

Aptamer/AuNP Biosensor for Colorimetric Profiling of Exosomal Proteins

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Abstract: Exosomes constitute an emerging biomarker for cancer diagnosis because they carry multiple proteins that reflect the origins of parent cells. Assessing exosome surface proteins provides a powerful means of identifying a combination of biomarkers for cancer diagnosis. We report a sensor platform that profiles exosome surface proteins in minutes by the naked eye. The sensor consists of a gold nanoparticle (AuNP) complexed with a panel of aptamers. The complexation of aptamers with AuNPs protects the nanoparticles from aggregating in a high-salt solution. In the presence of exosomes, the non-specific and weaker binding between aptamers and the AuNP is broken, and the specific and stronger binding between exosome surface protein and the aptamer displaces aptamers from the AuNP surface and results in AuNP aggregation. This aggregation results in a color change and generates patterns for the identification of multiple proteins on the exosome surface.

Exosomes are membrane-enclosed vesicles (30–100 nm in diameter) secreted by most cell types.^[1] They carry molecular information of their parent cells and are enriched in membrane proteins and genetic materials for cell–cell communication.^[2] For example, the protein patterns of responses to metabolic or oxidative stress were shown to be identical between parent cancer cells and the exosomes they produced.^[3] Notably, the exosome surface presents a quilt-like tapestry of protein markers, and a combination of such markers could predict the origin of parent tumors.^[4] Therefore, exosomes are emerging as noninvasive diagnostic biomarkers of cancer based on the profiling of their surface protein patterns.^[5] However, analyzing exosome phenotype information, in particular, the subtle variations in protein patterns among different cell types, poses a formidable challenge because of the lack of feasible and accurate

profiling methods. Currently, mass spectrometry and immunoassays,^[6] such as western blot and enzyme-linked immunosorbent assays (ELISA), can identify the most abundant exosomal proteins but these approaches require laborious sample pretreatment, thus limiting their adoption for rapid exosome biomarker screening. Nanoplasmonic sensing provides a powerful tool for exosomal protein analyses; however, it requires specialized equipment and complicated analysis procedures.^[7] In this context, a convenient and reliable method for exosome surface-protein profiling is highly desired for cancer diagnosis.

Aptamers are short nucleic acids oligomers selected by a process termed systematic evolution of ligands by exponential enrichment (SELEX).^[8] They are appealing alternatives to antibodies for targeted molecular recognition. In particular, these “chemical antibodies” exhibit excellent binding affinity and specificity toward cell-surface proteins.^[9] This characteristic of specific recognition has been harnessed as a versatile platform to develop biosensing and molecular imaging tools in the physiological environment.^[10] Inspired by these achievements, we hypothesized the generation of a predictive signature-based strategy based on the binding between aptamer and exosome surface protein, which constitutes the basis for profiling the subtle variations in exosome protein patterns among different cell types to collect phenotype information of cancers.

Herein, we report a novel multiplexed sensor platform created through the assembly of a gold nanoparticle with a panel of aptamers targeting ubiquitous or putative exosome surface proteins. We demonstrate that complexation of aptamers with AuNPs protects them from aggregation in a high salt solution. However, in the presence of exosomes, the non-specific and weaker binding equilibrium between aptamers and the AuNP is broken, and the specific and stronger binding between exosome surface protein and the aptamer takes place, resulting in a rapid displacement of aptamers from the particle surface and consequent aggregation of AuNPs. Under these circumstances, the aggregation of AuNPs results in a color change from red to blue, which can be monitored by absorption spectroscopy. Depending on specific aptamer–exosome surface-protein interaction, the aptamer/AuNPs can be detected both visually and quantitatively, in turn, generating patterns that allow identification of multiple proteins on different cancer cell exosomes (Figure 1).

The sensor was designed by the noncovalent conjugation of 13-nm AuNPs with a panel of five aptamers previously demonstrated to target cell-surface proteins with high specificity and affinity (Figure 1).^[11] The aptamers can bind exosome surface proteins and induce the aggregation of

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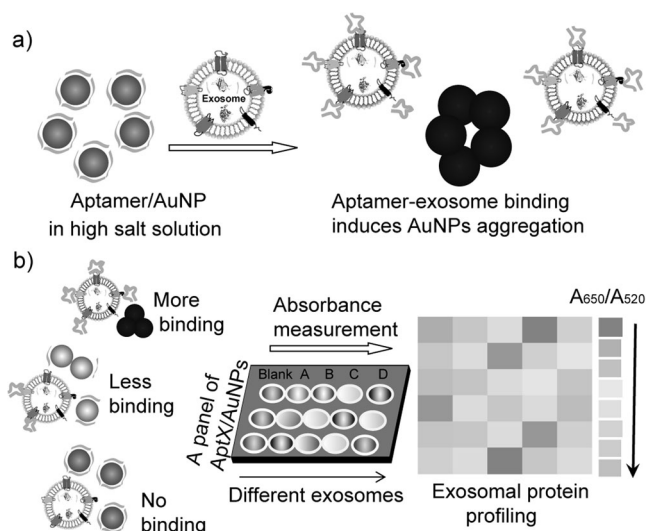


Figure 1. Working principle of the aptamer/AuNP complex for molecular profiling of exosomal proteins. a) Schematic of the displacement of aptamers from gold nanoparticles by binding with exosome surface protein and the concomitant aggregation of gold nanoparticles. b) Profiling of different exosome surface proteins with the aptamer/AuNP complex.

AuNPs for exosomal protein profiling. It is well known that AuNPs show a red-to-blue color change in response to a dispersion-to-aggregation state change,^[12] while single-stranded DNA (ssDNA) can prevent AuNPs from salt-induced aggregation by noncovalent AuNP–DNA complexation.^[13] Controllable ssDNA (aptamer)/AuNP interaction has previously been explored for protein detection^[14] and cancer cell discrimination.^[15] In this study, the competitive binding between aptamers and exosomes displaces aptamers from AuNPs, resulting in nanoparticle aggregation. The red-to-blue color change of AuNPs indicates the corresponding binding events between aptamers and exosomal proteins, while the intensity of AuNPs aggregation (A_{650}/A_{520}) reflects the relative abundance of target proteins present on the exosome surfaces (Figure 1). Therefore, by strategically selecting aptamers that interact with exosomal proteins and comparing the binding of aptamers with AuNPs in the presence of different exosomes, we are able to generate predictive signature-based exosomal protein patterns for potential cancer diagnosis.

We initially demonstrated the ability of the aptamer/AuNP platform to detect well-characterized exosome-enriched proteins. As a proof-of-concept, CD63, which is ubiquitously present on most cellular exosomes,^[16] was selected as our model target. To accomplish this, 13-nm citrate-coated AuNPs were first synthesized and characterized by transmission electron microscopy (TEM; Supporting Information, Figure S1). An aptamer targeting CD63 (hereinafter termed as Apt_{CD63}) was modified with hexaethylene glycol (Supporting Information, Table S1) to increase the biocompatibility of the aptamer and to avoid its potential nonspecific binding with biomacromolecules at high concentrations. Exosomes derived from human cervical carcinoma (HeLa) cells were isolated by ultracentrifugation according to

previous reports and further characterized by TEM (Supporting Information, Figure S2) before use.^[17] TEM analysis of the HeLa exosomes showed an average diameter of 80 nm, which is consistent with previous reports (Supporting Information, Figure S2).

As shown in Figure 2a, Apt_{CD63} (79 nm) or DNA with a random sequence (Library DNA or Lib, 79 nm) protects AuNPs (7.9 nm) from aggregation in the presence of 38 mM NaCl, as evidenced by the minimal color change of AuNPs (the 3rd and 6th tubes, respectively). However, the simultaneous addition of Apt_{CD63} and HeLa exosomes ($7 \mu\text{g mL}^{-1}$) to a AuNP suspension led to the aggregation of AuNPs and the change of color from red to blue in the presence of NaCl (5th tube). The aggregation of AuNPs was also confirmed by UV/Vis spectra of the AuNP solution (left-pointing triangles; Figure 2b), which showed an increased absorption at 650 nm. Meanwhile, the addition of exosomes alone to AuNPs had minimal effect on the dispersal of AuNPs (1st tube). Moreover, the replacement of Apt_{CD63} with Lib DNA did not induce significant aggregation or color change of AuNPs (4th tube). These observations confirmed the presence of CD63 on the HeLa cell exosome surface and revealed that the specific interaction between Apt_{CD63} and exosomal CD63 was sufficient to reduce the absorption of aptamer by AuNPs, resulting

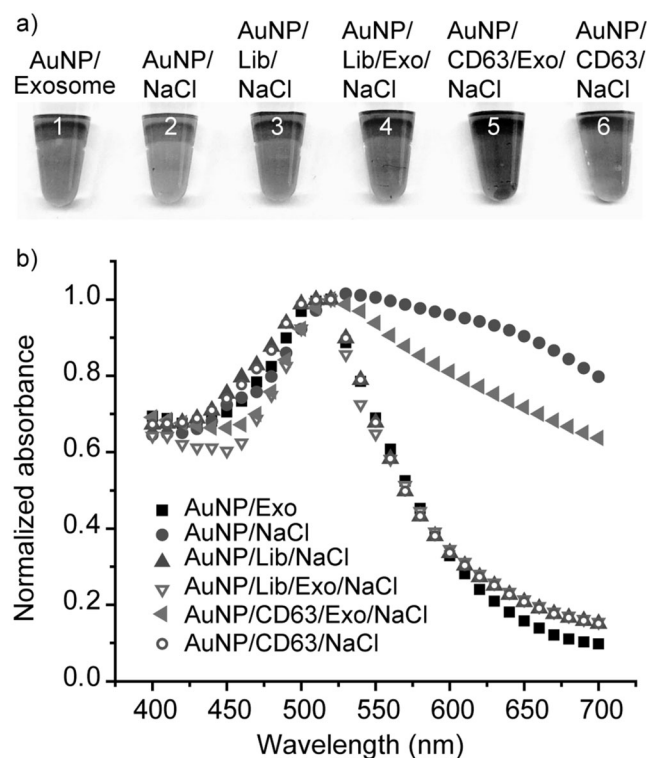


Figure 2. Aptamer-exosome interaction induces the aggregation of gold nanoparticles. a) Direct observation of the color change of AuNPs and b) The absorbance change of AuNPs (7.9 nm) with the addition of HeLa exosome (1st tube, ■), NaCl (2nd tube, ●), Lib/NaCl (3rd tube, ▲), Lib/Exo/NaCl (4th tube, ▽), CD63/Exo/NaCl (5th tube, ◀), and CD63/NaCl (6th tube, ○). For all studies, AuNPs were mixed with Lib, CD63, and Exo for 20 min before recording the absorbance intensity. AuNP absorption at 520 nm was normalized to 1. Exo = HeLa exosomes, Lib = Library DNA, CD63 = aptamer targeting CD63.

in the aggregation of AuNPs for exosome surface-protein detection.

Having demonstrated the ability of the Apt_{CD63}/AuNP conjugate to detect exosomal CD63 by a colorimetric assay, we next studied whether the Apt_{CD63}/AuNP conjugate could distinguish the subtle changes of CD63 level on different cancer cell exosomes. As shown in previous studies,^[16] CD63 is present on different exosomes. These exosomes may show binding toward the same aptamer but with different affinities, depending on their level of CD63 production. A reliable method able to analyze various cancer exosomes can lead to the development of an exosomal protein profiling and thus a better understanding of the exosomes' origin. To this end, three additional exosomes secreted from PC-3 (human prostate cancer), Ramos (human acute lymphoblastic leukemia), and CEM (human acute lymphoblastic leukemia) cells were collected and characterized (Supporting Information, Figure S2), and the presence of CD63 on these exosomes was verified and compared using Apt_{CD63}/AuNP. As shown in Figure 3 a–d, the addition of HeLa and PC-3 exosomes to the

Apt_{CD63}/AuNP suspension resulted in a similar trend of AuNP aggregation at all concentration ranges tested (ranging from 0 to 12.8 $\mu\text{g mL}^{-1}$). CEM and Ramos exosomes, however, induced less aggregation of AuNPs. In particular, Ramos exosomes displayed the weakest ability to induce AuNPs aggregation. These observations suggest the most abundant presence of CD63 on HeLa and PC-3 exosomes, with a medium level of CD63 on CEM exosomes, and the smallest level of CD63 on Ramos exosomes. As exosomes usually carry the molecular information of their parent cells, the observed exosomal CD63 level may reflect the different protein pattern of their parent cells, allowing non-invasive classification and identification of cancer cells at the molecular level.

Having compared the presence of the aforementioned ubiquitous exosomal marker in exosomes, we next explored whether the aptamer/AuNP conjugate could identify exosomal proteins that are restricted to a certain cell line. For example, protein tyrosine kinase-7 (PTK7) is overproduced in CEM cells but not Ramos cells, while its association with their respective exosomes remains unclear.^[18] To examine whether PTK7 is specifically enriched on CEM cell exosomes, we first incubated Apt_{PTK7} with AuNPs, followed by separately adding CEM and Ramos exosomes in the presence of NaCl (38 mM). As shown in Figure S3 (Supporting Information), the addition of CEM exosomes but not Ramos exosomes resulted in an obvious aggregation of AuNPs. With increasing concentration of CEM exosomes, the absorbance ratio (A_{650}/A_{520}) of AuNP (Figure 3 e) showed a linear increase. In contrast, no significant change of A_{650}/A_{520} was observed for AuNP/Apt_{PTK7} complex added with Ramos exosomes (Figure 3 f), suggesting PTK7 was present on CEM exosomes but negligibly present on Ramos exosomes. These findings are consistent with the PTK7 production profile in parent cells,^[11,19] highlighting the substantial ability of the Aptamer/AuNP conjugate to profile specific exosome surface proteins and relate them to the parent tumors.

Since a single type of the aptamer/AuNP complex can differentiate the subtle variation of a specific exosomal protein, we envisioned that using a panel of aptamer/protein interactions would allow us to create a pattern of exosomal proteins, serving as a comprehensive reference for predicting exosome origin and increasing the accuracy of using exosomes for cancer diagnosis. To test this hypothesis, we studied and compared the interaction of AuNPs with Apt_{CD63}, Apt_{EpCAM}, Apt_{PDGF}, Apt_{PSMA}, and Apt_{PTK7} in the presence of different exosomes. A Lib DNA with a random sequence was used to replace the aptamer and served as a negative control. This set of aptamers was selected to target ubiquitous markers present on exosomes (for example, CD63) and markers either absent or present on exosomes of specific cancer cell types (for example, prostate-specific membrane antigen or PSMA, PTK7).^[20] We also selected aptamer target proteins that had not been fully verified on cancer exosomes, for example, platelet-derived growth factor (PDGF) and epithelial cell-adhesion molecule (EpCAM).^[21] In this study, four different cancer exosomes with the same concentration (6.4 $\mu\text{g mL}^{-1}$) were added into each aptamer/AuNP suspension (7.9 nm/79 nm). The change of AuNP absorption (Figure 4 a–d) and

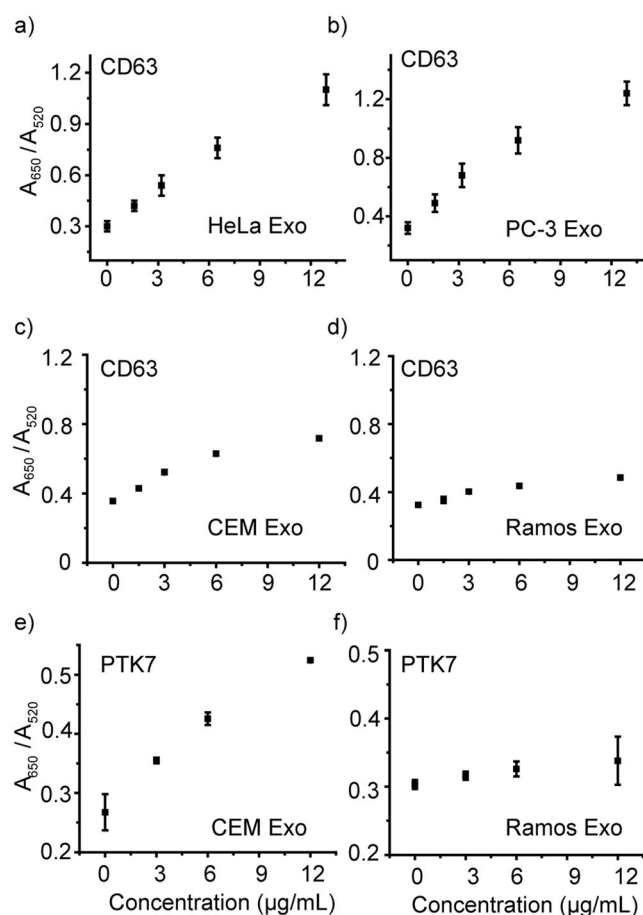


Figure 3. Aptamer/AuNP sensor verifies the ubiquitous exosomal presence of CD63 protein and confirms the specific exosomal production of PTK7 on CEM exosomes but not Ramos exosomes. Analyses of CD63 presence on a) HeLa exosomes, b) PC-3 exosomes, c) CEM exosomes, and d) Ramos exosomes using AuNP/Apt_{CD63}. Analyses of PTK7 presence on e) CEM exosomes, and f) Ramos exosomes using AuNP/Apt_{PTK7}. All measurements were performed in triplicate, and the data are displayed as mean values.

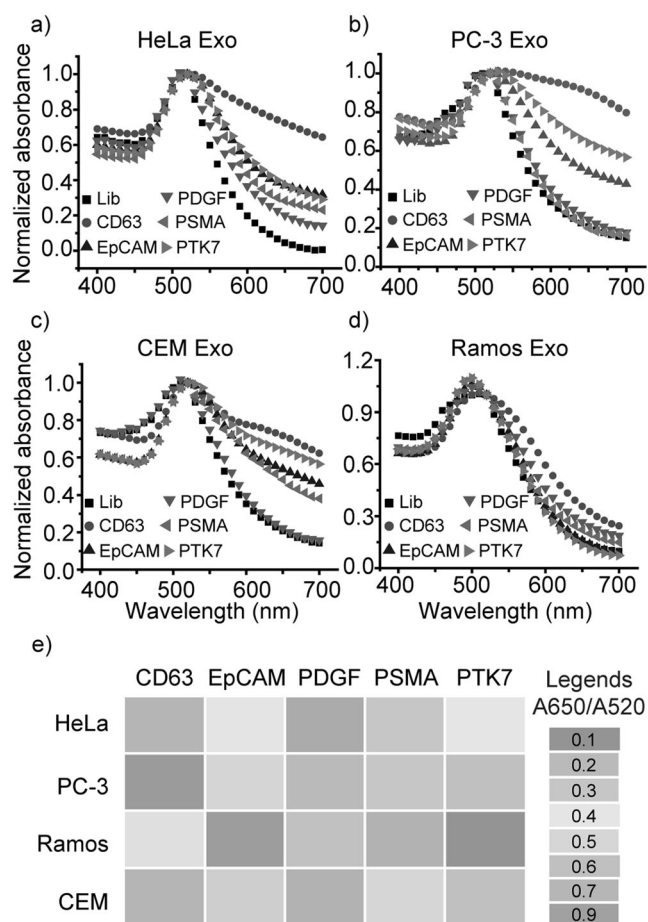


Figure 4. Molecular profiling of exosomal proteins using aptamer/AuNP sensor. Levels of five cancer protein markers, including CD63, EpCAM, PDGF, PSMA, and PTK7 were profiled on a) HeLa, b) PC-3, c) CEM, and d) Ramos exosomes. AuNPs complexed with Lib DNA (black squares) were used as control. Gold nanoparticle absorption at 520 nm was normalized to 1. e) Levels of five protein markers were determined using A_{650}/A_{520} and the data are represented as a heat map highlighting the profiling and comparison of exosomal proteins on each exosome.

the ratio of A_{650}/A_{520} for each aptamer/exosome combination were summarized and presented as a heat map, as shown in Figure 4e. It is obvious that CD63 is present at high levels on HeLa, PC-3, and CEM exosomes, as indicated by the high intensity of Apt_{CD63}/AuNP aggregation in the presence of these exosomes.

Substitution of Apt_{CD63} with the panel of aptamers resulted in noticeable AuNP aggregation patterns for exosome protein profiling. For example, Apt_{PSMA}/AuNP showed negligible response after the addition of PC-3 exosomes, indicating the low presence and, hence, low production of PSMA in PC-3 cells.^[22] In addition, most cancer exosomes showed moderate EpCAM level with Ramos cells as an exception. Furthermore, CEM exosomes showed a much higher level of PTK7 than that of Ramos exosomes, which is consistent with the above finding (Figure 3e,f). Surprisingly, none of the four cancer exosomes showed sufficient binding with Apt_{PDGF} indicating the absence or very low presence, of PDGF on cancer cell exosome surfaces. The as-generated

distinct pattern of responses from a set of Apt_x/AuNPs in the presence of various cancer cell exosomes enables the profiling and comparison of exosomal proteins associated with a particular cancer type and its parent tumor.

Compared to traditional screening methods that use genomic,^[23] transcriptional,^[24] and proteomic signatures^[25] to characterize exosomal proteins and require specialized equipment and extensive sample purification, the Apt_x/AuNPs colorimetric sensor platform, as herein demonstrated, can differentiate various exosomal proteins in minutes. In particular, the utilization of the array of Apt_x/AuNPs allows detection visually as well as quantitatively (by A_{650}/A_{520} ratio) for enhanced accuracy. Moreover, these information-rich results allow the generation of a distinct pattern for multiple types of cancer exosomes from a single measurement, making it widely and economically accessible for clinical use. The simplicity and effectiveness of the system underscores its potential to accelerate cancer biomarker discovery and ultimately to allow non-invasive early diagnosis of cancers.

In summary, we have demonstrated a colorimetric tool to capture and profile exosomal protein information in a simple manner. This sensor takes advantage of the dual nature of aptamers as recognition moieties and protectors of AuNPs from aggregation. We showed that Apt_x/AuNPs could differentiate and profile subtle exosome surface protein differences in minutes, making this strategy applicable to massive high-throughput screening, especially in the analysis of clinical specimens for point-of-care detection. In addition, with the ability to precisely identify the exosome protein pattern, the Apt_x/AuNPs platform opens the door to a better understanding of cancer development, offering the possibility for early detection of cancer and help in designing potential curative options.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: aptamers · exosome biomarkers · gold nanoparticles · molecular recognition · protein profiling

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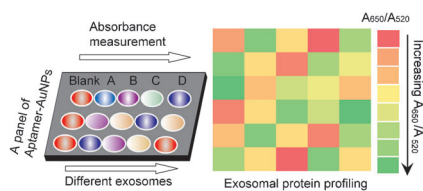
Communications



Analytical Methods

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L. Zhang, W. Tan* ———— ■■■■-■■■■

Aptamer/AuNP Biosensor for
Colorimetric Profiling of Exosomal
Proteins



A coat of many colors: A gold nano-particle/aptamer biosensor (AuNP/Apt_x) provides a colorimetric platform for rapid and multiplexed protein analysis of exosomes.