



Decoding tumor microenvironment-derived extracellular vesicles with molecularly imprinted polymer biosensors

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ABSTRACT

Early diagnosis and timely intervention of cancer are essential to improve patient survival; however, many cancers are still detected at advanced stages with limited therapeutic options. The tumor microenvironment (TME), characterized by its complex architecture and dynamic interactions between cancer cells and the surrounding milieu, plays a decisive role in understanding tumor progression, therapeutic response, and prognosis. Accurately mimicking the TME *in vitro* remains a challenge for developing diagnostic strategies. Herein, we presented an integrated approach that recapitulates the breast cancer microenvironment using a microfluidic platform, while simultaneously investigating the role of extracellular vesicles (EVs) as critical biomarkers of tumor progression. Complementary computational analyses—comprising density functional theory, molecular docking, and molecular dynamics simulations—were performed to elucidate the binding behavior of selected ligands to CD63, a key EV-associated marker in breast cancer. To overcome the instability inherent to conventional immunoassays, we developed EV-imprinted nanoparticles that preserve the molecular fingerprint of target EVs. The EV-imprinted nanoparticles were coupled with optical biosensors to enable precise, selective, and real-time detection of EVs. This integrated experimental–computational strategy not only enhances the understanding of EV-mediated cancer signaling but also provides a foundation for developing cost-effective, rapid, and sensitive biosensing platforms for early cancer diagnosis.

1. Introduction

Cancer remains a major global health challenge, ranking among the leading causes of mortality and significantly diminishing quality of life [1]. Among malignancies, breast cancer is the most prevalent, accounting for nearly one-quarter of all female cancer diagnoses and exceeding 2.3 million new cases worldwide in 2020 [2–4]. These trends underscore the critical need for effective prevention and early detection strategies, as timely diagnosis remains pivotal for improved prognosis [5]. Current screening methods—such as mammography, MRI, and ultrasonography—require biopsy confirmation and face limitations in sensitivity, operator dependence, and infrastructure demands [6–9]. While complementary assays like ELISA and immunohistochemistry enhance diagnostic precision, they still suffer from stability and sensitivity issues [10,11]. Hence, developing reliable, minimally invasive, biomarker-based diagnostic tools is of paramount importance.

On the other hand, the tumor microenvironment (TME) plays a central role in cancer progression, therapeutic response, and prognosis. Comprising immune and stromal cells, extracellular matrix (ECM) components, and signaling molecules, the TME dynamically interacts with tumor cells to regulate growth, invasion, and metastasis [12,13] [14]. Tumor-derived extracellular vesicles (EVs) further modulate the TME by transferring proteins, nucleic acids, and lipids that influence immune evasion, angiogenesis, and metastasis. Owing to their remarkable stability and molecular fidelity across diverse biofluids, EVs have emerged as promising biomarkers for noninvasive “liquid biopsy” applications [15–17]. However, conventional two-dimensional cultures fail to replicate the dynamic microenvironmental cues governing EV secretion. Integration of microfluidic systems with ECM components enables physiologically relevant three-dimensional models that better simulate *in vivo* conditions [18,19]. Current EV characterization techniques—including electron microscopy, western blotting, flow

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cytometry, and nanoparticle tracking analysis—offer detailed molecular profiling but remain resource- and time-intensive, limiting routine clinical application [20–25].

Biosensor technologies present a compelling alternative, providing rapid, sensitive, and real-time EV detection compatible with clinical settings [26,27]. Optical biosensors integrated with microfluidics enable label-free measurements with superior sensitivity [28,29]. Moreover, molecular imprinting techniques advance this field by generating synthetic polymers with highly selective recognition sites tailored to EV surface markers, delivering improved durability, reusability, and cost-effectiveness relative to antibody-based assays [30–33].

Here, we present a sensitive and selective molecularly imprinted optical biosensor for the real-time detection of breast cancer-derived EVs. EVs were isolated from three-dimensional microfluidic cultures incorporating ECM components that recapitulate the TME. Complementary molecular modeling elucidated ligand–CD63 interactions, guiding the design of imprinted nanoparticles integrated into the biosensor. This approach not only advances a highly specific and rapid diagnostic platform but also provides mechanistic insights into EV-mediated communication within the TME, offering transformative potential for early breast cancer detection and a deeper understanding of tumor biology in the future.

2. Materials and methods

2.1. Computational methods

Docking and molecular dynamics (MD) simulations were performed to understand the interactions between the studied structures. Details on the structures and MD simulation conditions were presented in the [Supplementary Material](#) file (Figure S1–S3 and Table S1, S2).

2.2. EV collection

2.2.1. 2D and 3D cell cultures

MCF-7, HEK293, and HeLa cells were cultured under standard conditions in their respective complete media supplemented with 10% FBS and antibiotics. Cells were maintained at 37°C with 5% CO₂ and subcultured at ~85% confluency. Detailed medium compositions and handling procedures were provided in the [Supplementary Materials](#) file. Microfluidic chips were fabricated using PMMA, DSA, and polystyrene layers with a final channel dimension of 10 mm × 60 mm × 250 μm, as previously described [34]. Collagen-coated channels (50 μg/mL) were seeded with 1.15×10^5 cells and maintained at 37°C, 5% CO₂. After 24 h of adhesion, continuous perfusion was applied at 2 μL/min. Fabrication and coating details were available in the [Supplementary Material](#) file.

2.2.2. Isolation and characterization of EVs

EVs were isolated from cell culture supernatants using an ultrafiltration-based microfluidic system with sequential 200 nm and 50 nm membranes at a flow rate of 40 μL/min [35]. After isolation, they were characterized by (i) Nanoparticle Tracking Analysis (NTA) for size and concentration, (ii) Scanning Electron Microscopy (SEM) for morphology, and (iii) Western blotting of CD63 and CD81 for surface markers. Detailed isolation parameters, imaging, and immunoblotting procedures were provided in the [Supplementary Material](#) file.

2.3. Optical biosensor studies

2.3.1. Preparation and characterization of nanoparticles

EV-imprinted nanoparticles were synthesized according to a previously reported protocol [36]. Briefly, a functional monomer methacrylic acid (MA, 168.8 μL) was pre-complexed with template molecule (EV, 1×10^8 particles/mL) by gentle rotation at 20 rpm for 30 min. A two-phase emulsion polymerization was employed. In the first phase, poly(vinyl alcohol) (PVA, 100 mg) and sodium dodecyl sulfate (SDS,

100 mg) were dissolved in 200 mL of deionized water, serving as a stabilizer and a surfactant, respectively, to generate the aqueous emulsion. The second phase consisted of 10 mL deionized water containing PVA (187 mg), SDS (28.35 mg), and sodium bicarbonate (NaHCO₃, 23.45 mg) as a pH-adjusting component. The third phase—comprising 2-hydroxyethyl methacrylate (HEMA, 1 mL) as a co-monomer and ethylene glycol dimethacrylate (EGDMA, 2.1 mL) as a cross-linker—was introduced. The resulting mixture was homogenized at 20,000 rpm for 15 min to obtain a stable emulsion. The pre-complexed EV-MA solution was then incorporated into the emulsion, and the mixture was transferred into the polymerization flask. Phase 1 was used to rinse the flask thoroughly and was subsequently added to the reaction system. Polymerization was initiated by the addition of a redox initiator pair: ammonium persulfate ((NH₄)₂S₂O₈, 126 mg, dissolved in 1 mL water) and sodium bisulfite (NaHSO₃, 115 mg). The reaction was maintained at 40°C for 24 h under continuous mechanical stirring at 500 rpm. The non-imprinted nanoparticles (NIP) and control NIP biosensor were prepared using the same polymerization conditions and monomer composition (MA-HEMA) as the MIP biosensor, but in the absence of the template molecule (EV). Thus, the NIP surface represents a structurally comparable polymeric layer lacking specific recognition sites, rather than an unmodified substrate. While this design enables direct assessment of the imprinting effect, additional control surfaces could further clarify the contribution of surface architecture. After polymerization, the nanoparticles were sequentially washed with ethanol, an ethanol–water mixture, and deionized water, each step followed by centrifugation at 14,500 rpm for 30 min to remove impurities, surfactants, and unreacted monomers. Template removal was carried out using 0.1 M NaCl as a desorption agent. The pellets were repeatedly re-dispersed in deionized water and centrifuged until removal of all EVs. The final pellets were dispersed in 100 μL of deionized water, sonicated, and prepared for further analysis. The characterization analyses of the nanoparticles were evaluated using dynamic light scattering (DLS), Fourier-transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM), and transmission electron microscopy (TEM). Hydrodynamic size distributions were determined using a Nano Zetasizer where nanoparticle suspensions were loaded into cuvettes and scattered light intensity was measured at a 90° detection angle. Measurements were performed in triplicate, and the mean particle size with standard deviations was reported. For SEM imaging, the nanoparticles were first dispersed in ethanol and then mounted onto SEM specimen stubs using conductive adhesive carbon tape. The samples were subsequently coated with a thin layer of gold under vacuum conditions to render the surface conductive. The coated samples were then placed into the SEM chamber, and images were captured at various magnifications. Likewise, for TEM imaging, the nanoparticles dispersed in ethanol were dropped onto TEM grids and imaged at different magnifications. The nanoparticles were lyophilized for 24 h and homogenized with potassium bromide, pressed into pellets, and analyzed by FTIR over a range of 400–4000 cm^{−1}.

2.3.2. Preparation and characterization of optical biosensors

Following nanoparticles characterization, 10 μL aliquots of the respective nanoparticle suspensions were drop-cast onto commercially available gold-coated surfaces. The suspensions were uniformly distributed by spin-coating for 10 s. Surface topography was examined by atomic force microscopy (AFM), and wettability was assessed via static contact angle measurements. AFM analyses were conducted on both unmodified bare gold and EV-imprinted optical biosensor surfaces. The cantilever tip was operated in non-contact mode, maintaining a nanometer-scale distance above the surface to detect weak intermolecular forces without direct interaction. Images were acquired over scan areas of $5 \times 5 \mu\text{m}^2$ at a scanning rate of 0.5 Hz. Morphological parameters, including nanoparticle distribution, particle size, and surface roughness, were quantified using Gwyddion software. Contact angle measurements were performed on bare gold and EV-imprinted optical biosensor surfaces to evaluate hydrophilicity/hydrophobicity. Using the

sessile drop method, deionized water droplets were dispensed at multiple positions across each surface, and the static contact angles were captured through digital imaging. Left and right angles at the liquid–solid interface were measured, and mean values were calculated with DSA2 software.

2.3.3. Real-time EVs detection

Kinetic interactions were evaluated using surface plasmon resonance (SPR) sensograms acquired with the SPRImager®II system. The EV-imprinted (MIP) and non-imprinted (NIP) optical biosensors were first equilibrated with phosphate-buffered saline (PBS) to establish baseline conditions. EVs at concentrations ranging from 1×10^6 – 2×10^8 particles/mL (shown as p/mL) were prepared *via* serial dilution in PBS and introduced into the flow system to construct calibration curves for the EV-imprinted optical biosensor. Binding of EVs to the biosensor surface was monitored in real-time by recording changes in reflectance (ΔR) as a function of time. For each run, a stable baseline was obtained by flowing PBS for 90 s, followed by sample injection for 270 s to allow EV detection and approach to steady-state binding. Surface regeneration was

achieved by rinsing with 0.1 M NaCl for 90 s after each measurement cycle. Moreover, the selectivity of the MIP and NIP optical biosensors was assessed using EVs derived from breast cancer cells (MCF-7), as well as competing EVs obtained from HEK293 and HeLa cells, and all tested at an equivalent concentration of 2×10^8 particles/mL. HEK293-derived EVs represent physiological, non-malignant vesicle profiles, whereas HeLa-derived EVs, originating from a malignant cell line, are enriched in tumor-specific microRNAs, oncoproteins, and proliferation-associated markers [37,38]. Accordingly, HEK293 EVs were used as controls, while HeLa EVs served to probe cancer-related specificity [37,39]. The selectivity coefficient (k) and relative selectivity coefficient (k') were calculated according to the following Eqs. 1–2:

$$k = \Delta R_{EVs} / \Delta R_{competitor} \quad (1)$$

$$k' = k_{MIP} / k_{NIP} \quad (2)$$

where ΔR_{EVs} and $\Delta R_{competitor}$ denote the biosensor responses to target and competing EVs, respectively.

In order to evaluate reusability, the EV-imprinted optical biosensor

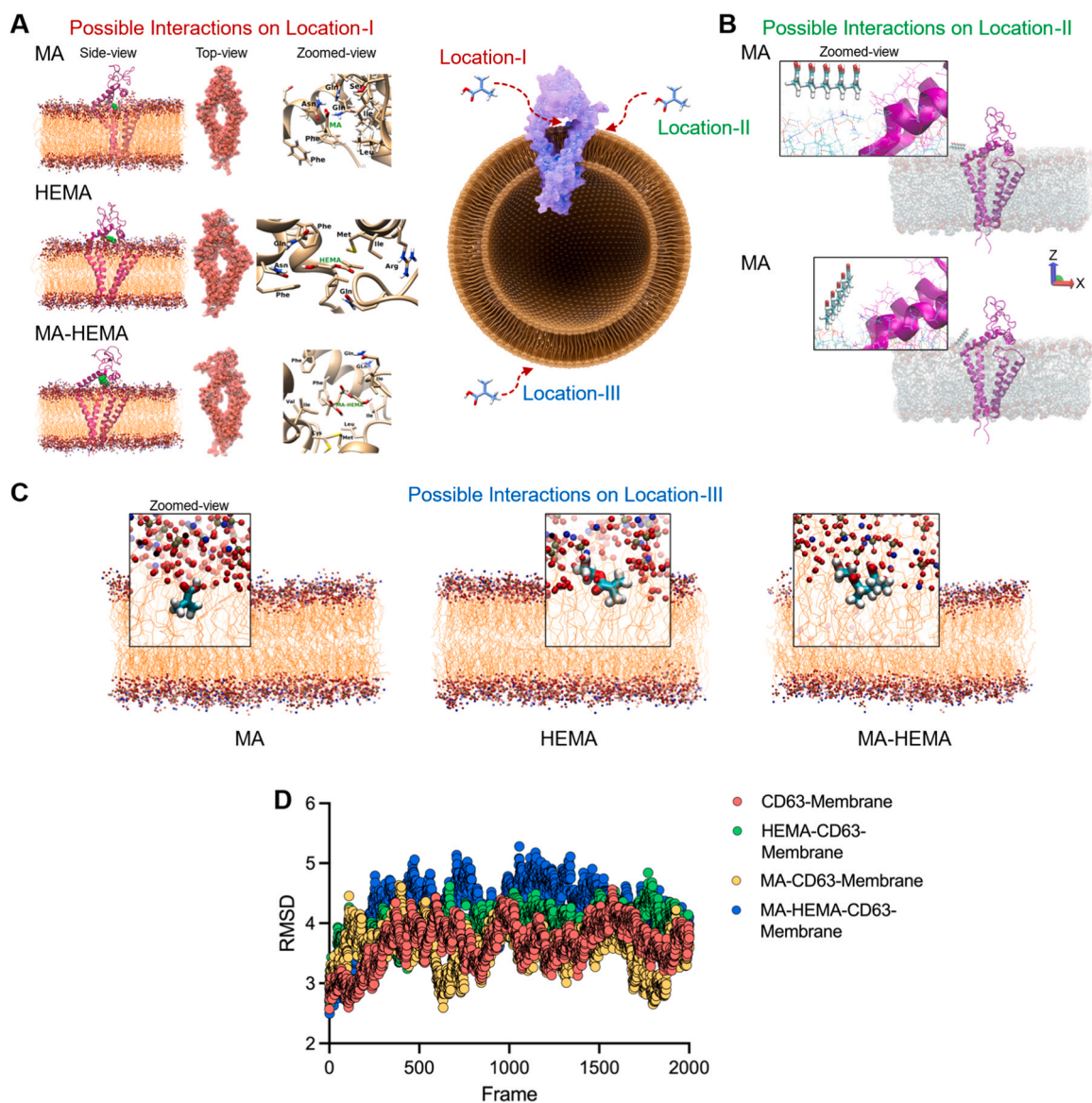


Fig. 1. A) On Location-I: the binding orientation of MA, HEMA, and MA-HEMA between the residues of CD63, B) on Location-II: the positions of MA when its distance changes in the X and Z direction, C) on Location-III: the binding orientation of MA, HEMA, and MA-HEMA in the structure of the membrane, and D) the RMSD of CD63 along the simulation time.

surface was exposed to an EV solution (2×10^7 particles/mL), followed by regeneration using a desorption solution. This adsorption–desorption cycle was repeated five consecutive times with the same sample to determine signal retention and performance stability. To assess performance in complex biological matrices, artificial samples were prepared by spiking 10 μ L of each EV stock solution into serum and plasma matrices to achieve a final concentration of 2×10^8 particles/mL in PBS buffer. The EV-imprinted optical biosensor response was then evaluated under these physiologically relevant conditions. All kinetic measurements were performed at a constant flow rate, and each experiment was conducted in triplicate to ensure reproducibility. Detailed mathematical calculation was provided in the [Supplementary Material](#) file.

3. Results and discussions

3.1. Computational methods

3.1.1. Orientation of ligands

Molecular docking identified the initial binding poses of MA, HEMA, and MA–HEMA on the CD63 protein (Location-I). Owing to their mixed polar (carbonyl, hydroxyl) and nonpolar (ether, hydrocarbon) functionalities, each ligand exhibits distinct affinities toward the protein and membrane. Polar groups engage in hydrogen bonding or electrostatic interactions with charged CD63 residues, whereas nonpolar segments preferentially associate with hydrophobic protein or membrane regions. MD simulations ([Fig. 1A, C](#)) revealed the ligands' orientations at Location-I and their conformations at Location-III, where interactions mainly occur with the lipid membrane. Carbonyl and hydroxyl groups of MA, HEMA, and MA–HEMA interact with polar residues (Asn, Gln, Ser, Arg, Met, Cys), while hydrocarbon and ether moieties bind to nonpolar ones (Ile, Leu, Phe, Val). This amphiphilic behavior persists in Location-III, with polar domains facing the hydrophilic membrane surface and nonpolar parts embedding within its core—facilitating stable membrane anchoring and penetration. Briefly, MA exhibits pronounced polarity, aligning its hydrophilic and hydrophobic regions with corresponding membrane layers. MA–HEMA shows a similar yet more spherical conformation, enabling multivalent interactions with both environments. In contrast, HEMA adopts a flatter geometry, allowing concurrent polar and nonpolar contacts that generate competing forces. These results suggest that the specific structural orientation of HEMA within the binding site results in a different interaction pattern compared with MA and MA–HEMA. To examine intermediate interactions (Location-II), molecular systems were constructed as shown in [Fig. 1B](#), using MA as a representative ligand. Ten simulations were performed per ligand—five along the X-axis (2–10 Å from the protein, Z = 5 Å) and five along the Z-axis (2–10 Å from the membrane, X = 5 Å). Ligand orientations in this region largely resembled those at Location-III but retained protein–ligand contacts, suggesting that protein proximity significantly modulates ligand orientation and binding behavior.

3.1.2. Interaction quality assessment

Hydrogen bonding (H-bonding) is a central determinant of molecular recognition, governing both specificity and stability of ligand interactions [40]. The average number of H-bonds formed by each ligand was analyzed across Locations I–III ([Table S3](#)), consistent with the interaction trends previously described. The symmetric structure of MA and the pseudo-spherical geometry of MA–HEMA, arising from their functional group distributions, enable stronger and more stable H-bonding. In contrast, the planar conformation of HEMA causes overlapping polar and nonpolar domains, generating competing forces that weaken H-bonding. At Location-II, CD63 proximity notably modulates H-bond formation, with maximal bonding observed at X = 5 Å and Z = 10 Å, where sufficient conformational freedom during the 50 ns simulation allows optimal orientation. Shorter distances introduce steric and electrostatic competition, reducing stability. Interaction energies (E_{int}) between ligands and their environments ([Table S3](#)) corroborate

these findings, confirming that MA and MA–HEMA exhibit more favorable electrostatic interactions than HEMA. However, considering the unfavorable contribution of HEMA to these interactions, it is necessary to further examine how HEMA maintains its interactions with the EV structure along the simulation time. Therefore, this aspect will be discussed in detail in the following sections.

3.1.3. Stability of CD63 during simulation

Protein structural stability during molecular dynamics simulations ensures reliable interaction analysis. In this context, the Root Mean Square Deviation (RMSD) assesses conformational flexibility over time [41]. RMSD values, calculated from CD63 backbone atoms (C α , C, N), were analyzed for two cases: (i) CD63 within the lipid bilayer and (ii) CD63 bound to ligands. As shown in [Fig. 1D](#), all RMSD values remained within acceptable limits, confirming that CD63 retained structural stability throughout the simulations.

3.1.4. Absolute binding free energy calculations

To further validate the ligand–CD63 interactions and address the questions raised in the previous analyses, the absolute binding free energies (ΔG) were calculated using the Free Energy Perturbation (FEP) method at Position A. The calculated values are summarized in [Table S4](#). Among the studied ligands, HEMA exhibited the most negative binding free energy ($-7.43 \text{ kcal}\cdot\text{mol}^{-1}$), indicating the strongest overall binding affinity toward CD63. The MA ligand showed a slightly weaker but still favorable binding free energy ($-6.29 \text{ kcal}\cdot\text{mol}^{-1}$), whereas the MA–HEMA complex displayed the least favorable value ($-4.51 \text{ kcal}\cdot\text{mol}^{-1}$). According to the thermodynamic relationship $\Delta G = \Delta H - T\Delta S$, the overall binding free energy results from the balance between enthalpic (ΔH) and entropic (ΔS) contributions. Although MA–HEMA interactions exhibit more favorable enthalpic contributions, likely arising from stronger H-bonding and E_{int} , this advantage appears to be partially offset by an unfavorable entropic contribution, resulting in a less favorable overall ΔG value. In contrast, the HEMA ligand, despite having a relatively smaller enthalpic contribution compared with MA–HEMA, benefits from a larger favorable entropy term, which likely originates from its higher conformational flexibility and dynamic behavior during the simulation. This entropic contribution allows HEMA to maintain stable interactions with CD63 while remaining dynamically mobile. The MA ligand exhibits an intermediate behavior, where both enthalpic and entropic contributions participate in stabilizing the interaction, leading to a binding free energy between those of HEMA and MA–HEMA. Overall, although all three ligands demonstrate favorable interactions with the EV structure, the free energy calculations suggest that HEMA has the most thermodynamically favorable binding, while MA–HEMA interactions appear to be primarily enthalpy-driven but entropically limited, resulting in a weaker overall binding free energy.

3.2. EV collection

3.2.1. 2D and 3D cell cultures

MCF-7 cells were stained with Hoechst 33342, Calcein-AM, and Ethidium Homodimer-1 to evaluate cell viability after passaging ([Fig. 2A–F](#)). Fluorescence imaging confirmed predominantly viable cells across the culture surface. [Fig. 2G](#) showed MCF-7 cells cultured within the collagen-coated microfluidic channels. 24 h after seeding, the cells were evenly distributed throughout the channel, and long-term dynamic culture was maintained for an additional 7 days under flow conditions.

3.2.2. Isolation and characterization of EVs

To justify the selection of 3D microfluidic culture for EV collection, EVs isolated from both 2D and 3D cultures were compared in terms of size distribution, concentration, and surface marker expression ([Figure S4](#)). NTA analysis revealed that EVs from 2D cultures exhibited a mean diameter of $125.0 \pm 4.7 \text{ nm}$ and a concentration of $5.27 \times 10^9 \pm$

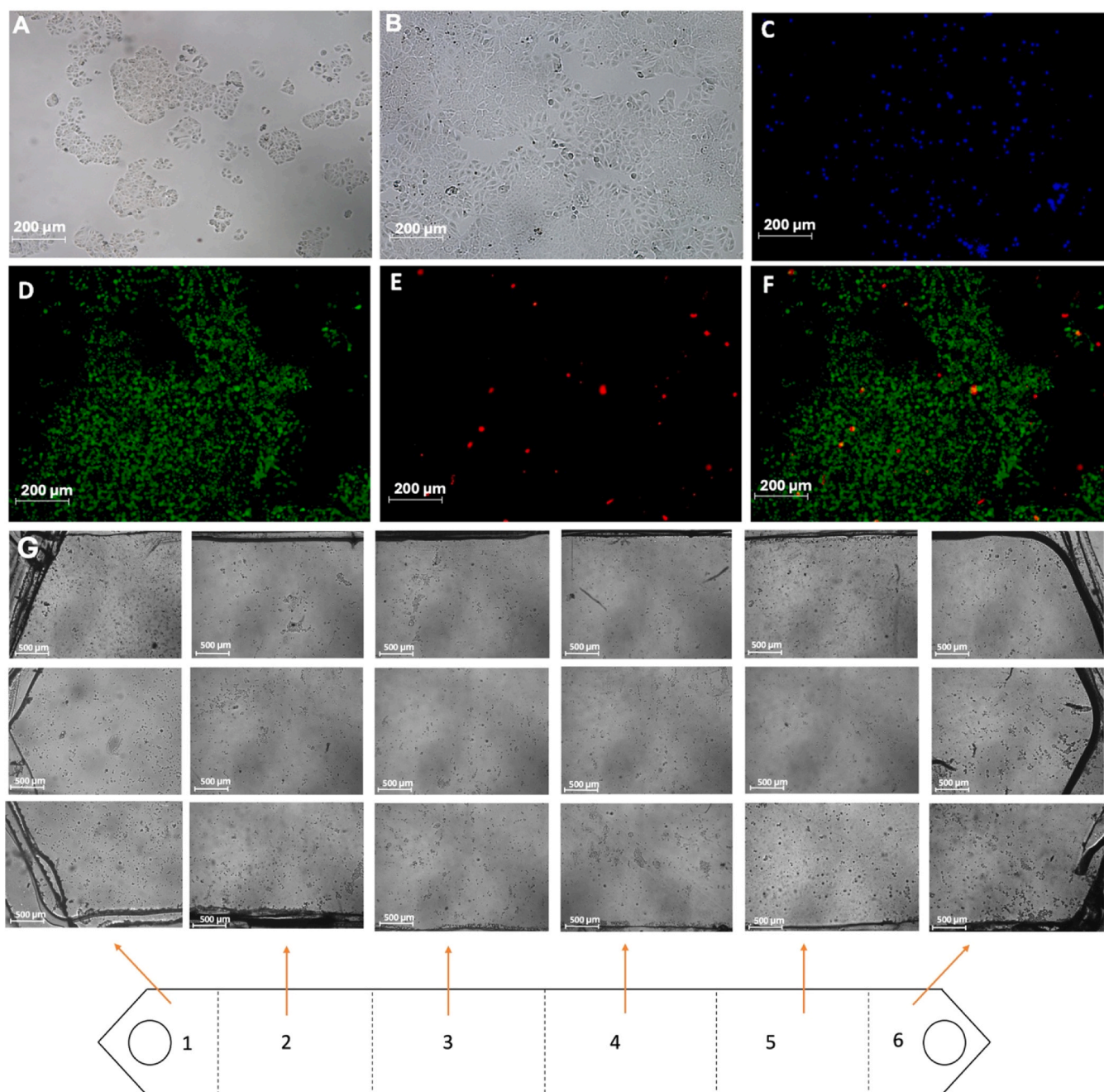


Fig. 2. Microscopic images of MCF-7 cells cultured in standard T-flasks prior to microfluidic seeding: A) bright-field image at day 3 and B) day 7 post-passaging, confirming sustained proliferation; C) fluorescence image of nuclei stained with Hoechst 33342, D) live cells stained with Calcein-AM, E) dead cells stained with Ethidium Homodimer-1, and F) merged fluorescence image showing both live and dead cells (C–F: day 3 post-passaging) and G) light microscope image of MCF-7 cells seeded into collagen-coated microfluidic channels.

1.42×10^8 particles/mL, whereas EVs from 3D microfluidic cultures showed a mean diameter of 129.2 ± 4.5 nm and a higher concentration of $6.49 \times 10^9 \pm 3.56 \times 10^8$ particles/mL. Western blot analysis confirmed the presence of CD63 and CD81 in both conditions. The higher EV yield observed in 3D cultures is attributed to the physiologically relevant flow conditions and collagen-based ECM environment, which better recapitulate the TME and enhance EV secretion [42]. Therefore, EVs derived from 3D microfluidic cultures were used for all subsequent biosensor experiments. Fig. 3A–D illustrated the design of the microfluidic chip developed for EV isolation. Supernatants collected from cultured cells were introduced into the isolation chip at a flow rate of $40 \mu\text{L}/\text{min}$. Fig. 3E presented the NTA results. According to the analysis, EVs isolated from cells cultured within collagen-coated

microfluidic channels at a flow rate of $2 \mu\text{L}/\text{min}$ exhibited an average diameter of 129.2 ± 4.5 nm and a concentration of $6.49 \times 10^9 \pm 3.56 \times 10^8$ particles/mL. The EVs displayed a homogeneous size distribution and oval morphology (Fig. 3F). In Fig. 3G, the presence of the transmembrane proteins CD63 and CD81—typical markers of EVs—was confirmed by Western Blot analysis, verifying the successful isolation of EVs.

3.3. Optical biosensor studies

3.3.1. Characterization of nanoparticles

EV-imprinted nanoparticles were characterized with a series of methods to investigate their size and size distributions, morphologies,

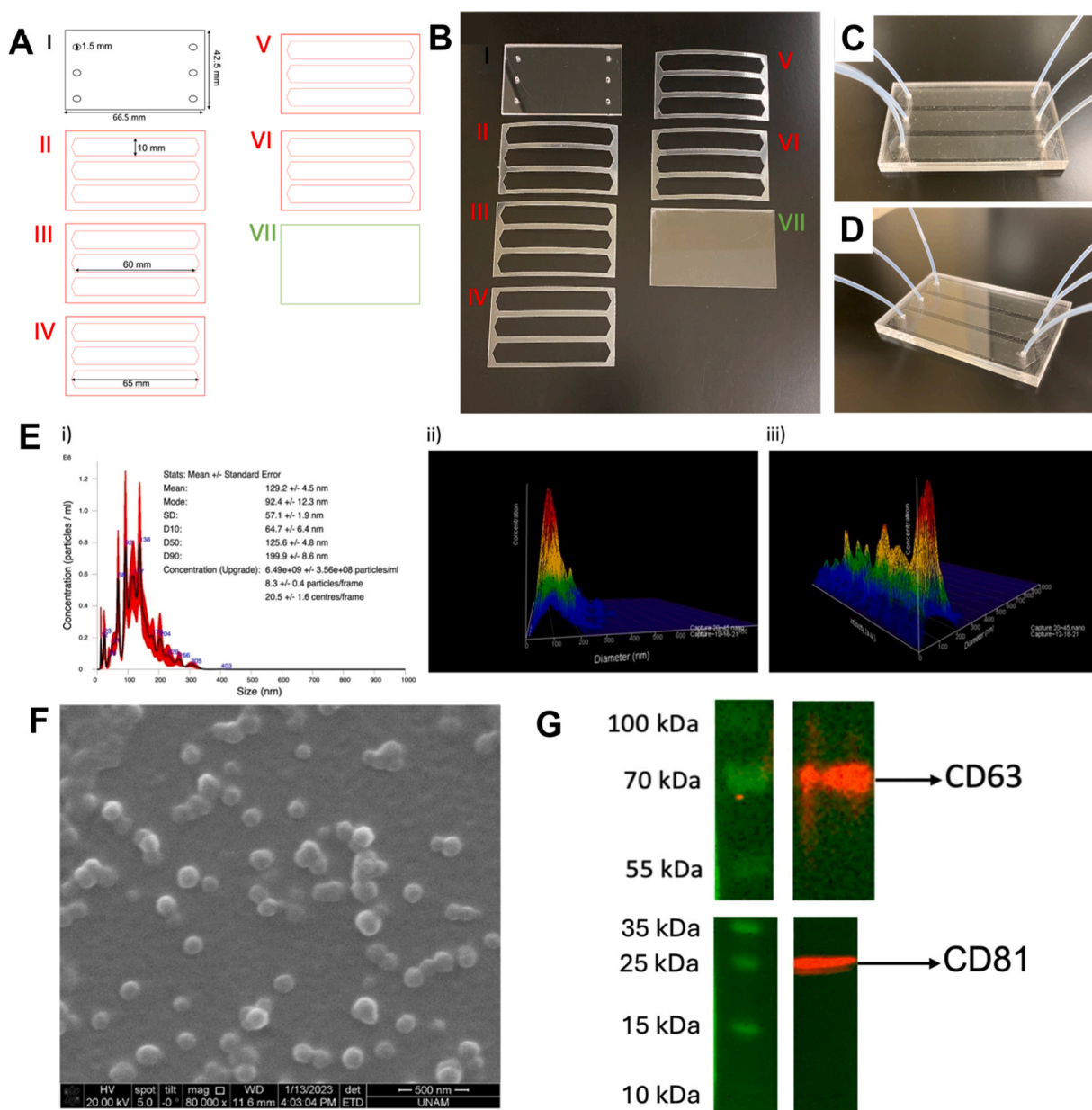


Fig. 3. A) Design parameters of the microfluidic chip, B) layered structure of the chip composed of PMMA, DSA films, and polystyrene layers, C) sample injection and collection of isolated particles through inlet and outlet ports connected via tubing, D) side view of the microfluidic chip, E) NTA results of EVs obtained from collagen-coated microfluidic surfaces, F) SEM images of the isolated EVs, and G) Western Blot analysis of the isolated EVs showing CD63 and CD81 protein bands.

and chemical characteristics. Initially, EV-imprinted nanoparticles were evaluated with the DLS method, and they exhibited a narrow size distribution with a polydispersity index (PDI) of 0.116 and an average diameter of 62.78 nm (Fig. 4A). The presence of a single, sharp peak confirms homogeneity and uniform particle size distribution. Second, the surface morphology of EV-imprinted nanoparticles was thoroughly analyzed using SEM and TEM imaging. SEM and TEM analyses revealed that the EV-imprinted nanoparticles appeared both as individual particles and as clustered aggregates due to polymeric interactions. These nanoparticles also exhibited a homogeneous, spherical, and monodisperse morphology (Fig. 4B-C). When compared with the DLS analysis results, the average particle sizes were consistent (~ 60 nm), confirming the successful synthesis of the nanoparticles. Third, chemical characterization was assessed through the FTIR measurements, and the spectrum of EV-imprinted nanoparticles showed characteristic absorption bands corresponding to ester carbon-oxygen bonds (C-O) at 1210 cm^{-1} ,

vinyl groups (C=C) at 1638 cm^{-1} , carbonyl groups (C=O) at 1723 cm^{-1} , and carboxylic acid O-H stretching vibrations at 2952 cm^{-1} (Fig. 4D).

3.3.2. Characterization of optical biosensors

Once EV-imprinted nanoparticles were immobilized onto the surface of the optical biosensor, we examined their wettability and topographical analysis. Initially, the hydrophilic-hydrophobic characteristics of the unmodified and EV-imprinted nanoparticles immobilized optical biosensor surfaces were also evaluated using a contact angle measurement system via the sessile drop method. The water contact angles for the bare gold and EV-imprinted optical biosensors immobilized optical biosensor surfaces were 94.2° and 88.5° , respectively (Fig. 4E-F). The decrease in contact angle values demonstrates an increase in surface hydrophilicity, pointing out the successful modification of the biosensor surface. Moreover, AFM analysis was carried out to compare the

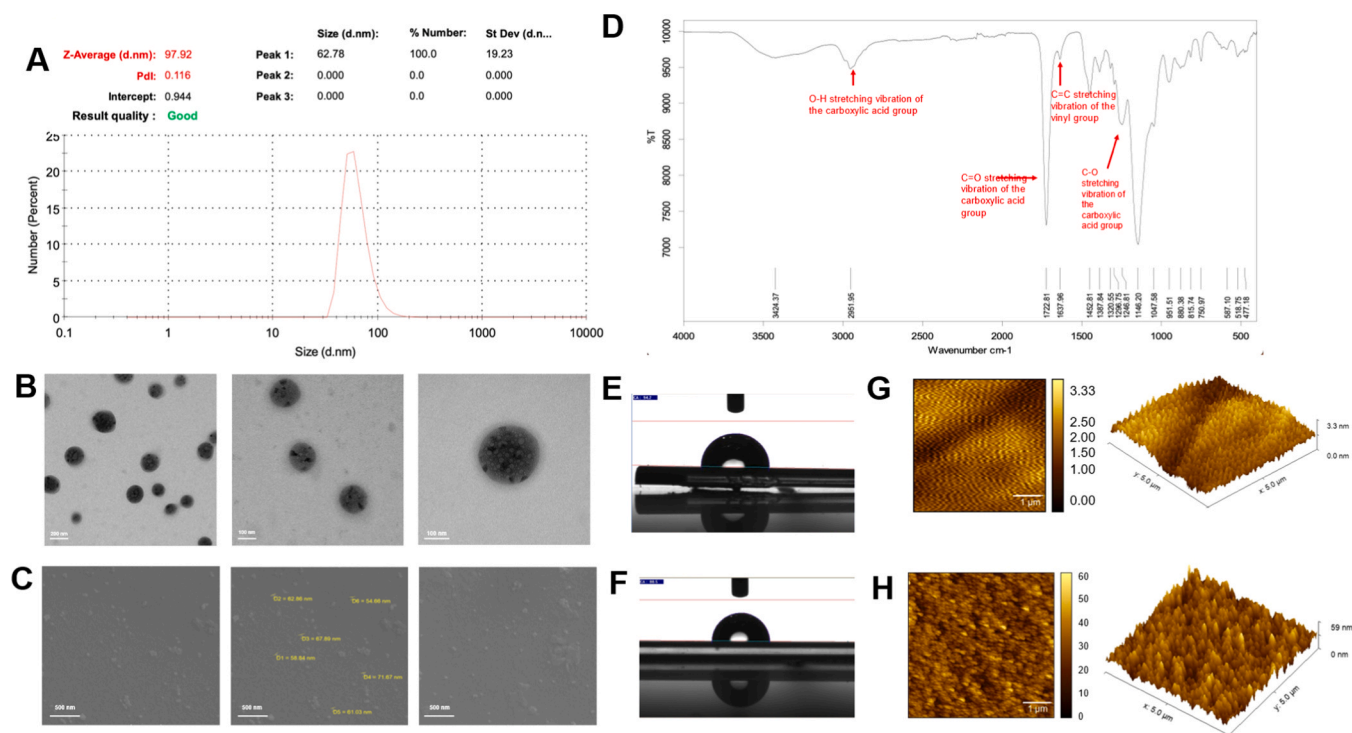


Fig. 4. A) DLS, B) SEM, C) TEM and D) FTIR results of EV-imprinted nanoparticles. Contact angle and AFM images of E, G) bare and F, H) EV-imprinted optical biosensors.

morphology of the unmodified bare gold surface with that of the EV-imprinted nanoparticles immobilized on the optical biosensor surface. Based on the two-dimensional and three-dimensional AFM images, the

bare optical biosensor surface exhibited a depth value of 1.2 nm and an average roughness (R_a) of 0.16 nm (Fig. 4G). In contrast, the EV-imprinted optical biosensor surface showed a significantly increased

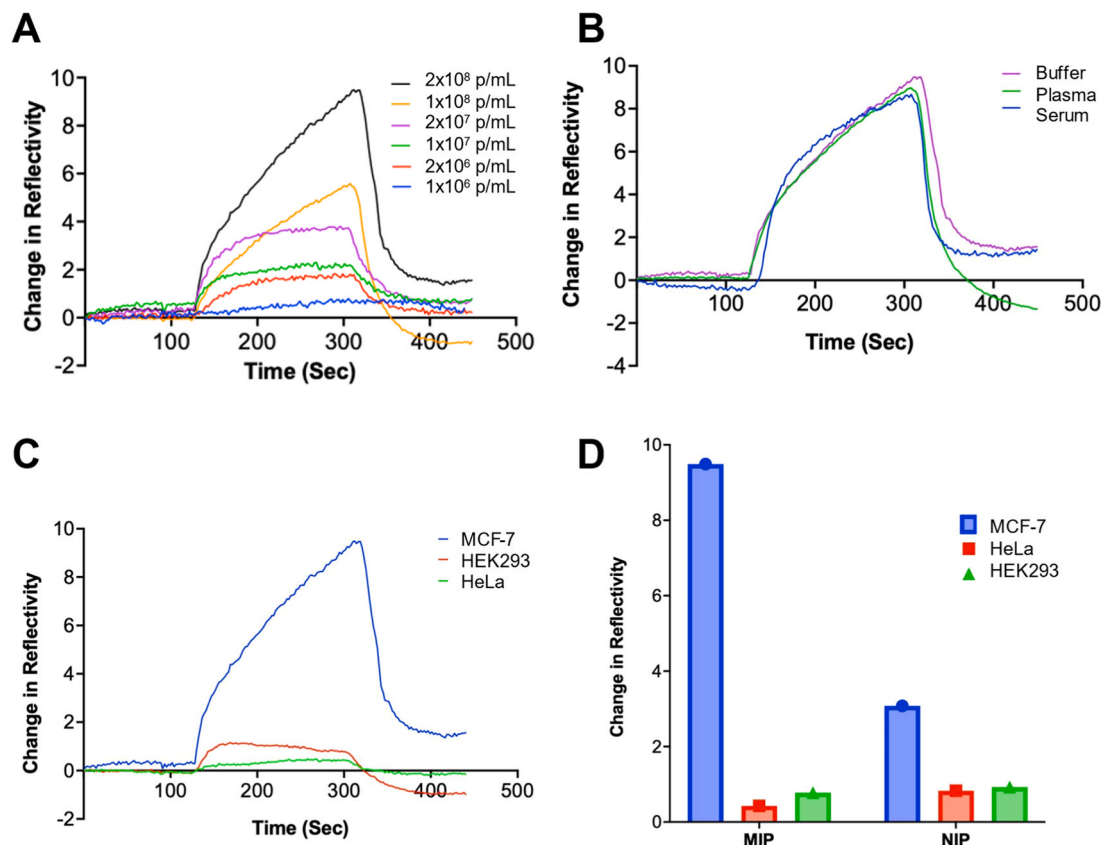


Fig. 5. A) Real-time EVs detection, B) applicability responses in complex matrices, C) selectivity responses, and D) comparison of MIP and NIP optical biosensors.

depth of 25.5 nm and an average roughness of 3.8 nm (Fig. 4H). These findings indicate that the EV-imprinted nanoparticles were uniformly immobilized across the biosensor surface, resulting in a topographically richer and rougher surface morphology.

3.3.3. Real-time EVs detection

Sensograms obtained at each concentration were shown in Fig. 5A, illustrating real-time ΔR values. Overlapping plots for triplicate runs at different concentrations are provided and obtained the calibration curve in Figure S5. The kinetic performance of the EV-imprinted optical biosensor was evaluated over a range of detection that spanned three orders of magnitude (1×10^6 – 2×10^8 particles/mL) with a low detection limit (8.7×10^5 particles/mL) and a high correlation coefficient ($R^2 = 0.9431$). The calibration curve for detection of EVs exhibited an R^2 value, reflecting moderate linearity. This outcome is largely attributable to the intrinsic heterogeneity of EVs, including differences in size, surface marker expression, and biomarker loading, which are inherent to biological samples and absent in a conventional analytical system. Despite this complexity, the platform demonstrated reproducible responses and reliably differentiated between EV concentrations within the tested range, supporting its robustness as a proof-of-concept analytical tool. Future studies would focus on further optimizing assay conditions and incorporating clinically derived EV samples to enhance quantitative accuracy and fully establish the platform's potential for diagnostic applications.

The kinetic responses of NIP biosensor was also investigated over an EV concentration range of 1×10^6 to 2×10^8 particles/mL. The kinetic analysis results were provided in Figure S6. To evaluate the applicability of the EV-imprinted optical biosensor in complex matrices, artificial sample analyses were conducted and demonstrated reliable detection in complex biological fluids, achieving recovery rates of 91.5–94.5% (Fig. 5B). Fig. 5C and Fig. 5D displayed ΔR values of MIP and NIP optical biosensors interacting with EV solutions from MCF-7, HEK293, and HeLa cells. The results revealed that the MIP optical biosensor produced significantly higher signals for MCF-7 EVs, with markedly reduced signals for competing EVs. The NIP optical biosensor yielded generally lower signals overall, indicating non-specific binding. These results demonstrate that the EV-imprinted optical biosensor selectively recognized binding sites exclusive to MCF-7 EVs with high specificity. It should be noted that the selectivity experiments were performed using EVs derived from different cell lines (MCF-7, HEK293, and HeLa), which may share overlapping surface marker profiles, particularly tetraspanins such as CD63, CD81, and CD9, due to common biogenetic pathways [39]. However, EVs are known to carry cell-type specific molecular signatures that reflect their cellular origin, including distinct proteomic profiles, surface topography, and lipid compositions [43,44]. As a result, these comparisons represent a stringent and biologically relevant test of selectivity rather than idealized, fully distinct targets. The observed preferential binding of MCF-7 EVs suggests that the molecularly imprinted platform can discriminate EV populations based on subtle differences in their overall physicochemical characteristics, including surface topography and spatial arrangement of binding sites [45]. Nonetheless, further studies with well-defined EV subpopulations and clinically derived samples are required to comprehensively validate the platform's selectivity under physiologically relevant conditions [46]. Mathematical calculations of selectivity and relative selectivity coefficients were presented in Table S5. Compared to the MIP biosensor, the signal decreased from 9.49 to 3.08 for the NIP biosensor. Relative selectivity coefficients (k') were 5.95 for HeLa and 3.67 for HEK293 EVs. In addition, the real-time reusability response of EV-imprinted optical biosensor was investigated by subjecting the system five repetitive equilibrium-adsorption-desorption cycles of same the EV sample solution (Figure S7). The reusability of the MIP biosensor was evaluated over five regeneration cycles. Quantitative analysis showed that the platform retained 96% of its initial ΔR signal after the fifth cycle, indicating stable performance and demonstrating its potential for repeated use in

analytical applications. This result indicated that EV-imprinted optical biosensor can be utilized in several times without any corruption in interaction sites and loss of response capacity.

Compared to recently reported state-of-the-art EV detection platforms, the performance of the EV-imprinted optical biosensor can be contextualized. Qin et al. reported a MOF corona-based SERS assay achieving single-particle sensitivity with > 95% clinical accuracy [47], and Zhang et al. developed a wash-free electrochemical platform with near single-EV detection capability validated in 111 plasma samples [48]. Wei et al. demonstrated a bubble multimer-enhanced fluorescence assay for streamlined colorectal cancer EV profiling [49]. While these approaches offer superior sensitivity, they typically require complex signal amplification strategies, specialized instrumentation, or multi-step fabrication workflows. In contrast, the MIP optical biosensor developed in this study achieves a detection range spanning three orders of magnitude (1×10^6 to 2×10^8 particles/mL) with a LOD of 8.7×10^5 particles/mL, label-free real-time monitoring, and reliable recovery rates of 91.5–94.5% in serum and plasma matrices — all without requiring signal amplification or complex surface chemistry. These characteristics highlight its potential as a cost-effective, operationally simple platform for EV-based cancer diagnostics, particularly in resource-limited settings.

3.3.4. Mathematical calculations

The acquired data were analyzed to evaluate binding kinetics. Commonly, the equilibrium and Scatchard kinetic analyses of the interaction between the EV-imprinted optical biosensor and EVs. Both Langmuir and Freundlich isotherm models were also applied to characterize the interaction. The corresponding plots were shown in Figure S8. As summarized in Table S6, the experimental results were best fitted to the Langmuir isotherm, indicating that the EV-imprinted optical biosensor surface can be approximated as a monolayer with uniformly distributed binding sites of equal affinity. In the analysis, all isotherm models yielded high correlation coefficients. The Scatchard model exhibited a lower correlation coefficient ($R^2 = 0.8586$), suggesting surface heterogeneity. In contrast, the adsorption data were well described by the Langmuir isotherm, which provided a higher correlation coefficient ($R^2 = 0.9854$), indicating monolayer adsorption on the gold biosensor surface. The surface heterogeneity observed in the Scatchard plot can be attributed to the presence of multiple binding sites with comparable affinities for EV binding. Although both the Freundlich ($R^2 = 0.9526$) and Langmuir ($R^2 = 0.9854$) models demonstrated strong correlation, the $\Delta R_{\max}$ value predicted by the Langmuir model ($\Delta R_{\max} = 4.89$) was in closer agreement with the experimental value ($\Delta R_{\max} = 5.52$) compared to the Freundlich model ($\Delta R_{\max} = 1.12$).

4. Conclusion

This study presents a novel and highly integrated biosensing strategy that combines microfluidic systems, MIPs, and advanced optical detection for the selective isolation and analysis of TME-derived EVs. The platform demonstrates promising analytical performance in terms of selectivity, sensitivity, and real-time monitoring capability under controlled experimental conditions, while also providing valuable insights into the complex biochemical dynamics of the TME.

By enabling the non-invasive detection and characterization of breast cancer-related biomarkers, this approach contributes to the growing field of next-generation biosensing technologies and offers a versatile tool for studying cancer biology. The integration of microfluidics with MIP-based recognition elements further enhances system controllability and analytical precision, distinguishing it from conventional methods that often rely on more labor-intensive and less selective workflows. Importantly, while the results highlight the potential of this platform for biomarker detection and translational research, the present study is limited to proof-of-concept validation. No patient-derived samples or comprehensive diagnostic performance metrics are

included in the present study. Future work would prioritize large-scale validation using well-characterized clinical cohorts, alongside rigorous evaluation of diagnostic performance parameters to robustly assess clinical utility. Within this framework, the current platform establishes a strong proof-of-concept and provides a solid foundation for the development of reliable, non-invasive diagnostic systems, with potential to support both fundamental investigations and subsequent translational efforts in oncology.

CRedit authorship contribution statement

Serok Devrim Aydoğan: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Fatih Inci:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition. **Eylul Gulsen Yilmaz:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Maryam Atabay:** Writing – original draft, Methodology, Investigation, Data curation. **Yeşeren Saylan:** Writing – original draft, Supervision, Resources, Methodology, Funding acquisition, Formal analysis, Conceptualization.

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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.snb.2026.139991](https://doi.org/10.1016/j.snb.2026.139991).

Data availability

Data will be made available on request.

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