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Multi-target polydiacetylene liposome-based biosensor for improved exosome detection

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ABSTRACT

Exosomes are extracellular vesicles 15–150 nm in size and serve as delivery vehicles for long-distance intercellular communication. Exosomes contain various biomolecules derived from the cell cytosol and have potential applications in disease diagnosis and drug delivery. However, due to their low density, small size, and scarcity, reliable methods for the selective capture or efficient isolation of exosomes are required to broaden their range of applications. Particularly, the conventional method for selective exosome capture is based on a ligand-receptor interaction using a receptor that targets tetraspanin on the exosome surface. Sensing platforms detect the signal generated by the binding ligand in the sensing region; therefore, efficient exosome capture in a receptor-based exosome sensor platform has a significant impact on the detection performance. The conducting polymer polydiacetylene (PDA) has unique optical properties resulting from the polymerization of self-assembled diacetylene molecules, and PDA based platform can be used to indicate the degree of ligand-receptor interaction. When the PDA backbone is disturbed by an external stimulus, it produces a blue to red colorimetric transition and fluoresces in the red region without a label. Herein, it was confirmed that exosomes were more efficiently captured by a multi-target platform compared to a single-target platform. The PDA array platform demonstrated significantly improved exosome binding efficiency with a 4–8-fold increase in sensitivity compared to the single-target system. Moreover, the binding efficiency was high in low exosome concentration environments. Therefore, the multi-target exosome platform increases detection sensitivity through efficient exosome capture and this strategy can be universally applied to applications that use exosome-targeting receptors.

1. Introduction

In a living organism where multiple cells divide and develop, different biomolecules carrying specific information are involved in long-distance intercellular communication. Among the signal transmitters, exosomes have emerged as a versatile mediator in the fields of medical diagnosis and therapeutics [1,2]. Exosomes are cell-derived vesicles 15–150 nm in size that are formed through the invagination process in an intracellular multi-vesicular body (MVB). In the process of exosome formation, intracellular biomolecules such as nucleic acids, proteins, and metabolites are encapsulated and the produced vesicles are released out of the cell by exocytosis [3,4]. The released exosomes circulate for intercellular communication in several body fluids such as blood, saliva, urine, and cerebrospinal fluid. By separating exosomes from a variety of body fluids and analyzing disease-related biomarkers in them, they can be used for diagnosing diseases such as cancer or Alzheimer's disease [5–8]. In therapeutic applications to alleviate or treat various diseases, exosomes containing drugs or biomolecules are also used as drug carriers [9,10]. Due to their high potential for a broad range of applications, effective and efficient methods for more selective and sensitive detection of exosomes are required [2,11]. However, complex procedures or delicate equipment are required for the selective isolation and screening of exosomes due to their low density, small size, and scarcity [12]. Conventional sensors for exosome detection require

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the selective capture of exosomes from bodily fluids. Typically, for the selective capture of exosomes, tetraspanin membrane proteins are the main targets. The tetraspanin family includes CD9, CD63, CD81, and CD82, and the tetraspanin type and composition usually depend on the origin of the exosome [13,14]. For the detection of exosomes, it is common to introduce receptors such as antibodies or aptamers to the sensing platform. The concentration of exosomes captured by the interactions between a receptor and the tetraspanin on the exosome membrane is a significant factor in triggering the sensing platform, and it influences the sensitivity of the detection platform [15,16].

Among the platforms that validate captured exosomes, polydiacetylene (PDA) is widely used in a variety of sensor platforms for fast and facile detection because of its peculiar optical properties caused by the ene-yne conjugated bond in its backbone structure [17,18]. The conjugated ene-yne in PDA is formed by the 1,4-topochemical reaction induced by ultraviolet irradiation on self-assembled diacetylene monomers. The newly formed conjugated bond absorbs a specific wavelength, leading to a PDA with intense blue color. When the conjugated backbone is deformed or disturbed by external stimuli, the absorption spectrum is shifted, leading to the blue to red colorimetric transition. In addition, since the red form of PDA exhibits fluorescence in the red region, the change in PDA structure can be confirmed through optical analysis such as colorimetric or fluorescence observation [19,20].

PDA is a suitable conductive polymer for sensing applications for several reasons. First, due to the nature of PDA derived from the self-assembly characteristics of diacetylene monomers, various types of structures such as films, filaments, crystals, and vesicles can be formed as required [19,21–24]. In addition, since polymerization by UV is easily performed without initiators or catalysts, a high-purity platform can be manufactured. Second, different factors such as temperature, pH, solvent, and electrical or mechanical stress can physically modify the conjugated ene-yne PDA backbone [25–29]. In particular, ligand-receptor interactions make the PDA platform advantageous for biosensors. By introducing a receptor for a specific target, selectivity can be assigned to the PDA supramolecules, making them suitable devices for measuring binding ability [30–32]. Finally, since PDA exhibits fluorescence under stimuli, it can be used to construct a label-free sensor platform. Currently, representative optical-based sensors such as surface plasmon resonance (SPR), electrochemical sensors, long period fiber grating (LPG) and surface resonance raman spectrometer are widely studied in the sensor applications [33–38]. In an optical method for the selective detection of exosomes, high accuracy and sensitivity can be achieved by introducing a receptor or analyzing the sensitive optical signal displayed by the exosome itself. However, for fine signal analysis, it has limitations in terms of versatility because customized equipment and complicated experimental procedures are required [39–43]. In contrast, the PDA-based sensor can be characterized with relatively universal spectrometers and can be transformed into various platforms.

In this study, instead of the conventional exosome detection platform, which has a single receptor, multiple receptors were introduced to target different tetraspanins on the surface of the exosome and enhance exosome binding. Exosome capture is validated by the label-free detection arising from the change in optical properties of PDA vesicles attached to a two-dimensional surface. We present, experimentally, a multiple target strategy for improving the sensitivity of the sensing platform by enhancing exosome capture efficiency and specificity. The multiple-target PDA array can be designed to sense a wide range of exosomes expressing different types of targets and biomarkers.

2. Materials and methods

2.1. Materials

Diacetylene monomer 10,12-pentacosadiynoic acid (PCDA) was obtained from GFS-Chemicals [Powell, OH, USA] and phospholipid 1,2-ditetradecanoyl-sn-glycero-3-phosphocholine (DMPC) was supplied by Tokyo-Chemical Industry [Tokyo, Japan]. (3-aminopropyl)triethoxysilane (APTES) and tween® 20 were purchased from Sigmaaldrich [MO, USA]. Sulfuric acid (95%) and hydrogen peroxide (35.0%) were supplied by Samchun Chemicals [Seoul, Korea]. N-hydroxysuccinimide (NHS) and N-3-Dimethylaminopropyl-N-ethylcarbodiimide hydrochloride (EDC) were purchased from Tokyo Chemical Industry [Tokyo, Japan]. Anti-CD63 monoclonal antibody [M38] and anti-CD81 monoclonal antibody [Ts63] were supplied by ThermoFisher Scientific (MA, USA). Anti-CD63 aptamer and anti-CD81 aptamer were supplied by Aptamer Sciences, Inc. [Gyeonggi-do, Korea]. The plasma protein fibrinogen (extracted from human plasma) was supplied by Sigma-Aldrich [MO, USA]. Bovine serum albumin (BSA) protein was purchased from MP Biomedicals [Santa Ana, CA, USA]. Lyophilized exosomes (from healthy human plasma) were obtained from Hansa-BioMed Life Science, Inc. [Tallinn, Estonia]. Human plasma for exosome isolation was purchased from Innovative Research, Inc [MI, USA]. DiO cell-labeling solution for exosome was obtained from ThermoFisher Scientific [MA, USA]. Phosphate-buffered saline buffer (PBS) was obtained from Welgene Inc. [Korea]. Other organic solvents were purchased from Samchun Chemicals [Seoul, Korea].

2.2. Manufacture of the amine-modified glass substrate

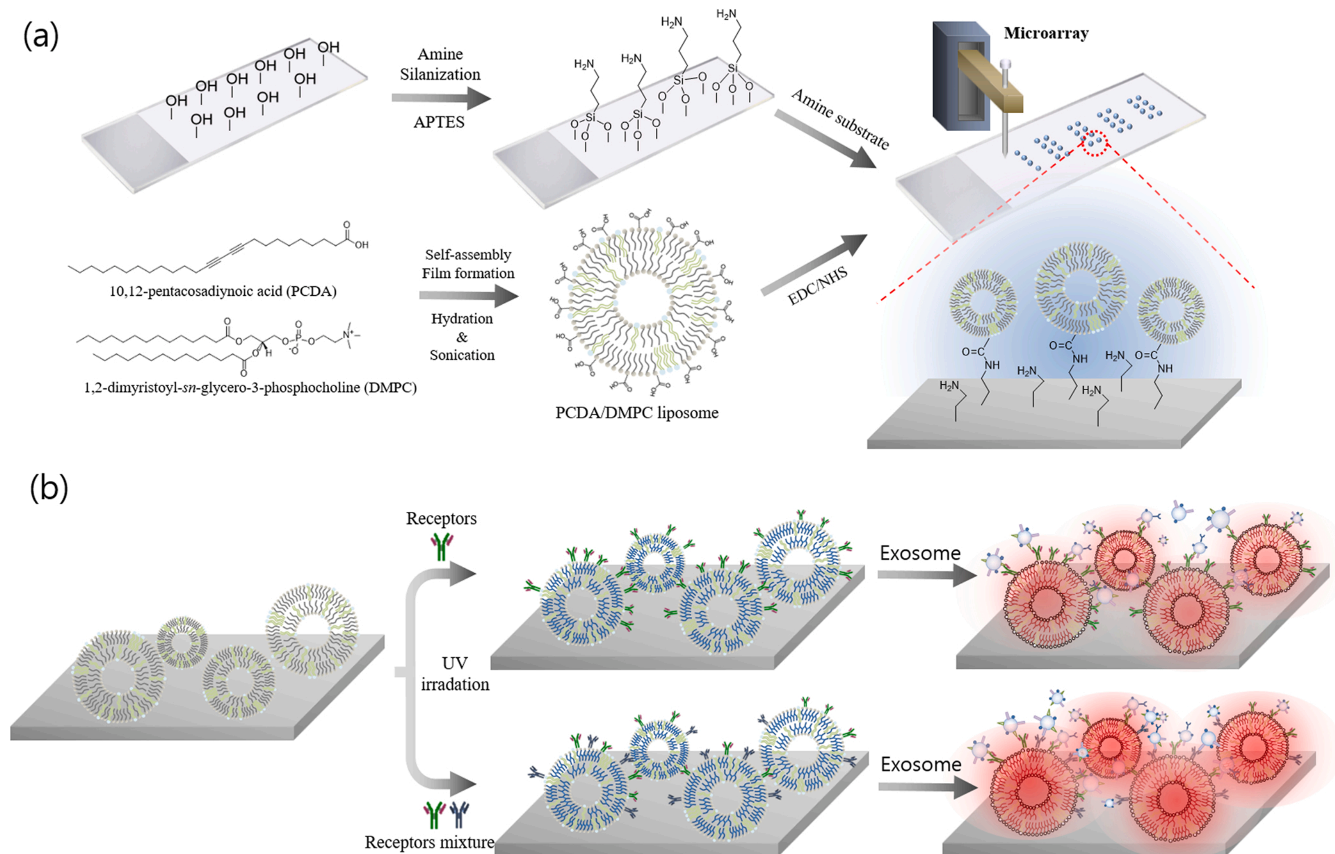
Glass slides were immersed and sonicated sequentially in chloroform, acetone, and ethanol for 5 min each in order to remove foreign substances or organic substances from the surface (Scheme 1). After the first wash, the glass was placed in a bath set at 80 °C in piranha solution (95% H_2SO_4: 35% H_2O_2 = 3:1) for 1 h. The hydrophilic glass substrate was washed several times with plenty of water and dried, followed by immersing in 2 wt% APTES-ethanol solution for 2 h with gentle stirring. After washing with a large amount of ethanol to remove the remaining APTES from the surface, the surface was dried and annealed at 110 °C under vacuum for 1 h.

2.3. Preparation of diacetylene/phospholipid liposome composite

The diacetylene/phospholipid liposome composite was prepared using the general thin film hydration method. Diacetylene monomer and phospholipid were individually dissolved in chloroform at amber glass vials. Each solution was extracted and evenly blended according to a 4:1 molar ratio of diacetylene monomer (PCDA) and phospholipid (DMPC). The mixed solution was slowly evaporated under nitrogen flow and the thin lipid film was hydrated by adding deionized (DI) water (Milli-Q® water, resistivity 18.3 MΩ•cm) to give a total lipid concentration of 0.5 mM. When the lipid film started to float after equilibrating the temperature for a few minutes in an 80 ℃ bath, the solution was sonicated using a probe sonicator (Sonics & Materials, CT, USA) for 20 min at 150 W, maintaining the bath at 80 ℃. As the vesicles became uniform and homogeneous, the suspension gradually became translucent and was filtered immediately through disposable 0.45-μm syringe filters (Advantec, CA, USA) to eliminate aggregated lipids or large impurities. The filtered lipid solution was cooled down to room temperature and placed at 4 ℃ to re-assemble the lipid vesicles overnight.

2.4. Vesicle immobilization and receptor conjugation

The crystallized lipid vesicles were immobilized on an amine-modified substrate by EDC/NHS chemistry. Separately dissolved EDC and NHS solutions were mixed with the vesicle solution, leading to a concentration of 100 mM. The mixture solution was arrayed on the substrate using a manual microarray spotter (Labnext Inc.) in a humidity chamber with a humidity of 80% (introducing more than six array dots per well). The microarray spotted glass was incubated at 4 ℃ overnight and washed thoroughly with 0.1% tween-20 solution and DI water and gently dried. To introduce the receptor on the liposome surface, 30 μL of 100 mM EDC/NHS solution was added per well and re-activation of



Scheme 1. Schematic illustration of the preparation of polydiacetylene (PDA) based array sensor for multi-targeting exosome detection. (a) PDA liposomes consisted of diacetylene monomer and phospholipid are activated by EDC/NHS reaction and immobilized on the amine modified substrate. (b) Receptors are conjugated onto the PDA surface, which confers specificity to PDA array platform for selective exosome detection.

vesicles was performed at room temperature for 30 min. After washing and drying, 30 μL of receptor solution of the required concentration was added per well to conjugate the receptor (overnight at 4 ℃ for the antibody and room temperature for 5 h for the aptamer). Then, washing with 0.1% tween-20 solution was done to remove unbound receptors, followed by drying. Before adding the exosome solution, the array dots were photo-polymerized by 254 nm UV irradiation for 15 min through a UV lamp (Vilber, France) with 400 $\mu W/cm^2$ intensity.

2.5. *Structural analysis of the PDA liposomes*

The morphology of PCDA/DMPC vesicle composites was imaged through a high-resolution transmission electron microscope (HR-TEM, JEM-3010, JEOL, Japan) and acceleration voltage was set at 300 kV. TEM samples were prepared by dropping and drying the diluted liposome solution on a 300 mesh copper grid. The size distribution and zeta potential of the vesicle solution were characterized by dynamic light scattering analysis (Zetasizer nano ZS, Malvern Instruments, U.K.). The vesicle solution was measured using disposable folded capillary cells (DTS1070) after dilution to a concentration of about 0.05 mM.

2.6. *Spectrophotometric and fluorescence analysis of PDA array dots*

The colorimetric change of PDA liposomes immobilized on the two-dimensional glass substrate was characterized by a UV–vis spectrometer (Lambda 35, PerkinElmer, USA). The incubated substrate was measured for each exosome concentration, and the spectrum was obtained in the 450–700 nm range.

The PDA vesicle array dots physically modified by exosomes were characterized by fluorescence microscopy (ZEISS, Carl Zeiss AG, Germany). For fluorescence measurements of the PDA array, filters with an excitation wavelength of 560/40 nm and emission wavelength of 630/60 nm were used. To optimize receptor conjugation on the PDA array, the part with the strongest fluorescence intensity within the array dot was photographed at 400 × magnification, and the fluorescence intensity of the array dot was photographed at 200 × magnification after exosome incubation. The exposure time was adjusted from 3000 ms to 10,000 ms according to the magnification and the base fluorescence of the control group, and the intensity of the red element in the image was quantified using ImageJ. As a background signal for LOD confirmation, the PDA fluorescence signal for distilled water without exosomes was used.

2.7. *Exosome labeling and characterization*

Exosomes for labeling were isolated from human plasma by ultracentrifugation (ProteomeLab XL, Beckman, USA). First, human plasma sample was centrifuged at 3000 g for 30 min to eliminate cell flakes, and the supernatant was filtered through a disposable 0.2 μm syringe filter (Advantec, CA, USA) for further removal of suspended materials in plasma. The filtered plasma was ultra-centrifuged at 170,000 g for 90 min at 4 ℃, obtaining an exosome pellet. To remove suspended plasma proteins, the supernatant was discarded, the exosome pellet was thoroughly dispersed in PBS, and the same washing procedure was repeated.

Exosomes isolated from plasma were immediately subjected to exosome labeling using DiO based on lipophilic carbocyanine dye. Dye solution (5 uL) was added to the exosome solution and incubated at 37 °C

for 20 min to penetrate the membrane. After the reaction was completed, the solution was ultra-centrifuged at 170,000 g for 90 min at 4 °C to remove the unreacted dye, followed by re-dispersal in PBS and then washing. The final labeled exosome solution was diluted 100-fold for characterization of the exosome size and concentration using nanoparticle tracking assay (NTA) (NanoSight LM10, Malvern, UK).

2.8. Characterization and quantification of captured exosome

Characterizations of the size and concentration of exosomes were performed through SEM, DLS, and NTA. The residual exosome concentration was measured by NTA using the 100-fold diluted exosome solution.

In order to calculate the efficiency of the exosomes attached to the surface, the exosome solution that underwent the incubation process was recovered from the PDA array well and the concentration of the exosome solution was measured through NTA. The adhesion performance of exosomes was calculated by:

$$\text{Capture efficiency} = \frac{C_0 - C_1}{C_0} \times \frac{1}{N}$$

Where C_0 is the exosome recovery concentration of the control group recovered from the PDA array dot into which the receptor is not introduced and C_1 is the remaining exosome concentration recovered from the PDA array dot into which the receptor is introduced. The exosome capture efficiency of the receptor was determined by the concentration ratio of the exosomes captured in one PDA array dot (normalized surface area, N) arrayed in one well.

The labeled exosomes captured on the surface of the PDA were observed using a fluorescence microscope. For fluorescence observations of the labeled exosome on the PDA array, a filter with an excitation wavelength of 470/40 nm and emission wavelength of 525/50 nm was used. The exposure time was adjusted to 10,000 ms according to the magnification and the base fluorescence of the control group. Next, the intensity of the green element in the fluorescence image was quantified using ImageJ.

3. Results and discussion

3.1. Principle of PDA array platform

To utilize exosomes for sensing, the specific biomarkers carried by the exosomes are treated as targets. As shown in Scheme 1, the PDA-based platform was fabricated with a micro-array of PCDA/DMPC vesicles on a two-dimensional substrate. Specifically, PCDA/DMPC vesicles are covalently immobilized through EDC/NHS reactions on amine-modified glass. The shape and size of PCDA/DMPC vesicles present in the solution are similar to those of the immobilized material on the substrate, so the chemical reaction between the amine substrate and the vesicles can be confirmed (Supplementary Material Fig. S1 and Fig. 1). In the case of amine-modified glass, silanization was confirmed by XPS analysis (Supplementary Material Fig. S3a). PDA arrays immobilized on amine-modified glass are much denser and evenly distributed than PDA arrays immobilized on unmodified glass substrates (Supplementary Material Fig. S3(b-c)). Receptors for exosome biomarkers are introduced onto the immobilized vesicles to obtain selectivity and reactivity for the exosomes. Due to the characteristics of the PDA vesicle, in which a signal is generated due to the interaction between the receptor and the exosome, the sensitivity of the PDA sensor platform can be modulated by the exosome capture efficiency of the receptor. The adhesion between the characterized exosomes and the receptor was confirmed by SEM (Supplementary Material Fig. S2). For a more effective exosome capture, a system targeting multiple exosome ligands can be designed to replace the conventional single receptor system. The multiple receptor model improves the capture efficiency by increasing the probability of interactions with the exosomes. Through the label-free PDA array platform, it is possible to verify the capture of exosomes and the results can be used to quantify the improvement in sensitivity of the multiple target sensing platform.

3.2. Optimization of receptor density on the PDA surface

For the PDA liposome platform to induce signals from selective exosome capture, receptor conjugation with the PDA backbone is required. In this study, protein-based antibodies and nucleic acid-based aptamers were used as receptors for ligand-receptor interactions

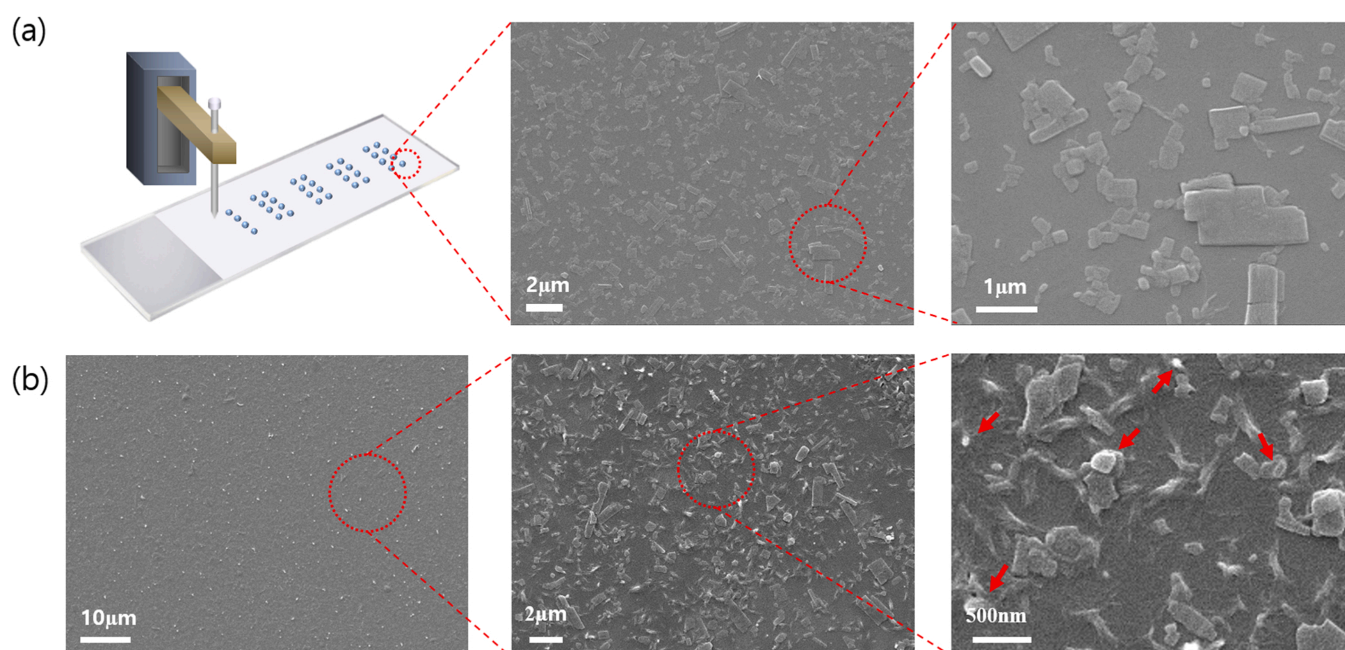


Fig. 1. SEM image of PDA vesicle arrayed on amine substrate. (a) Arrayed bare PDA vesicles and (b) exosomes attached to PDA vesicles with incorporated receptors.

between the immobilized vesicles and the exosomes. Ligand-receptor interactions modify the backbone of the PDA incorporated in the immobilized vesicles, causing changes in the optical properties of PDA. In order for the stimulus to be transferred effectively to the conjugated ene-yne bond of the PDA backbone, sufficient receptor density on the surface of the PDA is required. To optimize the concentration of exosome-targeting receptors, receptor solutions in the 0.1 ng/mL to 1000 ng/mL concentration range were evaluated. The exosome incubation was performed at the same exosome concentration (1×10^{10} vesicles/mL) and the receptor concentration showing the strongest signal was selected (Fig. 2). When the receptor density is too high, polymerization of the PDA vesicle does not proceed smoothly because steric hindrance by tightly bound receptor molecules on the vesicle surface inhibits the self-assembly of the diacetylene structure (Fig. S4). Conversely, when the receptor density is too low, both the probability of interaction with the exosome and the binding efficiency are reduced. Using the anti-CD63 receptor-based PDA platform, the fluorescence intensity of PDA was greatest with 1 ng/mL antibody solution and 10 ng/mL aptamer solution.

3.3. Spectrometric transition of PDA vesicles via exosome interaction

When a ligand-receptor interaction occurs on the surface of the receptor-PDA composite, the interaction stimulus is transmitted to the PDA backbone, disturbing the conjugated ene-yne bond. The modified conjugated bond shifts the energy absorption spectrum, causing a colorimetric change in the PDA supramolecules (Fig. 3). The blue PDA vesicles immobilized on the amine-modified glass substrate show a maximum absorption peak around 610 nm (Fig. 3a). When the exosome binds to the receptor on the surface of the PDA, the intensity of the maximum absorption peak decreases and a new absorption peak appears around 540 nm. In the 540 nm region indicating red-form disturbed PDA, the absorbance spectrum of blue PDA without exosomes does not deviate significantly from the baseline spectrum. However, when the exosome is introduced, the spectrum starts to deviate from the baseline, and the area of deviation from the baseline spectrum increases as the concentration of the exosome increases. In the abundant exosome environment (1×10^{10} vesicles/mL), a noticeable spectral change is observed in the 540 nm region (Fig. 3b). The formation of a new peak value due to an increase in exosome concentration means that the proportion of interaction-modified PDA increases.

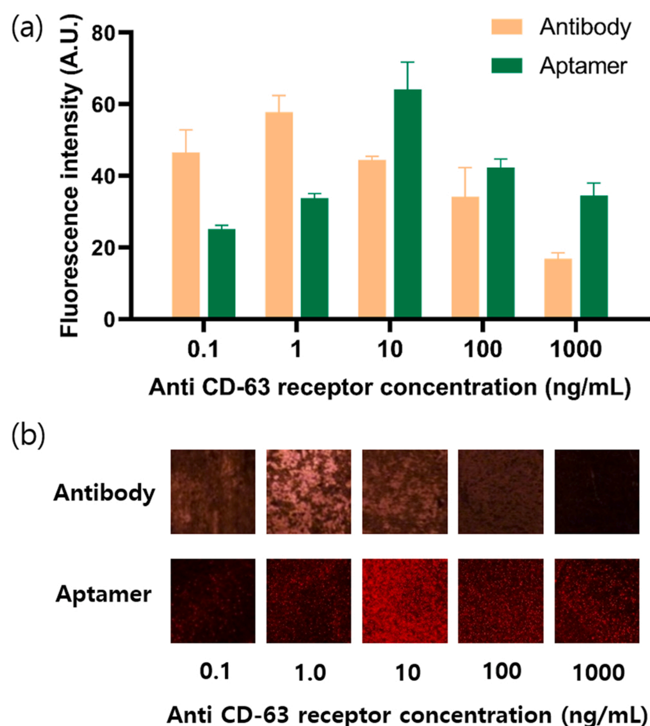


Fig. 2. Optimization of receptor concentration for efficient exosome detection. Both the antibody and the aptamer target the CD-63 of the exosome to determine the appropriate linkage concentration of the receptor. (a) The concentration of the antibody and aptamer to be incubated was from 0.1 ng/mL to 1000 ng/mL, and the fluorescence was measured after adding 1×10^{10} vesicles/mL of exosome to each sample. (b) Fluorescence image of PDA vesicle after exosome addition. The intensity of fluorescence was measured based on the largest fluorescence value with an excitation wave of 560 nm. (Derived fluorescence intensities are subtracted the signal from the background signal). (n = 4).

In addition to the colorimetric change, the fluorescence intensity derived from the PDA array was also measured according to the concentration of each type of exosome. Receptor-carrying PDA vesicles exhibited a fluorescence spectrum with a peak in the 640 nm region when they were transformed into the red form by the interactions induced by the addition of exosomes. The fluorescence intensity of PDA triggered by ligand-receptor interaction showed a tendency to increase as the concentration of exosomes increased (Fig. 4). This phenomenon confirms that the PDA platform is a suitable method for exosome detection and quantification.

3.4. Limit detection of PDA array platform

To confirm the change in the limit of detection (LOD) of the PDA platform due to multi-targeting, PDA signals for each exosome concentration were characterized (Fig. 5). For LOD calculations, the baseline was set to the sum of the background and three times the standard deviation. The LOD corresponds to the exosome concentration at the point where the signal starts to cross the baseline. In the case of antibodies, for the single-target array, the LOD was measured to be about 3.5×10^8 vesicles/mL on the CD63 target platform and 1.5×10^8 vesicles/mL on the CD81 target platform (Fig. 5a). On the other hand, in the CD63 and CD81 multi-target platform, 4×10^7 vesicles/mL was required, resulting in a LOD improvement of 4–8 times compared to the single target platform. In the case of aptamers, the LOD corresponds to 1.5×10^8 vesicles/mL in the CD63 target platform, 2.0×10^8 vesicles/mL in the CD81 target platform, and 3.5×10^7 vesicles/mL in the CD63 and CD81 multi-target platform (Fig. 5b). The LOD was improved by 4–6 times in the multi-target platform compared to the single target platforms. In summary, the performance of the sensor platform can be improved by simply introducing a receptor system that targets multiple ligands.

The graphs show the sensitivities for antibodies (top) and aptamers (bottom) according to the introduced target receptor type. The sensitivity (LOD) of the platform was determined as the value measured within 3 times the sum of the background and standard deviation of the PDA array signal without exosomes. Derived fluorescence intensities are subtracted from the baseline signal. (The fluorescence intensities were calculated at the maximum values and excitation wavelength was 560 nm, n = 4).

3.5. Influence of multiple receptors on exosome capture efficiency

To investigate the ability of the PDA array to capture exosomes with and without the introduction of multiple targets, the number of exosomes attached to the PDA array dot was quantified. First, after incubating the exosomes in the PDA array with each receptor condition, the remaining exosome solution was collected and the concentration of the solution was analyzed (Fig. 6a). In a relatively abundant exosome environment (3×10^9 vesicles/mL solution), the capture efficiency of exosomes was similarly measured regardless of the receptor type or target ligand. However, in an environment with sparse exosomes (1×10^8 vesicles/mL solution), the concentrations of the recovered exosomes showed differences despite the same density of the introduced receptor. The exosome binding efficiency was greater in multiple target platforms

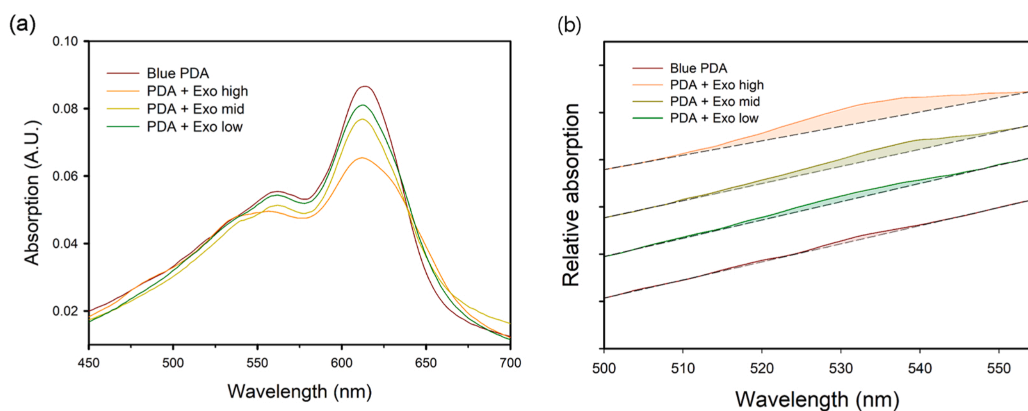


Fig. 3. Changes in UV–vis spectrum of PDA vesicle array according to the concentration of reactive exosomes. (a) The absorption spectrum of PDA vesicles that appears when each exosome concentration (1×10^{10} vesicles/mL, 1×10^9 vesicles/mL, 1×10^8 vesicles/mL) is added to Blue PDA with CD-63 and CD-81 antibodies introduced. (b) Absorption spectrum (500–550 nm) of red form PDA according to exosome incubation concentration. (Each spectrum was rearranged and each division means 0.01 A.U.).

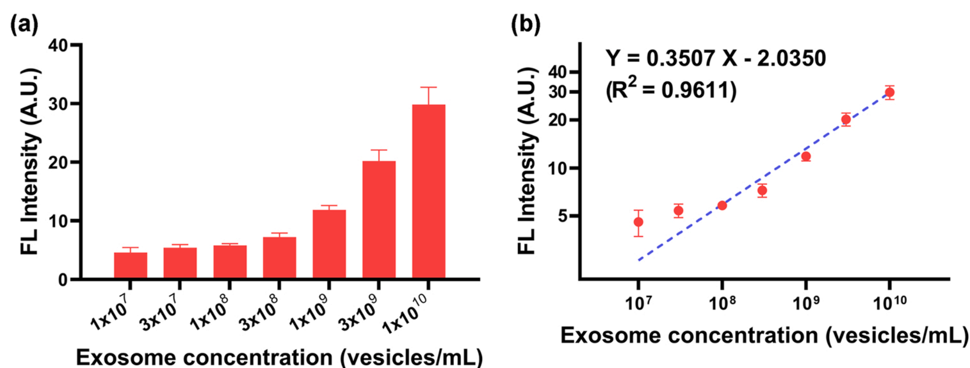


Fig. 4. (a) Fluorescence intensity of PDA array according to exosome concentration. (b) Correlation curve between the FL intensity of arrayed PDA vesicles and exosome concentration (fluorescence intensities were calculated at the maximum values, the excitation wavelength was 560 nm, n = 4).

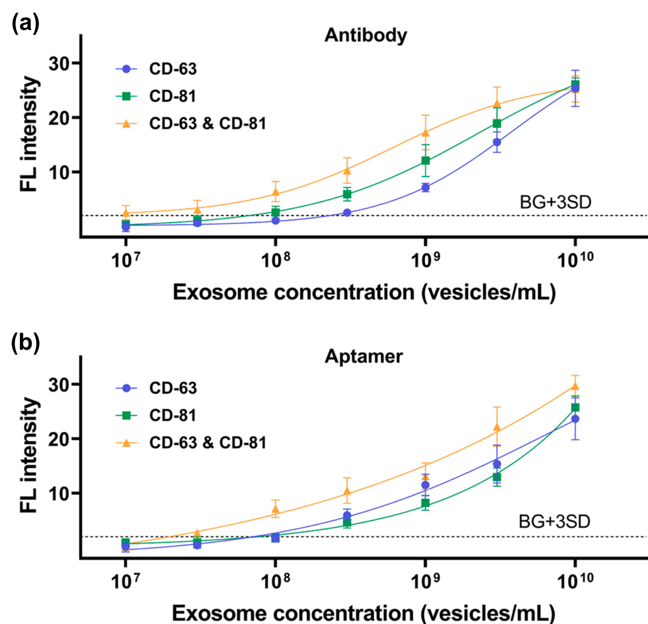


Fig. 5. Correlation curves between the fluorescence intensity and the exosome concentration.

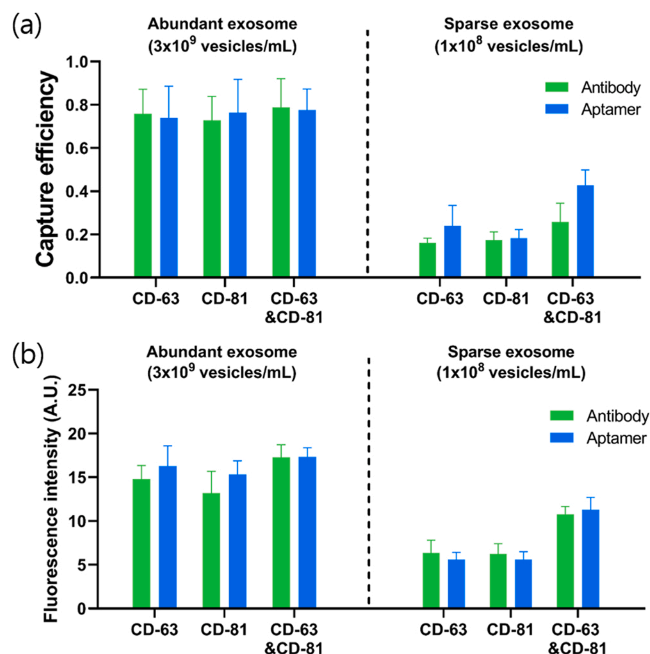


Fig. 6. Quantification of the captured exosomes on the PDA liposome surface. (a) The ratio of captured exosomes according to receptor type. After exosome solution incubation on the PDA array, the captured exosome was quantified based on the remaining exosome solution (n = 4). (b) Fluorescence intensity of the fluorescent exosome according to the type of receptor. Quantitative evaluation of exosomes captured in PDA array using DiO labeled exosomes (n = 4).

than in single target platforms. The exosome binding efficiency for the multiple target platform is close to the sum of exosome efficiencies for each single target platform. An increased exosome binding efficiency in the mixed receptor system was shown for both the antibodies and the aptamers. This is considered to be the effect of increasing the likelihood

of ligand-receptor interactions due to the PDA array recognizing multiple targets on the exosome.

As an additional quantification method, exosomes labeled with fluorescent dye were incubated following the same protocol and the binding density of the attached exosomes was evaluated using a fluorescence microscope. In the abundant exosome solution, a relatively strong fluorescence signal was shown even when CD63 or CD81 was present as a single receptor and the increase in fluorescence signal due to the mixed receptor system was not significant (Fig. 6b). On the other hand, in a low concentration of exosomes, a relatively low fluorescence signal was shown when a single receptor was present, which means that exosomes were not sufficiently attached to the surface of the PDA array. When multiple target receptors were present, the exosome fluorescence signal was increased compared to that of a single receptor, and the increase was larger than in the high concentration exosome environment. This phenomenon was exhibited by both aptamer and antibody-based systems. That is, regardless of the type of receptor used (antibody or aptamer), the multi-target platform increases the exosome capture efficiency and this effect is more pronounced in low concentration environments Supplementary Material Fig. S7. Through the adhesion experiment using fluorescent exosomes, exosomes isolated from plasma using ultracentrifuge can also be applied to the PDA array system, and exosome capture efficiency by multiple receptors can be increased. In particular, this can be advantageous in applications with low biomarker concentrations such as disease diagnosis.

3.6. *Exosome specificity of PDA array*

In order to verify the selectivity or specificity of the PDA array platform for exosome detection, we evaluated the response of PDA to various proteins present in plasma such as BSA, immunoglobulin G (IgG), and fibrinogen. Using the same protocol for detecting exosomes, 1 × PBS buffer, BSA, IgG, and fibrinogen solution were applied to the PDA array. Each plasma protein was injected into the array at a concentration of 20 μg/mL without exosomes, and after incubation, the PDA fluorescence was measured. The PDA fluorescence signals for various plasma proteins without exosomes did not show significant deviations near the LOD (Fig. 7). This implies that the plasma proteins did not interact with the receptors on the PDA surface, and the array has sufficient selectivity for exosome detection.

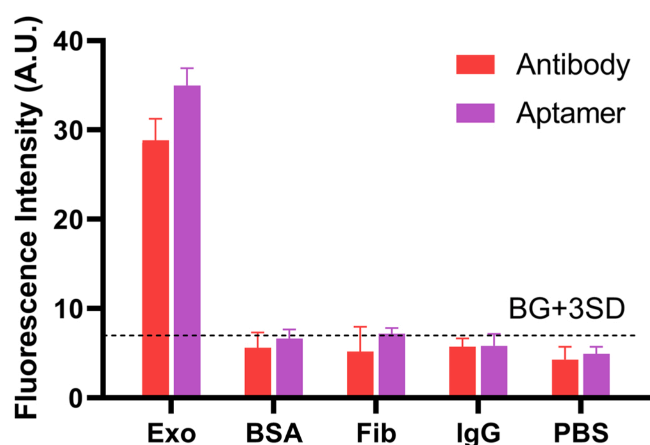


Fig. 7. Specificity test of the PDA vesicle array for exosomes with plasma proteins. Anti-CD63 and Anti-CD81 were simultaneously included for each antibody and aptamer for the exosome target. (Each plasma protein has a concentration of 20 μg/mL in 1 × PBS, n = 4).

4. Conclusions

Exosomes are important mediators for diagnostic and therapeutic applications, and biosensors with high exosome sensitivity are required. Sensor platforms utilizing highly specific receptor-based arrays with multiple targets can increase the efficiency of ligand-receptor interactions. In this study, a label-free polydiacetylene (PDA)-based vesicle array sensor platform was constructed. The changes in the optical properties, especially fluorescence, of the array due to its interactions on the PDA backbone can be used to indicate exosome capture. An exosome capture system that can bind to multiple targets was constructed to increase the capture efficiency of exosomes on the surface of the array and increase the fluorescence intensity of the PDA array. Exosomes with multiple biomarkers showed higher capture efficiency on the multi-receptor platform. Up to 8 × enhancement in the limit of detection was demonstrated with a multiple target array compared to an array with a single receptor. The multiple-target strategy for improving biosensor sensitivity is simple and the PDA-based array can be customized for different ligand-receptor interactions making it a very powerful technique for diagnostic and therapeutic applications.

CRediT authorship contribution statement

Changheon Kim: Conceptualization, Methodology, Data analysis, Writing – original draft, Writing – review & editing. **Bo Hoon Han:** Investigation, Visualization. **Dongwoo Kim:** Resources, Formal analysis. **Gyubok Lee:** Software, Validation. **Changgi Hong:** Data curation, Writing – review & editing. **Ji Yoon Kang:** Supervision, Funding acquisition. **Kangwon Lee:** Supervision, Funding acquisition, Project administration.

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.2021.131286.

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