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Electronic Supplementary Information (ESI)

An exosomal- and interfacial biosensing-based strategy for remote monitoring of aberrantly phosphorylated proteins in lung cancer cells

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MATERIALS

Lung cancer cell lines of NCI-H1666, NCI-H1975, and HCC827 were purchased from ATCC (USA) and the cell culture materials such as growth medium (RPMI 1640), serum-free medium (Ref. 12677027), fetal bovine serum and antibiotics were provided by Gibco, Life Technologies (Australia). Total exosome isolation reagent (Ref.4478359) was collected from Invitrogen, Life Technologies (Australia). Pierce Classic Magnetic IP/Co-IP Kit containing protein A/G magnetic beads, lysis buffer, elution buffer, and neutralization buffer were procured from Thermo Scientific (Australia). Western blot reagents such as NuPAGE 4-12% Bis-Tris Gel, NuPAGE MOPS SDS running buffer, nitrocellulose membrane were acquired from Novex, Life Technologies (Australia) and the Odyssey Blocking Buffer were from LI-COR (USA). EGFR polyclonal antibody (Ref.OPA1-10100), phospho-specific EGFR monoclonal antibody (Ref. ab32430) and ERK monoclonal antibody (Ref. 136200) were bought from Thermo Scientific (Australia), phospho-specific ERK monoclonal antibody (Ref. ab136926) from Abcam (Australia), and IRDye 680RD anti-rabbit (Ref. 926-68071) and IRDye 800CW anti-mouse (Ref. 926-32210) secondary antibodies from LI-COR (USA). Gefitinib (Ref. 4765) was collected from Cell Signaling Technology (Australia).

METHODS

Isolation and characterization of exosomes: The lung cancer cells, including $H1666^{EGFR/ERK}$, $H1975^{pEGFR/pERK}$ and $HCC827^{pEGFR/pERK}$, were grown in the standard medium until 70-80% confluency. The washed cells were then incubated in serum-free medium for 20 h and subsequently the medium was collected as a source of exosomes. Exosomes were then isolated via centrifugation (AllegraX-22 Series Benchtop Centrifuge, Beckman Coulter) at 10,000 × g for 1 hour using total exosome isolation reagent. Nanosight (NS300, Malvern Instruments, UK) measurements were performed to characterize size distribution of the exosomes under optimized dilution 1:1000 in PBS (1X) and 3 X 1 min measurement runs with standard settings (camera level =14, syringe pump speed = 100, viscosity = 0.9, temperature = 25^0C). (Figure S1 A)

Characterization of target proteins in exosomes derived from the lung cancer cells: Western blot was performed to verify presence, isoformity and phosphorylation status of exosomal EGFR and ERK isoforms derived from the respective cell lines. 20 µg of each exosome lysate was mixed with sample buffer and loaded on SDS-PAGE gel along with protein ladder. Samples were run in the gel with the MOPS running buffer (1X) at 200 V for 65 min. The gels were then transferred using the running buffer (1X) on the nitrocellulose membrane under 30 V for 65 min. The membranes were then washed with PBST (1X PBS + 0.1% Tween20) and blocked with the blocking buffer for 1 h. Subsequently, the blocked membranes were separately incubated for 2h with the respective primary antibody solutions (dilution ratio is 1:200) containing 0.1% Tween 20. After incubation, the membranes were washed (3×10 min) with the PBST and then blocked again for 30 min. Next, the membranes were dipped in the respective secondary antibodies (IRDye 680RD anti-rabbit for EGFR primary antibody and IRDye 800RD anti-

mouse for ERK primary antibody) solutions at 1/10000 dilution for 1h and rewashed (3x10 min) with the PBST. Finally, images of the membranes were taken using a fluorescence based infrared imager (Odyssey, LI-COR, USA) under channels 700 (for IRDye 680RD) and 800 (for IRDye 800RD) with intensity level of 3.

Magnetic immuno-purification (IP) of EGFR, pEGFR, ERK and pERK from isolated exosomes and purity evaluation of these proteins: Lysis of exosomes from each lung cancer cell line was performed by adding ice cold lysis buffer (25mM Tris.HCl pH 7.4, 150 mM NaCl, 1%NP-40, 1 mM EDTA, 5% glycerol), followed by centrifugation (AllegraX-22 Series Benchtop Centrifuge, Beckman Coulter) at 13,000 × g for 10 min to have total protein in the supernatant. Then 2 µg of generic EGFR and ERK antibodies was separately added to 1000 µg of the total protein lysates, and left for 2h incubation time at RT to form respective immuno-complexes. Each immune complex was added to the prewashed protein A/G coated magnetic beads (25 µg) and incubated at RT for 1h with mixing. Next 100 µL of elution buffer (Glycine.HCl, pH 2) was added to the mixture and incubated at RT with mixing for 10 minutes. Finally, the beads were separated magnetically and saved the supernatant-containing target proteins. To neutralize the low pH, 10 µL of neutralization buffer (Tris.HCl, pH 8.5) was added for each 100 µL of eluate and then stored it at -20 °C with necessary aliquots. The concentration of eluted protein for the three cell lines was quantified by BCA protein assay (Thermo Scientific, Australia) based on Microplate Reader (BioTek, Synergy HT)**.**

The purity of exosomal EGFR and ERK protein isoforms extracted from the three lung cancer cell lines was confirmed by silver staining experiment (Silver stain plus kit, Bio-rad, Australia). For this, we mixed 3 µg of each purified protein sample from exosomes with NuPAGE LDS sample buffer 4X and loaded on the SDS-PAGE gel (lanes 2-4) along with a protein ladder (lane 1). Samples were run in the gel with MOPS running buffer (1X) at 200 V for 65 min. After electrophoresis, the protein isoforms were separately fixed in the gels by incubation with fixative enhancer solution (contents: 50% methanol, 10% acetic acid, 10% fixative enhancer concentrate, and 30% water) for 20 min with gentle agitation. After rinsing the gels for 20 min, we then followed staining and development steps through incubation with a mixture of silver complex solution, reduction moderator solution, and image development reagent under gentle shaking for another 20 min. The developing stains on gels were then stopped by adding a stop solution containing 5% acetic acid for 15 min. After that the gels were rinsed in high-purity water for 5 min. (Figure S1 B)

Electrochemical quantification of protein adsorption levels on gold surface of screen-printed electrodes. All the electrochemical experiments were carried out using CH1040C (CH Instruments) with a three electrode system (screen–printed electrodes (C220BT, Dropsens, Spain)) consisting of a gold working electrode (4mm in diameter), Pt counter electrode, and Ag/AgCl reference electrode. Differential Pulse Voltammetric (DPV) experiments were conducted in PBS solution containing 2.5mM $[K_3Fe(CN)_6]$ and 2.5mM $[K_4Fe(CN)_6]$ electrolyte solution. DPV signals were obtained with a potential step of 5 mV, pulse amplitude of 50 mV, pulse width of 50 ms, and pulse period of 100 ms. The electrolyte solution was put on bare Au electrode for recording the baseline DPV signal. Afterwards 5 µL of the protein samples (protein amount: 75 ng, solution pH 7) were allowed to adsorb on these electrodes for a range of time intervals (15, 25, 40, 60 min) and DPV signals were then recorded after washing the Au surface with PBS. The relative percent current ($\%i_r$) of the DPV signals generated for phosphorylated and non-phosphorylated protein isoforms with respect to the baseline current were calculated using equation 1:

$$\%\, i_r\,(\%\text{ change in DPV}_{\text{protein}}) = \frac{I_{\text{Baseline}} - I_{\text{protein}}}{I_{\text{Baseline}}} \text{ X } 100 \quad \ldots\ldots 1$$

Where, $I_{Baseline}$ is the peak current of the bare gold electrode, $I_{protein}$ is the peak current after adsorption of the protein on the electrode.

Relative current difference between phosphorylated and non-phosphorylated isoforms:

$\Delta i_r = \%i_r^{(P\text{-}EGFR/P\text{-}ERK)} - \%i_r^{(EGFR/ERK)}$ 2

Optimization of factors affecting protein adsorption on Au SPEs. Some parameters including protein concentration, adsorption time and solution pH affecting its adsorption on Au surface were optimized for maximizing the DPV current gap between phosphorylated and non-phosphorylated EGFR isoforms. Phosphorylated EGFR showed non-linear increasing adsorption trend with protein concentration, whereas non-phosphorylated isoform followed a linear trend. A concentration of 15 ng/µL (75 ng) of was fixed for downstream analysis (Fig. S2 A). Adsorption also improved with increasing adsorption times in the similar manner (Fig. S2 B) and 15 min was selected as the optimum adsorption time for downstream analysis. The pH had little influence on the process and pH=7 was selected as the optimum value (Fig. 2S C). Under all the optimized conditions including 75 ng (15 ng/µL) EGFR isoforms, 15 min adsorption time and solution pH 7, we observed a DPV current difference, $\Delta i_r = \%i_r^{pEGFR} - \%i_r^{EGFR}$ = ~50%.

ESI FIGURES

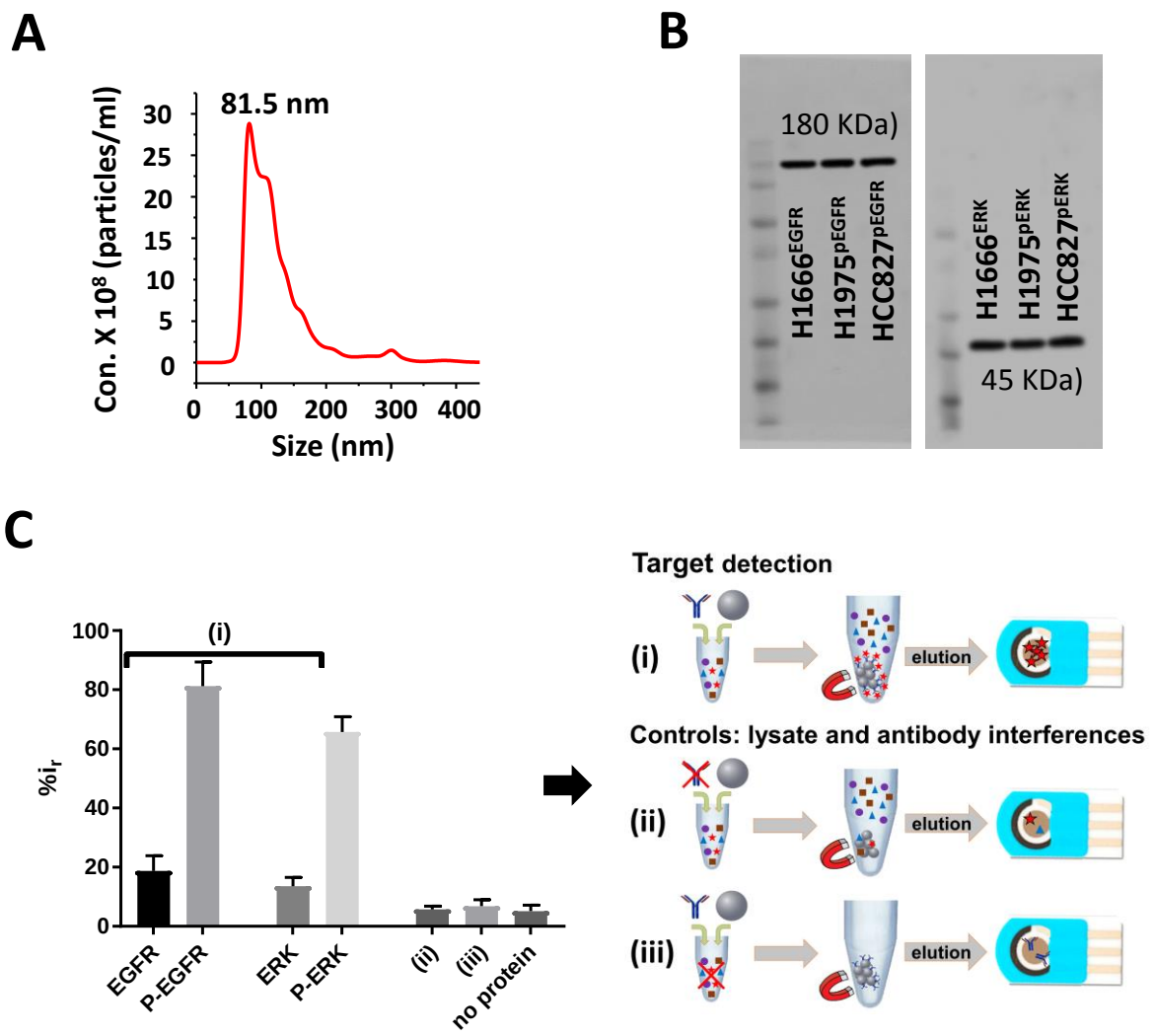

Fig. S1 (A) Particle size distribution of isolated exosomes as measured by Nanosight. (B) Purity test of IP-extracted EGFR, P-EGFR, ERK and P-ERK proteins by gel electrophoresis and then gel staining with silver stain. The visualization of an individual band on the gel (lanes 2-4 in both gels) is indicative of the presence of highly pure protein. (C) Specificity of the protein-gold phosphorylation assay. This assay can specifically detect phosphorylation status of target proteins (EGFR and ERK) from exosomal samples with minimal interferences from other molecules from lysate or antibodies that may have leaked from magnetic beads during elution step. (Left) Schematics of the target and control experiments and (Right) representative DPV current changes (%i_r) for adsorption of exosomal eluted fractions from target and control purification experiments. The IP-extracted non-phosphorylated and phosphorylated EGFR and ERK proteins were diluted by 1/20 to the working concentration of 20 ng/µL in Tris-glycine of pH 7. The same level of dilution was also done for the IP-extracted non-specific interferences. 5 µL of each 1/20 diluted solutions (EGFR, P-EGFR, ERK, P-ERK, sample buffer (no protein), lysate and antibody interferences) was then separately allowed to adsorb on Au SPEs for 15 min. Each data point represents the average of three separate trials and error bars represent the standard deviation of measurements. (P-EGFR: phosphorylated EGFR; P-ERK: phosphorylated ERK; EGFR: non-phosphorylated EGFR; ERK: non-phosphorylated ERK)

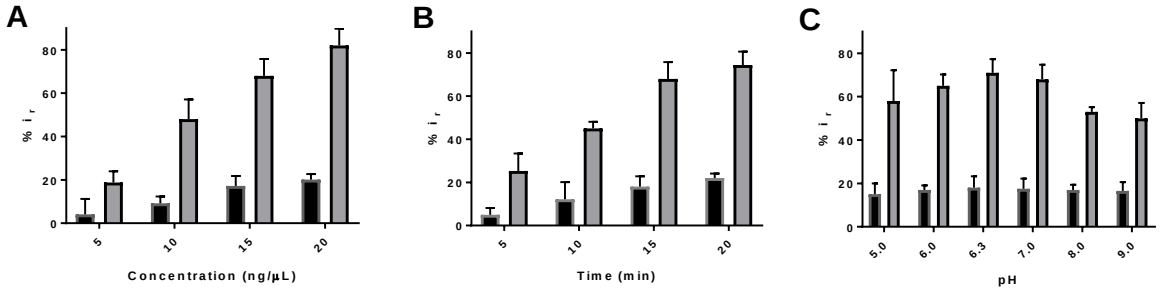

Fig. S2 Optimization of operating parameters including (A) protein concentration (B) adsorption time (C) sample solution pH. Each data point represents the average of three separate trials and error bars represent the standard deviation of measurements. (black bars: EGFR; grey bars: P-EGFR)