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Ultra-sensitive real-time detection of cancer-derived exosomes directly from cell supernatants by a large Goos–Hänchen signal generation on plasmonic sensing interface

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ABSTRACT

Exosomes have shown great potential in serving as a cancer biomarker over these years since they carry crucial information of their parent cells. Therefore, detection of exosomes is of vital importance to the early-stage diagnostics of multiple major diseases. In this paper, we have proposed a real-time and label-free sensing technique based on 2D Ge₂Sb₂Te₅ (GST) nanomaterial enhanced plasmonic substrate. We have achieved a detection limit down to 10⁴ exosomes/mL based on Goos–Hänchen (GH) shift measurement, which is more than two orders of magnitude superior to conventional SPR sensing techniques. Moreover, the detection of unpurified exosomes directly in cell culture supernatant (CCS) has been successfully demonstrated with a significant experimental GH shift signal of more than 120 μm been detected. Multiple control experiments using exosome-free solutions and control antibody coated substrates have also been performed, which validates the specificity of our device. The proposed plasmonic sensing scheme with enhanced sensing performance has the capability of detecting exosomes at low concentration levels. It also possesses the ability of direct detection of exosomes in CCS, which offers a convenient and efficient platform for exosome detection and analysis. We envision that this technique can serve as a promising tool in early-stage clinical diagnostics and treatment.

1. Introduction

Exosomes are small membrane extracellular vesicles (EVs) with characteristic size ranging from 50 to 150 nm, which are secreted by various cell types and carry key molecular information of parent cells (Kalluri and LeBleu, 2020; Théry et al., 2002). They are enriched in biofluids and have good stability over weeks. They play an important role in tumor aggressiveness transfer required for tumor progression, invasion, and metastasis (Mathivanan et al., 2010; Nie et al., 2017; Pinet

et al., 2016). These characteristics have made exosomes a promising biomarker in clinical applications (Azmi et al., 2013; Nawaz et al., 2014; Yu et al., 2021). Therefore, exosome detection is of prime significance to early stage diagnosis (Bu et al., 2019; Stobiecka et al., 2019) as well as to its prognosis value on therapeutic resistance in order to prevent recurrences in various diseases (Boelens et al., 2014; Tang et al., 2020; Thakur et al., 2020). In particular, the expression profile of the proteins carried by exosomes has a close relationship with the types and stages of cancer. The identification of exosomal proteins is of great significance to

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study the development and progression of cancer, which also has great potential in cancer diagnostic and prognostic (Li et al., 2017; Wang, X. et al., 2022). Currently, enzyme-linked immunosorbent assay (ELISA) and Western blot are classical detection approaches to detect and characterize exosomal proteins (Oliveira-Rodríguez et al., 2016; Olver and Vidal, 2007; Pfeiffer et al., 2022; Song et al., 2019). However, these methods present limited sensitivity detection. Moreover, they usually require large sample volumes, expensive labeling and are time-consuming. Since patients' liquid biopsies are limited and need precious materials, developing convenient, highly sensitive, low sample volumes, and real-time sensing techniques is still of major importance to improve diagnostic and prognostic applications in cancer.

Over the recent years, plasmonic sensors have experienced a rapid technological development in exosome detection and analysis (Chin et al., 2020). Various platforms including localized surface plasmon resonance (LSPR) (Thakur et al., 2017, 2021), SPR imaging (Zhang et al., 2022), nanoplasmonics (Im et al., 2014; Li et al., 2022; Song et al., 2022; Zhang et al., 2020; Zhang et al., 2018; Zhu et al., 2020), surface-enhanced Raman spectroscopy (Carmicheal et al., 2019) as well as plasmonic-enhanced fluorescence detection (Zhou et al., 2022) have been utilized to capture and detect exosomes. Among them, SPR possesses the merits of high sensitivity, label-free, and real-time sensing capabilities (Mao et al., 2021; Stobiecka and Hepel, 2011; Wang et al., 2019; Wang et al., 2022). The sensing mechanism of SPR sensors is to detect the optical signal change in the reflected light beam at the sensing interface. This signal change strongly depends on the evanescent field perturbation, which is caused by the molecular binding happened on the plasmonic substrate (Singh, 2016; Souto et al., 2019). Therefore, the label-free detection of exosomes can be realized by measuring this signal change. Traditional plasmonic sensors based on wavelength or angular interrogation still face challenges in terms of detection sensitivity, which makes it difficult to deal with the situation where target analytes have low concentration (Zeng et al., 2014). Moreover, since the purification of exosomes usually requires complex differential ultracentrifugation processes (Ayala-Mar et al., 2019; Zhang et al., 2016), the direct sensing of exosomes surface protein in the cell culture supernatant (CCS) without previous isolation and purification steps is largely needed, which raises a higher demand in terms of the detection sensitivity. Currently, most SPR sensing techniques for exosome detection need complicated isolation/purification steps in advance using either extraction kit or ultracentrifugation (Im et al., 2014; Sina et al., 2019; Wang et al., 2022). The incapability of direct detection of exosomes in CCS has largely limited their application scenarios.

To overcome this challenge, we have proposed a highly sensitive plasmonic sensing technique for enhanced exosome detection. The sensitivity enhancement has been achieved through adding an atomically thin layer of $\text{Ge}_2\text{Sb}_2\text{Te}_5$ (GST) material to tune the reflection state. Moreover, the exosome detection is based on phase-induced

Goos–Hänchen (GH) shift measurement instead of the traditional wavelength or angle-based measurement, which brings further improvement to the sensitivity. A detection limit of 10^4 exosomes/mL has been achieved, which is more than two orders of magnitude superior to the traditional SPR sensing scheme. We have also successfully demonstrated the detection of unpurified exosomes directly in CCS and achieved a significant GH shift signal change of more than $120 \mu\text{m}$. The proposed plasmonic sensing scheme provides a convenient and efficient platform for exosome detection, which is a promising tool in biological applications and clinical diagnostics.

2. Experimental

2.1. Design of plasmonic sensing system

Fig. 1 illustrates the schematic diagram of detecting exosomes using our designed plasmonic metasurface based on the typical Kretschmann configuration. The highly enhanced plasmonic sensing substrate consists of a 40 nm Au layer and an atomically thin layer of 2D GST material. Compared to other 2D materials such as MoS_2 or graphene, 2D GST materials possess a higher absorption rate in visible and near-infrared regions (Kumar et al., 2020; Li, C. et al., 2022). Through optimizing the thickness of GST layer deposited on Au, a “zero-reflection” state can be achieved, which will lead to an extremely sharp phase change at the resonance angle. This steep phase change will cause a giant lateral position shift which can be readily detected by a position sensor. The measurement of such phase-induced lateral position shift can largely enhance the detection sensitivity. In our application scenario, when the target exosomes are captured by the antibody, a giant phase-induced lateral position shift will be triggered, which can be used to detect exosomes at low concentration levels. Detailed information on the sensing mechanism and optimization of the plasmonic substrate can be found in supporting information.

2.2. Purification of exosomes

Exosomes were isolated from glioblastoma cell line (U87-MG). Cells were cultured at a concentration of $10,000 \text{ cells/cm}^2$ in Minimum Essential Medium (MEM) (Gibco, Life Technologies), supplemented with 10% fetal bovine serum (FBS), 1% sodium pyruvate, 1% Non-Essential Amino Acid (NEAA), and 0.75 g sodium bicarbonate. Cell supernatants were harvested after 72 h (h) in culture to purify exosomes by differential centrifugation. To eliminate the influence of EVs present in the FBS, it was ultracentrifuged for 16 h at $120,000 \times g$ (Optima XPN-80 Ultracentrifuge, Beckman Coulter) and then filtered using $0.2 \mu\text{m}$ membrane.

For SPR detection directly in CCS, the supernatant was first centrifuged at $300 g$ for 10 min to eliminate the remaining cells. Supernatant

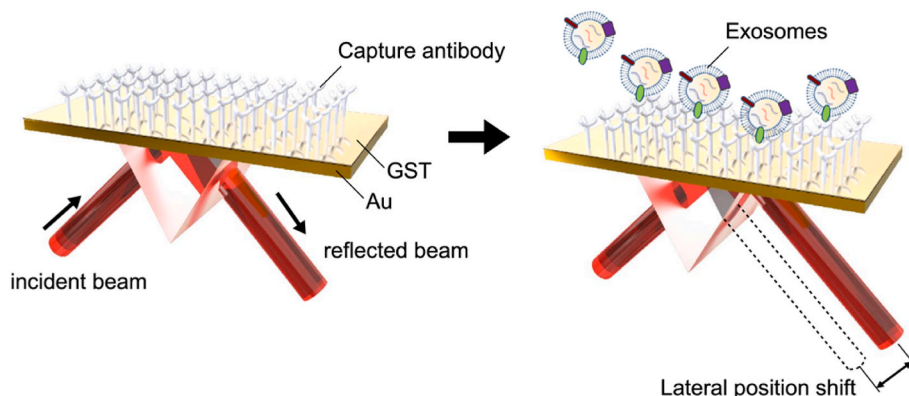


Fig. 1. Schematic diagram of exploiting plasmonic metasurface for exosome detection.

was then centrifuged at 2000g for 10 min to remove dead cells. For SPR detection of purified exosomes, the differential ultracentrifugation protocol was used (Théry et al., 2006). After the two initial centrifugations at 300 g and 2000g, the supernatant was centrifuged at 18000 g for 40 min. The remaining supernatant was then ultracentrifuged at 120000 g for 70 min for obtaining the pellet containing EVs. The pelleted EVs were then washed in phosphate-buffered saline (PBS) solutions (pH~7.4) before the second ultracentrifugation at 120000g for 70min. EVs were then resuspended in PBS for the following nanoparticle tracking analysis (NTA). For Western blot analysis, they were resuspended in cell lysis buffer.

2.3. Characterization of exosomes

2.3.1. NTA analysis

As shown in Fig. 2a, we have characterized the size and determined the concentration of purified EVs by using the NTA technique. NTA was carried out by NanoSight NS300 (Malvern Panalytical Ltd.) as previously described (Auger et al., 2022). Images and analysis were processed using the software NanoSight NTA3.3.301 (Malvern Panalytical Ltd.). Samples were diluted in 1 mL PBS. Their concentration was determined by respecting a rate of around 40–70 particles/frame. The size of EVs was in a range of 50–180 nm with a mean value of 126.1 nm and their concentration was around 10^8 nanoparticles/mL.

2.3.2. Transmission Electron Microscopy

Purified EVs were resuspended in filtered PBS and 1 drop (5 μ L) of EVs suspension were deposited on the formvar-carbon films coated 200 mesh copper grids (Agar scientific). EVs were then stained with uranyl acetate solution (1%). Analyses were made by using a Jeol 1011 electron microscope (JEOL) and images were acquired with AMT XR60B digital camera (Advanced Microscopy Techniques). Morphologies of the purified exosomes were shown in Fig. 2(b), which is consistent with the size measured by NTA and matches the value of reported exosomes.

2.3.3. Western blot analysis

Purified EVs (after differential ultracentrifugation) were lysed using a cell lysis buffer (Cell Signaling Technology) for 30 min on ice, followed by a centrifugation at 17,000 g for 20 min. The supernatant was then collected for evaluating the protein concentration using a Bradford protein concentration assay (BioRad). SDS-PAGE was achieved on 10 μ g and proteins were transferred on PVDF membranes (GE Healthcare). Membranes were incubated in 5% BSA in 1X TBS-0.1% Tween-20 (blocking solution) for 1 h. Then, they were exposed to primary antibodies (Anti-CD9, catalog number SAB1402143, Sigma-Aldrich; Anti-CD81 and Anti-HSC70 both from Santa Cruz Biotechnology, catalog

number SC-166029, SC-7298) in a blocking solution overnight at 4 °C. Anti-CD9 and anti-CD81 antibodies were used for their specific recognition of tetraspanins (Mathieu et al., 2021), present on EVs membrane, whereas anti-HSC70 targets a well-known intra-exosomal protein HSC70. Polyvinylidene difluoride (PVDF) membranes were washed in TBS-0.1% Tween-20 for three times and then incubated in the blocking solution again for 1 h with the secondary antibody targeting mouse immunoglobulins (Dako Cytomation). Membranes were then washed in TBS-0.1% Tween-20 for two times and once in TBS, followed by revelation using a G-Box (Fisher Scientific) and the GeneSys application suite. As shown in Fig. 2 (c), CD9, CD81 and intracellular HSC70 were all evidenced.

3. Results and DISCUSSION

3.1. System calibration

As a typical calibration process of plasmonic sensing systems, we first measured the lateral position shift signals under PBS solutions mixed with distilled water at different ratios. As shown in Fig. 3, the lateral position shift signals increased linearly with the increasing PBS/water weight ratios. The experimental measurement of the signal change for 100% PBS solutions compared to distilled water solutions (0.0012

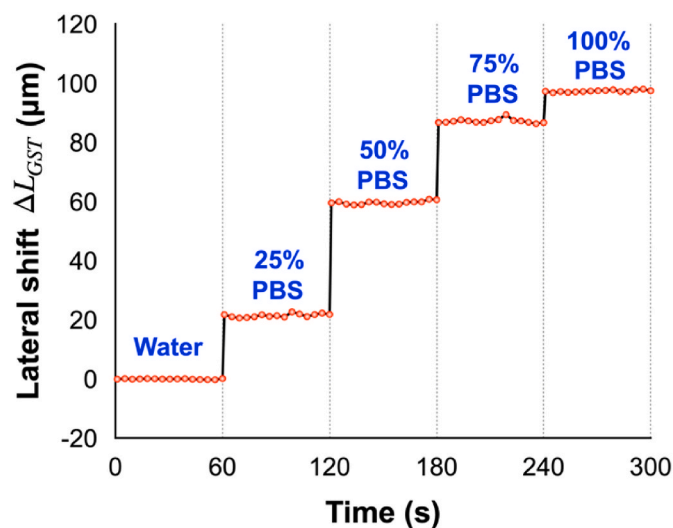


Fig. 3. System calibration results through measuring PBS solutions with different concentration.

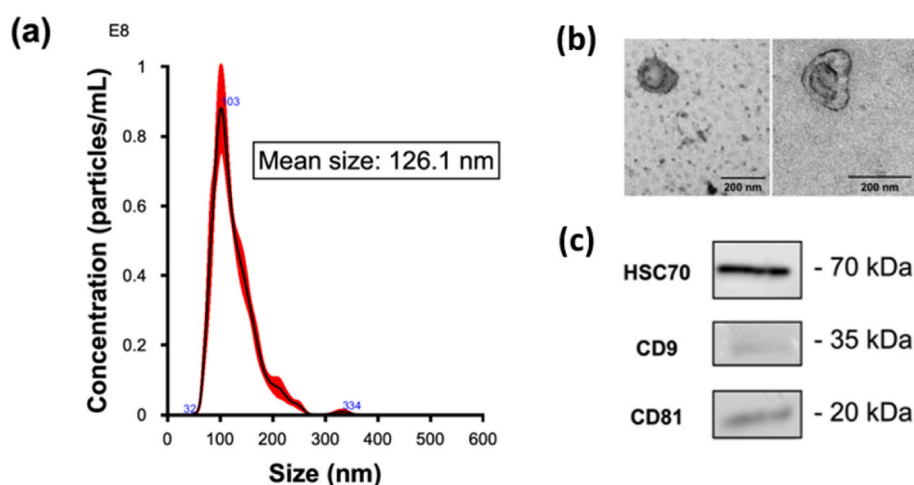


Fig. 2. (a) NTA analysis of extracellular vesicles from U87-MG cells, allowing an evaluation of the size repartition and the particles concentration. Most of the EVs population is between 50 and 180 nm, which is within the characteristic size range of exosomes. (b) Transmission Electron Microscopy images of purified EVs showing their size and their typical cup-shaped morphology. (c) Western blot analysis of common exosomes' markers on purified EVs: expression of membrane tetraspanins CD9 and CD81 and intracellular HSC70 were evidenced.

Refractive Index Unit, RIU change) using our GST-on-Au plasmonic substrate is $97.80 \mu\text{m}$ on average, which corresponds to a sensitivity figure-of-merit of $8.15 \times 10^4 \mu\text{m}/\text{RIU}$.

3.2. Detection of purified exosomes

The capability of our device in sensitive exosome detection has been demonstrated in Fig. 4. We have conducted experiments using both traditional Au-only substrate and our GST-on-Au substrate. As shown in Fig. 4 (a), the plasmonic sensing substrate was first coated with capture antibodies. Then, the exosomes solutions were injected to the sensing surface. If the exosomes contain surface protein which is specific to the capture antibodies, a large SPR sensing signal will take place. Then, after PBS wash, the detection antibody was introduced to the surface for further signal enhancement. Here, we used a target antibody (Anti-CD81) as both the capture antibody and the detection antibody. The sensing substrate was first coated with 1 nM Anti-CD81 antibodies, followed by injecting purified exosomes into the microfluidic chamber. Since tetraspanins like CD81 can serve to specifically characterize exosomes, anti-CD81 antibodies can bind to exosomes by recognizing specific epitopes in the CD81 protein structure. In our system, the increasing lateral position shift signal has indicated the binding process of exosomes to the antibody. For Au-only sensing substrate, the lateral

position shift signal upon injection of solutions containing 3×10^6 exosomes/mL barely showed any noticeable change. However, through exploiting the GST-on-Au substrate, exosome solutions with concentration down to 3×10^4 /mL can cause a significant increase (up to $77.68 \mu\text{m}$) in lateral position shift signal. Moreover, the signal experienced a further increase upon injection of 1 nM target antibodies. These results showed that our designed setup based on GST-on-Au substrate has the capability of detecting the membrane proteins on exosomes at much lower concentration levels compared to traditional Au substrate.

To verify the specificity of our system, we conducted two sets of control experiments, as shown in Fig. 5. First, we coated the same capture antibody on the plasmonic metasurface. Then, instead of flowing target exosomes like in Fig. 4, we flow exosome-free solutions into the microfluidic chamber and use PBS to wash the unbound molecules. The lateral position shift barely showed any change during this process in Fig. 5 (a). Furthermore, the signal remained stable after the second antibody injection. This shows that our system only responds to the situation where exosomes are present in the solutions to be measured. Besides this experiment, we have also conducted experiments based on anti-HSC70 as a control antibody. Since HSC70 is an intra-exosomal protein, no SPR signal will be triggered upon exosome injection when the sensing surface were coated with anti-HSC70. As shown in Fig. 5 (b), the lateral position shift signal showed an increase upon the injection of exosomes, followed by an immediate drop when injecting PBS buffer. The lateral position shift signal has returned to the initial level, which indicated the weak binding between exosomes and control antibodies. The second injection of the control antibody experienced a similar trend, which further confirmed the high specificity and reliability of our device. The experimental results also indicate that our plasmonic sensing scheme can effectively distinguish the protein type and identify whether it's a surface protein or an intra-exosomal protein.

3.3. Direction detection of unpurified exosomes in CCS

Furthermore, we demonstrated the capability of our system in detecting exosomes directly from CCS without any purification or enrichment process. The real-time detection of exosomes in CCS was shown in Fig. 6. Here, anti-CD9 antibody was used as the capture antibody. We can see that the injection of exosomes in CCS can cause a significant increase in the lateral position shift signal. Despite some decrease after PBS wash, the signal still exhibited an increasing trend upon the exosomes and antibody injection. The experimental results have shown the capability of our device in detecting unpurified exosomes directly in CCS using a sandwiched bioassay.

When detecting unpurified exosomes directly in CCS, it is important to make sure the protein aggregates in the solutions will not affect the sensing performance. Therefore, we have conducted several sets of control experiments and made a comparison based on these results. Please note that other than the exosomes, the exosome-free solutions contain the same content as exosomes-present CCS solutions including all the possible protein aggregates in the solutions, which can provide a reliable reference signal. As depicted in Fig. 7 (a), the control experiment of detecting exosome-free solutions showed a tiny change in lateral position shift signal and experienced a sudden drop to almost zero after PBS wash. For the control experiment based on anti-HSC70 coated substrate in Fig. 7 (b), the signal also experienced an increasing trend, except that the value is smaller than the one based on anti-CD81 coated substrate. Moreover, the decrease in signal upon PBS buffer injection is much larger. Compared to the detection of exosomes in CCS using anti-CD81 coated substrate, both control experiments showed an insignificant increase upon analyte injection, followed by a large decrease after PBS wash. These results showed that the presence of potential protein aggregates in the solutions will not give rise to any misleading signals in the lateral position shift. We have also performed t-tests between groups to show that there is a significant difference ($p < 0.0001$) in signal change between the experiment group and control groups (Supporting

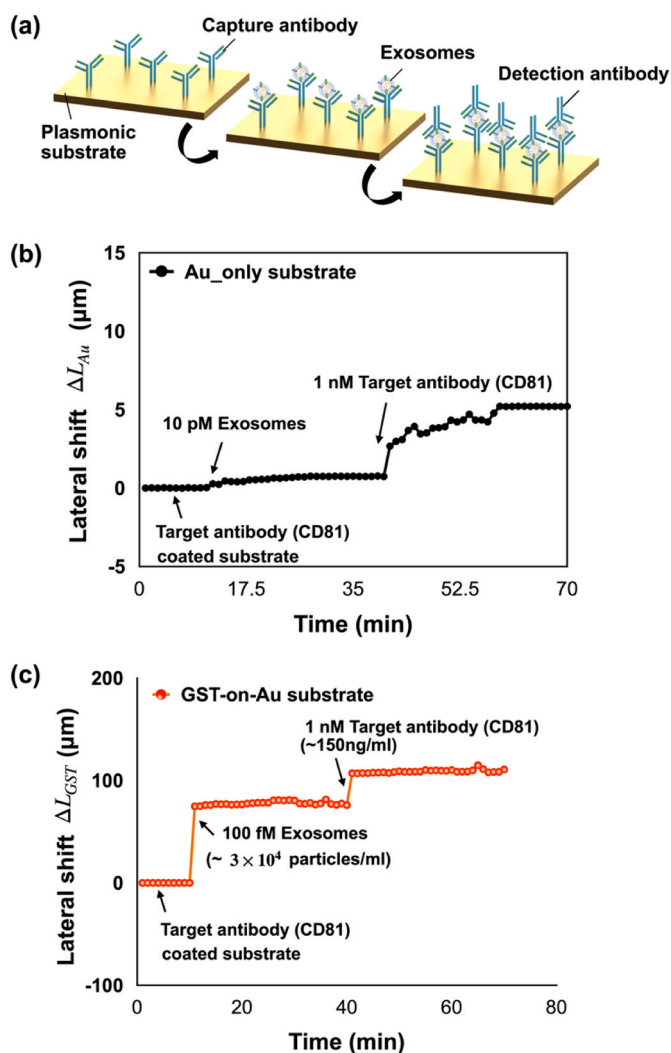


Fig. 4. Detection of exosomes: (a) Schematic diagram of the detection process; (b) Detection based on Au-only substrate; (c) Detection based on GST-on-Au substrate.

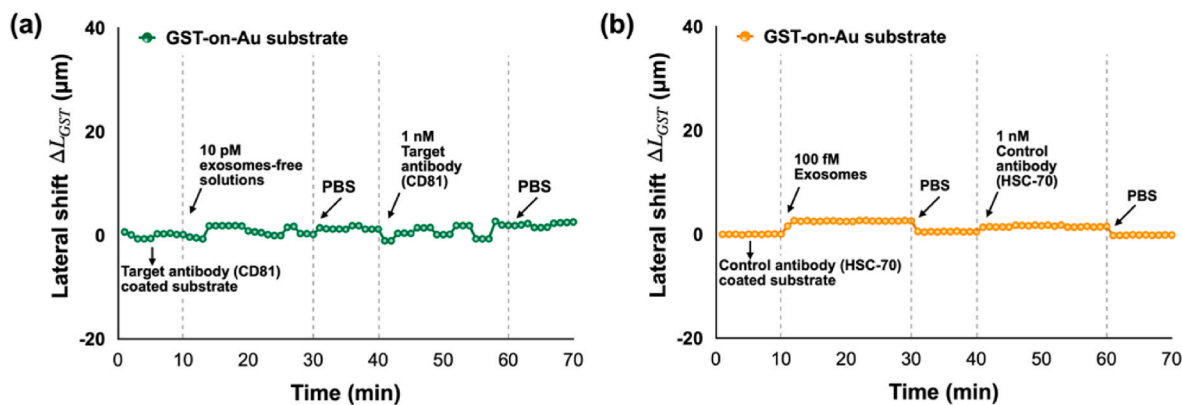


Fig. 5. Control experiments based on GST-on-Au substrate: (a) Detection of exosome-free solutions; (b) Detection of exosomes (10 fM) based on control antibody-coated substrate.

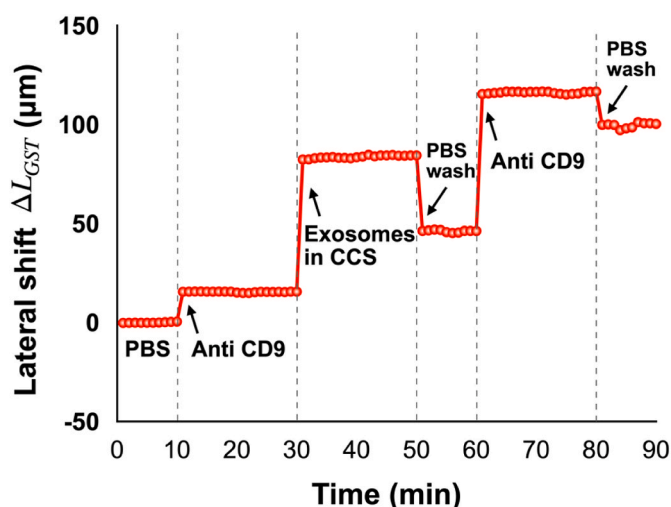


Fig. 6. Real-time detection of exosomes in CCS based on lateral position.

information). These results have shown the specificity and reliability of exploiting our device for direct exosome detection in CCS.

4. Conclusion

In conclusion, we have designed an ultrasensitive plasmonic biosensing scheme based on phase-induced lateral position shift for exosome detection. Our device possesses the merits of real-time, label-free, high sensitivity and specificity. The proposed biosensing scheme has successfully realized the detection of exosomes with a concentration level of 10^4 exosomes/ml, which has improved the detection limit by more than two orders of magnitude than the traditional one. Moreover, our device is also capable of detecting unpurified exosomes directly from CCS. The proposed biosensing scheme can serve as an effective tool for exosome monitoring and has great potential in the early-stage diagnostics of various diseases.

CRediT authorship contribution statement

Manel Hedhly: Methodology, Validation, Investigation, Writing – original draft. **Yuye Wang:** Formal analysis, Visualization, Methodology, Writing – original draft. **Aude Brunel:** Visualization, Investigation,

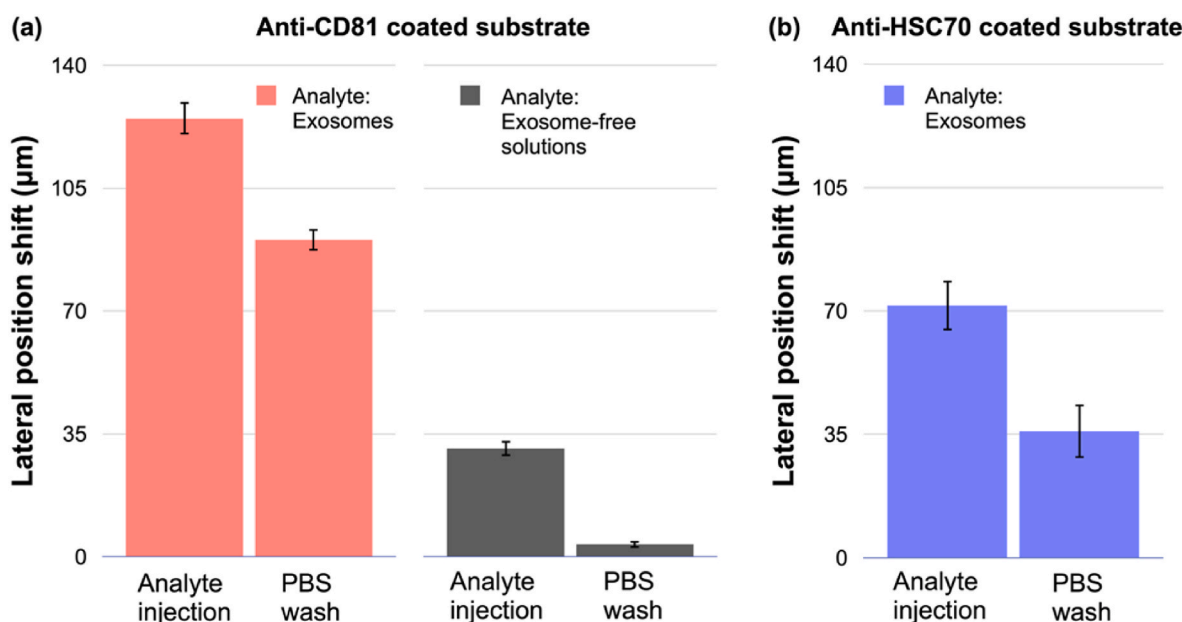


Fig. 7. Detection of exosomes in CCS: (a) Sensing signal of exosomes and exosome-free solutions. (b) Sensing signal based on anti-HSC70 coated substrate.

Formal analysis, Methodology. **Flavien Boffara**: Formal analysis, Methodology. **Hussein Akil**: Validation, Formal analysis, Investigation, Methodology. **Mireille Verdier**: Formal analysis, Funding acquisition, Investigation, Methodology. **Barbara Bessette**: Validation, Investigation, Formal analysis, Writing – review & editing. **Aurelian Crunteanu**: Methodology, Writing – review & editing. **Ho-Pui Ho**: Methodology, Writing – review & editing. **Georges Humbert**: Conceptualization, Formal analysis, Funding acquisition, Methodology, Writing – review & editing. **Fabrice Lalloué**: Resources, Conceptualization, Formal analysis, Funding acquisition, Methodology, Writing – review & editing. **Shuwen Zeng**: Conceptualization, Methodology, Supervision, Resources, Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.biosx.2023.100391>.

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