



Ultrasensitive detection of oral cancer biomarkers CD63 and CD44 in exosomes using janus particles: a rotational diffusometry-based assay

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Abstract

Exosomes containing biomarkers offer potential for non-invasive diagnostics. For screening purposes and to improve patient treatment outcomes, early diagnosis of oral cancer is essential. This study's goal is to create a non-invasive assay using Janus particles (JPs) for detecting exosomes biomarkers CD63 and CD44 derived from the H357 oral cancer cell line. The JPs were functionalized with CD63 and CD44 antibodies to enable specific binding with target exosomes. The binding events were monitored through changes in rotational diffusivity, analyzed using cross-correlation techniques. The Stokes–Einstein–Debye model was used to correlate increasing exosome concentrations with slower particle rotation. The presence of the target biomarkers is confirmed with an enzyme-linked immunosorbent assay (ELISA). The JPs detected significant differences in rotational diffusivity between exosome binding antibodies with control group (PBS only) groups ($p < 0.05$). The ELISA confirmed the presence of CD63 and CD44 biomarkers. The JPs based assay provides a high-sensitivity method for detecting oral cancer surface markers on exosomes. This technology shows promising potential as an effective and non-invasive method for oral cancer early screening and diagnosis.

Keywords Janus Particles · Oral Cancer · Exosomes · CD63 · CD44 · Biomarker Detection · Biosensor

1 Introduction

Oral cancer ranks as the sixteenth most predominant variety of cancer, and Asian populations have the greatest rates of incidence, death, and five-year prevalence (GLOBOCAN,

“LIP, ORAL CAVITY” 2022; Sun et al. 2023). Low public awareness and late-stage diagnoses are the main causes of the low survival rate. Human papillomavirus (HPV) infection, alcohol and tobacco usage, and the cultural practice of chewing betel quid are important etiological variables (Sun et al. 2023; Xie and Shang 2022).

Early detection is crucial for enhancing the survival rate of oral cancer patients. However, diagnosis is often delayed because early-stage lesions are asymptomatic or misdiagnosed. The current gold standard for diagnosis is tissue biopsy. It enables definitive histopathological assessment, but is invasive, time-consuming, and requires specialized expertise (Rebaudi et al. 2023). These disadvantages highlight the need for non-invasive, quick, affordable, and widely available alternative diagnostic instruments, especially for use in community-level or resource-constrained settings.

Existing non-invasive diagnostic modalities, including vital staining and oral cytology, are limited by subjectivity, inconsistent interpretation, and potential discomfort during sampling (Alsarraf et al. 2018; Kujan et al. 2018). Similar challenges include limited tissue penetration, environmental

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sensitivity, and variability resulting from electrode placement or tissue hydration for optical coherence tomography (OCT), fluorescence, Raman spectroscopy, and electrical impedance spectroscopy (Esperou et al. 2025; Murdoch, et al. 2014). Consequently, this further emphasizes the urgent need for objective, sensitive, and reproducible diagnostic technologies to detect early molecular alterations associated with malignant transformation.

Detecting molecular signatures from oral potentially malignant disorders (OPMDs) lesions could significantly enhance screening efficiency and patient outcomes. The OPMDs represent precursor lesions that precede oral cancer and offer a window for early intervention.

Disease-specific proteins and nucleic acids, which represent the molecular state of their parent cells are carried by exosomes (Cheng et al. 2019; Zhang et al. 2019). Among their surface proteins, CD44 is a known biomarker of the development of oral cancer, while CD63 is frequently linked to endosome-derived vesicles (Jeppesen et al. 2019)–(Singh et al. 2025). Exosomes have been successfully used to identify a number of cancer forms, such as glioma (Skouras et al. 2023), prostate cancer (Kohaar et al. 2021), pancreatic cancer (Qin et al. 2024), gastric cancer (Pan et al. 2017), and breast cancer (Wang et al. 2024; Alagundagi et al. 2023). Additionally, research indicates that exosomes can function as trustworthy biomarkers for oral cancer (Bano et al. 2023; Liu, et al. 2022). Salivary, serum, or plasma exosomes are examples of samples that are typical of biomarkers in cases of oral cancer (Zhao et al. 2025). According to the study by Xu et al. (Xu et al. 2025), tongue squamous cell carcinoma is the most prevalent type of oral cancer. Compared to cancer in the other parts of the oral cavity, oral cancer in the tongue region has worse clinical outcomes and is more biologically aggressive reflecting the tongue's heavy blood and lymphatic circulation as well as its constant mechanical activity.

Janus particles (JPs), consisting of fluorescent polystyrene microspheres partially coated with gold, present unique anisotropic properties that enable enhanced biosensing capabilities. Molecular diagnostics, imaging, and pathogen detection have all benefited from their high surface-to-volume ratio, biocompatibility, and functional flexibility (Jiang et al. 2018; Zhang et al. 2021). Recent research has shown that antibody-functionalized JPs may successfully detect tumor necrosis factor- α and pathogens like SARS-CoV-2 and *E. coli* with greater speed and sensitivity than real-time (Chen and Chuang 2020; Das et al. 2024).

This study aims to develop a non-invasive, rapid, and cost-effective biosensing platform utilizing antibody-functionalized JPs for detecting exosomal biomarkers CD63 and CD44 derived from H357 oral cancer cell line. By integrating immunoassay specificity with cross-correlation analysis of rotational diffusivity, this platform is designed to provide ultrasensitive detection of oral cancer exosomes. In the

end, our strategy aims to create a useful screening tool for OPMDs and oral cancer early detection, improving accessibility and diagnostic precision in clinical and community settings.

2 Materials and method

2.1 Materials

Janus particles (JPs) were prepared using 1 μm fluorescent polystyrene microspheres (Thermo Fisher Scientific, USA), with one hemisphere coated with a thin layer of gold following the method described by Chen and Chuang (Chen and Chuang 2020). The gold-coated surface provided anisotropic functionalization for biomolecular conjugation. A Gold Conjugation Kit (Abcam, UK) was used to covalently attach antibodies to the gold surface of the JPs.

To detect specific exosomes, monoclonal anti-CD63 antibody (Invitrogen, USA) and anti-CD44 monoclonal antibody (ABclonal, USA) were used. Exosomes from the H357 cell line, a squamous cell carcinoma of the tongue, served as the experimental model. For all tests, the suspension and washing buffer was phosphate-buffered saline (PBS; pH 7.4). Enzyme-linked immunosorbent assay (ELISA) kits specific for CD63 and CD44 (ABclonal, USA) were utilized in compliance with the manufacturer's recommendations to validate biomarker expression.

2.2 Methods

2.2.1 Exosome isolation

Oral squamous cell carcinoma cell lines (H357) were obtained from Integrated Research Laboratory, Faculty of Dentistry, Universitas Gadjah Mada. Dulbecco's Modified Eagle Medium (DMEM) with a high glucose supplement was used to cultivate the cells from passage 3 with the addition of 10% fetal bovine serum and 1% penicillin–streptomycin then incubated at 37°C with 5% CO₂ level and 95% humidity.

After H357 cell cultures achieved around 80% confluence, the conditioned media was extracted. The supernatant was initially transferred into tubes and centrifuged at 300 $\times$ g for 10 min at 4 °C in order to extract intact cells. The resultant supernatant was centrifuged at 10,000 $\times$ g for 30 min at 4 °C in order to eliminate big debris and dead cells. A 0.22 μm syringe filter was then used to filter the cleansed supernatant to produce an exosomes-enriched fraction.

To pellet the exosome, this filtrate was moved to an ultracentrifuge and centrifuged at 100,000 $\times$ g for two hours at 4 °C. The pellet was subjected to a second ultracentrifugation at 100,000 $\times$ g for two hours at 4 °C after the supernatant

was carefully disposed of, and then, a first cleaning with sterile PBS to get rid of any contaminating proteins was done upon that. After being cleaned, the last exosome pellet was resuspended in 1.5 mL of sterile PBS. The purified exosomes were aliquoted and kept at -80°C before being used again (Coughlan et al. 2021). A microdrop was used to measure the exosomes' concentration and size distribution.

2.2.2 Antibody-conjugated janus particle

A commercial gold conjugation kit (Abcam, UK) was used to covalently attach antibodies to the gold surface of Janus particles (JPs). In short, the antibodies ($1\text{ }\mu\text{g/mL}$) were reconstituted to a concentration of $0.1\text{ }\mu\text{g/mL}$ using the provided gold antibody diluent buffer. $12\text{ }\mu\text{L}$ of diluted antibodies were mixed with $42\text{ }\mu\text{L}$ of gold reaction buffer.

To guarantee uniform dispersion, the JP suspension was vortexed and sonicated for 30 s in an ultrasonic waterbath before conjugation. Next, $20\text{ }\mu\text{L}$ of the JPs suspension was added to the antibody mixture, and it was agitated at 400 rpm for 15 min at 25°C . After incubation, $5\text{ }\mu\text{L}$ of gold quencher buffer was added, and the mixed solution was gently vortexed. To stabilize antibody attachment, the reaction mixture was maintained at 4°C throughout the night. The conjugated particles were centrifuged at 13,500 rpm for 6 min at room temperature after three times washes using PBS to eliminate unattached antibodies. The final pellet was kept for later use after being resuspended in $70\text{ }\mu\text{L}$ of PBS.

2.2.3 Experimental setup

The rotational diffusivity measurements were used to detect CD63 and CD44 as biomarkers of exosomes derived from H357 cell line. To prepare the observation space, the edges of the microscope slide were sealed with tape to form shallow well that maintained the circular shape of the droplet. The glass slide was covered with a cover slip after $2\text{ }\mu\text{L}$ droplet of the JPs-exosomes suspension was put on it.

A fluorescence microscope (Nikon, Japan) fitted with a green filter (EX:510/DM:575/BA:590) was used to examine the JPs. To see the "blinking" fluorescence signal from the revolving JPs, the objective lens was tuned to $10\times$ magnification. The image sequences were captured using a camera (OptiLab, Indonesia) with a frame rate of 25 frames per second, or fps, and an image size of 1280×960 pixels (Fig. 1).

To determine the characteristic correlation time, a cross-correlation algorithm implemented in MATLAB (MathWorks, USA) was used to evaluate the blinking fluorescence signal, which represented the rotational movement of the JPs. An unpaired t-test with an acceptable threshold of 0.05 was used to statistically compare correlation times between groups.

2.2.4 Working principle of diffusometry particles

By computing the statistical correlation intensity, the rotational diffusivity of the JPs suspended in solution was examined. Rotational diffusivity is described by the Stokes–Einstein–Debye relation (Miller 1924), expressed as:

$$D_r = \frac{k_B T}{8\pi\mu r_p^3} \quad (1)$$

, where T is the absolute temperature, μ is the dynamic viscosity of the medium, r_p is the particle radius, and k_B is the Boltzmann constant. Rotational diffusivity is inverse to the cube of the particle radius under constant temperature and viscosity, as demonstrated by Eq. (1). In this study, antibody-functionalized JPs were used to produce a "blinking" signal under a fluorescence microscope, enabling the assessment of the rotational dynamics (Fig. 2 and Video S1). Instead of analyzing individual particles, the cross-correlation algorithm was used to compare sequential particle pictures over a specified of time period (Δt) in order to calculate the temporal correlation intensity. As paired particle orientations diverge over time, the correlation strength peak tends to decline (Das et al. 2022a). A gradual decline in peak intensity decreases corresponds to higher diffusional activity. The normalized cross-correlation intensity was fitted using an exponential decay model:

$$I = A \exp\left(-\frac{t}{\varnothing}\right) + B \quad (2)$$

, where $\varnothing$ represents the correlation time, t is the lapse of time, and A along with B are constants found from the data fit. Another way to express the correlation time is as:

$$\varnothing = \frac{\mu V}{k_B T} \quad (3)$$

, where V is the particle's volume. Equation (3) demonstrates that the correlation time ($\varnothing$) is inversely related to the viscosity of the surrounding medium, although particle volume and temperature remain constant.

2.2.5 Functionalized jps binding assay to the surface marker

The stock suspension of exosomes (1.5×10^5 exosomes/mL) was serially diluted with PBS to obtain 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , and 10^{-5} dilutions. For each dilution and undiluted stock, $10\text{ }\mu\text{L}$ of the JPs suspension were combined with $5\text{ }\mu\text{L}$ of the exosomes suspension. The PBS alone was used as a negative control. To promote exosome binding, the mixtures were shaken at 800 rpm for 11 h at room temperature. Following incubation, the suspensions were centrifuged at 13,500

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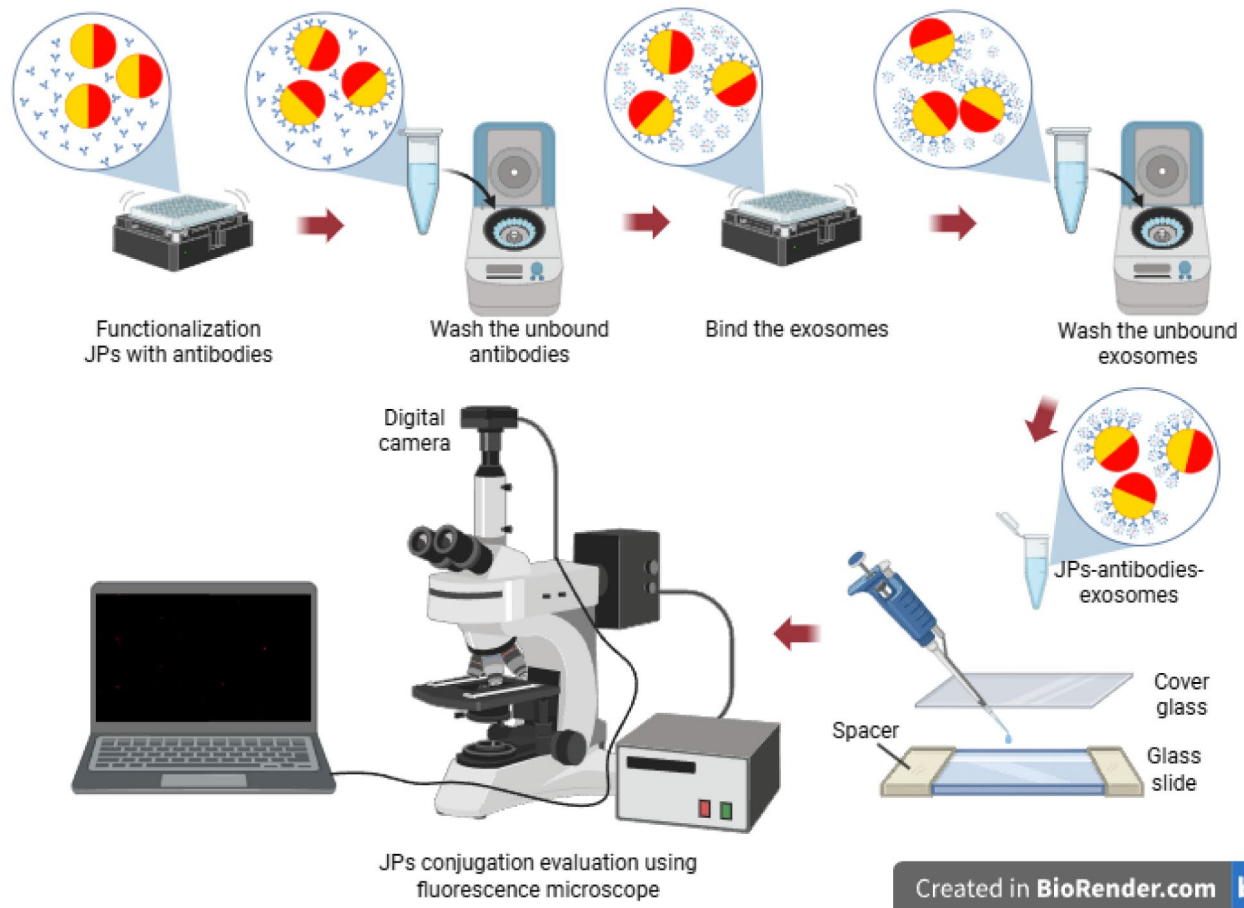


Fig. 1 Schematic illustration of the experimental workflow for exosome detection using antibody-functionalized Janus particles (JPs). The process begins with the functionalization of JPs, where antibodies specific to CD63 or CD44 are conjugated onto the gold hemisphere. The conjugated JPs are then incubated with exosomes derived from the H357 oral squamous cell carcinoma cell line. After incubation, unbound exosomes are removed through centrifugation and

washing steps. The resulting JP–exosome complexes are subsequently placed onto glass slides for fluorescence microscopic observation. Under the microscope, rotational motion of the JPs produces a “blinking” fluorescent signal, which is captured and analyzed by a computer using a cross-correlation algorithm to determine rotational diffusivity as an indicator of exosome binding. The schematic illustration was created by using BioRender

rotations per minute for 6 min at 25 °C to extract the unbound exosomes. These steps minimized random aggregation and ensured that subsequent analyses reflected specific exosome–JPs interactions.

2.2.6 ELISA measurement for cd63 and cd44 quantification

The interaction between antibodies and exosomes was verified using an ELISA test in black 96-well microplates. Before preparing the serial dilutions, the lyophilized standards were dissolved in 1.0 mL of sample solvent, gently mixed, and let it settle at 25 °C for 15 min. The wash buffer concentration was diluted with deionized water to create a 5% working solution.

Before the sample was added, microplates were cleaned three times using 350 µL of washing buffer. Then, 100 µL of standard solvent was filled to the blank well, while 100 µL of prepared samples with different concentrations was placed in the other wells. After that, the assay plate was incubated for two hours at 37 °C.

The 100× concentrate was diluted to 1:100 in diluent to create a biotin-conjugated antibody solution. Each well was then filled with 100 µL of diluted biotin-conjugated antibody and incubated for one hour at 37 °C. Following three rounds of washing with a wash buffer, each well was filled with 100 µL of streptavidin–HRP solution (diluted 1:100 in diluent), and the mixture was then incubated at 37 °C for 30 min.

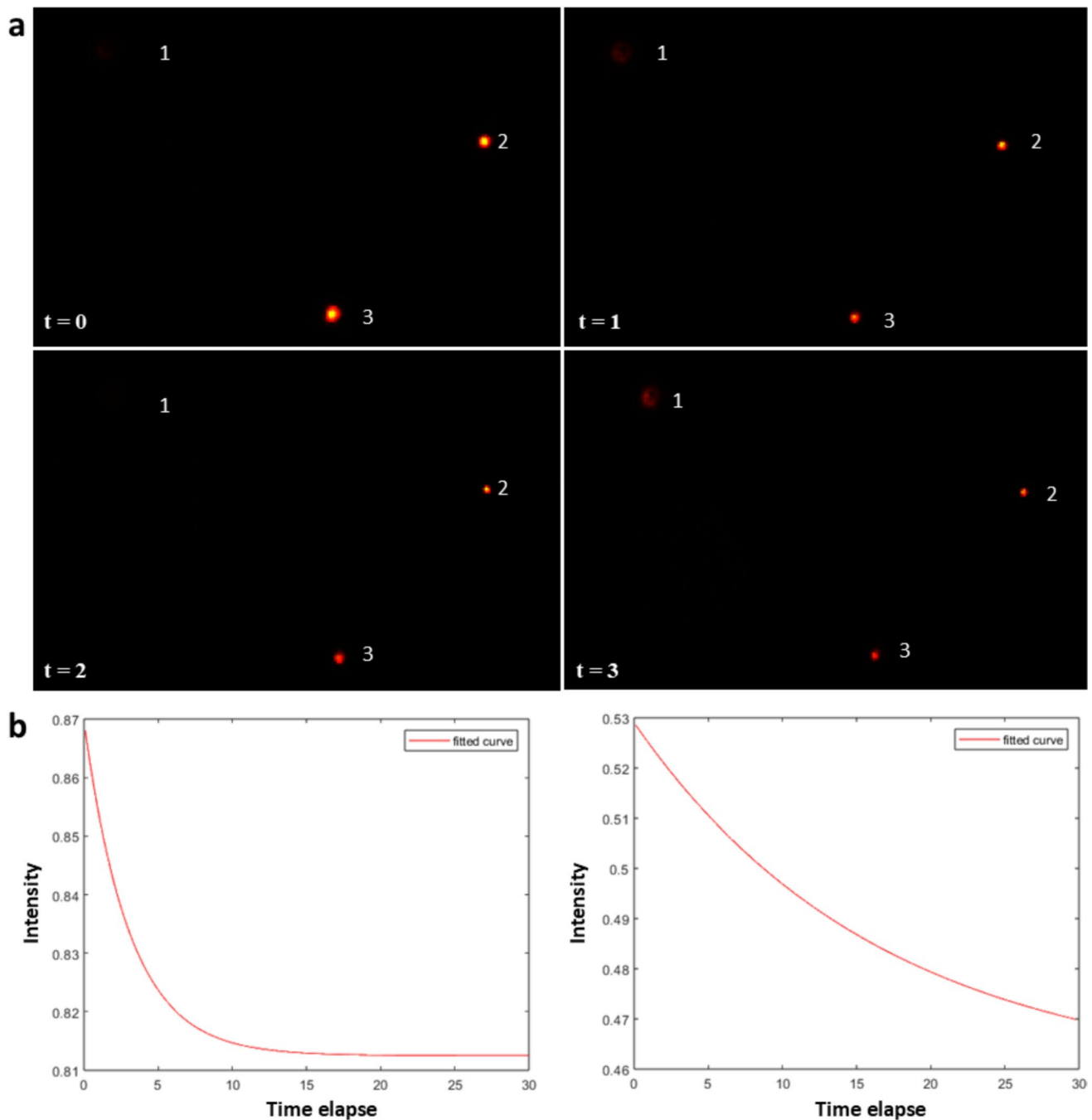


Fig. 2 (a) Time-lapse fluorescence images of 1 μm Janus particles (JPs) captured under a fluorescence microscope (10 $\times$ objective), showing characteristic “blinking” signals caused by rotational motion at different time intervals ($t = 0\text{--}3$ s). (b) Exponential decay curves

of crosscorrelation intensity for JPs at low exosome concentration (left) and high exosome concentration (right). The slower decay at higher concentrations indicates reduced rotational diffusivity due to increased exosome binding

After a final wash, each well received 100 μL of TMB substrate solution, and the microplate was then incubated at 37 $^{\circ}\text{C}$ in a dark conditions for 20 min. Each well received an injection of 50 μL of stop solution to halt the enzymatic process. Using a microplate reader, optical density (OD) at 450 nm was found in five minutes.

3 Result and discussion

3.1 Characterization of janus particles

The JPs were fabricated following a previously established protocol (Chen and Chuang 2020). Briefly, a thin gold film

was deposited onto one hemisphere of the spherical fluorescent polystyrene particles, while the opposite hemisphere remains uncoated. This asymmetric surface configuration produced the characteristic “blinking” fluorescence pattern resulting from the particles’ random Brownian rotational motion.

3.2 Detection of exosome h357 using janus particles

Exosomes from the H357 cell line, which is originated from squamous cell carcinoma (SCC) of the tongue, were detected using the suggested methods. In this assay, 1 μm JPs were functionalized with anti-CD63 and incubated with serially diluted exosome suspension from H357 cell line, ranging from 2 particles/ml to 1.5×10^5 particles/ml. Since CD63 is widely acknowledged as a universal exosome marker across a variety of cell types, it was chosen to illustrate the detection method's capacity to discover a general exosomal biomarker (Welsh, et al. 2024; Théry and “Minimal information for studies of extracellular vesicles 2018). Anti-CD44 functionalized JPs were used as a comparison marker to identify CD44, a particular biomarker linked to oral cancer (Mirhashemi et al. 2023; Singh et al. 2025).

Exosome detection was quantified by analyzing the blinking behaviour of individual JP (Fig. 2 and Video S1) using cross-correlation algorithm. The correlation intensity between consecutive image frames was calculated to account for the reduction in fluorescence signal due to rotational diffusion. In a 30-s recording, correlation values were calculated for image pairs with increasing time intervals (Δt) ranging from 0 to 500 frames. The second frame was gradually moved by Δt seconds, while the first frame was fixed at 0 s. The correlation time was retrieved as a measure of rotational slowing caused by exosome binding from the resulting correlation intensity–time curve, which showed an exponential decrease (Fig. 2).

The particles' rotating diffusion was identified as the cause of the correlation intensity's progressive decline over time. JPs showed faster rotational motion and a quicker decrease in correlation intensity at lower exosome concentrations (Figure SI 1). On the other hand, slower rotational movement and a more gradual decrease in correlation intensity were the results of enhanced exosome binding to the particle surface at higher exosome concentrations. The Stokes–Einstein–Debye theory (Eq. 1) states that the rotational diffusivity coefficient D_r of a spherical particle is inverse to the cube of its radius r_p , or $D_r \propto \frac{1}{r_p^3}$. Hence, exosome binding increases the effective hydrodynamic radius of the JPs–exosome complex, leading to reduced rotational diffusivity and slower motion (Chen and Chuang 2020; Hess et al. 2019). This correlation between particle size and

rotational dynamics confirms that specific interaction between antibody and exosome directly influence JPs behavior.

The control and exosome-treated groups revealed significant differences ($p < 0.05$) according to statistical analysis using an unpaired t-test. Significant variations in rotational diffusivity were observed for anti-CD63-functionalized JPs at all exosome concentrations. Anti-CD44-functionalized JPs, on the other hand, demonstrated significant differences at concentrations between 1.5×10^1 and 1.5×10^1 particles/mL, while lower concentrations did not produce significant change ($p > 0.05$), suggesting that exosome detection may be limited at very low levels.

The concentration at which the response is larger than three times the control standard deviation divided by the calibration curve's slope is the limit of detection (LOD) (Figure SI 2). For anti-CD63-functionalized JPs and anti-CD44-functionalized JPs, the calculated LOD values were 6.2×10^3 particles/mL and 5.5×10^3 particles/mL, each. The ELISA assay, which verified the specificity of antibody binding to the exosomes biomarkers, further validated the reliability of the proposed JPs-based detection platform Fig. 3.

3.3 Surface marker detection using ELISA

The expression of CD63 and CD44 on exosomes derived from H357 cell line measured using ELISA at a series of sequential dilutions. Figure 4 displays the quantitative results of surface marker expression, while Figure SI 3 displays standard curves. CD63 expression rose in direct proportion to exosome concentration, as seen in Fig. 4a. CD63 levels were almost undetectable at very low doses, but signals were apparent at greater quantities. The detection of oral cancer-derived exosomes utilizing both anti-CD63- and anti-CD44-conjugated Janus particles (JPs) was confirmed by a comparable trend for CD44 expression (Fig. 4b). These surface markers have a quantitative correlation with exosome abundance, as seen by the regular reduction in observed quantities of CD63 and CD44 with serial dilution.

The detection capability of the JPs-based platform has been verified using CD63, a universal exosome marker. On the other hand, oral squamous cell carcinoma (OSCC) is selective for CD44, a surface protein associated with tumors. Based on previous studies, CD44 expression increases gradually from normal oral mucosa to dysplastic lesions and OSCC (Tharani et al. 2024), indicating its involvement in tumor growth and metastasis. CD44 is known to mediate cell adhesion, migration, invasion, and intercellular communication, all of which are essential processes in OSCC progression (Chakraborty et al. 2023). Additionally, CD44 expression changes to tumor differentiation: it is weak in poorly differentiated OSCC, moderate in moderately differentiated OSCC, and high in well-differentiated OSCC

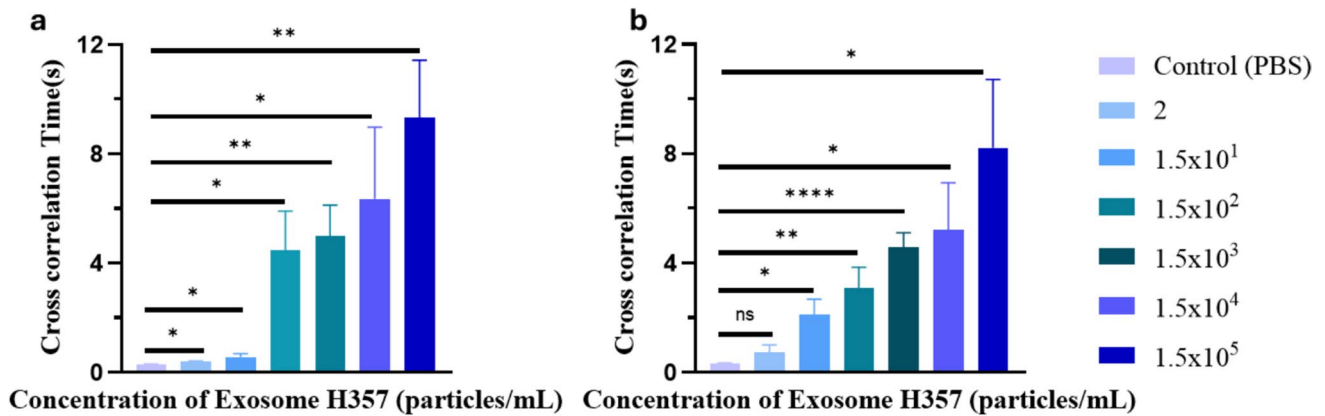


Fig. 3 Cross-correlation analysis of Janus particle (JP) conjugation with exosomal biomarkers. (a) JPs functionalized with anti-CD63 antibodies and (b) JPs functionalized with anti-CD44 antibodies show increasing cross-correlation time with rising concentrations of

H357-derived exosomes. As exosome concentration decreases, the cross-correlation time correspondingly declines, indicating reduced binding events. Data were analyzed using an unpaired t-test. **** $p < 0.0001$; ** $p < 0.01$; * $p < 0.05$; ns = not significant

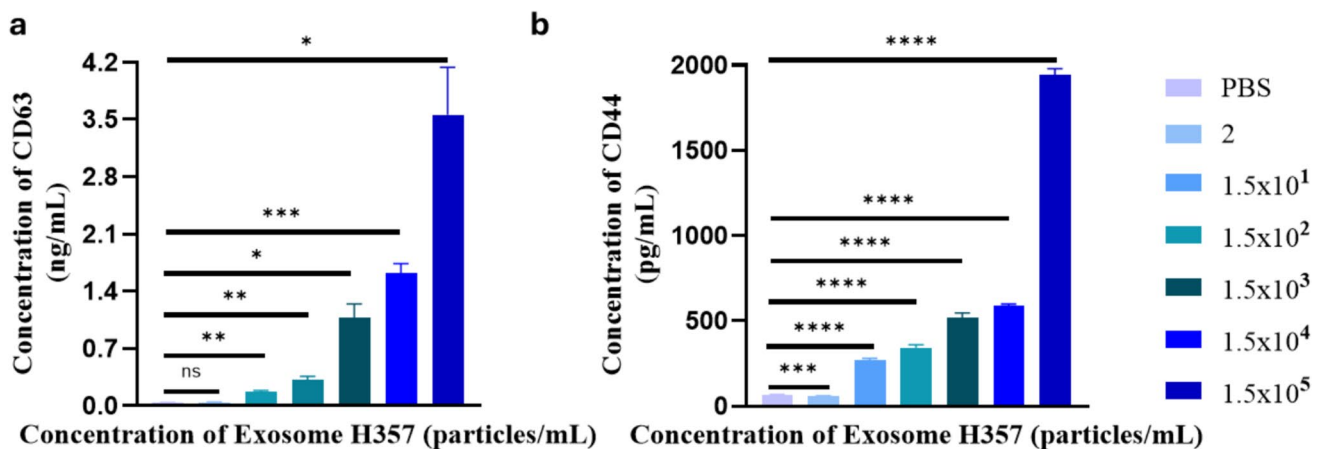


Fig. 4 Quantification of exosomal surface markers by ELISA. (a) Concentration of CD63 and (b) concentration of CD44 on H357-derived exosomes across serial dilutions. As exosome concentra-

tion decreases, corresponding marker levels of CD63 and CD44 also decline. Data were analyzed using an unpaired t-test. **** $p < 0.0001$; *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; ns = not significant

(Kumar et al. 2024). These results support CD44's potential as an oral cancer biomarker for prognosis and diagnosis.

Despite these promising results, several technical challenges were encountered. Variability in particle blinking and image acquisition sometimes affect the consistency of correlation measurements. This inconsistency is likely caused by particle aggregation, which can affect the uniformity of particle–exosome interactions. Future optimizations, such as integrating a microfluidic systems for high-throughput screening and developing multiple detection assays, could further improve the diagnostic performance of this platform. However, the measurable diffusivity and observed rotational motion in JPs demonstrate dependable performance, indicating its potential application as a fast and precise diagnostic

method for the preliminary identification of oral cancer biomarkers.

4 Conclusion

The successful separation of exosome-bound and non-exosome-bound samples was achieved by modifying the surfaces of Janus particles (JPs) with particular antibodies (anti-CD63 and anti-CD44), validating the efficient synthesis and operation of the biosensing platform. The limit of detection (LOD) for exosome identification for JPs–anti-CD63 and JPs–anti-CD44 conjugates was determined to be 6.2×10^3 particles/mL and 5.5×10^3 particles/mL, each.

The assay's capacity to detect the exosome surface markers CD63 and CD44 was validated by the ELISA results. These findings show that the developed rotational diffusometry approach offers an accurate and sensitive way to detect exosomes. The urgent need for non-invasive, quick, and affordable diagnostic techniques in clinical practice may be met by incorporating this technology into the diagnostic workflow, which could provide a useful tool for early oral cancer detection.

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1007/s44445-026-00144-0>.

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Author contribution FNL: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – Review & Editing; VS: Writing – Review & Editing; TTHP: Formal analysis, Investigation, Methodology, Resources, Writing – Review & Editing; YMG: Formal analysis, Methodology, Resources; DD: Formal analysis, Investigation, Methodology, Visualization, Resources, Writing – Review & Editing; AM: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Resources, Supervision, Validation, Project administration, Writing – Review & Editing; HSC: Conceptualization, Funding acquisition, Investigation, Methodology, Resources, Supervision, Validation, Project administration, Writing – Original Draft, Writing – Review & Editing.

Data availability Data will be made available on request.

Declarations

Competing interests 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.

Associated content Supplement Information: Simulation of rotational diffusivity of the JPs, Cross-correlation intensity comparison at different concentration, standard curve ELISA measurements of the surface marker quantification.

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