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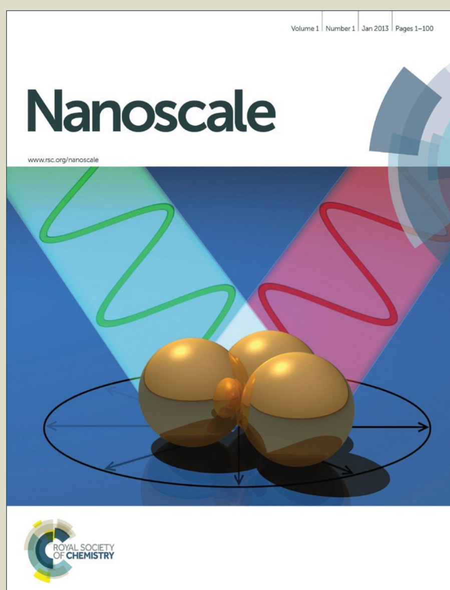

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Nanomechanical Sandwich Assay for Multiple Cancer Biomarkers in Breast Cancer Cell-derived Exosomes

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The use of exosomes as cancer diagnostic biomarkers is technically limited by their size, heterogeneity and the need for extensive purification and labelling. We report the use of cantilever arrays for simultaneous detection of multiple exosomal surface-antigens with high sensitivity and selectivity. Exosomes from breast cancer were selectively identified by detecting over-expressed membrane-proteins CD24, CD63, and EGFR. Excellent selectivity; however, was achieved when targeting the cell-surface proteoglycan, Glypican-1 at extraordinary limits (~200 exosomes mL^{-1}, ~ 0.1pg mL^{-1}).

Exosomes are nanoscale vesicles with sizes in the range of 30 nm – 100 nm shed by many cell types into the bloodstream[1]. As they harbour numerous bioactive receptors, nucleic acids, and signalling proteins for cell-to-cell communication, they have become increasingly attractive as diagnostic and therapeutic targets[2, 3]. Recent studies have shown the potential use of circulating exosomes as biomarkers for predicting and monitoring a number of complex diseases, including cancer. It has been reported that circulating exosomes may carry valuable information about their parental tumours,[3, 4] which make them ideal biomarkers for early detection of cancer. Selective detection of cancer specific exosomes; however, is currently limited by their size, their identical composition to exosomes from non-tumorigenic cells and most importantly, their lack of specific markers that can discriminate them from other extracellular vesicles. Therefore, developing a technique for selective isolation and characterization of cancer exosomes are very important to overcome the challenges towards their use as biomarkers for detecting cancer. The current available approaches for detecting tumour-derived exosomes are either inept or impractical. Ultracentrifugation for instance, is time consuming and lacks the ability to differentiate between tumorigenic and non-tumorigenic exosomes[5, 6]. ELISA and western blot analysis require large amounts of samples and extensive labelling; thus, they are impractical for routine screening with high throughput[6, 7]. The commercially available nanoparticle tracking analysis (NTA) is an ideal tool to sort nanoparticles in the size range of 10 nm – 2 µm. However, the system can only detect high concentrations of nanoparticles (10^6 to 10^9 particles mL^{-1}). Other novel approaches such as nano-plasmonic sensors[8], Raman scattering[9], miniature magnetic resonance[10] and others[11], are presently under development.

Here, we report the use of a sandwich technique of multiplexed cantilever array sensor for real-time, high-throughput screening of cancer cell-derived exosomes. We use the cantilever to discriminate between tumorigenic and non-tumorigenic exosomes and to detect ultra-low concentrations of breast cancer exosomes spiked in human serum. The technique has the potential to circumvent the limitations of other analytical methods in detecting low-abundant tumorigenic exosomes secreted into the blood stream by tumours at the initial stages of their development.

The first set of experiments was designed to assess the specificity of detecting exosomes in human serum spiked with breast cancer-derived exosomes using cantilever arrays. We targeted a number of biological markers including, CD24, CD63, EGFR and Glypican-1 (GPC1), that are believed to be over-expressed on surface-membrane of exosomes originated from cancer cells. Monoclonal antibodies (mAbs) were used as probes to target those membrane-associated proteins. Therefore, the antibodies were chemically immobilized onto the cantilever surface using a multiplexed capillary tube technique (See Methods).

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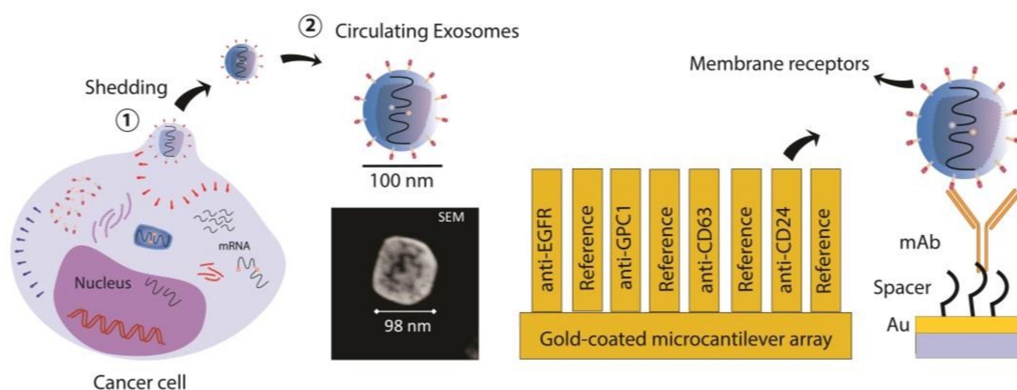

Fig 1. Tumour cells secrete exosomes through blending of the cell membrane to induce circulating extracellular vesicles in various biofluids with nanoscale sizes. These circulating nanovesicles carry arrays of biological markers including proteins, lipids and nucleotides, identical to that existing in their parental cells. High magnification scanning electron microscopy shows a single exosome from cancer cell lines (MDA-MB231) having a diameter of ~98 nm with no further characteristic details. The cantilevers in the array were either functionalized with exosome-targeting probes (monoclonal antibodies) as indicated, or with a reference control for differential detection of signal. The right-end diagram shows chemistry of surface coating, where a thiolated spacer was placed between the antibody and the Au-cantilever interface in order to enable the immobilization and reduce steric hindrance.

Exosomes derived from breast tumour cell lines (MCF7 and MDA-MB231) or non-tumorigenic cell lines (MCF10A), were isolated by multiple steps of ultracentrifugation and characterized by Dynamic Light Scattering (DLS) and Scanning Electron Microscopy (SEM) (supplementary). DLS and SEM results showed comparable extracellular vesicles with an average size of ~102 ±8 and ~89 ±5 nm diameter, respectively (supplementary Fig. 1 and 2). The isolated exosomes were suspended in 1 ml sterile-buffer/human serum samples (1:1 v/v) and introduced to the cantilever array functionalized as shown in Fig 1. Different cantilever bending signals were observed after injecting the exosomes (Fig. 2a, b, c) with bending amplitudes scaled according to the targeted antigens. Differential deflection of the mAbs functionalized cantilevers displayed selective response to cancer cell-derived exosomes (Fig 2a, and b) when compared to that of non-tumorigenic exosomes (Fig. 2c). The highest affinity (highest deflection) was observed when targeting the EGFR receptor (Fig. 2a, and b); however, the lowest selectivity was observed when targeting the same receptor (c). Interestingly, the best selectivity was achieved when detecting the cell-surface GPC1, as very low response to non-tumorigenic exosomes was observed (Fig. 2a, b and c). In addition, by targeting GPC1, the tumorigenic exosomes could still be detected in the presence of a 20-fold excess of non-tumorigenic exosomes (supplementary Fig. 3), reaching a detection limit of 10^{-9} g mL^{-1}. When we compared the relative expression of the above-mentioned antigens on the tested exosomes from cancerous and non-cancerous cells (Fig. 2d), the protein profiles showed significant discrepancy in their distribution with the best differential discrepant level with GPC1 (P = 0.00158), demonstrating its minute expression on non-tumorigenic exosomes. This compares very well with earlier evidence of proteins expression of breast cancer exosomes. The CD63, CD24 and EGFR are established exosomal markers and their expression is relatively higher in tumour exosomes than the levels on noncancerous-derived exosomes[11]. Recently, immunoblotting and FACS analysis have also identified GPC1 protein in much higher abundance in exosomes from breast cancer cells than in exosomes from noncancerous cells[4]. In addition, the relative concentration of GPC1 was found significantly higher in the sera of cancer patients compared to healthy donors[4]. Our nanomechanical results provide further support of elevated level of GPC1 on exosomes from breast cancer cells and raise the prospect use of this exosomal biomarker to identify breast cancer at its early stages of development.

To further understand response of the microcantilever to exosomes antigens, we carried out extra control experiments where GPC1-coated cantilevers were exposed to two samples; the first contained the exosomal bound antigen (GPC1) and the second one free of antigens. As in the previous experiments, following equilibration of the cantilever system with 1 ml of sterile-buffer/human serum sample of 1:1 v/v, the samples with or without the antigen was introduced into the sensor system and the response was monitored for 60 min. Results showed a substantial nanomechanical deflection (~104

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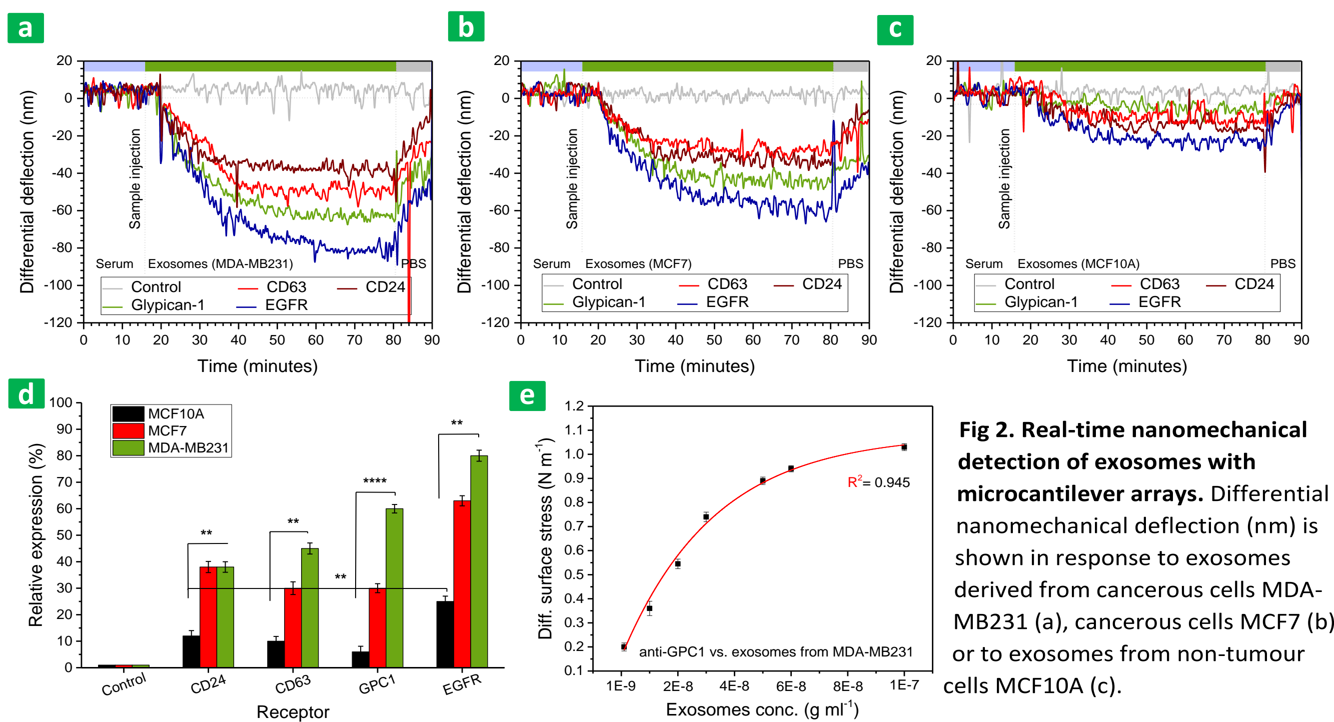

Fig 2. Real-time nanomechanical detection of exosomes with microcantilever arrays. Differential nanomechanical deflection (nm) is shown in response to exosomes derived from cancerous cells MDA-MB231 (a), cancerous cells MCF7 (b) or to exosomes from non-tumour cells MCF10A (c). The nanoscale deflection was measured in presence of 1 µg ml^{-1} exosomes in a solution of PBS/ human serum (1:1) using a 1000 µm-long and 1 µm-thick gold-coated silicon nitride cantilevers. (d) Surface stress measurement of the exosomes membrane-associated proteins with relative expression (%) in the tested cell lines, as indicated. Values represent mean ± s.d., n= 3 biological replicates. (e) Langmuir isotherm fit equilibrated according to surface stress values extracted from sensitivity experiments (supplementary). R2 = 0.94, indicating a consistent fit with the data. An average calculation of three replicates is presented with error bars indicate s.d.

± 8 nm) when the samples were spiked with GPC1 antigen. The cantilever, did not exhibit any deflection when exposed to antigen's free samples (supplementary Fig 4). These results provide further support to our previous results and clarify further the behaviour of the cantilever towards exosomal surface antigens.

Kinetics of real-time analysis showed exosomes quantification and protein profiling based on the cantilever's surface stress. The calculated dG value from GPC1 interaction to the cantilever (grafted with mAb) was found to be 197.9 kJ mol^{-1} calculated from a Langmuir isotherm fit (Fig. 3e). The observed binding constant was ~0.1 nM, which is slightly lower than that of individual antigen-antibody binding ~3 nM[12]. The observed steady binding may be due to the presence of multiple binding sites per exosome. Likewise, the calculated dG values of other ligands showed 231.8 kJ mol^{-1}, 139.6 kJ mol^{-1} and 121.8 kJ mol^{-1} for EGFR, CD24 and CD63 receptors, respectively, suggesting decent agreements with results previously reported for antigen-antibody experiments[8].

Next, to enhance the sensitivity and to detect ultra-low concentrations of exosomes based on GPC1 expression level, we used a sandwich cantilever assay, where a solution contains detective antibody (antiGPC1) grafted on 100 nm gold-nanoparticles was further introduced into the cantilever. The exosomes were captured first by the antiGPC1 mAb immobilized on the cantilever's surface, then further exposure to antiGPC1-tethered on the nanoparticles resulted in binding of the nanoparticles into the free region of the captured exosomes (Fig. 3a), amplifying the nanomechanical cantilever's deflection (the sandwich assay is detailed in the online methods). Fig. 3 illustrates the nanomechanical response of the cantilever to very low concentrations of exosomes (10^{-13} – 10^{-12} g mL^{-1}). As presented in Fig. 3b, adsorption of mAb-coated nanoparticles gives rise to an increase in the nanomechanical deflection, enhancing the mass limit of

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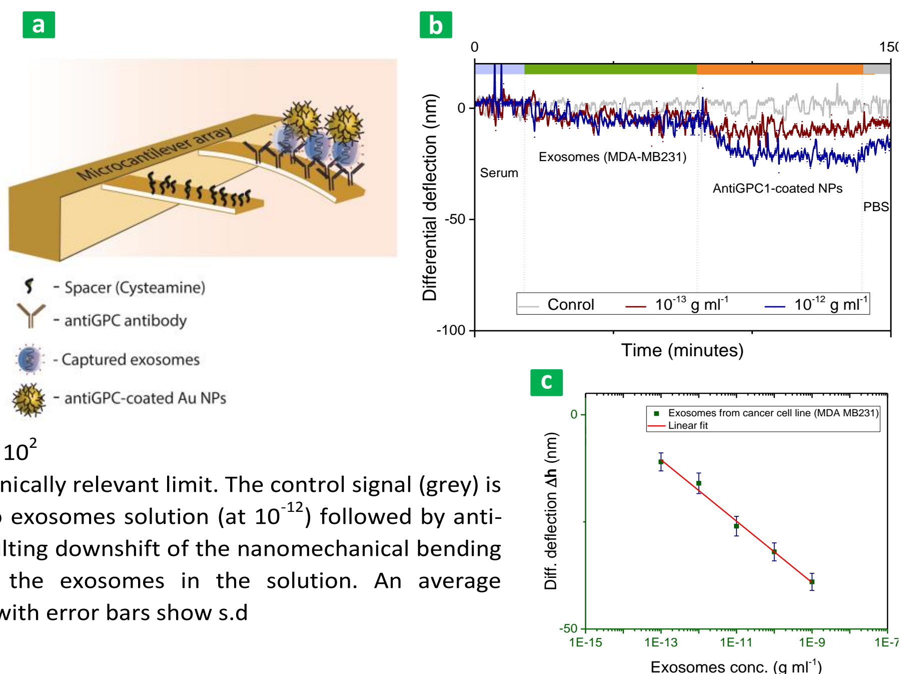

Fig 3. Sandwich assay for the cantilever and the effect on the nanomechanical deflection. (a) Schematic of the effect of the nanoparticle mass loading on the nanomechanical deflection of the cantilever. (b) Enhanced signals of the cantilever's nanomechanical deflections due to insertion of the mAb-coated nanoparticles (NPs). In the blue and wine signals, the cantilevers and NPs are bio-functionalized with antiGPC1 mAb. The sensitivity of the cantilevers before NPs binding (~10^{-9} g mL^{-1}) was increased by the sandwich assay to ~10^{-13} g mL^{-1} (~2×10^{2} exosomes mL^{-1}), which is higher than the clinically relevant limit. The control signal (grey) is cysteamine-coated cantilevers subjected to exosomes solution (at 10^{-12}) followed by anti-GPC1 coated NP at 2×10^{-12} g mL^{-1}. (c) Resulting downshift of the nanomechanical bending is proportional to the concentration of the exosomes in the solution. An average calculation of three replicates is presented with error bars show s.d

detection to 10^{-13} g mL^{-1} or/ 0.1 pg mL^{-1}. The nanomechanical bending (nm) is scaled proportionally with the exosomes concentration in the samples at fixed level of antiGPC1 NPs (supplementary Fig. 4). From the geometrical limit, the estimated number of nanoparticles on the surface is ~500 NPs and the bending response due to mass loading can be estimated as 10^{-7} nm. This comes in agreement with the experimental data that shows an estimate number of NPs on the surface to ~400 (supplementary Fig. 5). According to the results, the observed bending enhancement is most probably due to additional surface stress caused by NPs binding to more than one exosomes. The amount of observed stress is around 0.1mN/m^2. This limit is superior to the previously reported values due to the utilization of the functionalized NPs for mass enhancement[11]. Compared to the existing detection methods[7, 11], the observed sensitivity is 10^3 times higher than that observed for western blot and 10^2 times higher than the ELISA. Unlike the state-of-the art microscopies[13] and fluorescence based flow cytometry[14], the cantilever approach is label-free, adequate for routine clinical screenings and supports the detection of smaller size of extracellular vesicles (≥100 nm). The ultra-small size of the cantilever, which resembles a miniature diving board, allows the sensor to respond quicker to the biological and chemical binding in real-time and in-situ. The cantilever can also be assembled into a microfluidic device with an embedded microchannel for single particle detection[15, 16]. We anticipate these findings to provide a significant positive impact on the use of exosomeic sensors to detect cancer early and monitor its prognosis. Yet, a number of key studies are remained to be undertaken including a comprehensive stoichiometric investigation of binding between mAbs and their targeted exosomal antigens and the detection of cancer exosomes in complex biological fluids such as blood, urine, saliva, etc. obtained from cancer patients.

Conclusions

We have presented a simple and multiplexed approach that uses alternative biomarkers for highly sensitive and selective detection of breast cancer cell-derived exosomes. The approach detects exosomes at ultra-low concentrations, compares the expression level of exosomal-surface antigens, and discriminates in real-time tumorigenic from non-tumorigenic exosomes. The technique is simple, inexpensive and able to sort exosomes in short time. Unlike other methods, the sensor can be used in an array format to capture multiple exosomal markers simultaneously increasing the sensitivity and selectivity of the detection. This finding offers opportunities for the development of exosome isolation technique for future diagnosis of breast cancer and monitoring in real-time its progression.

Notes

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