On-line Protein Capture on Magnetic Beads for Ultrasensitive Microfluidic Immunoassays of Cancer Biomarkers

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Supporting Information

1. Instrumentation

The microfluidic immunoassay system incorporates an on-line protein capture chamber (Fig. S1) into our previously reported modular device (Chikkaveeraiah et al., 2011). This chamber features a layer of molded, flexible PDMS sandwiched in between two flat, machined PMMA plates. The assembly is bolted together tightly to form an oval cylinder channel 1.5 mm wide and 100 ± 2 µL in volume housing a tiny magnetic stir bar. Flow out of the channel is directed to waste or to the detection chamber by a switch valve. The detection chamber is a PMMA-supported PDMS slab, but with rectangular channel 1.5 mm wide, 2.8 cm long and 63 ± 2 µL volume placed on top of 8-electrode carbon printed array (Chikkaveeraiah et al., 2011). The top PMMA plates feature ports for connecting 0.2 mm i.d. PEEK tubing at the inlet and outlet. The detection chamber also has two holes 0.6 mm and 0.2 mm diameter directly above the microfluidic channel for inserting Ag/AgCl reference and Pt wire counter electrodes that run along the entire length of the chamber. A syringe pump (Harvard, no. 70 4504) was connected to 2 switch valves via sample injector (Rheodyne, 9725i) using 0.2 mm i.d. tubing. The 2 valves aid in changing direction of flow from reaction chamber to waste or detection chamber.

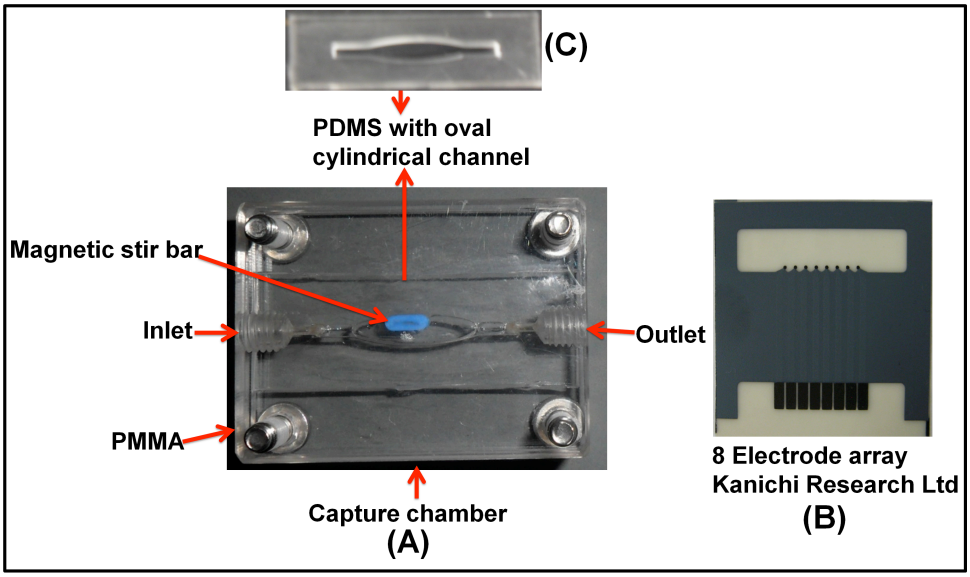

Fig. S1. Photograph of (A) Capture chamber in which target proteins are captured on-line from the sample by heavily labeled HRP-antibody-magnetic beads to form protein-bead bioconjugates. These are washed, and then flowed into the detection chamber housing the (B) 8 electrode array coated with Ab_1. (C) The PDMS that is sandwiched between two PMMA plates to form an oval cylinder channel which allow insertion of a tiny magnetic stir bar for mixing the bioconjugate during sample incubation and re-dispersion after washing for transfer of beads to the detection chamber.

2. Preparation of Ab_2 -magnetic particle -HRP (Ab_2-MP-HRP)

Biotinylated secondary antibodies (Ab_2) and biotinylated HRP labels were conjugated onto 1 μm diameter streptavidin-coated superparamagnetic beads (MPs) following a protocol supplied by Invitrogen as previously described (Malhotra et al., 2012). Briefly, 20 μL of MP (10 mg mL^{-1}) were washed three times with 0.1% BSA in PBS pH 7.4 and then reconstituted with 80 μL of 0.1% BSA in PBS pH 7.4. 40 μL of biotinylated-Ab_2 (20 μg mL^{-1}) and 80 μL of biotinylated-HRP (2.5 mg mL^{-1}) were then simultaneously added to the MP dispersion to link biotin to streptavidin. The reaction was done at 37°C for 25 min with slow tilt rotation in 1.5 mL microcentrifuge tubes. Ab_2-MP-HRP conjugates were separated magnetically, using an Invitrogen DynaMag-spin magnet, washed three times with 0.1% BSA in PBS pH 7.4 to remove unbound Ab_2 and HRP, reconstituted with 200 μL of 0.1 % BSA in PBS and stored at 4°C until use.

From enzyme activity assays, the number of horseradish peroxidase labels per MP was estimated to be 321,000 (± 23,000) using 2,2'-Azino-bis(3-Ethylbenzthiazoline-6-Sulfonic acid) as a reactant (Chikkaveeraiah et al., 2011; Putter, 1983). The average number of Ab_2 per MP was estimated by difference, from the amount left in solution, to be 38,000 (±7,000) using the bicinchoninic acid assay **(**BCA**)** kit (Chikkaveeraiah et al., 2011; Smith et al., 1985; Wiechelman et al., 1988).

3. Array Fabrication and Characterization

Glutathione-decorated gold nanoparticles (GSH-AuNPs) with diameter 5.0 ± 2 nm were prepared by reduction of gold (III) chloride trihydrate salt using sodium borohydride as reported previously (Mani et al., 2009)[Error! Reference source not found.]. The screen-printed carbon electrode was modified by electrostatic layer-by-layer deposition. Briefly, poly(diallyldimethylammonium chloride) (PDDA), a polycation was deposited on the electrodes. The array was then washed and AuNPs deposited to increase electroactive surface area and amplify the electrochemical signal of the arrays. The primary antibodies, Ab_1, were then attached onto the carboxylated AuNPs on the array via EDC-NHSS (400 mM EDC and 100 mM NHSS in water) amidization overnight. The electrode array was washed and incubated with 2% BSA in PBS for 1 hour to block non-specific binding (NSB).

Tapping-mode AFM images of uncoated carbon array sensors revealed a rough surface featuring hills and valleys with mean surface roughness of 17 ± 0.8 nm (Fig. S2A). AFM images of array sensors with successively deposited layers of poly(diallyldimethyl amine) (PDDA) and 5 nm GSH-AuNPs, revealed nearly complete coverage of the underlying layer resulting in decreased mean surface roughness of 14.5 ± 1.2 nm (Fig. S2B). AFM showed broader globular features after the immobilization of Ab_1 on the array (Fig. S2C), with mean surface roughness slightly decreased to 12.4 ± 0.9 nm. The globular features are characteristic of immobilized primary antibody on the AuNP underlayer (Mani et al., 2009). The electrochemical surface area after coating with AuNPs was estimated to be 2.53 (±0.26) × 10^{-3} cm^2 using cyclic voltammetry of 1 mM ferrocyanide and Randles-Sevcik equation (Chikkaveeraiah et al., 2011).

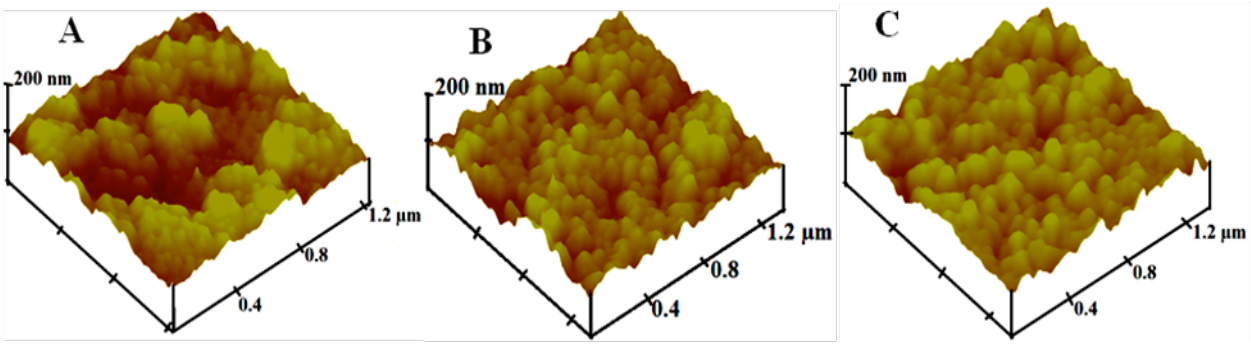

Fig. S2. High resolution tapping mode AFM images of one of the array sensor electrodes of (A) bare screen printed carbon, (B) Carbon/PDDA/GSH-AuNPs and (C) Carbon/PDDA/GSH-AuNPs/Ab_1.

4. NSB in On-line and Off-line protein capture protocol

In the off-line protein capture protocol, all the washing steps are done outside the microfluidic device and NSB of non-analyte proteins and other biomolecules in biomedical samples is eliminated before the analyte proteins are introduced into the measuring device. The immunoarray is exposed to only the washed bioconjugates that have captured the protein analytes. Thus, NSB background can only result from residual NSB from the MPs with no captured analyte. However, in the on-line protein capture, analyte proteins are introduced into the reaction chamber and washed inside the same chamber before being dispensed into the detection chamber. To test the NSB in this new protocol, a control was run using both protocols. Amperometric response for the controls developed after injection of a mixture of HQ and hydrogen peroxide is shown in Fig. S3. There were no observable differences in peaks produced by the two protocols, indicating that NSB is minimized equally by on-line and off-line capture methods.

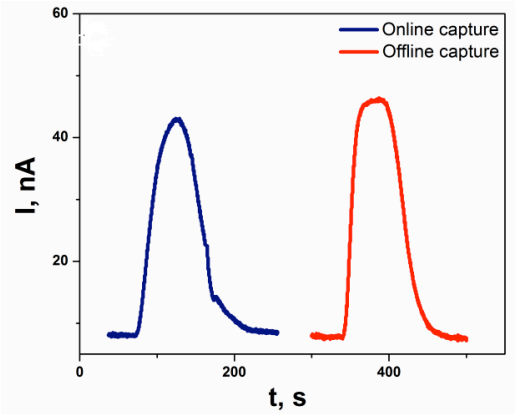

Fig. S3. Amperometric signals for on-line and off-line capture protocol for control developed by injecting a mixture of 1 mM HQ and 0.1 mM H_2O_2.

5. Immunoarray Reproducibility

Reproducibility was tested on 0 and 5 pg mL^{-1} of both IL-6 and IL-8 by running the immunoassay on different days with different arrays. The relative standard deviation (RSD) of

the measurements for the two concentrations was < 4 % (Fig. S4), confirming good day-to-day reproducibility of the immunoarray for both IL-6 and IL-8.

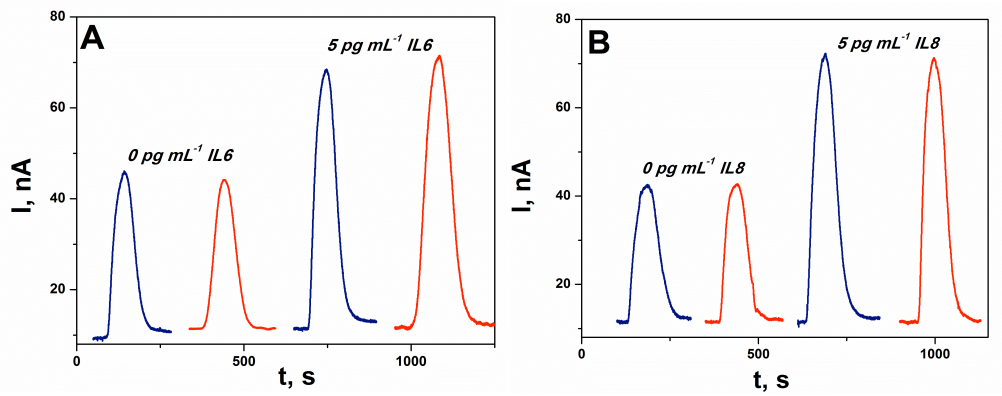

Fig. S4. Amperometric responses for individual standard solutions of IL-6 and IL-8 in undiluted calf serum at -0.2 V vs Ag/AgCl developed by injecting a mixture of 1 mM HQ and 0.1 mM H_2O_2 for (A) IL-6 and (B) IL-8 on different days with different arrays.

6. Determination of the cross-reactivity of the analytes

The immunoarrays were tested for cross reactivity of the protein with BSA and nonspecific antibodies before doing multiplexed detection. In these experiments, the arrays were coated with BSA and both IL-6 and IL-8 primary antibodies, however the Ab_2-MP-HRP-protein conjugates flown into the detection chamber were conjugated with only one type of protein. The amperometric measurements were recorded by injecting the mixture of 1 mM HQ and 0.1 mM H_2O_2. Amperometric responses for the cross reactivity of the protein with non-specific antibodies is illustrated in Fig. S5. Minimum cross reactivity between the analyte and non specific antibodies was observed. Cross reactivity between IL-8 (protein analyte) with IL-6 capture antibodies was 3 ± 2% (Fig. S5A) whereas the cross reactivity between IL-6 (protein analyte) with IL-8 capture antibodies was 4 ± 1% (Fig. S5B) and considered to be within acceptable limits for simultaneous detection.

For multiplexed detection, specificity of the protein analyte was tested first before obtaining the calibration curves. IL-6 capture antibodies were spotted on the first four electrodes while IL-8 capture antibodies on the last four electrodes. A mixture of 10 pg mL^{-1} IL-6 and 6 pg mL^{-1} IL-8, were mixed and injected into the reaction to be captured with Ab_2-MP-HRP conjugates for both IL-6 and IL-8. After 30 min incubation in the reaction chamber, the protein-Ab_2-MP-HRP conjugate was washed and dispensed onto the electrodes housed in the detection chamber. Amperometric signals were recorded by injecting a mixture of 1 mM HQ and 0.1 mM H_2O_2. Fig. S5C shows the amperometric differences between the response for the IL-6 on the first four electrodes and IL-8 on the last four electrodes indicating high selectivity.

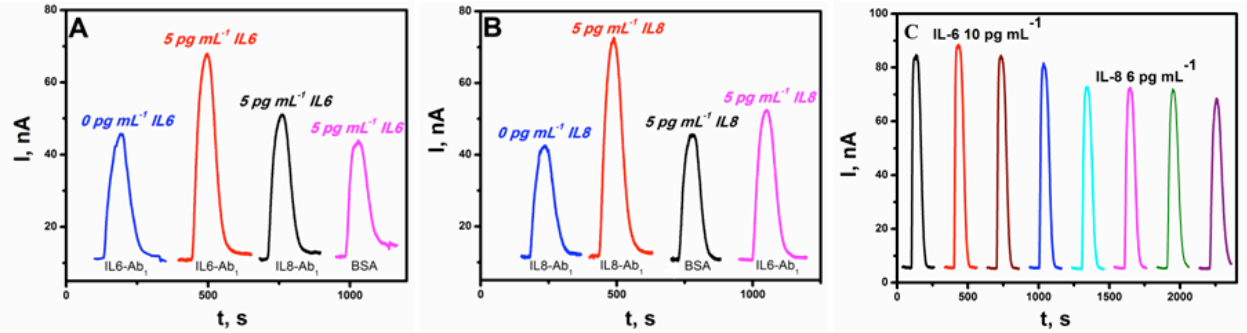

Fig. S5. Cross reactivity of the analyte with non specific antibodies. The array was coated with BSA, IL-8 and IL-6 primary antibodies. (A) 5pg mL^{-1} IL-6 and (B) 5 pg mL^{-1} IL-8 was injected and captured by the conjugate before being flown into the detection chamber. (C) Determination of specificity of the protein analyte on a single array. The first four electrodes show the amperometric response for IL-6 while the last four electrodes show the amperometric response for IL-8.

7. Assay Validation with conditioned cell media for Oral Cancer

To establish our method's accuracy, we used the microfluidic system to analyze levels of IL-6 and IL-8 in conditioned media for oral cancer cells. Conditioned media from heterogeneous populations of four different cell lines were analyzed to test the validity of the assay towards IL-6 and IL-8 detection. 5 µL of the samples were diluted in PBS to fit in the dynamic range prior to injection into the reaction chamber. Fig. S6 shows the concentrations of IL-6 and IL-8 in the cell lines tested detected via the new on-line capture protocol and standard ELISA assays. Cancer cell lines HN12, HN13 and Cal27 secreted large amounts of the biomarker proteins, >1000 pg mL^{-1}. Immortalized HaCaT cells established from normal non-cancerous epidermal cells showed low levels of IL-6 and IL-8. Good accuracy of the immunoarray is illustrated by the excellent correlation with standard ELISA. Linear correlation plots for the immunoarray versus ELISA gave slopes close to 1.0 and intercepts near zero confirming the excellent correlation (Table S1).

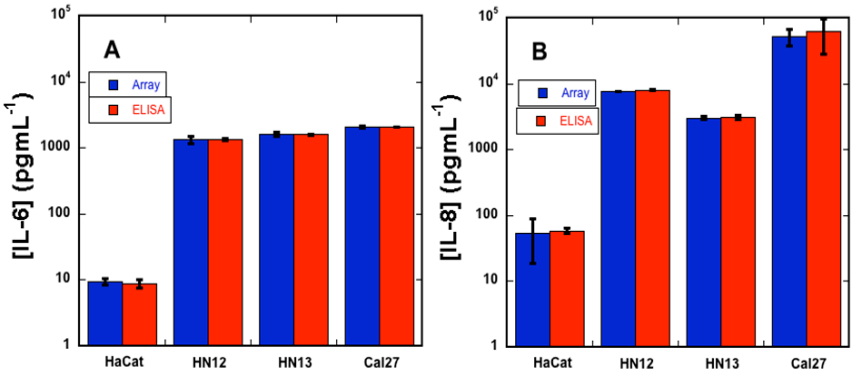

Fig.S6.Comparison of the immunoarray assay results for conditioned media for cells (HaCat, HN12, HN13 and Cal27) with standard ELISA assays for (A) IL-6 and (B) IL-8. Error bars represent standard deviation for immunoarray and average deviation for ELISA.

Table S1. Slopes and intercepts of correlation plots of immunoarray results for the conditioned media for cells against results obtained from ELISA.

Biomarker	Slope ± sd	Intercept ± sd
IL-6	1.00 ± 0.01	-0.59 ± 13.4
IL-8	0.83 ± 0.01	0.46 ± 0.29

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