An integrated multi-molecular sensor for simultaneous *BRAF*^{V600E} protein and DNA single point mutation detection in circulating tumour cells

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Abstract

The analysis of circulating cancer biomarkers in the form of liquid biopsies confers several potential benefits as compared to traditional surgical tissue sampling. As a common key anomaly strongly implicated across several cancer types, the *BRAF*^{V600E} mutation is one the most valuable oncogenic biomarkers available in liquid biopsies. Crucially, *BRAF*^{V600E} is also an actionable mutation which could be arrested by clinically beneficial drug inhibitors. Yet, as is true for most single base disease mutations, current *BRAF*^{V600E} detection in either its DNA or protein molecular state is still liable to false positive/negative outcomes, thus impacting patient treatment benefit. Here we present an integrated multi-molecular sensor (IMMS) for an entire sample-to-answer workflow from melanoma cell capture to simultaneous quantification of both intracellular *BRAF*^{V600E} DNA and protein levels on a single platform. The IMMS combines (i) specific capturing and release of circulating melanoma cells; (ii) electric field-induced cell lysis; (iii) simultaneous quantification of *BRAF*^{V600E} DNA and protein levels. We investigated the IMMS system's analytical performance in cell capture, release and lysis; and intracellular *BRAF*^{V600E} detection by ligase-mediated DNA amplification and antibody-based protein hybridization. As a proof-of-concept, we successfully demonstrated circulating *BRAF*^{V600E} detection at both DNA and protein molecular levels in simulated melanoma plasma samples. With its capabilities in integrated and miniaturized analysis, the IMMS could lead the emergence of a new generation of multi-molecular lab-on-chip biosensors for enabling more accurate and extensive analysis of powerful circulating biomarkers in patient liquid biopsies.

Introduction

Liquid biopsies refer to the non-invasive analysis of cancer mutations, often utilizing circulating tumor cells (CTCs) and circulating tumor (ct)DNA in patient biofluid samples such as blood, urine or saliva.[1, 2] The main motivation of using liquid biopsies is the potential to overcome shortcomings associated with tissue biopsy, such as i) surgical invasiveness; ii) limited small area evaluation of tumor at a single timepoint; iii) failure to represent complex, heterogeneous, and constantly-evolving tumor microenvironment. Most importantly, several recent groundbreaking studies have demonstrated the feasibility of using liquid biopsies as a possible tissue surrogate for detecting notable oncogenic aberrations, including circulating single point mutations in *BRAF*.[1, 3-6]

BRAF encodes for a Raf kinase protein that is involved in cell growth signalling, and has been shown to be frequently mutated in cancers, including malignant melanoma, colorectal cancer, and non-small-cell lung carcinoma.[7, 8] The majority of *BRAF* single point mutations consist of a single T→A base change at nucleotide 1799, resulting in an amino acid change of valine to glutamate in the encoded protein (now termed as "V600E"). The *BRAF*^{V600E} mutation is a valuable prognostic and predictive biomarker that is presently implemented in the clinic for late-stage malignant melanoma. Critically, *BRAF*^{V600E} is also a clinically-actionable aberration with the administration of *BRAF* inhibitors such as FDA-approved drugs: verufenib and debrafenib.[9, 10] Therefore, molecular analysis of circulating *BRAF*^{V600E} in liquid biopsies is of great clinical interest in both non-invasive cancer screening and drug response monitoring.

Over the past years, there have been a variety of developed techniques for molecular *BRAF*^{V600E} analysis in DNA or protein forms.[11-13] For molecular *BRAF*^{V600E} detection on the DNA level, a variety of techniques including Sanger sequencing, pyrosequencing, and PCR-based assays have been demonstrated with exceptional detection sensitivity. Yet, as is true for all single base disease mutations, DNA-based *BRAF*^{V600E} detection faces poor detection specificity due to cross-reactivity with highly similar wildtype/other V600 mutation sequences.[14] More recently, the emergence of mutation-specific monoclonal antibodies has enabled *BRAF*^{V600E} detection on the protein level. Although showing 100% detection specificity, protein-based *BRAF*^{V600E} analysis is still liable to false negatives due to insufficient sensitivity. Hence, this has led to a potential concept of combining protein- and DNA-based *BRAF*^{V600E} detection,[15] in order to increase the accuracy of *BRAF*^{V600E} testing with both superior detection sensitivity and specificity. Ideally, this could serve as a useful cross-verification tool for circulating *BRAF*^{V600E} status in individual patients, especially if combined DNA and protein detection are achievable simultaneously at reduced assay time. To address

this need, we were thus motivated to develop a single biosensing platform which is capable of the difficult task of simultaneously detecting both circulating *BRAF*^{V600E} DNA and protein in liquid biopsies.

Microfluidic biosensors are ideal for merging both DNA and protein detection techniques due to the possibility of miniaturizing laboratory-based bioassays onto a single device platform.[16] Apart from the distinct advantages of minimal liquid sample requirement, rapid bioassay analysis, and potential point-of-care use; microfluidic biosensors also enable integration of all sample preparation, target amplification, and detection readout to realize lab-on-a-chip applications. Microfluidic sensors are therefore an attractive option for conceiving an integrated platform capable of combined *BRAF*^{V600E} DNA and protein analysis for potential clinical liquid biopsy utilization.

Herein, we developed a novel integrated multi-molecular sensor (IMMS) for the combined analysis of *BRAF*^{V600E} in melanoma cells at both DNA and protein levels. Specifically, the IMMS platform consists of individual microfluidic zones with customized electrode patterns in microchambers for i) specific capturing and release of melanoma cells via melanoma-associated chondroitin sulfate proteoglycan (MCSP) expression; ii) electric field-induced cell lysis; iii) selective ligase-mediated amplification and antibody-based hybridization of intracellular *BRAF*^{V600E} DNA and protein targets respectively; and iv) eventual electrode-based electrochemical readouts of *BRAF*^{V600E} DNA and protein levels. It is envisaged our described IMMS platform could allow more reliable circulating biomarker testing in liquid biopsies by interrogating the same mutation status in differing DNA/protein molecular forms.

Materials and methods

Materials

All reagents (Sigma Aldrich, Australia) were of analytical grade and used without further purification unless otherwise stated. UltraPure^{TM} DNase/RNase-free distilled water (Invitrogen, Australia) was used throughout the experiments. Oligonucleotides (Integrated DNA Technologies, Singapore) sequences used in this work are shown in Table S1.

IMMS design and fabrication

The IMMS design consists of cell capture and release; lysis; and combined detection microfluidic zones. The zone for capture and release has a long serpentine microchannel with an array of 264 parallel electrode pairs (electrode size: 100 μm (d_1) and 400 μm (d_2), distance between electrodes within pairs: 50 μm and between pairs: 150 μm) along the channel length.

The lysis zone contains a pair of square symmetric electrodes (2 mm) within a proximity of 3 mm.

The combined DNA and protein detection zone contains three sets of circular working (1000 μm in diameter) and 120 μm thick ring electrode pairs. Both circular and ring electrodes are connected separately to a pair of common connection pads, and the inner circular and ring electrode are separated by 1000 μm.

The IMMS was fabricated following standard photolithography procedure at Australian National Fabrication Facility-Queensland Node. Initially, the IMMS design was prepared using L-Edit V15 (Tanner Research, USA) and translated to photomask by direct laser writer. Then the design in the photomask UV exposed to a thin AZ nLOF 2070 negative photoresist (MicroChemicals, Germany) coated silicon wafer. After hard baking at 110 ºC for 2 min, design was revealed by dipping the wafer in AZ 726 developer (MicroChemicals, Germany) for 2 min. The wafers were then loaded to a Temescal FC-2000 electron beam evaporator (Ferrotec, USA) machine for the layer by layer deposition of Ti (10 nm) and Au (200 nm) and after exposure, left in ethanol overnight for lift-off.

The photomask for microfluidic channel, lysis and protein detection chamber was UV exposed at 380 mJ/cm^2 to a silicon wafer coated with 1 mm negative SU-8 215 photoresist layer and hard baked on hot plate (from 65 °C for 5 min, 95 °C for 20 min, 65 °C for 3 min). Following that, wafers were kept in propylene glycol methyl ether acetate (PGMEA) solution for 45 min and then cleaned with 2-isopropanol solution to reveal the structure. After that, prepolymer was mixed with its cross-linker Sylgard 184 (Dow Corning, USA) in a ratio of 10:1, and poured on the newly prepared SU8 master mould for incubation at 65 ˚C for 1 h. An individual PDMS layer was then placed on the gold-patterned silicon wafer to complete the IMMS fabrication.

Cell culture

SK-MEL-28, SK-MEL-35, SKBR3 and MD-MBA-231 were cultured in RPMI 1640 growth media supplemented with fetal bovine serum (10%), glutamax (1%) and penicillin/streptomycin (Thermo Fisher Scientific, Australia) in a 37° C, 5% CO_2 incubator. Culture media was changed every third day and cells were harvested upon 80% confluences in the culture flask. Prior to imaging experiments, the membranes of harvested cells were stained by incubating with DiO (3,3'-Dioctadecyloxacarbocyanine Perchlorate) fluorescence dye (Invitrogen, UK) at 37 ˚C for 10 min (5 μL of DiO dye for 100 000 cells/sample). Post-staining,

labeled cells were washed three times with phosphate buffer saline (PBS) by centrifuging at 300 *g* for 5 min and resuspended in 1 mM PBS solution.

IMMS cell capture, release, and lysis

The microchannel of the capture and release microfluidic zone was modified with anti-MCSP antibodies following layer by layer functionalization processes. For antibody functionalization, 250 µL of 200 µg/mL of biotinylated bovine serum albumin (Thermo Fisher Scientific, Australia) was added to the bare surface of the microchannel and incubated for 2 h at 37 ˚C. Then, the surface modified microchannel was washed gently to remove unbound molecules and subsequently incubated for another 1 h at 37 ˚C with a solution of 100 µg/mL streptavidin in PBS, Invitrogen). In a final step, biotinylated anti-MCSP antibodies (Invitrogen, Australia) were hybridized onto the streptavidin coated surface by incubation for 1 h at 37 ˚C. A signal generator (Agilent Technologies 33510B, USA) was used for all subsequent on-chip workflow steps which required current field generation.

For general cell capture experiments, designated number of SK-MEL-28 cells were prepared in 1 mM PBS to 200 µL and flown through the anti-MCSP functionalized microchannel using an optimal alternating current (ac) field[17] (frequency (*f*) = 600 Hz, amplitude (V_{pp}) = 100 mV). For cell capture experiments using simulated patient plasma samples, designated number of target cells were reconstituted in healthy human plasma (200 µL) and driven through anti-MCSP functionalized device under optimal ac-EHD field (*f* = 600 Hz, V_{pp} = 100 mV). Then, the microchannel was gently washed with 1 mM PBS to remove unbound and/or non-specifically adsorbed molecules.

For fluorescence cell imaging and enumeration experiments, captured cells were initially fixed by filling the microchannel with cold methanol for 10 min, and then permeabilized by filling the microchannel with 0.2% Triton X-100 in PBS for 10 min. After permeabilization, fixed SK-MEL-28 cells were nuclear-stained with 4′,6-diamidino-2-phenylindole (DAPI) solution for 15 min. Following this step, the microchannel was gently washed with 1 mM PBS solution to remove excess DAPI solution. Finally, DiO- and DAPI-stained captured cells were imaged and enumerated with a multichannel fluorescence microscope (Nikon Ti−U, USA). Captured melanoma cells were then released using a direct current (dc) field as previously described.[17]

For cell lysis and release of intracellular contents, captured and released melanoma cells were transferred to the lysis microfluidic zone. The application of dc electric potential produced a strong electric field required to disrupt the transmembrane of cells for lysis.[18, 19] Under an

optimal dc potential of 5 V as latterly described, cells were lyzed and their internal molecular contents were released for downstream *BRAF*^{V600E} DNA and protein analysis.

IMMS *BRAF*^{V600E} DNA detection

The detection of *BRAF*^{V600E} DNA on the IMMS was started in a ligation microchamber of the detection microfluidic zone. An aliquot of cell lysate from the lysis microfluidic zone was first magnetically purified for nucleic acids within the microchamber by using the Agencourt AMPure XP SPRI kit (Beckman Coulter, USA) according to manufacturer's instructions and placing a permanent magnet below the microchamber.

The isolated nucleic acids were resuspended in 5 µL RNase-free water, and then transferred into the ligation microchamber to make up a 10 µL ligation mixture containing 100 nM of each ligation probe (each with unique primer recognition site), 40 U T4 DNA Ligase (New England Biolabs, Australia) and 1x T4 DNA Ligase Buffer. The mixture was heated to 95 °C for 3 min by placing the IMMS on a heating block, and then left to cool down to room temperature for 15 min to facilitate hybridization and ligation[20] under alternating current-electrohydrodynamic (ac-EHD) fluidic mixing (f = 500 Hz, V_{pp} = 800 mV).

Before isothermal recombinase polymerase amplification (RPA)[16, 21] on magnetic bead surfaces, primer functionalized magnetic beads were prepared beforehand. 10 µL of streptavidin-labeled magnetic beads (New England Biolabs, Australia) was first washed with 2× washing and binding (B&W) buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA; 2 M NaCl) and resuspended in 25 µL of 2x B&W buffer. Then, the resuspended magnetic bead surfaces were functionalized by thorough mixing and incubation with 5 µM biotinylated forward primer sequences (Table S1) on a mixer for 30 min at room temperature. After surface functionalization, excess unbound primers were removed by magnetic washing with 2x B&W buffer thrice, and functionalized magnetic beads were resuspended in 5 µL RNase-free water.

For solid-phase RPA in the amplification microchamber, the TwistAmp® Liquid Basic kit (Twist-DX, UK) was used with slight modifications to manufacturer's instructions. Briefly, 12.5 µL of 2x Reaction Buffer, 1.8 mM dNTPs, 20 nM biotinylated dUTPs (Thermo Fisher Scientific, Australia), 2.5 µL 10x Basic E-mix, and 500 nM of reverse primers (Table S1) were added to the amplification microchamber, and mixed via ac-EHD for 30 s to make a pre-master mix volume of 22 µL. Next, 1.25 20x Core Reaction Mix was added and mixed via ac-EHD for 30 s; before 5 µL of pre-functionalized magnetic beads, 1.25 µL 280mM MgOAc, and 1 µL ligation mixture (from the ligation microchamber) were added to the amplification microchamber for incubation at 43°C for 15 min. During solid-phase RPA, ac-EHD fluidic

mixing was induced through application of an ac field (f = 800 Hz and Vpp = 800 mV) to enhance molecular reaction kinetics.

Post-amplification, the magnetic beads were washed with 2x B&W buffer as described before, incubated with 1 µL 1:1000 diluted streptavidin-horse radish peroxidase (HRP) in 10 mM PBS with 0.5% triton-X for 5 min to label bead surface-bound amplicons, and washed again before being magnetically loaded onto the working electrode surface of the microchamber. For amperometric measurements, 15 µL of 1-Step™ TMB (3,3',5,5'-Tetramethylbenzidine) solution (Thermo Fisher Scientific, Australia) was added to the surface-loaded magnetic beads before 500 mM H_2SO_4 was added after 5 min to stop the reaction and activate TMB for measurements. Amperometric measurements were carried out using a CHI650D electrochemical workstation (CH Instruments, USA) at 150 mV, 30 s. All measurements were performed at room temperature.

IMMS *BRAF*^{V600E} protein detection

Prior to the electrochemical *BRAF*^{V600E} protein measurements, the gold electrode in the protein detection domain of the IMMS was functionalized with *BRAF*^{V600E} antibodies using biotin-streptavidin chemistry (Fig. S1). In brief, the following solutions were incubated in the following order: 2 ng/mL biotinylated bovine serum albumin (Pierce Biotechnology, USA) for 2 h, 1 ng/mL streptavidin (Sigma Aldrich, Australia) for 1 h, 0.1 µM 6-mercapto-1-hexanol (Sigma Aldrich, Australia) for 10 min, and 0.1 ng/mL monoclonal *BRAF*^{V600E} ab200535 (Abcam, Australia) for 1 h. To remove excess reagents, the electrode was washed after each incubation step with 10 mM PBS.

After functionalization, an aliquot of cell lysate (diluted to the required concentration in 10 mM PBS) from the lysis microfluidic zone was transferred onto the functionalized electrode and subjected to ac-EHD fluidic mixing frequency (f = 500 Hz, V_{pp} = 800 mV) for 3 min to promote the movement of protein molecules to the electrode surface as reported previously.[22] After fluidic mixing, the electrode was washed with PBS and DPV measurements were performed using ferri/ferrocyanide redox couple (i.e., 2.5 mM $[Fe(CN)_6]^{3-}$ / $[Fe(CN)_6]^{4-}$ (1:1) and 0.1 M KCl in 10 mM phosphate buffered saline at pH 7.4) with a CHI650D electrochemical workstation (CH Instruments, USA). The DPV measurements were recorded from -1.0-1.0 V with a pulse amplitude of 50 mV, a pulse width of 50 ms, a potential step of 5 mV, and a pulse period of 100 ms. The peak intensity of the DPV signal before (i_{before}) and after (i_{after}) target incubation was used to calculate the relative current change ($\%i_r$) as given by $\%i_r = [(i_{before} - i_{after})/i_{before}] \times 100\%$.

Results and discussion

IMMS platform for combined *BRAF*^{V600E} DNA and protein analysis

The IMMS platform has three independent microfluidic zones for 1) cell capture and release; 2) cell lysis to release intracellular content; and 3) combined detection of *BRAF*^{V600E} DNA and protein targets (Fig. 1). Previously, our research group has described the use of asymmetrical electrode design on microfluidic devices to generate an ac-EHD fluid flow for diverse biomolecular applications.[22-25] Here, we exploited various miniaturized electrode designs on our IMMS platform to manipulate fluidic movement of biomolecules at several steps along the IMMS workflow to enable on-chip sample-to-DNA/protein detection.

The cell capture and release microfluidic zone consists of a serpentine microchannel with an array of 264 parallel asymmetric gold electrode pairs. The electrodes are pre-functionalized with anti-MCSP antibodies for selective capture of *BRAF*^{V600E}-positive melanoma cells under an ac-EHD effect. This facilitates unidirectional sample flow through the functionalized microchannel without any external pump, and assists the removal of non-specifically adsorbed molecules (Fig. 1a). After cell capturing and washing, a reductive dc potential is applied across the electrode array to break anti-MCSP antibody-electrode surface thiol bonds. This dc potential releases the captured melanoma cells for transfer to the cell lysis zone.

The lysis zone has a pair of symmetric square electrodes to induce an electric field for lysis of released cells (Fig. 1b). The application of a dc electric potential across the square electrodes disrupt and rupture the cellular membranes, allowing intracellular contents (including nucleic acids and proteins) for downstream *BRAF*^{V600E} analysis. The combined *BRAF*^{V600E} detection zone consists of a series of microchambers with each containing an identical asymmetrical circular and ring electrode pair, and is designed to simultaneously detect *BRAF*^{V600E} DNA and protein targets from the intracellular contents of lyzed melanoma cells (Fig. 1c).

For *BRAF*^{V600E} DNA detection (Fig. 1c-i), an aliquot of cell lysate is first magnetically purified on the IMMS for nucleic acids. Then, *BRAF*^{V600E} ligation probes (with unique primer recognition sites) are added for complementary binding to purified *BRAF*^{V600E} DNA, and successfully bound probes are subsequently ligated by T4 DNA ligases. A fraction of ligated probes is then transferred to a separate amplification microchamber containing primer functionalized magnetic beads for solid-phase isothermal RPA. Importantly, we use the asymmetrical circular and ring electrode design on the IMMS for ac-EHD fluidic mixing to

increase molecular kinetics during both solution-based ligation and solid surface-based amplification processes. After amplification, bead surface-bound amplicons are tagged with streptavidin-HRP enzymes through randomly-inserted biotin-uracil bases during amplification.[26] The magnetic beads with bound amplicons are then washed and concentrated onto the electrode sensor surface by use of a permanent magnet. The addition of TMB chromogenic electron mediators gives rise to a HRP-catalyzed amperometric current signal to reflect initial *BRAF*^{V600E} DNA quantity.

For *BRAF*^{V600E} protein detection (Fig. 1c-ii), we introduce cell lysate onto a electrode surface which is pre-functionalized with anti-*BRAF*^{V600E} capture antibodies. We monitored the stepwise functionalization using differential pulse voltammetry (DPV) whereby each successful stepwise functionalization resulted in a further decrease in DPV current (Fig. S1) due to increased retardation of redox reaction on the electrode surface. For target capture, ac-EHD fluidic mixing is likewise performed to promote *BRAF*^{V600E} protein target movement towards the sensor surface. After target capture and washing, a $[Fe(CN)_6]^{3-/4-}$ redox system is used to generate a detectable Faradaic current signal that corresponds to initial *BRAF*^{V600E} protein level.

IMMS melanoma cell capture and release

A key aspect of the IMMS is its capacity to perform the entire sample-to-answer workflow on a single platform. This process starts with the specific capture of melanoma cells for downstream *BRAF*^{V600E} DNA and protein analysis. We first tested the melanoma cell capture efficiency on the IMMS by flowing various PBS-diluted amounts of SK-MEL-28 melanoma cells (50, 100, 250, 500, 1000 cells) through the anti-MCSP functionalized microchannel under an ac-EHD-driven flow, and enumerated the DiO- and DAPI-stained captured cells via fluorescence microscopy (Fig. 2a). Under the applied electric field, the average capture efficiencies for SK-MEL-28 cells (Fig. S2) were consistent for different cell count: 82.0±3.46% (50 cells), 81.0±1.73% (100 cells), 82.6±0.93% (250 cells), 81.6±1.58% (500 cells) and 85.4±0.83% (1000 cells). Next, to replicate cellular heterogeneity in patient blood samples, we mixed SK-MEL-28 target cells with a high background of 10^6 peripheral blood mononuclear cells (PBMCs) to further evaluate capture efficiency in a biologically-complex environment. For these mixed samples, we noticed an expected slight ~10% decrease in capture efficiency which was consistent for different amounts of SK-MEL-28 cells: 72.66±1.76% (50 cells), 70.33±1.45% (100 cells), 71.20±0.61% (250 cells), 71.66±0.81% (500 cells) and 74.7±0.61% (1000 cells) (Fig. 2b). This level of capture efficiency is similar to our previous report on

specific capturing of breast cancer cells.[17] Hence, we demonstrated consistent capturing efficiency at differing amount of melanoma cells. CTC counts in blood could range from 10-100 cells/mL,[27] we thus reasoned that our proposed IMMS could be feasible for liquid biopsy applications by relying on a rapid external pump-free ac-EHD-driven flow through the microchannel to capture a clinically-relevant amount of cancer cells.

Next, we investigated the capture specificity in the anti-MCSP functionalized microchannel by driving 10^6 pre-stained MCSP-negative breast cancer cells (SKBR-3 and MD-MBA-231) through the anti-MCSP functionalized microchannel. We observed low capture rate (Fig. 2c) for both non-specific cell lines to verify minimal nonspecific cell adsorption within the microchannel.

It is essential to release captured melanoma cells for downstream cell lysis and analysis of intracellular contents on the IMMS. To evaluate the release efficiency of captured cells through the application of an applied dc potential, we quantified released cells for comparison against starting cell count. A consistent release efficiency of 89.38±0.48% was established for each different starting cell amount (Fig. 2d) to demonstrate substantial cell release performance for downstream analysis.

To further investigate the capability of IMMS for isolating target cells in liquid biopsies, the capture and release efficiency in diluted blood was studied. We titrated 100 SK-MEL-28 cells in diluted human blood (i.e. 10-fold dilution in 1 mM PBS) and performed capture experiments with and without ac-EHD (pressure driven flow, flow rate = 8.3 µL min-1). Under ac-EHD conditions (f = 600 Hz and Vpp = 100 mV), the average capture efficiency was recorded at 69±1.73% (Fig. S3) as similar to cell capture experiments from simulated plasma samples and approximately 18% higher than cell capture efficiency from the blood sample without ac-EHD. We also tested release efficiency of the isolated cells under optimized DC field and found similar cell retrieval efficiency (approximately 89% for cells isolated from diluted blood sample). These data demonstrate that IMMS is potentially suitable for cellular and molecular detection from complex heterogeneous samples.

IMMS cell lysis optimization

Efficient cell lysis requires the generation of an optimal electric field for rupturing the cell membrane. An insufficient dc potential cannot disrupt the cell membrane bilayer effectively for lysis, and an excessive electric potential can damage the electrodes in use.[18, 19] We optimized the applied potential on the IMMS platform for efficient lysis of captured and released SK-MEL-28 cells. To evaluate the optimal applied dc potential, we first engaged a

range of dc potential from 3-5 V for 6 min, and observed the number of unlyzed cells through the fluorescence microscope (Fig. 3a). At 3V, cell lysis efficiency was poor with only 40.63±0.96% of lyzed but improved to 93.36±0.59% with increased applied potential up to 5 V. Beyond 5 V, cell lysis efficiency did not show significant increase and the resultant high current started to damage the cell lysis electrodes on the IMMS. We thus opted to use 5 V for the ensuing optimization of electric field duration. We found that 4 min of applied dc potential led to 93.23±1.16% of lyzed cells in the lysis zone (Fig. 3b), and no advancement in lysis efficiency was noted with longer durations. Therefore we chose to apply a dc potential of 5V potential for 4 min for efficient electrical lysis (Fig. 3c) of captured and released melanoma cells.

IMMS detection specificity

Specific *BRAF*^{V600E} detection is crucial for accurately informing mutation status and subsequent clinical decisions. To ensure high accuracy of combined *BRAF*^{V600E} DNA and protein detection on the IMMS platform, we evaluated the detection specificities of probes and antibodies on cultured cells with known *BRAF*^{V600E} expression status: two melanoma (*BRAF*^{V600E}-positive SK-MEL-28 and *BRAF*^{V600E}-negative LM-MEL-35) and one breast cancer (*BRAF*^{V600E}-negative SKBR3) cell lines. Each cell line underwent capture, release and lysis procedures on the IMMS platform before integrated downstream *BRAF*^{V600E} analysis.

On the DNA level, *BRAF*^{V600E} detection is often compounded by specificity issues as oligonucleotide primers/probes tend to cross-react with wildtype/other V600 mutation DNA in the background. To improve *BRAF*^{V600E} DNA detection specificity, we utilized the IMMS to perform a T4 DNA ligase-mediated ligation of *BRAF*^{V600E}-specific probes prior to isothermal solid-phase RPA of the ligated probes on magnetic beads. This dual target-specific ligation and RPA steps ensured selective *BRAF*^{V600E} detection (Fig. 4a) as T4 DNA ligase confers the ability of single base discrimination to ligate probes in target presence, whilst RPA occurs only for successfully ligated probes.[20] It is noteworthy that both ligation and RPA molecular kinetics were enhanced by ac-EHD fluidic mixing instead of relying on slow diffusion kinetics.[24] We demonstrated enhanced detection specificity toward *BRAF*^{V600E}-positive SK-MEL-28 with eight-fold higher amperometric signals (Fig. 4b) as compared to *BRAF*^{V600E}-negative LM-MEL-35 and SKBR3. These findings were successfully validated with previously-reported cell line *BRAF*^{V600E} characterization results,[28] and standard gel electrophoresis (data not shown) in which successful *BRAF*^{V600E} amplification was observed for SK-MEL-28 with *BRAF*^{V600E} PCR primers (Table S1).

On the protein level, we evaluated the *BRAF*^{V600E} detection specificity of the anti-*BRAF*^{V600E} capture antibodies used in our study. We first investigated the possibility of non-specific *BRAF*^{V600E} adsorption on the sensor surface by performing several control experiments in absence of anti-*BRAF*^{V600E} capture antibodies and *BRAF*^{V600E} protein targets, as well as in presence of non-target wildtype *BRAF* proteins and non-target anti-CD63 capture antibodies. The presence of *BRAF*^{V600E}-specific antibodies and targets generated a five-fold higher relative current change than the controls (Fig. 4c), thus demonstrating that high signal was achieved by specific capturing of target protein through sensor surface functionalized antibodies. Notably, the functionalized asymmetrical electrode format was also able to be engaged for ac-EHD fluidic mixing, thereby resolving the slow diffusion of protein targets towards the sensor surface. Furthermore, as similar to the DNA analysis, we also found that *BRAF*^{V600E} protein detection was highly specific for SK-MEL-28 and resulted in a four-fold relative current change in contrast to *BRAF*^{V600E}-negative cell lysates (Fig. 4d) which contained a high abundance of non-target cell debris. This suite of experiments showed that *BRAF*^{V600E} protein detection with excellent specificity was obtained through effective capture antibody-functionalized sensor surface on the IMMS platform.

IMMS detection sensitivity

BRAF^{V600E} DNA detection requires high detection sensitivity for early disease detection and need to discriminate low copy amounts of *BRAF*^{V600E} in presence of high wildtype background. We opted to use a magnetic bead-based enrichment strategy to load amplicons carrying HRP molecules onto the electrode surface and concentrate the amperometric signal sources for enhanced sensitivity. To evaluate detection sensitivity on the DNA level, we prepared a dilution series (0-100%) of *BRAF*^{V600E} sequences in a background of wildtype *BRAF* sequences (10 000 copies in total) (Fig. 5a). To simulate the biological complexity of patient-derived samples, we further included 5 ng of salmon sperm DNA into each preparation. With this experimental construct, we detected as low as 0.1% (10 copies) of mutant *BRAF*^{V600E} sequences (Fig. 5b).

To assess *BRAF*^{V600E} protein detection sensitivity, we first sought to investigate the sensitivity and dynamic range of *BRAF*^{V600E} protein in buffer to establish the conditions for subsequent cell lysate detection. The IMMS platform detected as low as 10 pg/mL of *BRAF*^{V600E} protein (Fig. 5c), and showed a dynamic response from 0-100 pg/mL. We then analyzed cellular lysate from a range of SK-MEL-28 cell concentration (0-100 cells/mL). A gradual increase in relative current change associated with *BRAF*^{V600E} levels was observed with

increasing cell concentration, and the platform was capable of *BRAF*^{V600E} protein detection from a prepared lysate concentration of 10 cells/mL (Fig. 5d).

Altogether, these results demonstrated that our IMMS platform is capable of clinically-relevant amounts of circulating *BRAF*^{V600E} DNA and protein biomarker detection for potential liquid biopsy applications.[27]

Combined *BRAF*^{V600E} quantification in simulated patient liquid biopsies

After extensive analytical performance testing of the IMMS as described above, the rational final step is to investigate its designed use for combined circulating *BRAF*^{V600E} quantification in clinical samples. To evaluate the feasibility of the IMMS platform for patient liquid biopsy applications, we performed the whole IMMS workflow on simulated plasma samples. To this end, we prepared known concentrations (25, 50, 100 cells/mL) of SK-MEL-28 cells in *BRAF*^{V600E}-negative healthy human plasma samples, and started with melanoma cell capture to eventual analysis of *BRAF*^{V600E} DNA and protein levels on the IMMS. The IMMS was able to quantify *BRAF*^{V600E} at both DNA (Fig. 6a) and protein (Fig. 6b) levels of different cell counts in the simulated plasma samples. This outcome is highly encouraging as a key indication that our IMMS is suitable as a lab-on-chip platform for processing raw clinical samples without off-chip purification. Pointedly, the capture and release microfluidic zone is robust in specific capturing of SK-MEL-28 melanoma cells from a biologically-complex matrix of biomolecules present in unprocessed plasma. This allowed for accurate downstream analysis as observed from the quantitative BRAF^{V600E} DNA and protein detection. The IMMS also demonstrated high detection reproducibility as %RSD values for DNA (13.3, 8.6, and 5.9%) and protein (10.9, 11.2, and 8.9%) detection in 25, 50, and 100 cells. From a clinical perspective, the IMMS could be of use in quantifying mutational burden for treatment decision-making or monitoring BRAF^{V600E} levels to evaluate treatment efficacy.

Finally, we also observed a strong correlation (Pearson correlation coefficient = 0.98) between matching *BRAF*^{V600E} DNA and protein expression levels in the same simulated plasma sample (Fig. 6c) on our single IMMS platform. This observation reinforced the concept of using simultaneous combined *BRAF*^{V600E} DNA and protein quantification to reduce false detection outcomes via analysis of a single *BRAF*^{V600E} molecular form. In addition, as concerns still currently persist about the accuracy of liquid biopsy testing (as compared to tissue sampling), the ability for *BRAF*^{V600E} quantification at both DNA and protein levels on the IMMS may also serve to provide better-validated interrogation of circulating *BRAF*^{V600E} status in liquid biopsies.

Conclusions

We have demonstrated an IMMS for simultaneous molecular analysis of a clinically actionable circulating cancer biomarker, *BRAF*^{V600E}, at both DNA and protein levels. Through tailored electrode design, we successfully achieved an entire sample-to-answer protocol encompassing melanoma cell capture in sample source, on-chip cell lysis, and combined quantification of intracellular *BRAF*^{V600E} DNA and protein amounts on a single miniaturized platform. It is noteworthy that the IMMS's detection performance is shown to be highly feasible for circulating biomarker analysis in liquid biopsies, notably for the challenging *BRAF*^{V600E} single base mutation which demand highly accurate and sensitive detection techniques for effective therapeutic actions.

As current cancer detection strategies mostly focused on biomarker detection at only a sole molecular level (eg. DNA, RNA or protein), we envisioned that our novel IMMS could lead the emergence of a new generation of biosensors which uniquely provide combined molecular analyses. The main advantages of such combined analyses on a single miniaturized platform are (i) cross-validation of a single oncogenic mutation by its different molecular forms for enhanced diagnostic accuracy; (ii) comprehensive simultaneous analysis of a panel of different biomarkers spanning across DNA/RNA/protein molecular forms to allow "multi-omic" testing for precision care.

Beyond the comprehensive proof-of-concept work in this paper for *BRAF*^{V600E} detection, the IMMS is amenable to advancements such as further sensor surface miniaturization and scale-up for high-throughput combined detection of more clinically useful circulating cancer biomarkers in liquid biopsies. Therefore, we believe that our proposed IMMS platform may find its potential in the in-depth analysis of rare tumor biomarkers in different molecular forms, thereby enabling better disease progression understanding and/or therapeutic decision making.

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Figure Captions

Fig. 1 Overview of the IMMS platform. (a) Specific melanoma cell capture on anti-MCSP functionalized microfluidic zone under ac-EHD and subsequent release of captured cells by applied dc potential. (b) Electrical lysis of released cells under dc potential. (c-i) *BRAF*^{V600E} DNA is targeted by mutant-specific ligation probes which are enzymatically ligated upon hybridization under ac-EHD fluidic mixing. The successfully ligated probes are then captured onto primer functionalized magnetic beads and isothermally amplified under fluidic mixing to enhance amplification molecular kinetics. Lastly, the surface-bound amplicons are then labelled with HRP enzymes before being magnetically loaded onto the electrode surface for amperometric detection. (c-ii) Cell lysate containing *BRAF*^{V600E} proteins is incubated on an anti-*BRAF*^{V600E} functionalized electrode under ac-EHD fluidic mixing. After target capture, the electrode is washed and the *BRAF*^{V600E} proteins are detected by voltammetry with a ferri/ferrocyanide redox couple.

Fig. 2 Melanoma cell capture and release performance on the IMMS platform. (a) Representative fluorescence cell images of captured SK-MEL-28 cells: (left) DiO stained cell membranes and (right) DAPI stained cell nuclei. Scale bar 10μm. (b) Capture efficiencies of different SK-MEL-28 cell counts in healthy plasma. (c) Capture efficiencies of non-target cells. (d) Release efficiencies of captured SK-MEL-28 cells under applied dc potential. Error bars represent standard deviation of three independent experiments.

Fig. 3 Optimization of IMMS electrical cell lysis. Cell lysis efficiencies (a) under different applied dc potentials, and (b) for different time durations. (c) Representative images of single SK-MEL-28 cell before (left) and after (right) electrical cell lysis. Scale bar 20μm. Error bars represent standard deviation of three independent experiments.

Fig. 4 IMMS *BRAF*^{V600E} DNA and protein detection specificities. (a) HRP-catalyzed amperometric *BRAF*^{V600E} DNA signals for *BRAF*^{V600E}-postive SK-MEL-28, and *BRAF*^{V600E}-negative LM-MEL-35 and SKBR3 cell lines. (b) Corresponding average current levels reflecting cell line *BRAF*^{V600E} DNA quantities. (c) Average $\%i_r$ obtained from positive capture antibody-specific target protein capture (anti-*BRAF*^{V600E}, blue); and negative controls without target protein (no *BRAF*^{V600E}, red), without capture antibody (no anti-*BRAF*^{V600E}, green), non-target protein (wildtype *BRAF*, purple), non-target capture antibody (anti-CD63, orange). (d) Average $\%i_r$ for *BRAF*^{V600E} protein detection in SK-MEL-28, LM-MEL-35 and SKBR3 cell lines. Error bars represent standard deviation of three independent experiments.

Fig. 5 IMMS *BRAF*^{V600E} DNA and protein detection sensitivities. (a) HRP-catalyzed amperometric *BRAF*^{V600E} DNA signals for a dilution series (0-100%) of *BRAF*^{V600E} sequences in a background of wildtype *BRAF* sequences (10 000 copies in total). (b) Corresponding average current levels reflecting *BRAF*^{V600E} DNA quantification. Average $\%i_r$ obtained for the detection of (c) designated *BRAF*^{V600E} protein concentrations in buffer, and (d) cell lysates of *BRAF*^{V600E}-positive SK-MEL-28 cells. Error bars represent standard deviation of three independent experiments.

Fig. 6 IMMS Combined *BRAF*^{V600E} quantification using simulated patient plasma samples. (a) *BRAF*^{V600E} DNA and (b) protein quantification of known concentrations (25, 50, 100 cells/mL) of SK-MEL-28 cells in *BRAF*^{V600E}-negative human plasma samples. (c) Correlation plot

showing concordance between matching *BRAF*^{V600E} DNA and protein expression levels. Error bars represent standard deviation of three independent experiments.