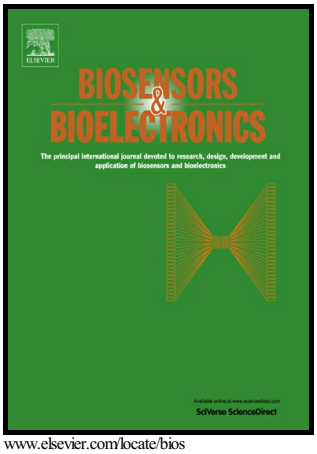

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Mohammad Saeed Nikshoar, Safoora Khosravi, Mojtaba Jahangiri, Ashkan Zandi, Zohreh sadat Miripour, Shahin Bonakdar, Mohammad Abdolahad

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Mohammad Saeed Nikshoar[a,b,1], Safoora Khosravi [a,b], Mojtaba Jahangiri [a,b], Ashkan Zandi [a,b,1], Zohreh sadat Miripour [a,b], Shahin Bonakdar[c], Mohammad Abdolahad[a,b,1,*]

[a] Nano Electronic Center of Excellence, Nano Bio Electronic Devices Lab, School of Electrical and Computer Eng, University of Tehran, Tehran, Iran, P.O. Box 14395/515, Tehran, Iran

[b] Nano Electronic Center of Excellence, Thin Film and Nanoelectronic Lab, School of Electrical and Computer Eng, University of Tehran, Tehran, Iran, P.O. Box 14395/515, Tehran, Iran

[c] National Cell Bank, Pasteur Institute of Iran, P.O. Box 1316943551, Tehran, Iran

***Corresponding Author: m.abdolahad@ut.ac.ir**

[1] Authors with same collaboration

Abstract

Determining the migratory and invasive capacity of cancer cells as well as clarifying the underlying mechanisms are most relevant for developing biosensors in cancer diagnosis, prognosis, drug development and treatment. Intravasation of metastatic cells into blood stream initiated by their invasion to vascular layer would be a significant characteristic of metastasis. Many types of biochemical and bioelectrical sensors were developed for early detection of metastasis. The simplicity of the setup, the ease of the readout, detection of the trace of rare metastatic cells and the feasibility to perform the assay with standard laboratory equipment are some of the challenges limiting the usability of the sensors in tracing the metastasis. Here we describe a biosensor based on recently reported metastatic diagnosis assay; Metas-Chip, with the assistance of nanoroughened Poly-methyl methacrylate (PMMA) layer to diagnose populated metastatic breast cells from primary cancerous ones. Retraction and detachment of Human Umbilical Vein Endothelial Cells (HUVECs) invaded by metastatic cells as a recently found phenomena is the mechanism of the action. A population of HUVECs would be detached from the gold microelectrodes, patterned on nanoroughened surface, which would lead to large changes in impedance. Here, applying biocompatible and patternable nanoroughened surface instead of using adhesive layers which might produce electrical noises resulted in great sensitivity and detectivity of the sensor. Apart from the tight interaction between endothelial cells and nanocontacts of the electrodes, using low concentration (10%) of tumor cells in this invasion assay, might enhance its application in clinical trials.

Keywords: Cancer invasion, HUVECs, Breast cancer cells, Impedance sensor, nanoroughened PMMA, Endothelial barrier.

1. Introduction

Malignant tumors exhibit some special indications such as transformed cell-cell and cell-matrix adhesions as well as reduced contact responsiveness [1] [2]. Metastatic strength of cancer cells could be graded by single tumor cells' invasion ability to the surrounding tissue initiated by intravasation into blood or lymph vessels [3]. Intravasated cells, named circulating tumor cells (CTCs)[4] , could extravasate into the connective tissue which leads to higher grades of metastasis [5] [6]. Many proteins implicated in tumor metastasis have been identified to act either as negative, (such as E-Cadherin) [7], or positive, (such as αvβ3,4,6, CD24, and CXCR2) [8] [9] [10] [11] factors. Expression of positive proteins is initiated by disruption in basement membrane, adhesion of tumor cells to vascular endothelium, retraction of endothelial junctions or necrosis of some endothelial cells [12] [13].

So, the invasive strength of the cancer cells to endothelial barrier could be a well indication for metastatic ability of tumor. However, investigating the possible expression of metastasis related proteins requires complicated biochemical processes with expensive biomarkers and skillful operators specially when the number of metastatic cells are so rare [14] [15] [16]. As a diagnostic indication, various components of tumor-endothelial cells interactions can be simulated *in-vitro* by challenging a monolayer of human umbilical vein endothelial cells (HUVECs) with removed tumor cells from a patient. Investigations revealed that the *in-vitro* sequences of events fairly represent the *in-vivo* metastatic process [17] [18]. However, fabricating an integrated diagnostic system to evaluate metastatic ability of unknown tumors based on their invasion to endothelial barriers is still a challenge which limits the entrance of vascular invasion assay in clinics. Recently, Some microfluidic biochips were introduced based on changed electrical [19] [20] and mechanical [21] responses of the HUVECs after their

interaction with tumor cells. Affecting angiogenesis after drug treatment [22], capability in producing blood vessels [23] and any chages in biochemical behavior are some of the features investigated on endothelial barrier after being invaded by tumor cells [24] [25] [26] [27] . However, some major limitations restricted their developments. First, an adhesive layer such as Fibronectin, Laminin or complicated 3D collagen matrix is required to enhance the attachment of HUVECs. Some mechanical and electrical characteristics of the mentioned layers such as elasticity and electrical capacitance might affect the measuring results [28] [29]. Second, minimum concentration of cancer cells required to induce perturbation in HUVEC barrier and measurable changes in the response of the sensor must be further than their real value in patients' tumor. Number of cancer cells used in one type of such biosensors were 1 cancer cells per 2.5 HUVECs [25]. It wouldn't be applicable in clinical trials in which tracing the rare concentration of metastatic cells would be so crucial. Applying advantages of new technologies such as nanotechnology [30] [31] [32] [33], photonics [34] [35] and tissue engineering [36] [37] helped to provide new assays with better efficiencies.

Here, we introduced a biosensor based on recently published metastatic diagnosis assay named Metas-Chip [17] to detect populated invasive cancer cells from primary ones. We used a Poly-methyl methacrylate (PMMA) layer being nanoroughened by reactive ion etching (RIE) system as sensing substrate to improve the electrical signals of attached HUVECs and distinguish the interactive effect of populated metastatic breast cells from primary cancerous ones. PMMA is a biocompatible hydrophobic surface widely used in cellular experiments [38].

In this device, nanoroughened PMMA substrate was covered with a thin layer of gold microelectrode array. Nanorough surface has been applied to enhance cellular proliferation by increasing the hydrophobicity and cell-substrate contact sites instead of applying adhesive layers

to improve the direct electrical contacts between cell and surface. After confluent attachment of HUVECs on the microelectrodes, the impedance of the sensor was measured as the background signal (10h after dropping the HUVECs). Subsequently, populated breast metastatic cells (MDA-MB231) were added to each sensing wells had been covered by HUVECs. The ratio of added metastatic: endothelial cells was 1:10. The experiments were repeated by non-metastatic breast cancer cells (MCF-7) on individual sensors. Results were compared to elaborate any probable diagnostic electrical patterns. In addition, optical and FESEM images were taken to verify the impedance changes induced by probable invasion of cancer cells to endothelial layer.

2. Materials and Methods

2.1 Fabrication of the Biosensor

The sensor is a PMMA chip (3cm in diameter) that contains an array of 6 circular shape microelectrodes with the diameter of 200μm (as cells sensing region) fabricated according to a simple and cheap process flow (fig 1A). First, a thin layer of photoresist (Microposit 1813) was spin coated on the PMMA substrate as the mask for making nanoroughened structures. The photoresist layer was then patterned by photolithographic process to obtain the desired region for producing nanofeatures. Subsequently the sample was held in RIE (Sensiran Co.) system. Patterned area was processed by SF_6, H_2 and O_2 gases (with typical flows of 100, 80 and 85 Sccm (standard cubic centimeter per minute)) in the presence of radio frequency (RF) Plasma (13.56MHz) to form nanoroughened surface. SF_6, which was ionized in the presence of RF Plasma, plays the key role as etching radical. The plasma power and period of the bombarding sub-cycles were 190W and 50S, respectively. The combination of H_2/O_2 and SF_6 during the settling step resulted in the creation of a protecting layer over the side walls of the formed nanohills in each sub cycle. Using this method, nanoroughened array were obtained on PMMA, with the wall sizes down to 60nm in width and 110nm in depth. FESEM images was taken to investigate the formation of such features. The resist layer was then stripped by acetone. In next step, Au/Ti bilayer (30/5 nm), was

deposited on the PMMA using sputtering process. The process was carried on by patterning the photoresist using reverse of the same mask containing the electrode design. Then, the metal bilayer was removed from the surface except electrodes and connection lines. So the metallic microelectrodes were formed exactly on nanoroughened region. The prepared device was then bonded to readout printed circuit board (PCB) and held in a cavity for biological tests (figure1B-C). More details were discussed in supplementary (fig S1).

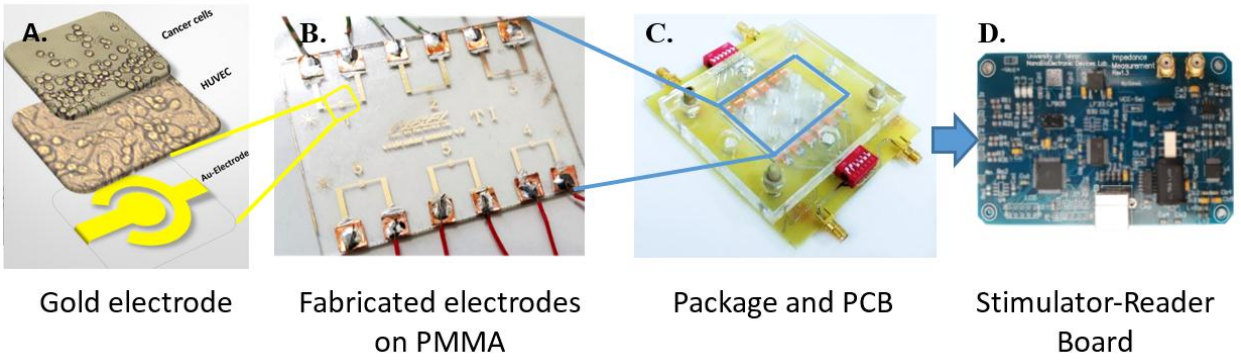


Figure 1 - Optical image of our invasion sensing system. A) The HUVECs and cancer cells were attached on sensing circular electrodes which were B) been integrated in an array C) The array was bonded to a PCB and D) connected to readout board.

2.2 Cell culture

MCF7 and MDA-MB231cell lines, were isolated from grades I and IV of human breast tumors, respectively. These cells were obtained from the standard cell banks of the National cell bank of Iran (NCBI) located in the Pasteur institute of Iran and they were maintained at 37 ^{o}C (5% CO_2, 95% 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. Human Umbilical Vein Endothelial Cells (HUVECs, ScienCell) were cultured in EC basal medium (EBM;

Scien Cell) with additional 10% FBS, and guaranteed to sub-cultured for three population doublings.

To start cellular experiments, HUVECs were cultured on the surface of the device for 6hr to form confluent layer. Subsequently, the metastatic and primary cancerous cells were individually added (by the concentration of 1/10 vs endothelial cells) to each HUVEC covered sensing wells and electrical impedance as well as optical images of the sensors were recorded in time-lapse images.

2.3 Signal extraction

The electrical impedance of HUVEC covered electrodes before and after interaction by primary and progressive cancer cells were carried out using impedance stimulator-reader board (fig 1D) designed by our group. The extraction mechanism was similar to NI-DAQ USB6323. Measurements were performed with applied voltage of 400 mV. The real time monitoring of the cells adherence was performed by measuring the impedance at the frequencies ranged from 1kHz to 100kHz in the time interval between 4h after dropping the HUVECs to 6 h after addition of cancer cells (whole time duration of signaling: 12h). To eliminate the effect of medium and clarify the invasive response of metastatic cells, the differentiated impedance value was calculated by comparing the response between the beginning of adding cancer cells and 6h later.

3. Results and discussion

3.1 Adhesion of HUVECs on nanoroughened PMMA

Figure 2 presents the surface morphology of nanoroughened (fig 2-A) and smooth (fig 2-D) PMMA. Formation of the nanostructures, increased the contact sites of the surface and resulted in improved adhesion and extension of HUVEC on the surface without requirement to any adhesive proteins (fig2-B vs. fig2-E). Optical microscopy images taken from the cells attached on nanoroughened (fig 2-C) and smooth (fig 2-F) PMMA layers, indicated better proliferation of the cells on nanotextured surface as the cells look more extended.

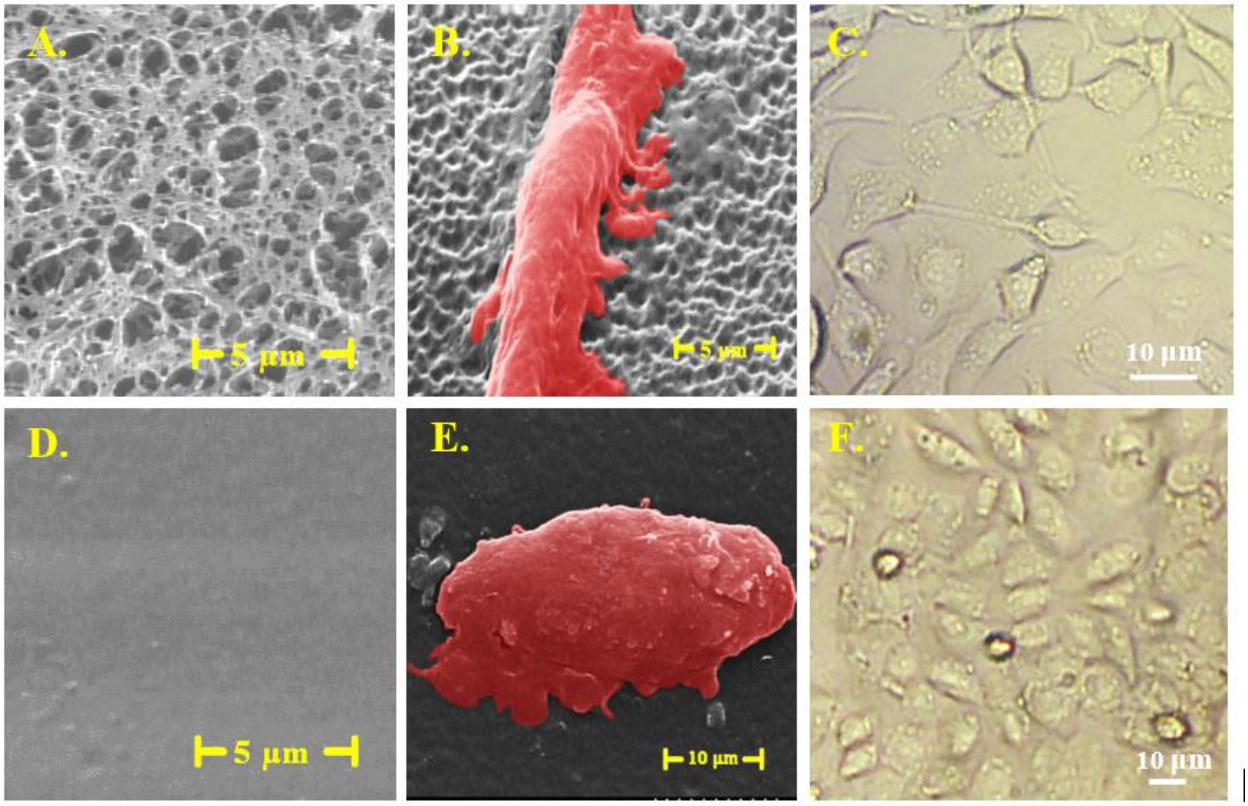


Figure 2 - FESEM images of A) nanoroughened PMMA produced by RIE system and B) A single HUVEC attached and extended on the surface. C) Optical microscopy image from the morphology and shape of HUVECs on nanoroughened PMMA which indicated their well extension on the surface. Also FESEM image of D) Smooth PMMA surface and E) a single cell attached on it could be observed the extension of the cell is much more on nanoroughened substrate. F) optical image of the HUVECs adhered on smooth substrate. The extension of attached cells are observably lower than those cultured on nanoroughened surface.

3.2 Electrical characterization of HUVECs covered electrode

Figure 3 presents the impedance diagrams of gold/ nanoroughened electrodes covered by HUVEC barrier in 6 (fig 3-A) and 16 (fig 3-B) hours after dropping the cells on the sensor. Attachment of HUVECs on nanoroughed PMMA resulted in impedance spectrum ranging from approximately 12kΩ to 1.5kΩ in the frequencies scanned from 0.1kHz to 100kHz (fig 3-A). After that, the impedance maintained stable and our measurements in 16th hour indicated non varied electrical response of HUVECs (fig 3-B). It means that the confluency of adhered endothelial barrier (fig 3-C & fig 3-D)) was suitable enough.

Presence of nanoroughened structures, produced on PMMA surface, prepared contact sites for fast and stable adhesion of HUVEC barrier on the sensor.

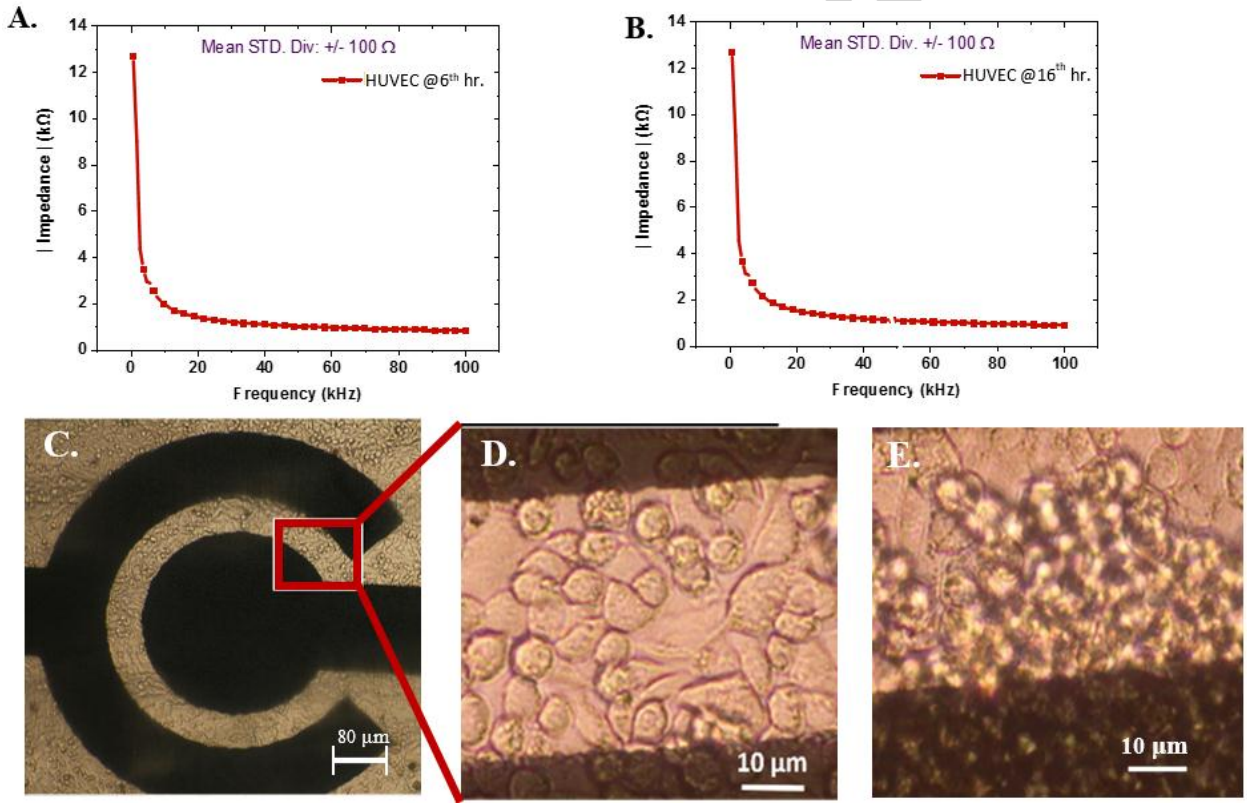


Figure 3 - Electrical impedance of sensing well 6hr (A) and 16hr (B) after culturing the HUVECs on the electrodes. Morphology of attached cells is observable in Optical image of the electrode (C), Confluent (D) attachment of HUVECs on PMMA surface made such stable responses in time evolution.

3.3 Assaying the invasion of cancer cells

Figure 4-A presents the percent of impedance changes in each HUVEC covered sensing well after 4 hours of interaction by known concentration (10%) of breast metastatic cells (-■- tagged curves). It is observable that about 50-80% impedance changes induced in the sensors after invasion of metastatic cells. This result was completely matched with the recent finding on selective invasion of a metastatic cell to single endothelial sensing trap [17]. Impedance changes recorded from similar individual microwells being interacted by similar concertation of low grade cancerous breast cells (MCF-7) plotted by circular tagged curves in figure 4-A (-●- tagged curves) were less than 10%. Calibrated impedance changes ($\frac{\Delta Z}{Z_0}$) in the wells defined as:

$$\frac{\Delta Z}{Z_0} = \frac{|Z_{\text{HUVEC covered before adding cancer cells}} - Z_{\text{HUVEC covered 12hr after adding cancer cells}}|}{Z_{\text{HUVEC covered before adding cancer cells}}} \times 100$$

More than 40% reduction in the impedance of HUVEC covered sensors could be named as invasion. Also the mean values of impedance changes in each well were presented in bottom panel of figure 4-A. Comparing the impedance of a microwell covered by HUVEC before and 6h after interaction by 10% of metastatic MDA-MB 231cells confirmed observable reduction in the impedance as the result of metastasis meanwhile no noticeable effects were recorded on electrical response of HUVECs after their interaction by non-metastatic MCF-7 cancer cells.

Raw impedance data recorded from HUVEC covered sensing wells exposed to MDA-MB 231MCF-7 cells exhibited more than 12kΩ reduction in impedance 12h after interaction (fig 4-B) meanwhile this value for MCF-7 cells was less than 100Ω (fig 4-C).

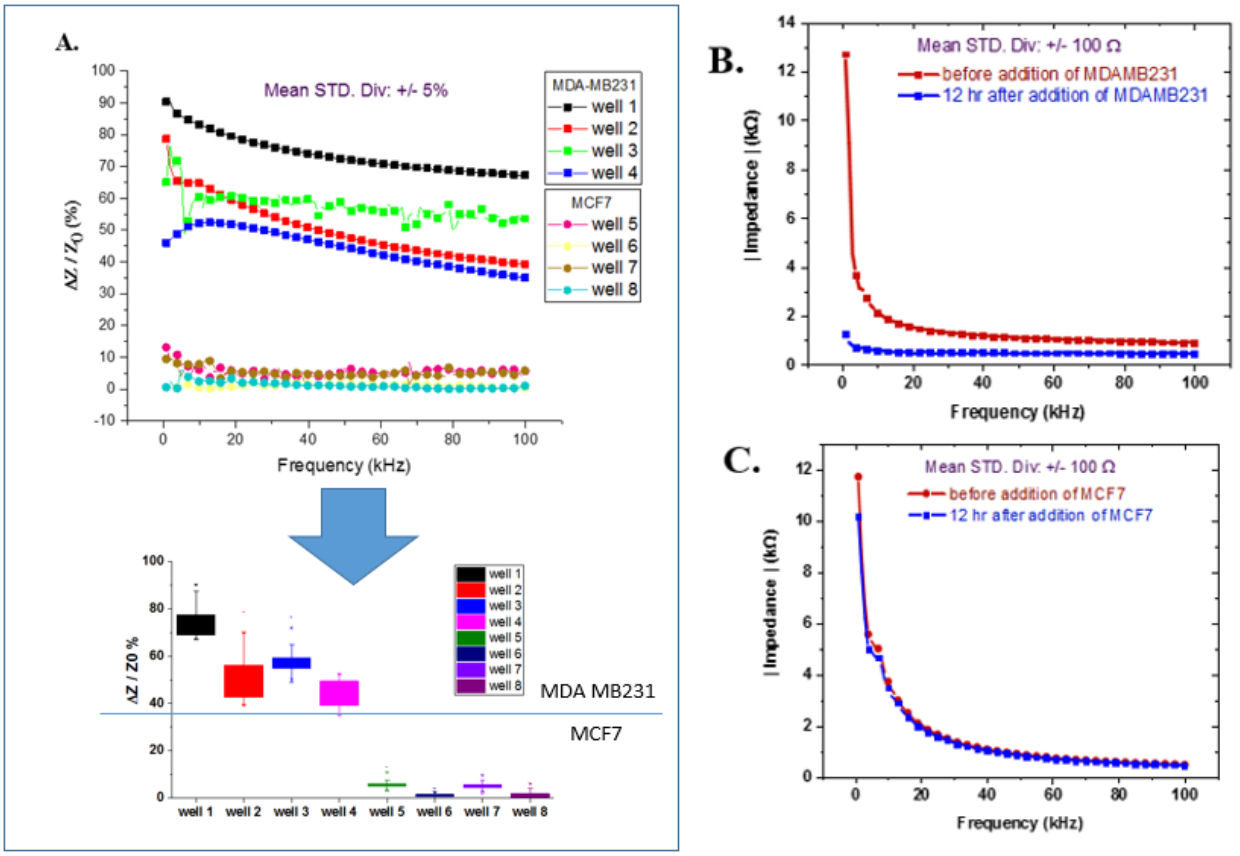


Figure 4 - A) Normalized Impedance changes of the HUVEC covered sensing wells interacted by 10% of MDA-MB231 metastatic (rectangular tagged curves) and MCF-7 primary cancerous cells plotted from four individual wells for each cell line. More than 60% changes in mean impedance is observable for all individual wells interacted by metastatic cells. In contrast , less than 10% changes in the impedance responses were recorded for the wells interacted by MCF-7 cells. Raw curves showed more than 12kΩ reduction in the impedance of HUVECs covered electrodes after 12h of interaction by MDA-MB 231 (B) but such changes were less than 100Ω after similar interaction with MCF-7 cells (C).

Field emission scanning electron microscopy (FESEM) taken from the HUVECs being interacted by MDA-MB231 (fig5-A) and MCF-7 (fig5-B) cells greatly confirmed the invasive behavior of populated metastatic cells to the endothelial barrier in which the extension of MDA-MB231 cells' filopodias into the HUVECs and their entrance into endothelial layer were observable. In contrast, MCF-7 cells exhibited contracted shape and didn't affect the proliferated state of HUVECs as endothelial cells didn't let MCF-7 to be proliferated on the surface.

Such interactions completely justified the different responses of the biosensors to the metastatic cells in comparison with low grade ones as schematically was presented in figure5-C. Proliferation of HUVECs on the electrodes blocked the passing current through the membrane due to beta dispersion phenomena (fig 5-C1) [31]. After addition of metastatic cells, they started to invade HUVECs. As the result, endothelial barrier was affected and contracted which resulted in degradation in current blocking and penetration of the current through the sensing electrode and finally drastic reduction in the impedance of the sensor (fig5-C2). But interaction of the endothelial layer by primary cancerous breast cells didn't induce any retraction (such as contraction and detachment from the surface) to HUVECs. So, the impedance response of the sensor remained unchanged (fig 5-C3).

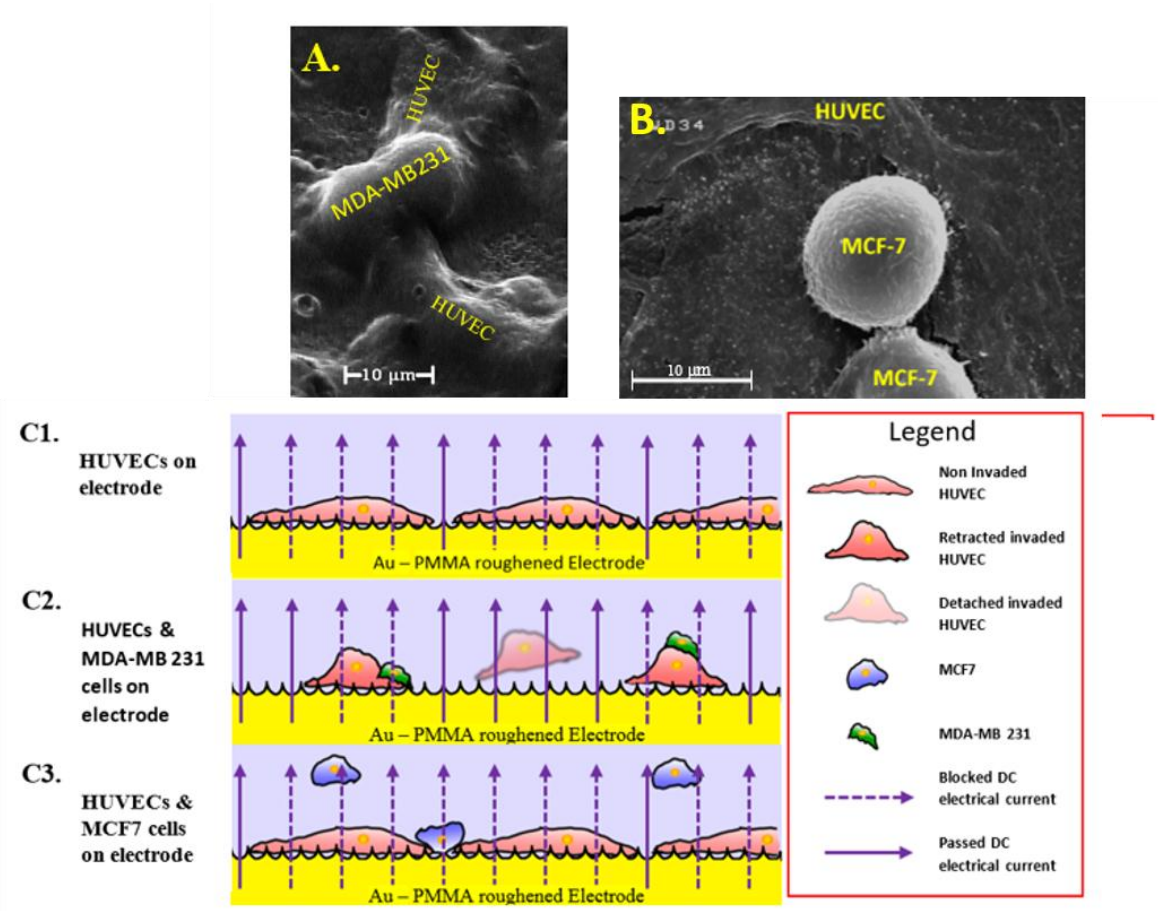
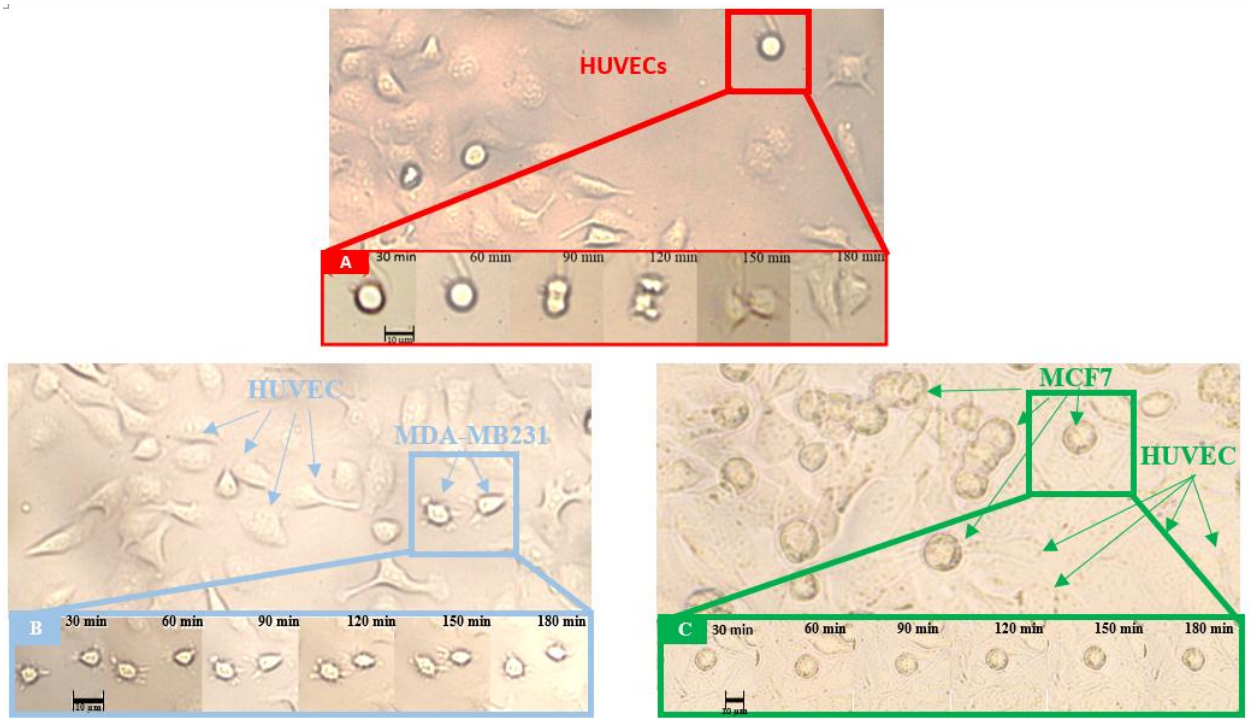


Figure 5 - (A) FE-SEM images of HUVECs invaded by MDA-MB231 cells. Retraction of endothelial layer resulted in some spaces on the surface. Also the extension of invaded cell's filopodia is observable on the endothelial cells. (B) similar SEM images taken after the interaction of HUVECs barrier and MCF-7 cells. no invasion has been occurred and the endothelial cells remained their natural morphology. Also the primary cancerous cells weren't exteneded and remained contracted on the surface. (C1) Schematic of current path blocked by confluent endothelial layer formed on nanoroughened sensing electrode before interaction with cancer cells. (C2) After invasion of metastatic MDA-MB231cells, the HUVECs were retracted and the currents penetrated through endothelial barrier. (C3) Addition of non-metastatic cancer cells (MCF-7) didn't induce any perturbation in endothelial barrier and the current were still blocked by confluent HUVECs layer.

Finally, time lapse microscopy images taken from a HUVEC which weren't interacted by any cancer cells indicated continues natural growth and division of the cells in the capturing time interval (fig6-A). Metastatic cells on the surface of HUVEC barrier showed their invading state by extending their conduit (fig6-B). In contrast, non metastatic cells weren't extended to invade HUVECs (fig6-C).



F)

Sample No.	Types of electrode/substrate	Adhesive layer	Time duration of HUVEC confluent attachment(h)	Ratio of HUVEC : Metastatic cells	Type of Metastatic. cells	Invasion time for diagnostic response (h)	Ref
1	Au / ECIS micro well	Fibronectin	40	1:1	SKOV3	50-60	[39]
2	Au / Boyden chamber	Gelatin	10-12	1:1	HEPG2	12-32	[26]
3	Au / XCELL well	Gelatin	25	1:2.5	K7M2	6-8	[25]
4	Au / Nanoroughened PMMA	No Adhesive layer	6	10:1	MDA-MB231	3-4	Our sensor

Figure *6* - (A) time laps monitoring of HUVEC layer which weren't exposed to any cancer cells. they continued their natural growth and division. (B) time laps microscopy image taken from MDA-MB 231 cells in first 3hours after dropping on HUVEC layer. Extension of their filopodia (conduits) for invasion have been started after 90min. (C) similar images taken from MCF-7 cells. no invasive activity could be observed. (F) Table of comparative parameters between invasion electrical assays.

Some crucial parameters of our device were compared with those of other impedimetric invasion assays tabled in figure 6-F. The ratio of metastatic cells /HUVECs in our device was 5 times lower than the best of previous reports. Although no adhesion layer was applied in our sensor, the adhesion time of HUVEC layers was less than 5h and the required challenging time between metastatic and endothelial cells to achieve diagnostic responses was less than 4h.

4. Conclusion

In summary, an impedimetric invasion biosensor was fabricated by the application of nanoroughened PMMA as substrate. HUVECs were cultured without any external adhesive layer on Au electrodes patterned on nanoroughened surface. Nanoroughening not only improved the proliferation of HUVECs but also enhanced their electrical contacts with Au electrodes. The response of each HUVECs covered sensing well to the presence of primary or metastatic breast cancerous cells after known intervals of time were measured. Noticeable changes in the impedance (about 65%) was observed just 4h after interaction of rare metastatic breast cells with endothelial barrier (with the concentration of 1:10). Metastatic cells invaded to confluent endothelial barrier, retract them from the surface. Penetration of the current from the perturbed endothelial layer observably reduced the impedance of the sensor. In contrast, primary cancerous cells couldn’t invade to HUVEC layer and no noticeable changes were observed in the impedance of the sensor. FESEM images corroborated the different interactive mechanisms of MDA-MB231 from MCF-7 cells in interaction with HUVEC layer. Fast and precise response of the device in low concentration of metastatic cells shed new lights for applications of electrical invasion assay in clinical trials.

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Highlights:

An electrical biosensor based on Nano roughened PMMA was designed and fabricated

Invasion of populated metastatic cells to a cellular layer of endothelial cells covered on the electrodes was recorded

Drastic changes in the electrical response was recorded between the interaction of invasive and primary cancer cells

Nano toughened PMMA facilitate the physical and electrical interface between endothelial barrier and sensing surface.