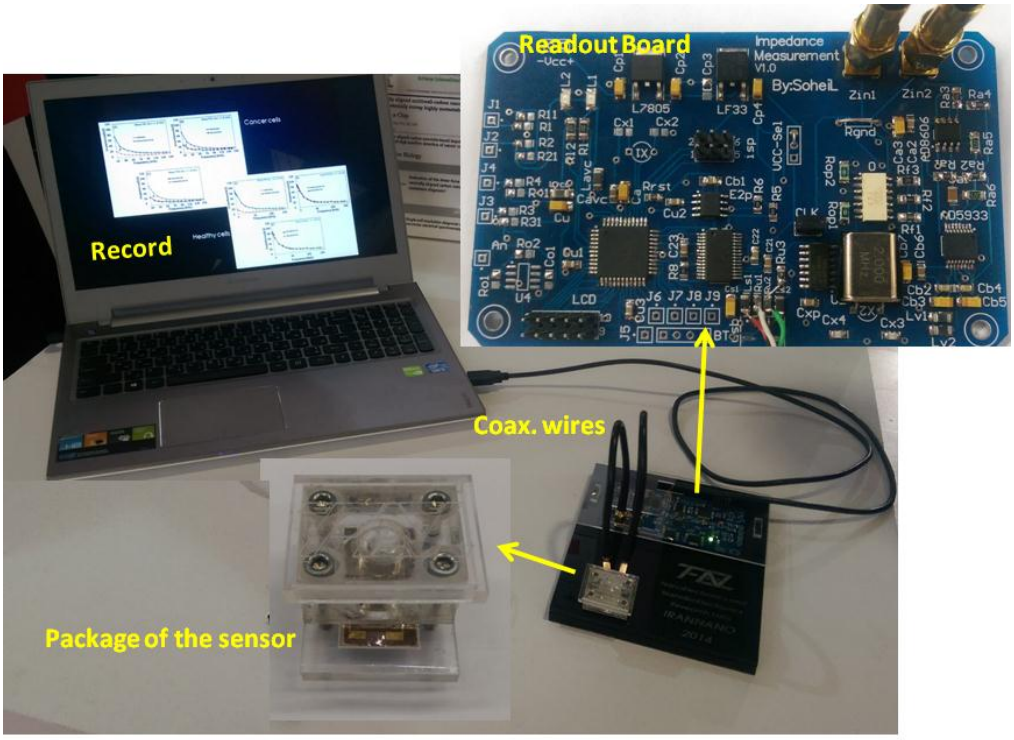

Fig. 2

2.5 Florescent microscopy

MRC-5 and QUDB cells were seeded on SINW ECIS surface in RPMI1640 complete medium. Phase contrast images of the cells were taken for the cultured cells after desired times of incubation by using JENUS florescent microscopy with a CCD camera using monochromatic phase contrast mode. Live florescent imaging was individually done on experimented cells

stained with A/O as per the manufacturer's instructions and held in incubator for 20 min. Then the cells were washed by PBS for six times and held under the microscope for imaging using the appropriate wavelength.

2.6 MTT assay

To investigate the biocompatibility of silicon nanowire, we used a MTT (3-(4,5-dim ethyl thiazol – 2-yl)-2,5-dipheny l.tetra zolium bromide) assay. The surface of the device has been sterilized by autoclave before cell seeding process. The QUDB cells were attached on SiNW surface and after 24hrs, they were removed from the substrate by trypsin and the culture media was added to the cell solution. Subsequently, the cells were placed in the wells of a sterile 96 well micro plate with the same concentration and the MTT protocol was applied on each well. This assay verifies the viability of the cells based on colorimetric measurement. The reduction of yellow tetrazole to purple formazane is related to the ratio of remained live cells. Metabolic activity of the cells depends on the density deviations of this color in the QUDB cell solution. In this regard, 10µl of MTT solution with the concentration of 5 mg/µl was added to each well. The wells were incubated for 4h in 5% CO_2 ambient in 37^{o}C. Next, the float materials were removed from the surface of the wells and 100µl of dimethylsulfoxied was added to each well. After 20 min stirring of each well (in order to solving the formazane), the optical absorption of each cell contained well was calculated in 493 nm by micro plate reader system. The percent of viable cells versus the control well and slope variation versus absorption diagram was indicated (Saura C. Sahu, 2009). The cell concentration gradient to absorption was calculated by the following equation (Saura C. Sahu, 2009).

(Absorption = (0.0172 × Cell number)+94) (1)

3. Results and discussion

3.1 Investigation of biocompatibility of SiNW

The Biocompatibility results of SiNW investigated by MTT assays (Fig. 3A) show 60% and 40% further viability of the seeded MRC-5 cells on doped and undoped SiNWs after 24h in comparison with control sample respectively. This study indicates that such nanostructured surface improved the growth and proliferation of cells in respect to well plate surface. Also the florescent images taken from the QUDB cells covered on individual devices before (Fig. 3B) and after (Fig. 3C) proliferation stages of the seeded cells (taken 6 h and 12 h after the cells culturing on the surface respectively) corroborate the vitality of the cells as well as their stable biological metabolism after attachment on SiNWs.

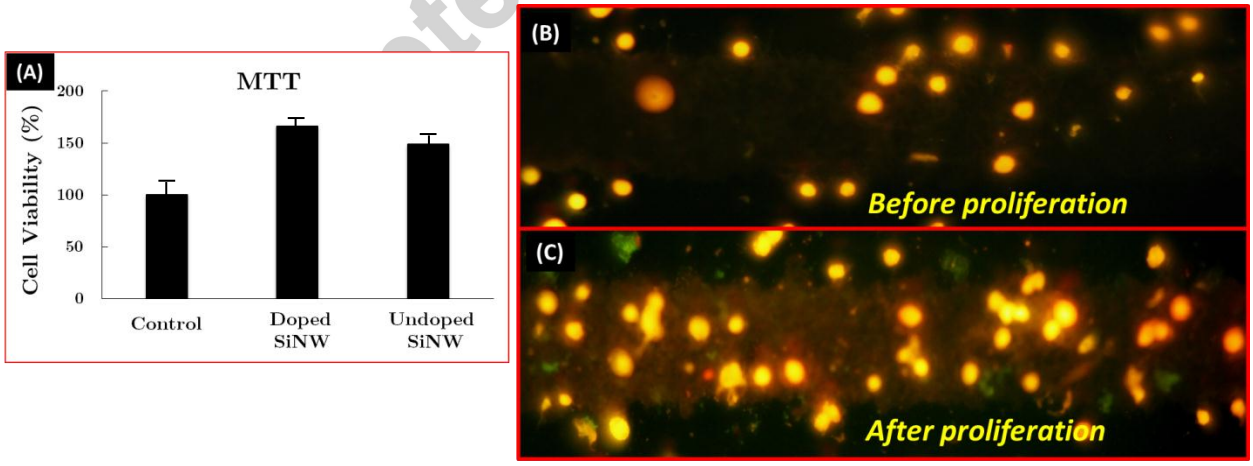

Fig. 3.

3.2 Diagnostic electrical pattern of SiNW-ECIS between lung normal and malignant cells in time evolution

Fig. 4 shows the comparative real time electrical monitoring of normal (MRC-5) and malignant (QUDB) lung cells. The first interval of culturing time (1^{st} -3.5^{th} h) represents the cell attachment stage while the second phase signifies the spreading (3.5^{th} -6.5^{th} h) and subsequently the cells entered to proliferation stage (6.5^{th} – 9.5^{th} h) (Pradhan et al., 2014a). From the figure, it is inferred that the impedance values increased during the cells attachment stage on the surface of nanowires for both malignant and normal cells (Figs.4A and 4D respectively). It is in a well demand with dielectric properties of the cells resulted in current blocking and impedance increment after direct attachment of the cells on nanowires (Mohammad Abdolahad et al., 2014).

In initial interaction of the cells solution with the surface of SiNW-ECIS, the current path was passed through the electrolyte solution and no observable difference between the electrical pattern of cancer and healthy cells was observed (Initial drop curves in Fig. 4A and 4D). In the time interval between initial drop and cells attachment on NWs, the impedance value increased gradually due to adhesion of the cells on SiNW covered electrode surface.

During spreading stage (from 4^{th} h to 7^{th} h), impedance changes is mostly affected by the cells seeded on the electrode surface region (Pradhan et al., 2014b) and the type of the electrode would be crucial in the quality and diagnostic impact of extracted signals. In this period of time a drastic reduction in impedance value of SiNW-ECIS was observed for malignant cells meanwhile no noticeable impedance variation was measured for normal cells (Fig. 4B and 4E respectively). An interesting mechanism might have played the key role in such different

behaviors. As we know, current path is blocked at the electrode surface due to the cells attachment.

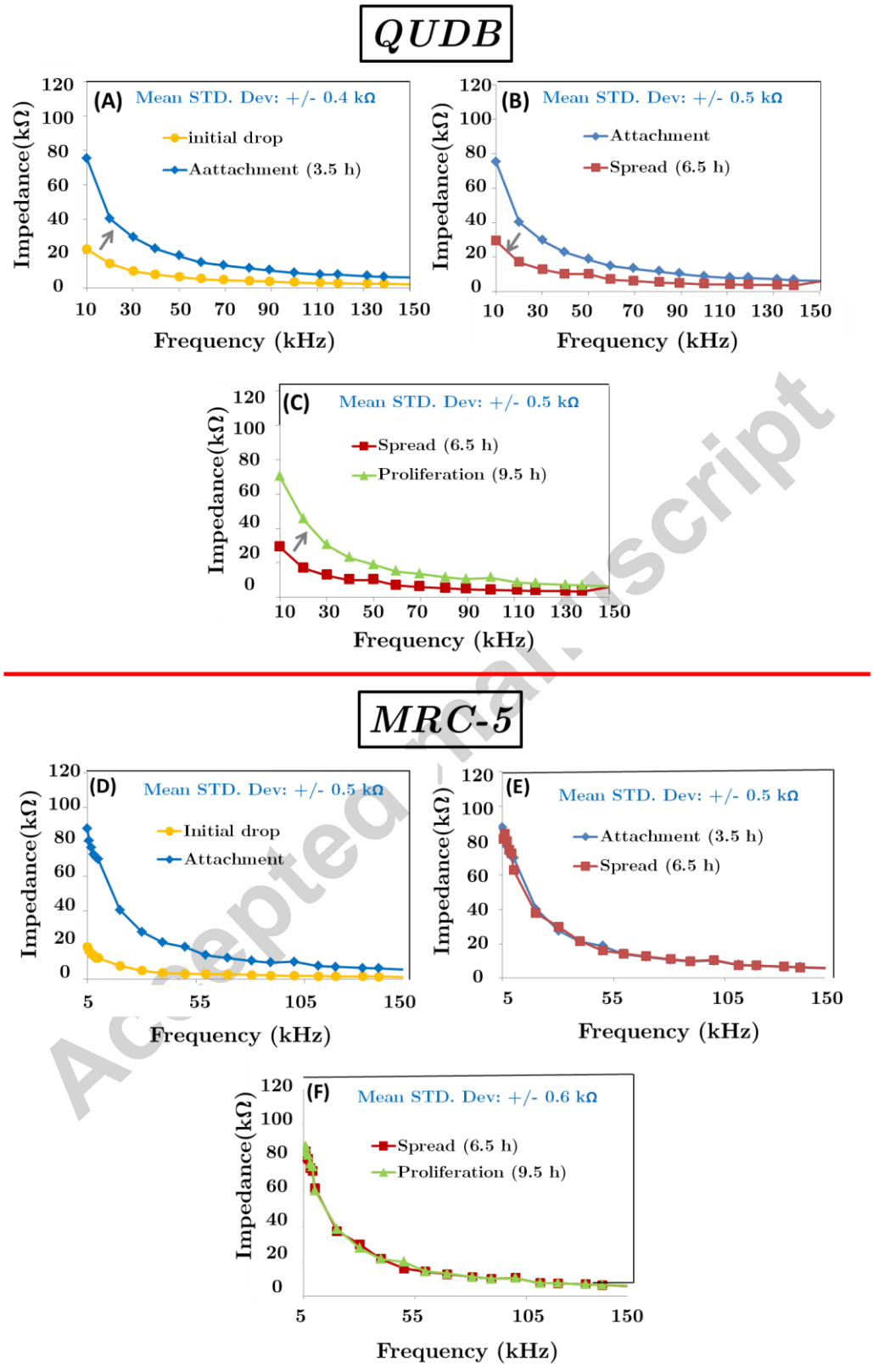
QUDB
(A) Mean STD. Dev: +/- 0.4 kΩ
initial drop
Aattachment (3.5 h)
(B) Mean STD. Dev: +/- 0.5 kΩ
Attachment
Spread (6.5 h)
(C) Mean STD. Dev: +/- 0.5 kΩ
Spread (6.5 h)
Proliferation (9.5 h)
MRC-5
(D) Mean STD. Dev: +/- 0.5 kΩ
Initial drop
Attachment
(E) Mean STD. Dev: +/- 0.5 kΩ
Attachment (3.5 h)
Spread (6.5 h)
(F) Mean STD. Dev: +/- 0.6 kΩ
Spread (6.5 h)
Proliferation (9.5 h)
Impedance(kΩ)
Frequency (kHz)

Fig. 4.

After interaction of QUDB cells with NWs (Fig. 5A), they started to attach on nano sites of SiNW (Fig. 5B and florescent image in Fig. 5E) and their presence resulted in current flow blocking between interdigital transducers (IDTs) due to beta dispersion phenomena (Mohammad Abdolahad et al., 2014). When the attached cancer cells entered to spreading sequence, their membrane would be extended (Fig. 5C). As the membrane of malignant cells are degraded and their dielectric parameters were disrupted during cancerous transformation (Szachowicz-petelska et al., 2010) (Abdolahad et al., 2013), stretching of such cells reduced their membrane ability to block the current flow. So, the impedance value of SiNW-ECIS decreased in spreading sequence of malignant cells (Fig. 4B).

Such phenomena weren't observed for normal lung cell. Attachment of the normal cells (Fig. 5F and florescent image Fig. 5I) led to the current flow blocking similar to cancer cells (Fig. 4D). During the spreading sequence of normal cells, similar extension of the membrane on nanowire was occurred (Fig. 5G), but as their membrane contains non degraded phopholipids and fatty acid (Szachowicz-petelska et al., 2010) their ability in current flow blocking wasn't disrupted. So, the current couldn't penetrate toward their extended membrane and no impedance reduction was measured in SiNW-ECIS covered by MRC-5 cells during spreading sequence (Fig. 4E). On the other hand, the proliferation rate of a normal cell is so slower than malignant one (Roland T. Skeel, 2011). Thus, the MRC-5 cells didn't complete the spreading stage after 6.5h. We named "Spread1" to this sequence for normal cells. Also Other reports confirmed that most of impedance variations of another kind of epithelial normal cells (MDCK) seeded on conventional ECIS surface had been occurred in first 4h and the impedance of the system didn't change during spreading stage (Wegener et al., 2000). Also no impedance reduction was reported in spreading stage of normal Sf9 cells (a type of animal cell) (Luong et al., 2001).

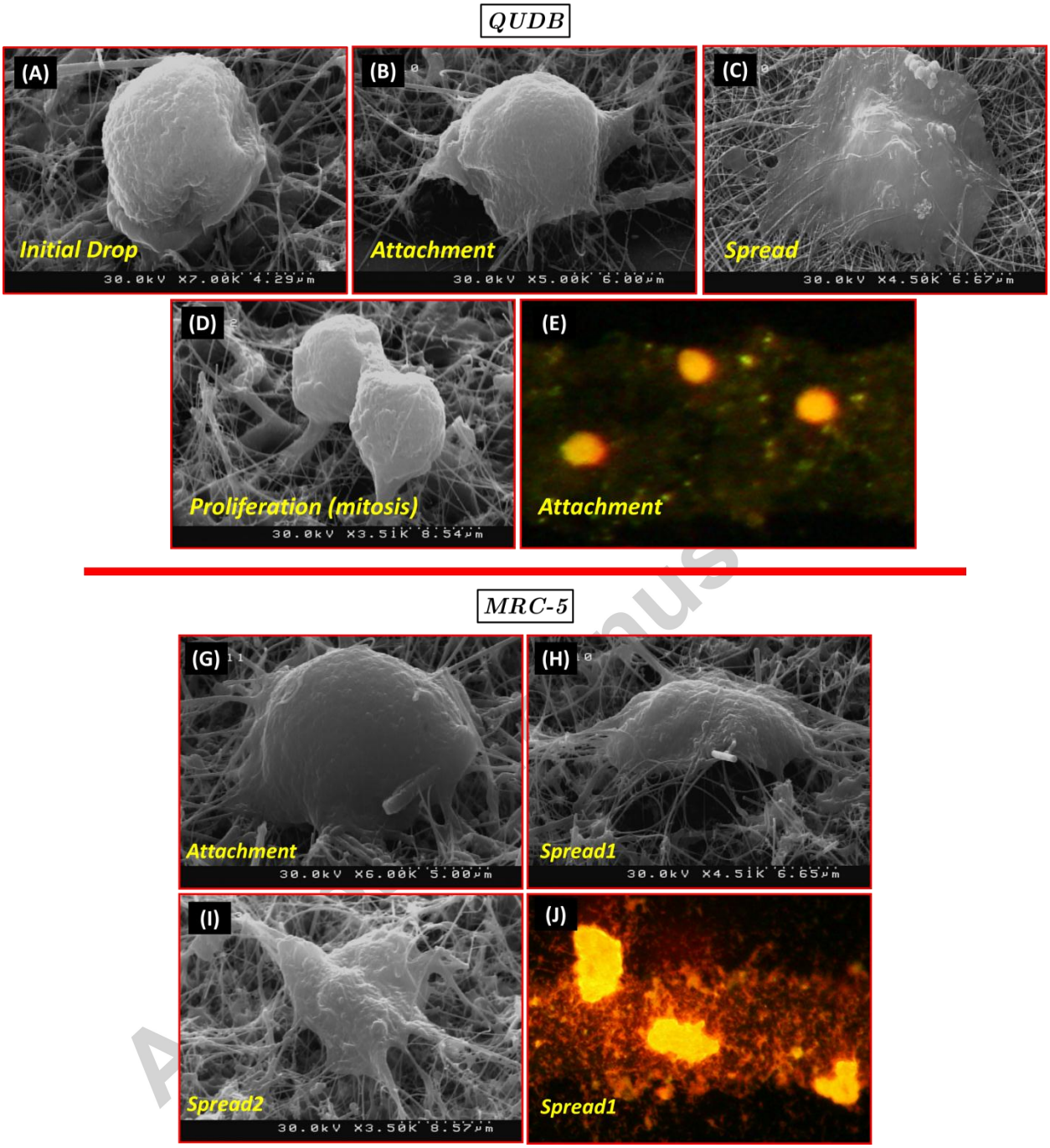

Fig. 5.

When the malignant cells entered the proliferation state (from 7th h to 10th h), the mitosis was occurred and again the membrane of the mitosed cells were contracted (Fig. 5D). Hence, the enhanced current flow blocking abilities of proliferated QUDB cells (mitosed cells with

contracted membrane) increased the impedance of the biosensor in respect to spreading stage (Fig. 4C). As the mitosis rate of normal cells are so slow, MRC-5 cells still didn't enter to proliferation stage in mentioned period of time and just an enhanced membrane extension was observed for normal cells in this stage (we named it "Spread2") (Fig. 5G). The strong dielectric parameters of normal cells membrane inhibited from current penetration towards them even after such extension. As the result, the impedance value of the SiNW-ECIS covered by MRC-5 cells didn't change in spread2 stage in respect to spread1 (Fig. 4F). Also the sensitivity and detection limit of the sensor were discussed in support information.

3.3 Impedance phase

Fig. 6 presented the impedance phase diagram of the SiNW-ECIS devices covered by QUDB and MRC-5 cells in their various seeding stages. The results are in a complete confirmation with impedance value diagrams. When the culturing time pass the 4^{th} h (entering to spreading stage) further increment in current penetration into the stretched membrane of cancer cells decreased their phase in negative values in the range of 10^{o} (Fig. 6B). As the current penetration into the membrane of normal cells didn't change during the extension, no observable variation in phase values in all of measured frequencies were measured for them (Fig. 6D). Such measurements indicated the ability of doped SiNW-ECIS in label free diagnosis of metastatic cells from normal ones faster than the time needed for their proliferation.

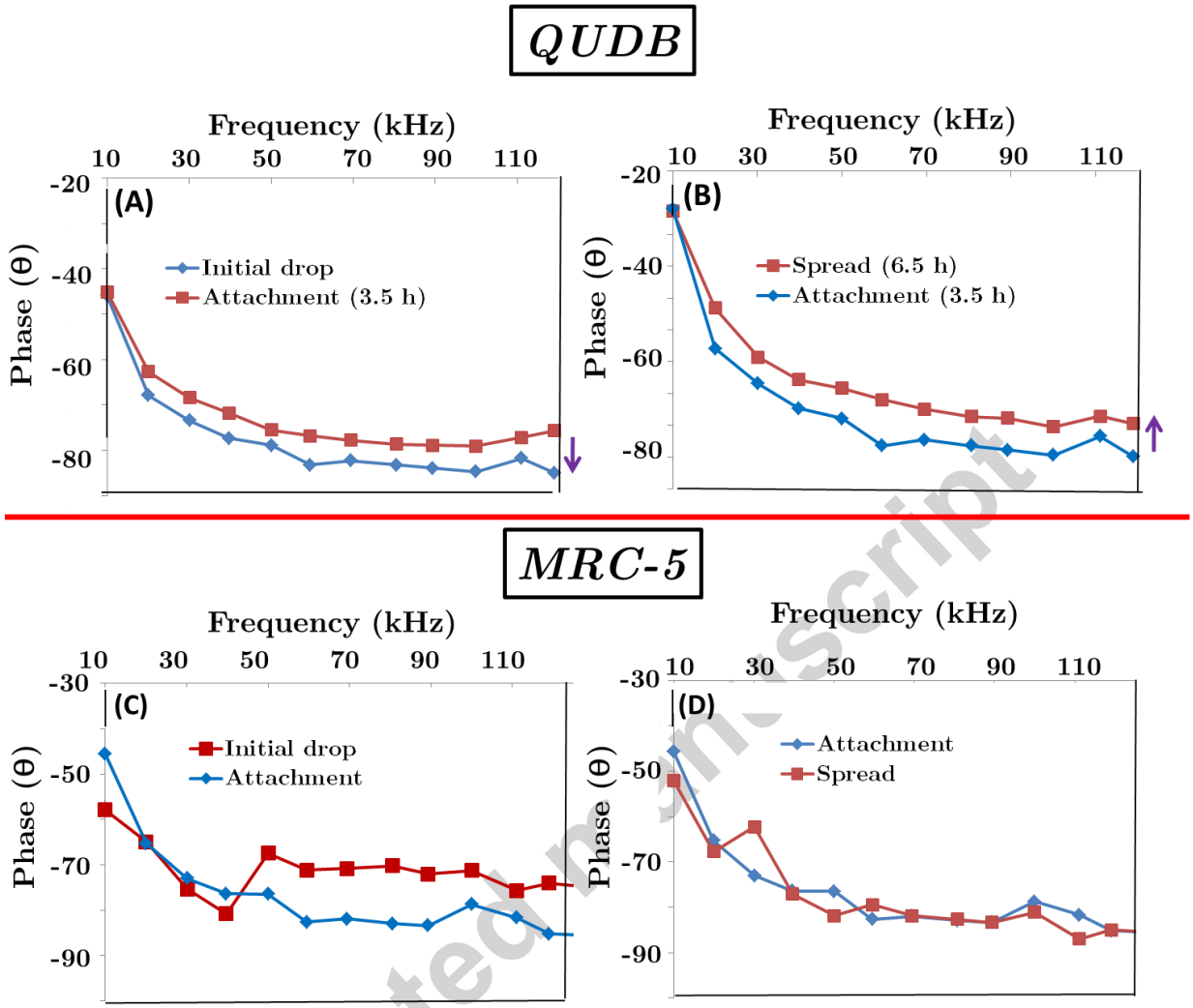

Fig 6.

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4. Conclusion

In summary, a silicon nanowire based electrical biosensor (SiNW-ECIS) has been developed to study the electrical response of malignant and normal lung cells in spreading stage (as a pre proliferation subsequence). Doped SiNW applied direct and great physical interaction sites with cell membrane with great electrical signal transduction abilities. The degraded membrane of cancer cells resulted in enhanced current penetration into them during spreading on the surface of

SiNWs-ECIS. As a result, a reduction in the impedance of the device through cancer cells spreading in comparison with their attachment stage was measured. Such decrease wasn't measured for normal cells because of their rigid membrane content (which strongly blocked the current flow toward). These comparative electrical measurements between stretched normal and malignant cells with same primary concentrations during spreading stage led to diagnostic patterns. In addition this sensor could be applied to investigate the differences in proliferation rate between normal and malignant cells in the early hours of culturing process. SiNW-ECIS is suitable for different purposes such as monitoring the effect of anticancer drugs on cells spreading as well as the external stimulating agents on cells proliferation.

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Fig 1. Schematic of fabrication process and FESEM of SiNW-ECIS. Schematics from the fabrication process of SiNW-ECIS started from: (A) oxide growth on Si wafer and followed by: (B) Deposition of the gold catalyst layer over the oxide: (C) patterning and etching the Au layer

in the region of the sensor and finally: (D) SiNW array was grown by LPCVD system on the sensor region. FESEM images of SiNW-ECIS before (E-G) and after the interaction between the cells (H).

Fig. 2. Images of the device and readout circuit. Images from the integrated design of SiNW-ECIS (package, signal readout board and data recording by computer).

Fig. 3. Biocompatibility assays of SiNWs. A) MTT results of doped and undoped silicon nanowires. Florescent images of QUDB cells seeded on SiNW-ECIS before (B) and after (C) proliferation stage.

Fig. 4. Diagnostic impedance diagram. Comparative impedance from Si-NW-ECIS biosensor covered by QUDB (A-C) and MRC-5 cells (D-F) between their different culturing stages (Initial drop, Attachment and Spreading). The impedance has been obviously increased during the attachment for both types of cells (A and D). Spreading stage of malignant cells reduced the impedance of the sensor meanwhile their proliferation had increasing effect on the impedimetric response of SiNW-ECIS. Attachment and spreading didn't affect the response of normal cells covered biosensor. When the QUDB cells entered to proliferation stages, normal cells (MRC-5) still stayed in spreading stage (Spread2) because of their so slower proliferation rates.

Fig. 5. FESEM images from seeded cancer and healthy cell in different stages. FESEM images of single QUDB cell cultured on SiNW-ECIS in various stage: A) Initial drop, B) Attachment, C) Spreading and D) Mitosis. (E) Florescent image from A/O tagged live malignant cells in attachment stage. Similar images were taken from normal lung cell (MRC-5): (F) Attachment, (G) Spread 1 and (H) Spread 2. (I) Florescent image from A/O tagged live normal cells entered

to stage of spread1. Normal cells have slower proliferation rate and required further time for spread and membrane extension in respect to malignant ones.

Fig 6. Impedance phase diagram. Impedance phase results of malignant (A-B) and normal (C-D) lung cells cultured on SiNW-ECIS reported from 5 individual tests at different frequencies. The impedance phase of both QUDB and MRC-5 cells increased (in negative values) during the attachment stage (A and C respectively). The phase of QUDB cells decreased during spreading stage (B), meanwhile we observed no changes in such stage for normal cells (D).

highlight

Silicon nanowire based impedance biosensor has been evaluated to diagnose the lung cancer cells from healthy ones.

A discernible impedance change was elicited during the spreading stage of cancer cells on nanowires

Stretched membrane of cancer cells couldn't block the current in respect to normal cells

morphology and architecture of silicon nanowires owing their superior nanocontacts with cells membrane can detect any slight variations in current penetration into cancer and normal lung cells.