



Label-free detection of exosomes using a surface plasmon resonance biosensor

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Abstract

The development of a sensitive and specific detection platform for exosomes is highly desirable as they are believed to transmit vital tumour-specific information (mRNAs, microRNAs, and proteins) to remote cells for secondary metastasis. Herein, we report a simple method for the real-time and label-free detection of clinically relevant exosomes using a surface plasmon resonance (SPR) biosensor. Our method shows high specificity in detecting BT474 breast cancer cell-derived exosomes particularly from complex biological samples (e.g. exosome spiked in serum). This approach exhibits high sensitivity by detecting as low as 8280 exosomes/ μL which may potentially be suitable for clinical analysis. We believe that this label-free and real-time method along with the high specificity and sensitivity may potentially be useful for clinical settings.

Keywords Exosome · Surface plasmon resonance · Biosensor · Cancer biomarker · Cancer diagnosis

Introduction

With a variety of vesicles secreted by eukaryotic cells, exosomes have attracted much attention for their ability to carry vital molecular information representative of the parent cell [1–5]. This discovery has spurred enormous interest in realising their potential as non-invasive disease markers and subsequently in the development of exosome-based diagnostic platforms [6–10]. However, a major bottleneck in the development of such platforms has been the specific isolation of exosomes from complex biological fluids. Over the years,

several methods utilised for the isolation of exosomes including ultracentrifugation [11], density gradient separation followed by electron microscopy [12], enzyme-linked immunosorbent assays (ELISA) [13], and western blotting [14] resulted in low purity yield, tend to be time consuming or involve extensive labelling procedures [15]. Similarly, several commercially available exosome isolation protocols or kits also co-isolate several non-exosome debris or biological material of similar physical characteristic rendering them to be ineffective.

Recent integration of microfluidics [16–18], immunosensors [19–23], and plasmonic sensors [24–29] has enabled the development of promising point-of-care devices for disease detection. These technology enhancements have significantly improved the capability of isolating exosome populations in a sensitive manner. For instance, a nanoplasmonic assay developed by Im et al. enabled profiling of exosomes based on membrane proteins and proteins present in the lysates [24]. The approach involved the use of periodically placed antibody-functionalised nanohole arrays and exosome profiling was performed by using transmission surface plasmon resonance system. To avoid the nanostructure fabrication complexity for nanoplasmonic sensor development, Liu et al. developed a compact SPR biosensor for exosomal protein detection [30]. The assay involved conventional SPR biosensing mechanism but does not require a nanostructure fabrication. Similarly, Picciolini et al. reported a

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surface plasmon resonance imaging assay that detected exosome subpopulations in blood derived from neurons and oligodendrocytes and demonstrated the heterogeneity in EV populations in terms of phenotypic expression and abundance [31]. Rupert et al. recently developed another surface plasmon resonance-based method that utilised dual wavelengths to accurately measure the size and concentrations of exosome subpopulations [32]. Apart from the SPR-based methods, Duraichelvan et al. reported a label-free method based on UV-VIS spectrophotometer that utilises an exosome capturing synthetic polypeptide to detect exosomes in liquid biopsy [33]. A high-performance microfluidic platform was developed by Shao et al. and colleagues which utilised magnetic particle tagged antibody to detect exosomes isolated using a nuclear magnetic resonance system [34]. In addition to these, numerous other microfluidic approaches based on microfabricated electric field-induced fluid micromixing, constricted nanochannels to induce cell-mediated exosome release, micropillars, or geometric features sequentially arranged with lateral displacement have been utilised for the successful isolation of exosomes using exosomal membrane-based cancer biomarkers [15–18, 35]. Although these techniques are highly sensitive, time-consuming procedure and expensive instrumentation limit their applicability in clinical settings. More recently, we have developed a surface plasmon resonance (SPR)-based method that can enumerate the proportion of tumour-derived exosomes within the bulk exosome population isolated from patient serum. The method required a sandwich assay where bulk exosome population (both normal and tumour-derived exosome) was initially captured in the sensor surface domain using a generic marker (e.g. CD63 or CD69) and then used to detect the tumour-specific exosomes using a secondary detection antibody such as HER2. Since this method used a sandwich assay with secondary detection antibody, it required long analysis time. Thus, we believe that a simple and direct assay without a labelling with secondary detection antibody might have potential application in point-of-care diagnostics.

Herein, we report a simple approach for the specific and sensitive detection of clinically relevant exosomes from biological fluids. Our proof-of-concept study involves a simple custom-built surface plasmon resonance (SPR) readout system that enables a rapid, real-time, and label-free monitoring of exosomes without relying on labelling with a secondary antibody. While the removal of the secondary antibody label resulted in a reduction in assay time and the use of custom-built SPR reduces the system cost, we successfully demonstrate the ability of our method to detect human epidermal growth factor receptor 2 (HER2)-specific exosomes derived from BT474 breast cancer cell line. Our approach exhibits high sensitivity by detecting as low as 8280 exosomes/ μL from complex biological samples (e.g. exosome spiked in serum) which is potentially suitable for clinical analysis. We

believe that this simple method may potentially enable its integration as a clinical or point-of-care testing tool [36].

Materials and methods

Chemicals and reagents

Analytical grade chemical reagents were purchased from Sigma-Aldrich (Australia). UltraPure™ DNase/RNase-free distilled water (Invitrogen, Australia) was used to carry out all the experiments. Reagents for immunoassay were purchased from R&D/Life Technologies (Burlington, ON), Thermo-Fisher Scientific (Australia), Abcam (Australia), and Invitrogen (Australia). Phosphate buffer saline (PBS, 10 mM, pH 7.4) was used to prepare all reagents and washing solutions. Breast cancer cell lines such as BT474 and MDA-MB-231 and prostate cancer cell line PC-3 were purchased from ATCC (USA).

Cell culture and isolation of exosomes

Breast cancer (HER2 (+): BT-474; HER2 (–): MDA-MB-231) and prostate cancer (PSA (+): PC3) cell lines are maintained in micro-vesicles depleted serum free Media 171 (Gibco, UK) supplemented with mammary epithelial supplement (Gibco, UK), 1% penicillin/streptomycin. The cells were grown in 5% CO₂ at 37 °C and after 60 h, the cell culture medium from 10⁶ cells were collected for exosome isolation. The cell debris and other contaminations were eliminated by centrifuging at 2000×g for 30 min. Total Exosome Isolation kit (Life Technologies) was used to isolate exosomes as per manufacturer's instructions. Briefly, the isolation reagent was added in a ratio of 1:2 to the cell culture supernatant. The samples were then incubated for overnight at 4 °C and exosome pellets were obtained by filtrating the solution using a 0.22- μm filter followed by centrifugation at 10000×g for 1 h. Isolated exosome pellets were re-suspended in 100 μL PBS (10 mM, pH 7.0) and stored at – 20 °C for future use.

Cryo-transmission electron microscopy

Prepared exosome samples (4 μL) were adsorbed on lacey carbon grids (Quantifoil, Germany) and then by using an FEI Vitrobot Mark 3 (FEI Company, The Netherlands), plunged into liquid ethane. Grids were blotted at 100% humidity at 4 °C for about 3–4 s. Frozen/vitrified samples were imaged using Tecnai T12 Transmission Electron Microscope (FEI Company) operating at an acceleration voltage of 120 kV. Images were taken at × 30,000 magnification (approximate dose of 13.6 electrons/ $\text{\AA}^2$), using an FEI Eagle 4k CCD (FEI Company) and Serial EM image acquisition software.

Device functionalisation

The experiments were carried out in a custom-made SPR platform (see Electronic Supplementary Material (ESM) Fig. S1) that was controlled and the signals monitored by LabVIEW Software (National Instruments Pty Ltd., Australia). The fabrication of the SPR sensor chips was by the deposition of 5 nm Ti and 50 nm Au on a 4-in. boroflat glass wafer using a Temescal BJD-2000 e-beam evaporator. The gold-deposited wafer was then diced into the SPR sensor chips followed by washing of the chips in acetone, ethanol, and purified water. Next, the chips were dipped quickly (2–3 s) into piranha solution (70% H₂SO₄–30% H₂O₂), rinsed with water, and dried with a stream of nitrogen gas. Finally, the chip was inserted into the SPR platform and conditioned with PBS buffer at a flow rate of 0.6 mL/h. To functionalise the chips, 250 µL of biotinylated BSA (100 µg/mL in PBS, Invitrogen) was flown through the platform at 0.6 mL/h prior to the blocking of the gold surface with 3% BSA. Next, 100 µg/mL streptavidin (Invitrogen) was conjugated to the biotinylated BSA gold surface. The chip functionalisation was completed by incubation with 10 µg/mL biotinylated anti-HER2 antibody.

Exosome capture and detection

Tunable resistive pulse sensing using qNano (iZON Science, New Zealand) was used to determine the concentration of exosomes as described previously [37]. The concentrations were measured by calibrating particle count rate against a reference particle (polystyrene beads, $d = 115$ nm) suspension. Exosome samples were then prepared by spiking exosomes in PBS (1 mM, pH 7.0, 250 µL) or undiluted human serum (250 µL) to obtain desired concentrations (3.31×10^4 exosomes/µL, 1.66×10^4 exosomes/µL, 1.10×10^4 exosomes/µL, and 0.828×10^4 exosomes/µL). Serum samples of healthy individuals were obtained from Ventyx Wesley Research Institute Tissue Bank, Brisbane, Australia, under the UQ HREC ethical approval number 2011001315 and Bellberry application number 2015-12-817. The serum samples were stored in -80 °C until further use. The target samples containing HER2 (+) exosomes were finally captured by passing through the previously functionalised SPR chip using SPR system.

Results and discussion

Assay principle

Figure 1 represents the methodological approach for the isolation and detection of tumour-specific exosomes from biological samples. The SPR chips were functionalised using

standard biotin-avidin chemistry to harbour an antibody targeting tumour-derived exosomes. We utilise this approach to demonstrate the specific capture and detection of tumour-derived exosomes expressing human epidermal growth factor receptor 2 (HER2) which is a potential breast cancer biomarker. In a typical assay, biological samples containing target HER2(+) exosomes were driven through the SPR chip, with exosome isolation being monitored in real-time from the resulting SPR spectral shift.

Isolation and characterisation of exosomes from cancer cell line

To investigate the potential of our approach in capturing exosomes, we initially obtained populations of breast cancer cell line (e.g. BT474)–derived exosomes that potentially over express HER2 biomarker [38]. Next, we characterised the isolated exosomes using cryo-transmission electron microscopy (cryo-TEM). cryo-TEM analysis verified the presence of nanosized (50–200 nm) vesicles in the sample and suggested that these vesicles contain double-walled lipid membrane layers (Fig. 2a). These observations are in agreement with previous demonstrations on characterisation of these nanosized vesicles, thereby suggesting these to be of exosomal origin [39, 40]. We then utilised these exosome populations for further experiments on the SPR chip.

Specificity of detection

Prior to validating the capture performance it is important to determine the specificity and accuracy of capture. In this regard, spiked samples containing 1.66×10^4 exosomes/µL of BT474-derived exosomes in PBS (10 mM, pH 7.0) were driven through SPR chips functionalised using anti-HER2 antibody. Control experiments to validate the specificity were performed using SPR chips (i) without any capture antibody, (ii) functionalised with non-cognate PSA antibody, and (iii) functionalised with anti-HER2 and tested with spiked samples containing HER2(–) MDA-MB-231 cell–derived exosomes. Figure 2b represents the SPR sensograms for capture and control experiments (replicate SPR sensograms are provided in ESM Fig. S3). The SPR signal (i.e. spectral shift = 0.4 nm) obtained for capture experiments (Fig. 2b; blue) suggest that our method is capable of detecting HER2-specific exosomes from the samples spiked in PBS. Further, the negligible SPR signals obtained for each of the control experiments (Fig. 2b; magenta, green, and red) suggest that our approach is highly specific and can potentially be utilised for the analysis of exosomes from biological samples.

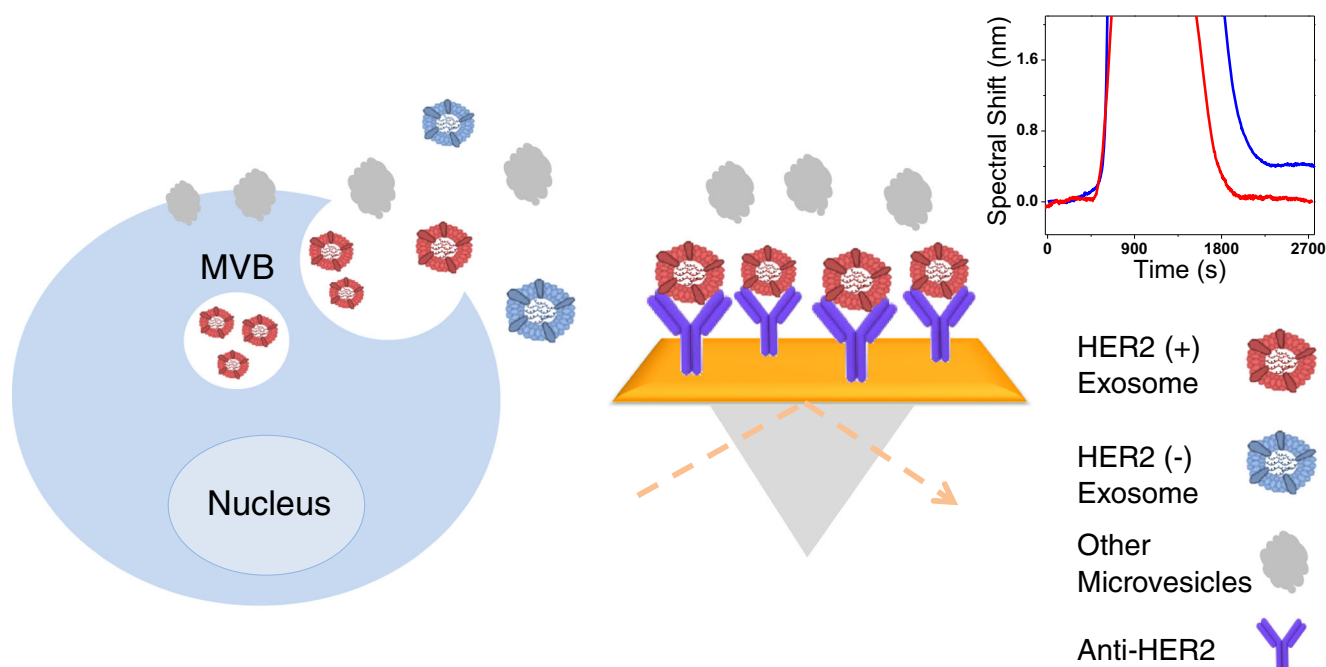


Fig. 1 Schematic representation of the detection of tumour cell-derived exosomes by SPR biosensor. The chip was first functionalised with biotinylated anti-HER2 (violet) using biotin-avidin chemistry. The target

samples were run through the SPR chips and the BT474 breast cancer cell-derived exosomes were detected in real-time by the functionalised anti-HER2

Sensitivity of detection

It has been previously determined that the average number of exosomes in biological sample is in the range from 1.0×10^5 to 3.0×10^9 exosome/ μL [40, 41]. Thus, it is important that any approach developed for exosomes is sensitive and specific to detect exosome populations in this desired range. To this end, we determined the sensitivity and dynamic range of detection of our approach by spiking BT474-derived exosomes in desired concentrations (3.31×10^4 exosomes/ μL , 1.66×10^4 exosomes/ μL , 1.10×10^4 exosomes/ μL , and 0.828×10^4 exosomes/ μL) in PBS and tested these samples by driving them through an anti-HER2-functionalised SPR chip. The

SPR signals were measured at 2750 s as the dissociation signals were constant after 2750 s. The SPR data in Fig. 3a clearly indicates that the assay is capable of detecting as low as 0.828×10^4 exosomes/ μL in PBS. The linear dynamic range for the detection of exosomes was found to be 0.828×10^4 to 3.31×10^4 exosomes/ μL (Fig. 3a and ESM Fig. S2). This level of detection is comparable with existing exosome detection techniques based on microfluidics and plasmonic biosensors. More importantly, our approach demonstrates the detection of exosomes well within the clinically relevant range of exosomes, thereby indicating that our approach may potentially be applicable to detect tumour-derived exosomes from biological samples.

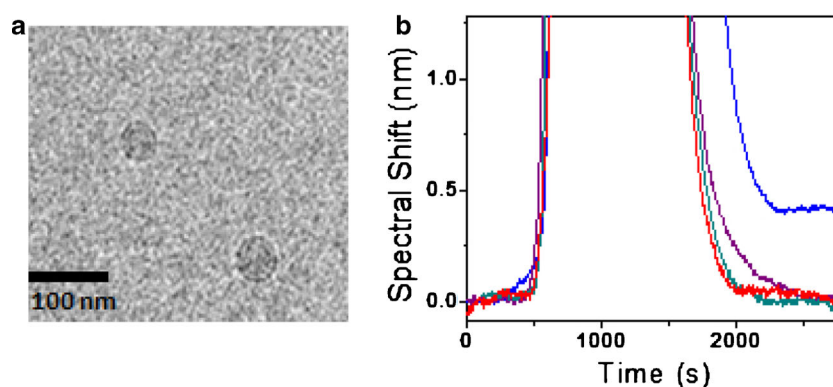


Fig. 2 Characterisation and capture specificity of the cell-derived exosomes. **a** Cryo-TEM image of the BT474 cell-derived exosomes. **b** SPR signals showing spectral shift generated by the BT474 cell-derived exosomes (1.66×10^4 exosomes/ μL spiked in PBS) driven through the

SPR chip functionalised with anti-HER2 (blue), without anti-HER2 (magenta), with non-specific anti-PSA (green), SPR signal for the MDA-MB-231 cell-derived exosomes (1.66×10^4 exosomes/ μL spiked in PBS) run through the anti-HER2 functionalised SPR chip (red)

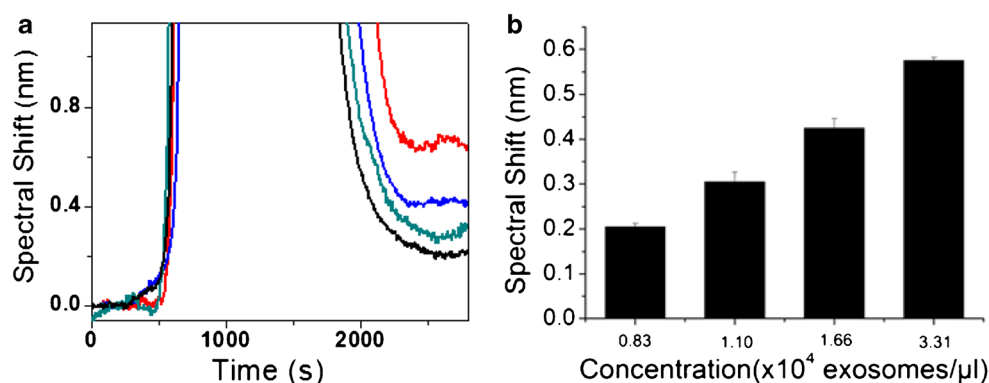


Fig. 3 Assay sensitivity and linearity. **a** SPR signal showing spectral shift generated by the designated concentration of BT474 cell-derived exosomes (3.31×10^4 exosomes/ μL (red), 1.66×10^4 exosomes/ μL (blue), 1.10×10^4 exosomes/ μL (green), 0.828×10^4 exosomes/ μL (black)) spiked in PBS. **b** Bar graphs showing the mean SPR spectral

shift values for each of the concentrations. Each data represents the average of three separate trials ($n = 3$). Error bars represent the standard deviation of measurements (relative standard deviation (%RSD) was found to be $< 5\%$ for $n = 3$)

Detection of exosomes spiked in serum

Finally, we tested the ability of our assay to detect tumour-specific exosomes spiked in serum sample. Since serum is a complex matrix containing a large number of biomolecules, including exosomes from most viable body cells, we initially performed a negative control study by driving serum samples without any target exosomes through an anti-HER2-functionalised SPR chip and determined the background response. This resulted in an increase in SPR signal (i.e. SPR spectral shift = 4.83 nm shown in SPR sensogram and in the bar graph) due to the non-specific adsorption and this was considered as the background noise for exosome capture experiment. Designated proportion of exosomes were then spiked in serum samples to get desired concentrations (3.31×10^4 exosomes/ μL , 1.66×10^4 exosomes/ μL , 1.10×10^4 exosomes/ μL , and 0.828×10^4 exosomes/ μL) and driven through the anti-HER2-functionalised SPR chip. The SPR

signals were measured at 2750 s as the dissociation signals were constant after 2750 s. However, since serum is a complex sample, the dissociation signals for the serum samples were monitored for longer time. The data presented in Fig. 4 shows the sensor response to detect exosomes spiked in serum samples. The SPR signal increased proportionally with the increased number of exosomes spiked in serum samples. These data corroborate our previous experiments with exosome samples spiked in PBS and showed that our capture was uncompromised despite the complexity of the sample evident from the detection of as low as 0.828×10^4 exosomes/ μL in serum samples. The capture performance of our approach is comparable with existing microfluidic technologies, conventional exosomes isolation methods and methods utilising nanoplasmonic sensors [13–18]. However, the sensitivity could be further be improved using plain silver [42] or gold-silver-gold trilayer [43] metallic SPR chip instead of plain gold chip. The

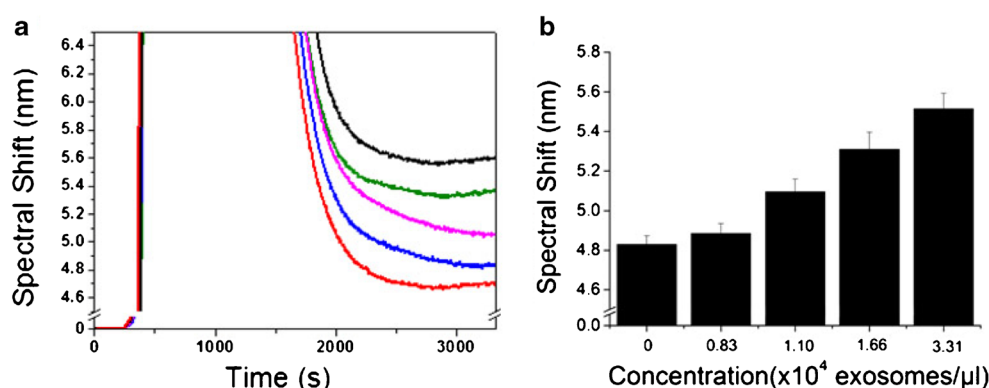


Fig. 4 **a** SPR signal showing spectral shift generated during the capture of exosomes spiked in serum samples in different proportions 3.31×10^4 exosomes/ μL (black), 1.66×10^4 exosomes/ μL (green), 1.10×10^4 exosomes/ μL (pink), 0.828×10^4 exosomes/ μL (blue), and serum without exosome (red). **b** Bar graphs showing the mean SPR spectral shift

values for each of the concentrations. Each data represents the average of three separate trials ($n = 3$). Error bars represent the standard deviation of measurements (relative standard deviation (%RSD) was found to be $< 5\%$ for $n = 3$)

sensitivity and specificity of the method could also be improved by integrating alternating current electrohydrodynamic fluid flow and micro-mixing instead of conventional fluid pump. The existing methods involving nanoplasmonic sensors [24] and surface plasmon resonance imaging (SPRi) [25] demonstrate remarkable capture performances and also facilitate specific isolation of exosomes from clinical samples. However, nanostructure fabrication for nanoplasmonic sensors and expensive set-up for SPRi restricted their integration into the clinical settings. In contrast, the alternative proof-of-concept approach presented here involves a simple custom-built readout system that enables a rapid, real-time, and label-free monitoring of exosome capture and offers potential as a simple diagnostic tool. We believe further improvements to the assay parameters, experimental protocol, and potential advancement towards a custom-built multiplexed detection platform could improve the applicability of our approach in clinical system.

Conclusion

We have developed a label-free and real-time technique for the detection of tumour-derived exosomes using SPR biosensor. As compared to the conventional method, this method allows highly specific, sensitive, and rapid detection towards detecting exosomes. Using this proof-of-concept method, we quantified as low as 8280 exosomes/ μL in PBS which is quite comparable with existing conventional techniques and immuno-affinity-based approaches. In addition, we also demonstrate the potential of our approach to identify tumour-derived exosomes from complex biological fluid (i.e. serum) thereby remarking its potential for non-invasive diagnostics. However, this platform is still limited by the non-specific adsorption of biomolecules to the sensor surface which could possibly be obviated by using efficient surface blocking. This can also be improved by introducing alternating current electrohydrodynamic micro-mixing of the biomolecules which can increase antigen-antibody interaction and simultaneously shear off the loosely bound biomolecules from the sensor surface. We believe that this simple and low-cost method with further improvements may potentially find numerous applications in cancer diagnostics.

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Compliance with ethical standards

The authors declare that they have no conflict of interest

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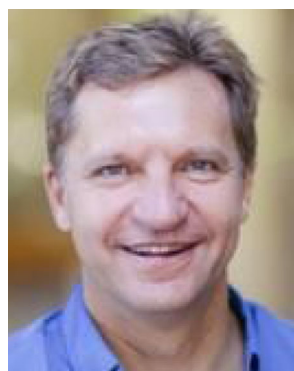
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direct interaction of these biomolecules with metal surfaces.



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