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A Graphene Oxide-interfaced Microfluidics System for Isolating and Capturing Circulating Tumor Cells and Microemboli

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***Abstract*—Circulating tumor cells (CTCs) and circulating tumor microemboli (CTM) are rare cell species present in peripheral blood and appear in circulatory system during cancer metastasis. As phenotype of single or aggregated CTCs can be different and may present different levels of potential aggressiveness, detecting and capturing both of them are crucial for preventing recurrence as well as achieving early-stage diagnosis. This research presents a microfluidics system aiming at isolating and highly sensitive capturing of CTCs and CTMs. A serpentine channel and a series of bifurcating micro-channels were use to separate CTCs and CTMs from other blood cells. A graphene oxide interface was patterned on glass slide to facilitate antibodies conjugation via click chemistry for capturing CTCs and CTMs, thus achieving multiplex detection in a high specificity and bio-compatibility manner.**

I. Introduction

Circulating tumor cells are tumor cells originated from tumor sites. They enter the blood stream after epithelial-mesenchymal transition (EMT) [1], one of the most important mechanism during cancer metastasis. In addition to CTCs, clusters of CTCs, which also known as circulating tumor microemboli (CTM), are recently discovered in cancer patients, and shows higher metastatic potential. CTM also play a crucial role in cancer metastasis and provide insights for tumor heterogeneity [2]. Studying CTCs and CTM could help understanding cancer biology and their value in diagnosis. However, CTCs and CTM detection are difficult due to their rarity. Only 1-10 CTCs in 10 mL blood [3], while CTMs is only 2-5% of CTCs [4]. Numerous of methods have been developed for isolating and detecting CTCs and CTMs. These methods could be mainly classified as physical properties based and affinity based.

Affinity based methods target cell surface markers by using antibodies against them. Epithelial Cell Adhesion Molecule (EpCAM) is the most commonly targeted surface marker due to their high expression in many cancers [5]. However, due to tumor heterogeneity, tumor cells show different levels of expression in different stages. For example, after cancer cells undergo EMT, their phenotype would alter from epithelial-like to mesenchymal-like and tend to have lower EpCAM expression. Targeting multiple cell surface biomarkers could enhance CTCs and CTM detection [6]. Graphene oxide (GO) is a nanomaterial which has shown its ability in enhancing selectivity of detection through surface functionalization with its rich carbon and oxygen framework. Due to it bio-compatibility and inexpensive production, it has been widely implemented for biomolecule detection, such as cell capturing from whole blood [7].

Physical properties-based microfluidics techniques for cell separation have been utilized in many biological assays for upstream sample preparation [8]. Separation of plasmas and leukocyte from whole blood had been demonstrated based on Zweifach-Fung effect [9] and leukocyte margination [10] without affect properties of cells. Inertial particles focusing [11] has been used in high-throughput cell separation based on their size without incorporating external forces.

In this work, we aim at multiplex detection for CTCs and CTM. We developed a graphene oxide-interfaced two-stage microfluidics system for cell isolation and cell capture. Microfluidics structures were designed to isolate CTCs and CTMs from other blood components in Stage I; while Stage II was designed for affinity-based cell capture on a GO patterned interface, targeting both EpCAM and Vimentin on cell surface.

II. Methods and Materials

A. Graphene oxide interfaced microfluidics device

1) Microfluidics system design and fabrication

A two-stage microfluidic system was designed for CTCs and CTMs isolation and detection (Fig 1a), soft lithography was used to create a single layer of microchannels on a silicon wafer as a mold. Microfluidics channels were created with polydimethylsiloxane (PDMS). PDMS was poured onto the mold at a ratio of 10:1 and was placed in a vacuum chamber to eliminate air voids and baked at 60 °C for 1.5 hours. Cured PMDS layer was peeled off from the mold. A biopsy punch (1.0 mm) was used to create holes for inlet and outlet.

2) Graphene oxide coating and patterning

Graphene oxide was coated on specific area on a glass slide (Fig. 1b). Polyimide (PI) silicone adhesive tape was used to isolate regions for GO patterning (area shaded in green in Fig. 1a). GO was coated as previously described [7]. In brief,

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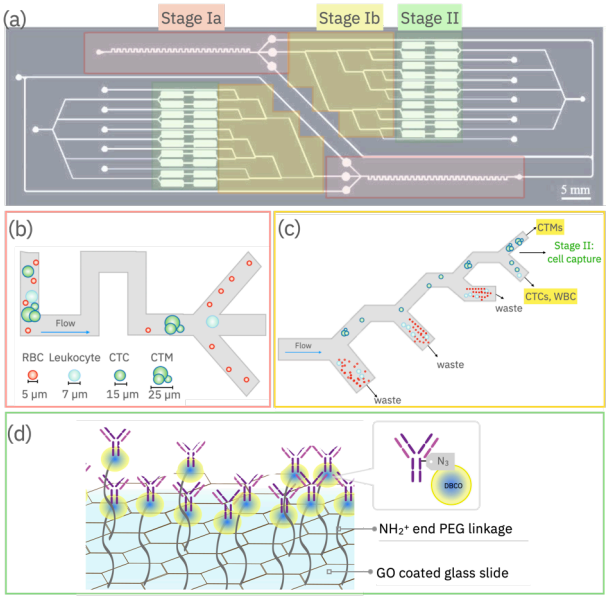

Figure 1. (a) Design of a two-stage microfluidics system for circulating tumor cells (CTCs) and circulating tumor mircoemboli (CTM) isolation (Stage 1) and capture (Stage 2). (b) Illustration of cell separation in Stage Ia along a serpentine microchannel. (c) Illustration of cell separation in Stage Ib using a branching structure. (d) Illustration of cell capture on a graphene oxide pattern surface.

cleaned glass slides were immersed in 3% 3-aminopropyltriethoxysilane (APTES) in toluene for 30 minutes. Followed by washing with 95% alcohol and deionized water and dried by nitrogen gas. Amine-functionalized glass slides were immersed in GO solution (1 mg/mL) for 1 hour on a rocker for gentle shaking to ensure uniform coating.

3) Bonding and device operation

Channels were enclosed by bonding diced PDMS to a GO-patterned glass slide via treating with oxygen plasma in a plasma cleaner. The microfluidic system is powered by a syringe pump via sanitary silicone tubing connection.

4) Antibodies modification - Azide group attachment on heavy chain

Per-Cy5.5 anti-human CD326 (EpCAM) (Biolegend Inc) and Cell-Surface Vimentin (CSV) Monoclonal Antibody (Abnova Co.) were used for cells capturing. Each of them was modified separately with an identical approach. By using SiteClickTM Antibody Labeling Kits (ThermoFisher). Modified antibodies were then purified and stored in 4°C.

5) Antibodies conjugation on GO-coated area via click chemistry

1-ethyl-3-[3-dimethylaminopropyl] carbodiimide)(EDC)/ N-hydroxysulfosuccinimide (NHS) chemistry was used to activate COOH- group on coated GO surface. A PEG linker, Di-benzocyclooctyne-polyethylene glycol-amine (DBCO-PEG4-amine) (BroadPharm©) was covalently bonded to the surface after reacting for 6 hours in room temperature. Azide-attached antibodies from the previous step were loaded into the channels (area shaded in green in Fig. 1a). DBCO conjugated with azide-labeled antibodies via click chemistry, hence antibodies were immobilized on the GO-coated regions (Fig 1d).

B. Device characterization

Scanning electron microscope (SEM) and X-ray photoemission spectroscopy (XPS) were used to confirmed GO was coated on the substrate. Atomic force microscopy (AFM) was used ensure the success conjugation of antibodies by detecting the reaction of the probe to the forces that the sample imposes on it, the surface topography was displayed as a pseudo color plot.

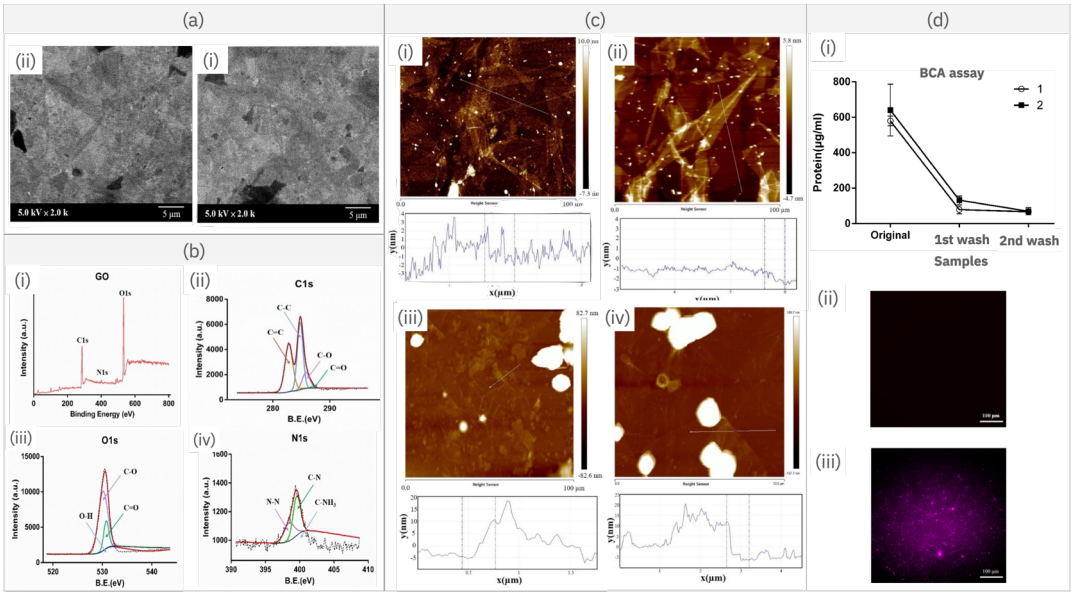

Figure 2. (a) SEM images of graphene oxide coated surface. Nano-layered structure was indicated by flaky texture capture. (b)(i) XPS spectra of graphene oxide. (ii-iv) XPS spectrum for C_{1s}, O_{1s} and N_{1s}. (c) AFM height images and the respective height profile for GO substrate before (i, ii) and after (iii, iv) antibody conjugation. (d) (i)Result of BSA assay, showing decrease in antibodies concentration after conjugation. (ii) Fluorescent image of antibody functionalized on GO coated surface. No aggregation of fluorescent signal suggest antibodies were evenly distributed.

A BCA Protein Assay Kit (Thermofisher) was used to quantify the amount of antibodies conjugated on the chip. Working reagents (WR) was prepared by mixing 50:1 Reagent A:B. Sample was then mixed with WR in 1:20 (v:v) and at 60°C for 30 minutes. After cooling to room temperature, absorbance of all the samples were measure with the spectrophotometer (NanoPhotometer NP80) at 562 nm.

C. Cell line and cell culture

1) Cell Culture

Murine lymphoma cell line (A20) cells were routinely maintained in suspension culture with RPMI1640 (Gibco) medium supplemented with 10% fetal bovine serum (FBS). Human carcinoma epithelial-like cells, A549-VIM cell line (ATCC® CCL-185EMT™) were cultured with F-12k cell culture medium (Gibco) and 20% fetal bovine serum (FBS, Sigma-Aldrich). All cells were maintained at 37°C in a humidified incubator with 5% CO_2.

2) Cell clusters culture

A549 cell line were cultured according to condition as mentioned above before being plated in 96-well Ultra Low Attachment Microplate (Corning). 40-100,000 cells were seeded in 100 µL of medium per well. Cell were further cultured for 24-72 hours until desired size of clusters formed.

3) Cell staining

To observe and distinguish different cells under fluorescent microscope during cell spiking test, A20 cells were stained by using Green Cell TrackerTM fluorescent probe (ThermoFisher), A549 cells were stained by using Red Long-Term Cell Tracer (ENZO life science) and Hoechst 33342(BD Biosciences according to the suggested protocols.

D. Cells spiking test

1) Cell spiking in glycerol

To mimic the viscosity of blood sample, 22% (w/v) glycerol in PBS was used. Stained A549 cell clusters, A549 and A20 were spiked in glycerol solution at concentration of 100 clusters/mL, 3333 cells/mL and $1x10^6$ cells/mL respectively.

2) Cell spiking in healthy human whole blood

Healthy donor's blood was collected in a EDTA tube and stored at 4°C upon usage. Stained A549 cell clusters, A549 and A20 were spiked in blood sample at concentration as above mentioned. After samples flowed to cell capturing regions (Stage II), chips were washed with PBS trice after 60 minuets incubation. Pre-stained cells were counted under microscope.

III. Results and Discussions

A. Device characterization

1) Graphene oxide coating characterization using SEM

To elucidate the efficiency of GO modification on the substrate, we used SEM analysis to observe the surface morphology. GO coated substrates from different batches were shown Fig. 2a(i) and 2a(ii) . Flaky texture indicated the nano-layered structure of graphene oxide. Similar results were obtained from different batches, this suggests that our coating method was robust and able to obtain uniform results.

2) Graphene oxide coating characterization using XPS

To further examine the functional groups on substrate, from the elemental survey spectrum obtained from XPS (Fig 2b), the images mainly presented element contains C and O. Peaks were de-convoluted and fitted into single element spectrums, the chemical states of the element were determined by the change of binding energy. Strong peaks of C=C (284.5 eV), C-C (284.8 eV), C-O (286 eV) and C=O (288 eV) in C_{1s} spectrum (Fig. 2b(ii)), C=O (531.6 eV), C-O (532.4 eV) and O-H(533.5 eV) in O_{1s} spectrum (Fig. 2b(iii)) and pyridine in N_{1s} (Fig. 2b(iv)) suggested the existence of graphene oxide.

3) Validating antibodies conjugation using AFM

From AFM images and the respective height profiles, height difference between GO substrate before (Fig. 2c(i) and 2c(ii)) and after (Fig. 2c(iii) and 2c(iv)) antibody conjugation, average height increased from 1.195 nm to 15nm,

4) Validating antibodies conjugation by BSA protein assay

BSA assay was used to measure the antibodies concentrations before and after conjugation. Flow throughs after antibodies binding reaction had a concentration 10 times lower than its input, suggesting 90% of antibodies were conjugated on GO surface (Fig. 2d(i)). In addition, from the fluorescent image of antibodies conjugated surface (Fig. 2d(ii)), no aggregation of signal was observed. Indicating that antibodies were evenly distributed on the GO-coated surface.

B. Cell isolation

1) Size-based cell separation - Stage Ia

A serpentine micro-channel was used to separate particles with inertial microfluidics (Fig. 1b, 3a). Particles' migration in a serpentine channel was dominant by both Dean drag force and particle centrifugal force [11]. We used different lengths

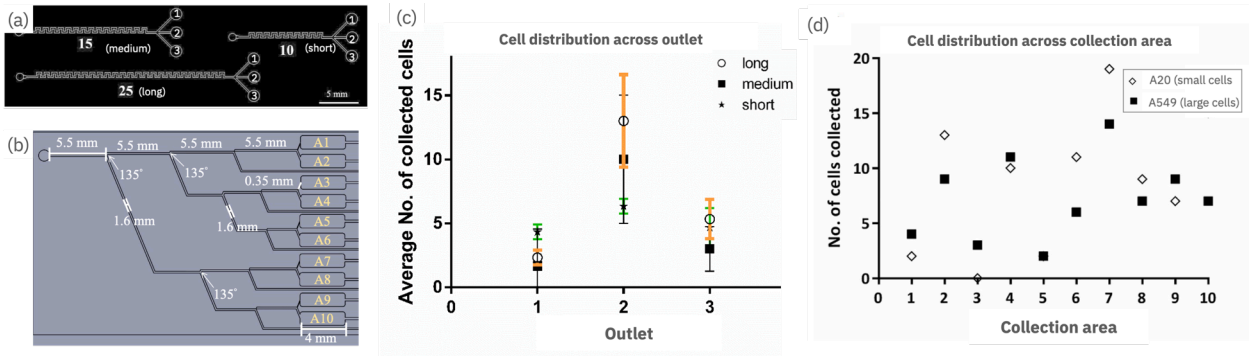

Figure 3. (a) Different length of serpentine channels. (b) Design of branching microfluidic channels. (c) Cell separation by using various lengths of serpentine channels. (d) Cell separation after Stage Ib, larger cells were found to flow along the slower channel and enter collection area: 1, 3, 5, 7, 9.

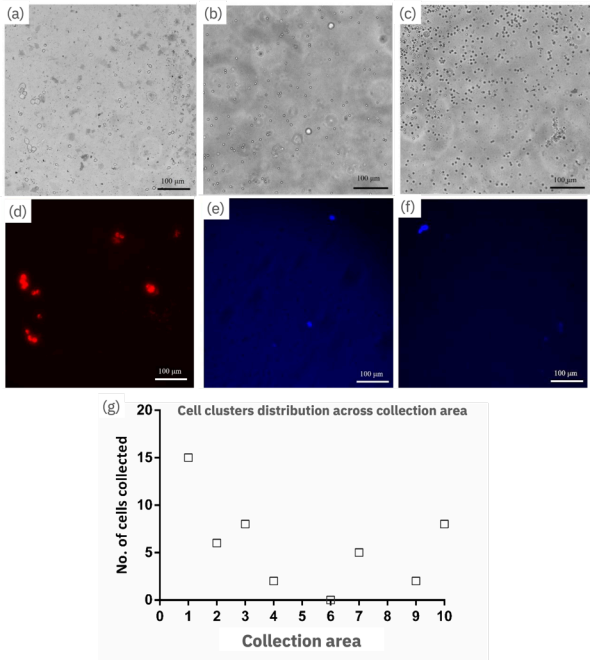

Figure 4. Bright field microscopy and fluorescent image for cell clusters captured from spiking in (a, d) glycerol solution, (b, e) and (c, f) healthy human blood sample. (g) Clusters count in each region

(10, 15, 25) of the serpentine channel (Fig. 3a) to test separation efficiency. Using cells spiking in glycerol solution as testing sample, at 33 μL/min, we observed the longer the serpentine channel, the better the separation performance (Fig. 3c). Number of larger cells collected from outlet 2 was higher than outlet 1 and 3, this shows that hydrodynamic forces focused larger particles at the center of the channel and successfully.

2) Size-based cell separation - Stage Ib

Stage Ib: Structure was designed based on bifurcation law, which also known as Zweifach-Fung effect [9] and leukocyte margination [10]. When red blood cells flow through a bifurcating region, they tend to travel into vessel with higher flow rate. Flow rate was adjusted by tuning the ratio of the width of the channels, we used 4:1 channel width to separate plasma and other blood cells. In addition, the larger the cells, the slower they flow than the rest of the blood, thus they are more likely to be pushed towards to walls of the channel and tend to travel along the slower channels. By implementing these theories, we designed a branching system for enriching CTMs and CTCs. Larger cells traveled along the channels and reached designated regions, A1, A3, A5, A7, A9 (Fig. 3b). In experiment using cell spiking in glycerol solution, by counting the number of cells in each region under fluorescent microscope, higher number of large cells were found in most of the designated regions (Fig. 3d).

C. Cell capture

1) Cell spiking in glycerol solution

A549 cell clusters (stained with red tracer) were captured by antibodies on GO-coated area (Fig. 4a, d). Sample was also tested in a chip without antibodies (only GO-coating), results showed that none of A549 cells or A549 cell clusters were captured on the surface. This shows low false positive rate in our system.

2) Cell spiking in healthy human blood sample

Cell clusters (stained with Hoechst) were capture in designated collection area (Fig. 4e, f). 65% of cell clusters were collected in designated area (Fig. 4g). However, after capturing cells from the spiking blood sample, small amount of other blood components (RBC, WBC) remained in the cell capture regions (Fig. 4b, c). This could be further improved by incorporating surface blocking techniques.

IV. Conclusion

In this work, we developed a microfluidics system for isolating and capturing CTCs and CTMs by integrating hydrodynamics mechanisms and graphene oxide interface. Targeted cells and cell clusters were captured and detected in designated regions. In addition, we also demonstrated a 90% efficiency antibody conjugation method on GO surface.

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