Microfluidic device for trapping and monitoring three dimensional multicell spheroids using electrical impedance spectroscopy

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In this paper, we report the design, fabrication, and testing of a lab-on-a-chip based microfluidic device for application of trapping and measuring the dielectric properties of microtumors over time using electrical impedance spectroscopy (EIS). Microelectromechanical system (MEMS) techniques were used to embed opposing electrodes onto the top and bottom surfaces of a microfluidic channel fabricated using Pyrex substrate, chrome gold, SU-8, and polydimethylsiloxane. Differing concentrations of cell culture medium, differing sized polystyrene beads, and MCF-7 microtumor spheroids were used to validate the designs ability to detect background conductivity changes and dielectric particle diameter changes between electrodes. The observed changes in cell medium concentrations demonstrated a linear relation to extracted solution resistance (R_s), while polystyrene beads and multicell spheroids induced changes in magnitude consistent with diameter increase. This design permits optical correlation between electrical measurements and EIS spectra. © 2013 AIP Publishing LLC. [<http://dx.doi.org/10.1063/1.4809590>]

INTRODUCTION

Three-dimensional (3D) multicellular spheroids (MCSs) have gained significance as a model of choice over two-dimensional (2D) cultures for investigating cytotoxic effects of anti-cancer drugs.^{1–4} The shift from 2D to 3D is due to the ability of the former to better simulate cell-cell and cell-matrix complexities native to *in vivo* 3D tumor models. Furthermore, these characteristics are essential for accurate investigations of tumor-drug interactions. For example, the 3D shape of MCS produces a differential oxygen gradient throughout the tumor resulting in an unequal distribution of cell nutrients while 2D cultures experience equal distribution of oxygen due to lack of phenotypic physiological orientation into a 3D structure. Also in 3D cultures, heterogeneity is expressed through increased cell differentiation and self-organization as opposed to most homogeneous monolayer cultures. Sutherland *et al.* observed that unlike 2D cultures human colon adenocarcinoma spheroids produced gland-like organizations similar to *in vivo* tumors.⁵ Due to the *in vivo* like characteristics,⁶ investigators utilized cell-line derived microtumors which are formed through the compact aggregation of cancerous cells in microgravity culture environments.^{7–9} The conventional methods of spheroid growth include cultures within agar non-adherent plates, suspension in spinning flasks, or within hanging drops.^{10,11} Aggressive efforts are focused on spheroid growth within microfluidic chip environments yielding arrays of spheroids with tightly controlled dimensions for repeated pharmacological evaluation.^{12–15}

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Significant research has been published in electrical impedance spectroscopy (EIS) device design and implementation to monitor single cell and 2D planar cell cultures reporting culture behaviors, e.g., adhesion, spreading, motility, and apoptosis.^{16–22} EIS measures the dielectric properties of materials as a function of frequency. The main advantage of EIS versus traditional optical microscopy is the ability to monitor cell systems semi-autonomously without the need of optical dyes, which tend to affect cell viability. The non-invasive, non-destructive, real time capability of EIS has made it a popular quantitative tool for investigations such as cell flow impedance cytometry^{23–27} and impedimetric analysis of trapped single cells.^{28–31} Cima *et al.* compiled recent works regarding label-free approaches to isolate and manipulate circulating tumor cells (CTCs) in which they included reviews on the present EIS-microfluidic approaches.³² As of date, few EIS investigations have been performed on 3D spheroid cultures.

The use of EIS to monitor microtumor-drug interaction has been a subject of much interest, and effort is being placed on how to maintain a microtumor between electrodes for long term evaluation.^{33–35} Molckovsky and Wilson devised a test setup where a spheroid between 500 μm and 700 μm in diameter was placed in a trench while an impedimetric electrode was positioned close to the spheroid using a micrometer. Though effective, this method is time intensive dismissing the ability to observe many samples in little time. Thielecke *et al.*, fabricated a micro-electrode array to monitor spheroids. Each electrode was fabricated with a small hole allowing suction as the method of trapping spheroids. This particular approach solves the throughput issue in the previous example; however, the asymmetrical forces on the spheroids and isolation of small portions of the spheroids from bulk medium could induce premature negative effects over time. Kloß *et al.* fabricated an array of micro-cavities in silicon in which a spheroid would come to rest the shallow well due to gravity. Each of the four angled sidewalls of the cavity included an electrode allowing impedimetric measurements across spheroids. Solving throughput from the first example and asymmetric forces as well as partial isolation issues from the second example this design relies heavily on minimal motion of the device itself as one could envision any slight motion could cause the spheroids to be displaced from the wells as they are only slightly heavier than neutrally buoyant in solution. These designs while attractive lack the ability to carefully control the microenvironment surrounding the spheroid culture.

This study presents a device design capable of in-flow trapping of multicellular spheroids ranging from 50 to 300 μm diameters between opposing (top and bottom) sets of electrodes for real-time electrical characterization using EIS. The geometry of channel and trap automatically guides and stops the spheroid between electrodes when perfused into the device. The device can be manipulated and potentially transferred from one station to another while seeded with a spheroid. Multiple channels and electrodes can be fabricated on one chip for higher throughput and multiple chips can be implemented. The transparent substrates (glass) and polymers (polydimethylsiloxane (PDMS) and SU-8) were used in the microfabrication process enable simultaneous visualization with either an inverted (bottom up) or traditional (top down) microscope allowing optical correlations to EIS measurements. The reported microfluidic system tolerates static (no flow) or perfusion (flow) conditions allowing for the introduction of nutrients (cell culture media) and/or anticancer therapeutics enabling pharmacodynamic evaluation of MCS drug response over long times. We characterized the microfluidic chip to differing conductivities of cell culture medium to determine effects on EIS curves. Further demonstrate the ability to size particles using polystyrene beads as a control and pinpoint the frequency values which demonstrated maximum change. In addition, MCF microtumors were trapped and evaluated using EIS.

MATERIALS AND METHODS

Concept

COMSOL simulations were performed on the cross section of the electrodes and channel (250 μm high $\times$ 500 μm wide), both with and without a 200 μm insulating particle for comparison (Figures 1(a) and 1(b)). The conductivity of the channel medium was set to 1.5 S/m while the particle boundary was set as an insulator. Figures 1(a) and 1(b) illustrate the potential

FIG. 1. Top left (a) is a cross sectional COMSOL simulation depicting the potential gradient into the channel. Middle left (b) is a cross sectional COMSOL simulation illustrating the extension of the potential intensity into the channel due to the dielectric properties of the polystyrene particle.

gradient across the channel and variation of the field intensity into the channel due to the dielectric properties of the particle.

AUTOCAD was used to virtually build and evaluate the final device. Figures 2(a)–2(c) are different viewpoints of the same rendering. Figure 2(a) illustrates the entire device while Figure 2(b) shows a cross sectional view of the trap and electrode area going the length of the channel, while Figure 2(c) is looking from the top down at a slight angle. The two gold (yellow) electrode planes of the Microelectromechanical System (MEMS) device were fabricated on Pyrex (glass) substrates. A $10\text{ }\mu\text{m}$ layer of SU-8 (magenta) was used to coat the connecting tracks of the electrodes to the pads. The top and bottom substrates were bonded together with a pre-formed PDMS gasket (blue) in-between to define the micro-fluidic channel. The fluidic chip utilizes a horseshoe shaped arrangement of vertical SU-8 photoresist posts (magenta) which were designed to trap particles directly between the two sets of electrodes as seen in Figures 2(b) and 2(c).

Fabrication

A process flow detailing the process steps to form the device is shown in Figure 3. First, wafers were cleaned using isopropanol and deionized (DI) water rinse, dehumidified at $200\text{ }^{\circ}\text{C}$ for 20 min, followed by a 5 min period to cool down to room temperature. Both wafers were

FIG. 2. Top left (a) is an AutoCad rendering of the final microfluidic EIS device. (b) is an AutoCad rendering of the channel cross section including, trap (posts) top and bottom set of electrodes and PDMS gasket outlining the channel. (c) is an AutoCad rendering of the channel from a top-skewed view illustrating trap (posts) top and bottom set of electrodes and PDMS gasket outlining the channel.

coated with a 20 nm sacrificial aluminum (Figure 3(A)) used to prevent UV light from scattering throughout the Pyrex wafer and overexpose resist during the lithographic process. The aluminum side of each wafer was coated with 3 μm thick negative photoresist (3000PY: Futurrex, Inc.) lithographically patterned with the electrode (Figure 3(B)). After lithographic patterning, the aluminum is etched from the electrode pattern (Figure 3(C)) using 16:1:1:2 Barker Al etchant, rinsed with DI water, and dried using nitrogen. The wafers were coated with 20 nm chrome and 150 nm gold via electron beam evaporation (Figure 3(D)), cooled for a minimum of 10 min at room temperature then placed in acetone for liftoff. Following the conformation of the electrode geometry via optical microscopy, the remaining aluminum was etched from the wafers (Figure 3(E)).

The wafers were rinsed in isopropanol/DI water, dried with nitrogen, and ashed in O_2 plasma for 5 min, in order to remove residual photoresist. The wafers were coated with a 10 μm thick SU-8 3010 (Microchem Industries). This photoresist is used to coat the streets of the electrodes which should not be exposed to the fluidic channel. The SU-8 is exposed and developed to uncover the sensing area of the electrodes and the outer probe pads (Figure 3(F)). This thin, high footprint layer permits a strong SU-8 to SU-8 adhesion for the tall, high aspect ratio SU-8 pillars which will form the MCS trap discussed later.

The wafer consisting of the bottom slides undergoes a two part SU-8 3050 spin. Each spin yields a 125 μm thick layer resulting in a 250 μm thick Su-8. The thick SU-8 is lithographically patterned to form an array of posts (Figure 3(G)) in a horseshoe pattern which are located in the channel and around the electrodes (Figure 2(c)). There are two types of 250 μm tall posts, type one is oval in shape with two axis of symmetry, they sit at the apex of the horseshoe trap where flow forces would be maximum and the second type of post is circular forming the periphery of the trap and act to guide or funnel 3D objects between opposing electrodes. The long axis of the oval is 100 μm , while the short axis is 30 μm ; the circular posts are 20 μm in diameter.

Both wafers are diced into predefined 1 in. long $\times$ 1/2 in. wide slides for assembly. Once the wafer consisting of the top slides is diced two holes are drilled through each slide for a fluidic

FIG. 3. Illustration of the first six fabrication steps (A–F) performed on both top and bottom sections of the device, illustrates additional fabrication steps of bottom section 250 μm SU-8 posts (step G), illustrates additional fabrication steps of top section allowing for channel inlet and outlet (step H), illustration of SU-8 master mold to form PDMS gaskets and final assembly procedure (step M) illustrating the bottom, gasket, and top sections. The offset assembly allows access to the contact pads

inlet and outlet at alignment marks located at each end of the channel using a CO_2 laser (Figure 3(H)).

In order to form the PDMS gasket, a 250 μm thick master mold was made using the same two part 125 μm thick SU-8 spin parameters as discussed previously except on a silicon wafer (Figure 3(I)). After the lithographic process, the wafer was cleaned and silanized (Figure 3(J)) for replica molding. Sylgard 184 polymer base and curing agent were mixed in the weight ratio of 10:1. After mixing, the PDMS is degassed in vacuum ambient then poured into the master mold. The PDMS mixture and SU-8 master mold are laminated between Kapton and held under pressure until cured. Once fully cured, the PDMS layers are gently lifted out of the mold (Figures 3(K) and 3(L)).

In the final assembly, the bottom slide is placed under a stereo microscope, a drop of DI water is placed on the slide to allow motion of the oxygen plasma treated PDMS gasket during alignment. The gasket, which forms the channel, is aligned to flats of the electrode streets on the bottom slide. The top slide is then placed over the PDMS gasket. The top and bottom slides are slightly offset overlapping by $\frac{1}{4}$ in. in order to expose top and bottom contact pads (Figure 3(M)). Precaution is taken to align top and bottom electrodes without breaking the SU-8 posts under a stereo microscope. The flats of the electrode streets on the top slide allow rough alignment with the PDMS channel; however, smaller alignment marks, less than 20 μm , located on the top slide are aligned to the tips of the 20 μm diameter SU-8 posts of the bottom slide yielding both precision and accuracy in top and bottom electrode positions in relation to each other. To ensure a robust assembly and to prevent future leaks, a bead of PDMS is injected around the edges of the system with a syringe. The assembly is placed inside an oven at 67 $^\circ\text{C}$ and left

FIG. 4. (a) Photograph of the final device. Note the yellow area is the gold contact pad (where the probe makes contact), while the dark areas on the right is the chrome layer between the glass and gold contact. The device is shown next to a US 10 cent piece for size comprehension. (b) Optical magnification of electrode and trap area of the device looking top down slightly skewed.

to cure for 12 h. Figures 4(a) show the final device next to a dime. Figure 4(b) is an optical magnification of the trap and electrode area from the top down and at a slight angle.

Measurement apparatus

In order to minimize noise associated with faulty electrode connection, a chip holder was designed (Figure 5) specifically to interface the device under test (DUT) with the Impedance Analyzer. Spring loaded contact pins (Pogo pins) were incorporated to allow for consistent pin to pad contact pressure and placement. The Pogo pins are connected to SMB connectors via copper streets on a printed circuit board (PCB). The SMB connectors are further attached to the impedance analyzer through SMB to BNC cables.

Protocol for spheroid formation

MCF-7 cells are pipetted into 15 ml tube with 9 ml of Eagle's minimal essential medium (EMEM) (Earle's balanced salt solution) increased bicarbonate concentration to handle 5%

FIG. 5. Image of the chip in a Jig which is held together with thumb screws; Pogo pins make contact to the chip and are connected to SMB using a PCB to the Agilent 4294a impedance analyzer.

CO₂, non-essential amino acids along with essential L-glutamine w/o CaCl₂, containing 10% fetal bovine serum (FBS). Contents are centrifuged at $200 \times g$ for 5 min. Supernatant is discarded and cells are resuspended in 10 ml of new EMEM media. Solution is mixed by pipetting and transferred to 20×100 mm tissue cultured grade plates and incubated at 37 °C inside a 70%–80% water-jacketed humidified incubator. Media is changed every 2 to 3 days. Cells are passaged once cells reach 80%–90% confluence.

To form spheroids, cells are first trypsinized and then diluted using culture medium to form 25 μ l droplets containing ~ 150 cells. MCF-7 droplets are added to sterile Petri dish lid and then inverted onto plate containing 10 ml of PBS for hydration. Plates are incubated for at least 5 days without disturbance. After which viable spheroids are examined under microscope. Spheroid diameters range from 300 to 450 μ m. To allow spheroids to proliferate, viable spheroids are carefully transferred into a new droplet (20 μ l) containing fresh media (EMEM + 10% FBS).

RESULTS AND DISCUSSIONS

Dilution studies

The operational frequency range for EIS measurements was selected to scan from 10 kHz to 10 MHz. Impedance was measured using an Agilent 4294a impedance analyzer. EMEM with 10% FBS was used as a baseline. Solutions of the baseline medium were diluted with DI water from the standard EMEM-FBS mixture (100%) down to 60% to verify dilution effects on impedimetric measurements. The conductivity of the EMEM-FBS ranged from 15 mS/cm (100%) to 9 mS/cm (60%). As expected, the impedance magnitude of the EMEM-FBS solution increased with the decrease of conductivity (Figure 6(a)). The percent change of magnitude ranged from 8% to 35% (Figure 6(b)) corresponding to 90% medium down to 60% medium, respectively, using 100% medium as zero baseline. The phase of the solution also shifted closer to zero as the ionic concentration decreased (Figure 6(c)), where the overall shift change ranged from 2.3° to 8° from 100% medium (Figure 6(d)) corresponding to 90% medium to 60% medium, respectively.

FIG. 6. (a) Impedance magnitude of cell culture medium and dilutions of medium. (b) Percent change in impedance magnitude due to the decrease of molarity. (c) Phase of medium dilutions throughout frequency spectrum. (d) Phase change throughout frequency spectrum demonstrating maximum change centered around 40 kHz.

FIG. 7. Chart of the inverse relationship between solution resistance and diluted cell medium. Lumped element model of electrode electrolyte system.

The impedance spectra from the EMEM-FBS conductivity experiment was fitted to a lumped circuit model (Figure 7) using the Z-VIEW modeling software.

Figure 7 also illustrates a general equivalent circuit model (inset) which includes the polarization resistance (R_p) in parallel with a constant phase element (CPE) all in series with the solution resistance (R_s). The R_p is resistance due to the corrosion potential (E_c) over the corrosion current (I_c) of the gold electrodes. CPE is a frequency dependent capacitive component formed by the interfacial charge separation (double layer) caused by the ionic layers at the electrode-electrolyte interface. While the equation for impedance (Z) due to an ideal capacitor is $1/(j\omega C)$ and the formula for a constant phase element (Z_{cpe}) is $1/(Q(j\omega)^\alpha)$, where α is a factor between 0 and 1, which is equal to the impedance (Z) of an ideal capacitance (C) when α equals 1. R_s is equal to $\rho l/A$, where ρ is the resistivity of the ionic solution. Table I presents the parameter extractions relating to medium dilutions and the variables described above.

An inverse linear relationship is shown to exist between the solution resistance (R_s) component and conductivity further verifying expected results as the molarity changes linearly with dilutions and so the resistance must follow the same trend.

Polystyrene beads

The impedance of polystyrene beads (96, 122, and 225 μm diameters) was measured to verify full-system functionality and structural integrity. Single beads were perfused into the channel and measured. Measurements were monitored optically with a stereo microscope during perfusion. Once a bead was trapped and EIS measurements were taken, the bead was removed by reversing the flow of the syringe, allowing for the next size bead to be collected and perfused for trapping and measuring.

Results indicated that impedance magnitude increased as bead diameter increased. Impedance magnitude increased as particles increased in diameter, due to their dielectric properties and the displacement of the conductive medium from between the electrodes (Figure 8(a)).

TABLE I. Extracted parameter details following the equivalent circuit model represented in Figure 8. These parameters demonstrate the change in solution resistance due to dilution of medium with DI water.

% Medium	CPE-Q	CPE- α	R_p	R_s	% Delta R_s
100	2.42×10^{-10}	0.9119	2.87×10^7	4938	0
90	2.44×10^{-10}	0.91097	2.83×10^7	5419	9.74
80	2.42×10^{-10}	0.91132	2.80×10^7	5843	18.32
70	2.43×10^{-10}	0.91104	2.85×10^7	6383	29.26
60	2.32×10^{-10}	0.91414	2.74×10^7	6818	38.07

FIG. 8. (a) Impedance magnitude of cell culture medium and polystyrene particle size. (b) Phase diagrams of medium and polystyrene beads. (c) Percent change in impedance of bead size normalized to cell culture medium. (d) Phase change due to bead size normalized to cell culture medium.

Table II presents the parameter extractions relating to medium and polystyrene beads. Since R_s is a term used for solution resistance the term R_{s+} is introduced to describe the solution resistance plus resistance due to particle introduction.

Multicell spheroids

MCSs of differing diameters were cultured from MCF-7 cell line using the protocol mentioned previously. Three spheroids were chosen whose diameter ratios somewhat resembled those of the polystyrene tests above. The average diameters of the spheres were evaluated to be $129\ \mu\text{m}$, $161\ \mu\text{m}$, and $320\ \mu\text{m}$ (Figures 9(a)–9(c), respectively) using IMAGEJ software.

Following the same protocol of the bead test, a spheroid was removed from the culture syringe and perfused inside the EIS device. An optical microscope monitored and verified spheroid perfusion and trapping, once trapped, EIS measurements of the spheroid were taken. Figure 9(d) demonstrates the trapping of the larger spheroid from Figure 9(c). A minimum of three measurements was acquisitioned per spheroid. Between each single spheroid measurement, the MCS was slightly back perfused to allow background medium measurements then reintroduced between the electrodes to allow reorientation and hence a mean value using same MCSs.

Figure 10(a) exhibits the percent change of magnitude relative to baseline medium, while Figure 10(b) shows degree change in phase relative to baseline medium. Evaluation of the change in magnitude due to the presence of the 129 , 161 , and $320\ \mu\text{m}$ MCSs yielded maximum

TABLE II. Extracted parameter details following the equivalent circuit model represented in Figure 8. These parameters demonstrate the change in resistance due to introduction of polystyrene beads of differing size.

Diameter	CPE-Q	CPE- α	R_p	R_{s+}	% Delta R_{s+}
Medium	1.34×10^{-9}	0.88313	6.28×10^6	5617	0 ± 1
$96\ \mu\text{m}$	1.34×10^{-9}	0.88264	4.25×10^6	6024	7.25 ± 1
$122\ \mu\text{m}$	1.38×10^{-9}	0.87940	8.09×10^6	6545	16.52 ± 1
$225\ \mu\text{m}$	1.33×10^{-9}	0.88355	6.40×10^6	7158	27.43 ± 2

FIG. 9. (a) Image of the 129 μm diameter spheroid in culture before perfusion. (b) Image of the 161 μm diameter spheroid in culture before perfusion. (c) Image of the 320 μm diameter spheroid in culture before perfusion. (d) The same spheroid from Figure 9(c) trapped between the electrodes of the fluidic chip. Only the top electrodes are seen in this image.

FIG. 10. (a) Percent change of magnitude due to the introduction of the MCSs using cell medium as zero baseline. (b) Phase change due to the introduction of the MCSs using cell medium as a zero baseline.

increases of $6.1\% \pm 1\%$, $12.3\% \pm 2\%$, and $19.5\% \pm 6\%$ with respect to cell medium centered around 2 MHz (Figure 10(a)). The large deviation is notably due to the reorientation of the spheroid resulting from the removal and reintroduction of the same spheroid between each measurement. Maximum change in phase due to MCSs centered around 100 kHz yielding a 1.4° , 3.2° , and 4.2° change (Figure 10(b)). It is noteworthy to mention that although the MCSs are larger than the polystyrene bead counterparts, the impedance changes due to the beads are larger than the MCSs. Similarly, the maximum phase change was greater in the beads. These

TABLE III. Extracted parameter details following the equivalent circuit model represented in Figure 8. These parameters demonstrate the change in solution resistance due to the introduction of MCF-7 MCS.

Spheroid	% Delta Rs+
Medium	0
129 μm	6.1 ± 1
161 μm	12.3 ± 2
320 μm	19.5 ± 6

FIG. 11. Data comparing diameter relative change in magnitude with respect to diameters of both spheroids and polystyrene beads.

results are attributed to a number of factors associated with the MCS. Namely, the MCS volume is mostly ionic in nature, the cell membranes are ionically leaky, and intracellular space permits pathways. Table III presents the numerical percent change of the MCF-7 MCSs relative to medium, while Figure 11 illustrates the MCSs numerical data alongside the polystyrene bead data.

CONCLUSIONS

A microfluidic device which correlated its electrode-electrolyte system to a lumped element model demonstrating the linear relation between cell culture medium dilution (conductivity) and extracted solution resistances (R_s) values modeled in zVIEW software has been fabricated. We have successfully demonstrated the trapping of 3D particles in order to evaluate the dielectric properties between opposing electrodes via EIS. The device has distinguished particle diameters verse impedance spectrum of both polystyrene beads (diameter ranging from 96 to 225 μm) as well as microtumor spheroids (diameter ranging from 129 to 320 μm). It was observed that one frequency (2 MHz) which demonstrated maximum change in magnitude and another frequency (100 KHz) which elicited a maximum change in phase while evaluating the impedance changes due to the presence of polystyrene beads, and MCS and dilutions of cell medium. A noteworthy observation is found while comparing impedance changes of dilutions of cell medium versus those of the polystyrene bead and MCS diameter changes. It can be seen that divergence from cell medium standard occurs about a half decade sooner in both magnitude and phase with polystyrene and MCSs than by dilutions (ionic) changes alone thereby shifting the frequency in which maximum change is observed by approximately a half decade. This would suggest localized conductivity changes may be isolated from physical changes in particles.

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