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Mini review

Recent Progress in Electrochemical Detection of Tumor-Derived Exosomes

Xiaohua Ma

Henan Key Laboratory of Biomolecular Recognition and Sensing, College of Chemistry and Chemical Engineering, Shangqiu Normal University, Shangqiu, Henan 476000, People's Republic of China
E-mail: maxhsqnu@aliyun.com

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Tumor-derived exosomes are tiny vesicles released from tumor cells to the extracellular environment. The sensitive and selective detection of tumor-derived exosomes is helpful for early diagnosis, clinical monitoring, curative effect evaluation and prognosis judgment of some tumors. In this paper, the detection of tumor-derived exosomes by electrochemical techniques are discussed, mainly including the direct, sandwich-like and magnetoelectric detection.

Keywords: exosomes; electrochemical biosensors; tumor cells; early diagnosis

1. INTRODUCTION

Exosomes are extracellular cystic bubbles with a diameter of 30 ~ 150 nm. They are cup-shaped membrane-wrapped phospholipid nanovesicles. All eukaremia cells can secrete exsomes through the internal lysosome pathway, including a variety of tumor cells and normal cells. Exosomes from different tissue sources have different physiological functions, composition, physical and characteristics. They are composed of phospholipid bilayers and stable in body fluids, including blood, urine, saliva and so on [1,2]. Several studies have confirmed that exosomes play important roles in tumor development, invasion, metastasis and immune escape [3]. Thus, tumor-derived exosomes have been considered as the biomarkers and targets for tumor diagnosis and treatment [4,5]. However, the small size and low level of exosomes in biological fluids bring great challenges to their separation and detection. Biomacromolecules and proteins in biological fluids can also interfere with the determination of exosomes. Therefore, it is necessary to separate and remove cell debris, DNA and other types of vesicles to obtain pure exosomes. There are four traditional isolation techniques for exosomes: ultracentrifugation, size-based isolation techniques, chromatography, and polymer coprecipitation [6]. Many kinds of characteristic proteins were expressed on the surface of tumor

exosomes. At present, antibody- or aptamer-based technologies corresponding to target proteins are often used in detection, such as colorimetry, fluorescence, surface enhanced Raman, surface plasmon resonance and electrochemistry [7-9]. Among these methods, electrochemical biosensors have been developed because of their simplicity, rapid response, low cost, and high sensitivity [10-12]. Based on the current status and progress of exosomes as tumor biomarkers and therapeutic targets, this paper summarized the electrochemical methods for exosome detection, which were classified as direct detection, sandwich-like detection, magnetoelectric detection with magnetic separation and so on. Their analytical performances are shown in Table 1.

2. DIRECT DETECTION

Direct detection is usually achieved by changing the electrical conductivity of the electrode with or without targets to cause electrical signal change [13]. Eelectrochemical assays of exsomes have been developed to improve the sensitivity of traditional ways in the format of direct detection. The methods of such detection by limiting the electron transfer can be classified according to the types of receptors modified on electrode surface. The two commonly used receptors for direct detection of exosomes include antibodies and aptamers since a large number of membrane proteins are overexpressed on the surface of exosomes. For example, Kilic's group reported an unlabeled electrochemical biosensor to monitor the release of MCF-7 cell exocystic in the breast cancer cell line [14]. The biosensor is based on the signal change caused by the recognization reaction of anti-CD81 to CD-81 on the lipid membranes of small extracellular vesicles (EVs) from breast cancer. This biosensor provided a limit of detection (LOD) of 77 vehicles/mL. Compared to other electrochemical biosensors, the biosensor has a lower LOD and can be used not only to detect EVs in blood samples, but also to integrate with a platform that simulates tumor microenvironments for chemotherapy drug testing.

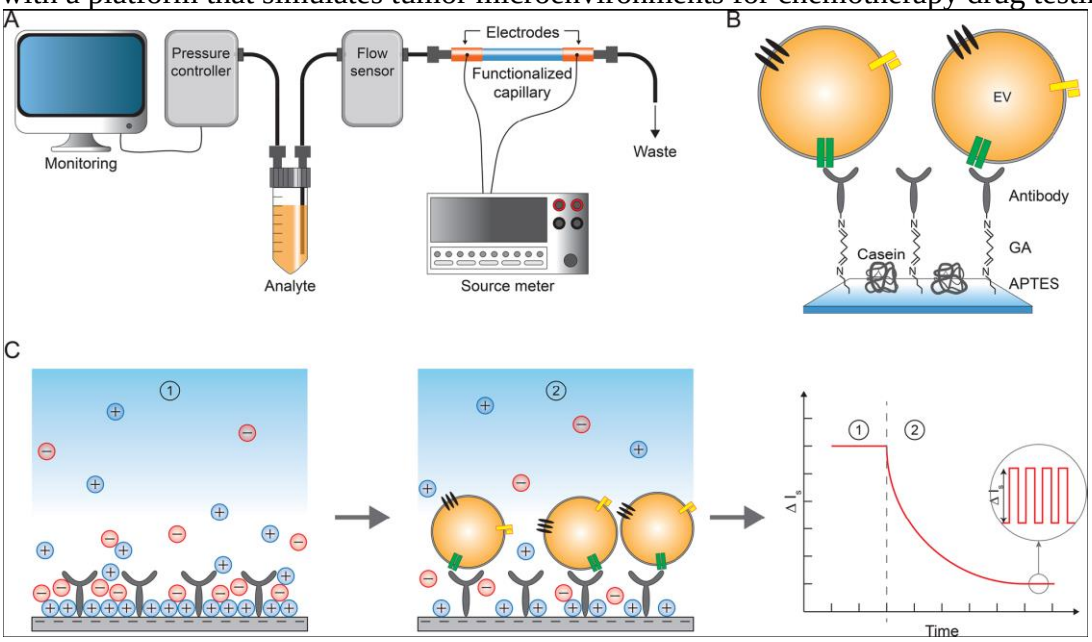

Figure 1. Electrokinetic platform and detection principle. Reprinted with permission from reference [15]. Copyright 2019 American Chemical Society.

CD63 antibody can capture exosomes for early diagnosis of cancers. Thus, exosomes can be distinguished from other vehicles by antibody-antigen interaction. Cavallaro's group first introduced the detection of sEVs based on a representative set of surface markers, including tumor-related protein epidermal growth factor receptor (EGFR) and exosome tetrapolymer family proteins of CD9 and CD63 (Figure 1) [15]. In their work, the sEVs samples were taken from non-small-cell lung cancer (NSCLC) H1975 cell lines. Good selectivity was achieved by fixing specific target affinity reagents. sEV samples containing different levels of EGFR and CD63 were analyzed, in which siRNA reduced the expression of EGFR in NSCLC H1975 cells and stabilized transfection of CD63eGFP fusion protein overexpressed in HEK 293T cells.

In contrast to antibodies, aptamers have the advantages of good chemical stability, easy preparation and modification, and low cost [16]. Recently, electrochemical aptasensor has attracted more and more attention in the field of cancerous exosome research. This biosensor has the advantages of sensitive recognition, fast response, convenient carrying, less sample volume and so on. Based on the advantages of DNA nanostructures and portable electrochemical devices, Wang et al. constructed a nanotetrahedron(NTH)-assisted aptasensor to directly capture and detect exosomes from hepatoma cells (Figure 2) [17]. The electrode was first modified with tetrahedral DNA for the specific recognition of exosomes. The tetrahedral DNA contains the aptamer DNA sequence. Compared to the single modification of LZH8 aptamer, the NTH supportor significantly improved the sensitivity for HepG2 exosomes. The LOD was found to be 2.09×10^4 particles/mL. A wide linear range from 10^5 to 10^{12} particles/mL was achieved.

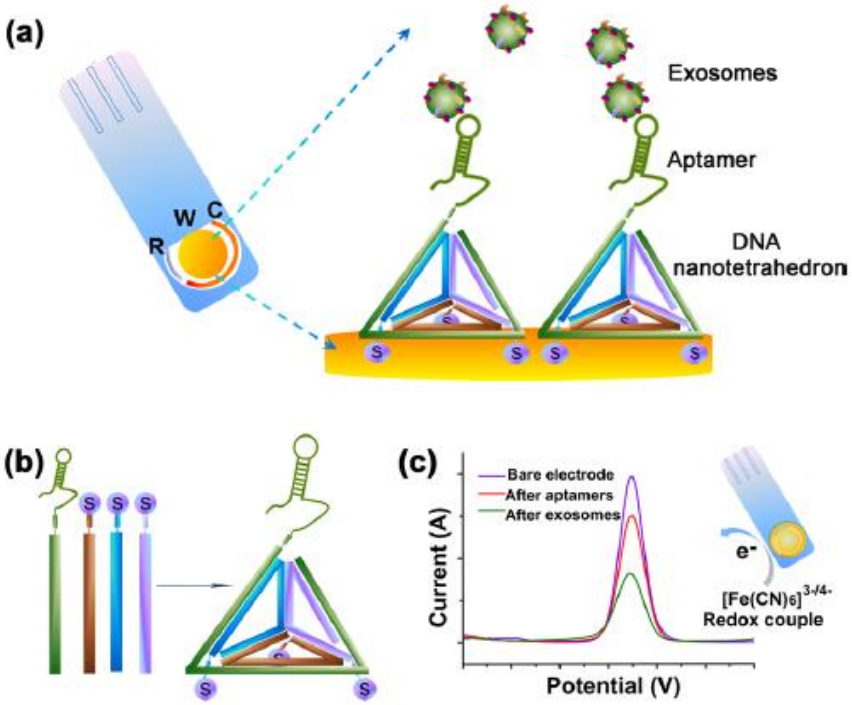

Figure 2. Schematic illustration of the NTH-assisted electrochemical aptasensor. Reprinted with permission from reference [17]. Copyright 2017 American Chemical Society.

Moreover, Yu's group explored the direct and label-free quantification of exosomes by CD63-functionalized reduced graphene oxide (rGO) FET biosensor (Figure 3) [18]. The FET device was

designed by standard semiconductor technology. CD63 antibody was immobilized on the surface of GO-casted sensor channel. Exosome was then detected by the CD63-functionalized biosensor. Li et al. developed an electrochemical impedance spectroscopy to quantify both external (tetraspanin) and internal (syntenin) exosome-specific markers (Figure 4) [19]. In their work, the detection limit for exosome are 1.9×10^5 particles/mL (equivalent to 320 aM or 9500 exosomes in 50 μL sampple) for intact exosomes.

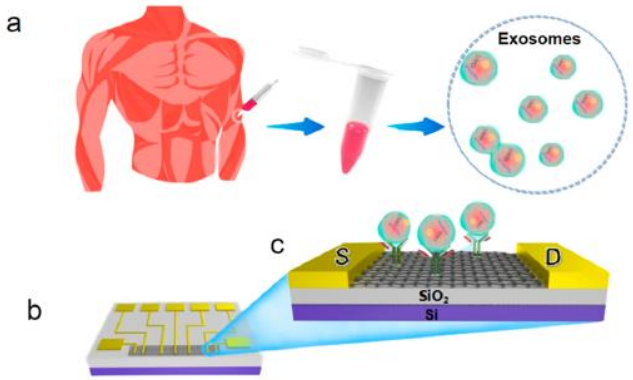

Figure 3. Schematic diagram of a CD63 antibody functionalized rGO FET biosensor for detection of exosomes. Reprinted with permission from reference [18]. Copyright 2019 American Chemical Society.

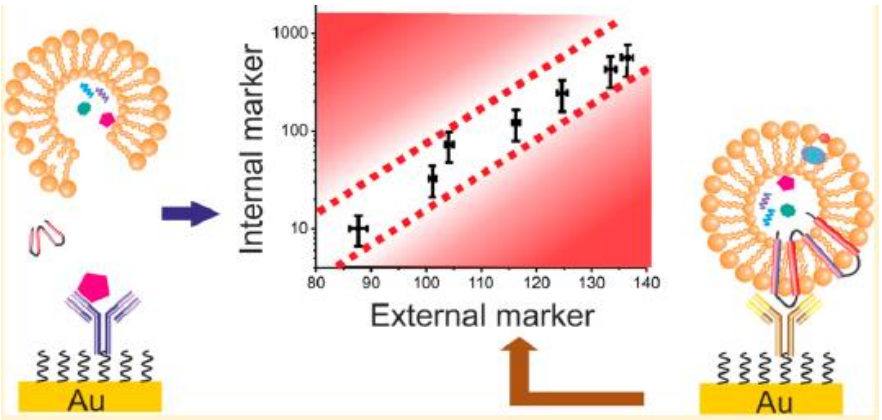

Figure 4. Concentration-normalized electroanalytical assaying of exosomal markers. Reprinted with permission from reference [19]. Copyright 2019 American Chemical Society.

3. SANDWICH-LIKE DETECTION

Sandwich-like detection format is widely used for designing of electrochemical biosensors and amplifying the electrical signal [20,21]. Aptasensors have been widely used in the quantitative determination of various analytes [12,22]. They can be easily integrated into a variety of DNA-based reactions to amplify the signal of biosensors, such as rolling cycle amplification (RCA), polymerase chain reaction (PCR), hybrid chain reaction (HCR) and cascaded hybrid reaction (CHR) [23]. In 2016, Zhou's group developed a biosensor by immobilization of Tetraspanin CD63-specific aptamer on a gold electrode [24]. The redox-labeled complementary oligonucleotide was used as the probe for signal output. The probe competed with exosome for the immobilized aptamer. The exosome-mediated

release of reporters led to the reduction of electrochemical signal. The method achieved a LOD of 1×10^6 particles/μL. After that, an increasing number of reports utilized CD63 aptamer as the recognition unit to capture or detect exosomes based on the interaction between aptamer and CD63 overexpressed on the surface of exosomes.

An's group developed an electrochemical aptasensor for sensitive detection of tumor exosomes based on click chemistry and signal amplification of HCR [25]. CD63-specific aptamers were assembled on the electrode to capture exosomes. 4-Oxo-2-nonenal alkyne (alkynyl-4-ONE) molecules were modified on the surface of exosomes through the reaction between amino and aldehyde. Azide-labeled DNA probes were then linked to the exosomes by copper (I)-catalyzed click chemistry. The signal was amplified by HCR and horseradish peroxidase (HRP). The oxidation of o-phenylene-diamine (OPD) by H_2O_2 was catalyzed by HRP. The signal was recorded by monitoring the electro-reduction of 2,3-diaminophenazine (DAP). The biosensor facilitated the quantification of exosomes in the range of 1.12×10^2 to 1.12×10^8 particles/μL with a LOD of 96 particles/μL. It showed great potential for clinical analysis such as human serums.

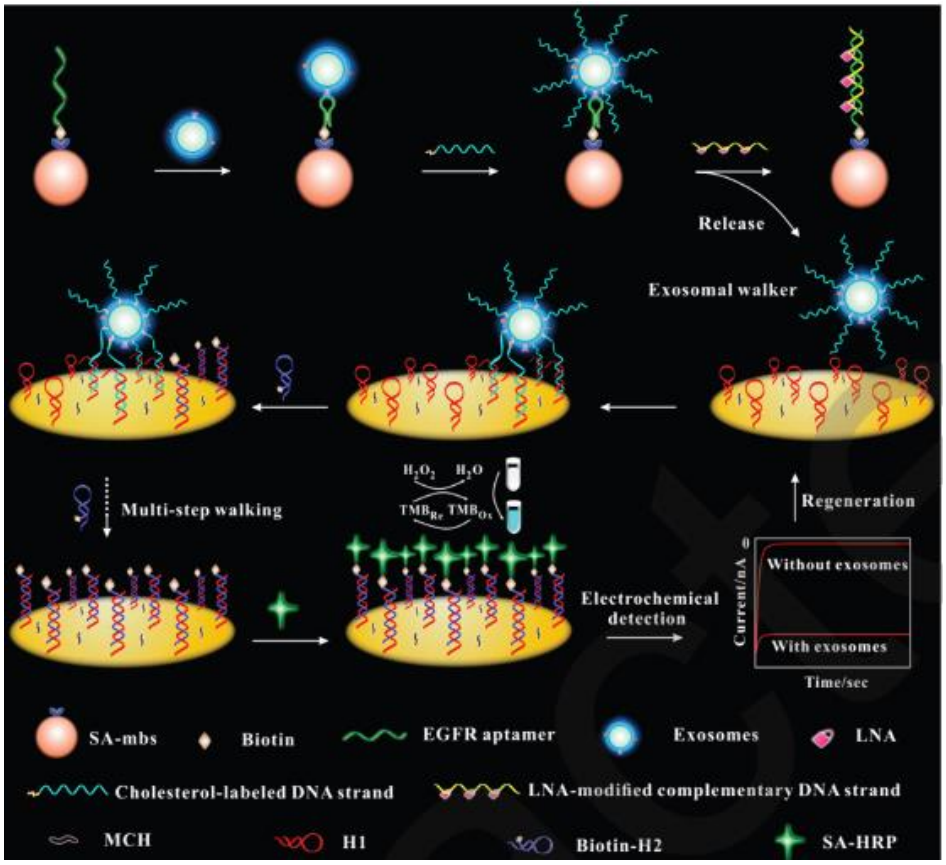

Figure 5. Schematic principle of the electrochemical sensing based on multilegged exosomal walkers for exosomes detection. Reprinted with permission from reference [26]. Copyright 2019 American Chemical Society.

To increase the selectivity and sensitivity, a cascade amplified electrochemical biosensor based on multilegged exosomal walkers was developed for sensitive and selective detection of exosomes by

Wang and co-workers (Figure 5) [26]. Exosomes were captured specifically by its membrane protein aptamer. Multilegged exosomal walkers with a large amount of cholesterol-labeled DNA strands plunged into their lipid bilayers. After being released efficiently by lock nucleic acid (LNA)-modified DNA strands complementary to the aptamer, the walkers were propelled by toehold mediated strand displacement reaction (TMSDR) and "walked" along with DNA "track", introducing considerable signal molecules attached on the "track" and achieving sensitive detection with a LOD of 29 exosomes/μL. Additionally, it is very thrilling to design a novel method for detecting exosomes wtih a rapid, simple and specifical procedure. Yin et al. have proposed an aptamer-trigged label-free homogeneous electrochemical method for highly selective and sensitive detection of cancer-derived exosomes [27].

Doldán et al. reported the detection of exosomes with α-CD9 antibody-modified gold electrode (Figure 6) [28]. When the electrode was incubated with sample, the captured exosomes were captured and then recognized by another α-CD9 antibody. HRP-conjugated anti-IgG was used to recognize the captured α-CD9 antibody for electrocatalytic reduction of oxidized TMB. Because large numbers of α-CD9 proteins are expressed on the surface of each exosome, the signal was greatly amplified. The method achieved a LOD of 200 particles/mL.

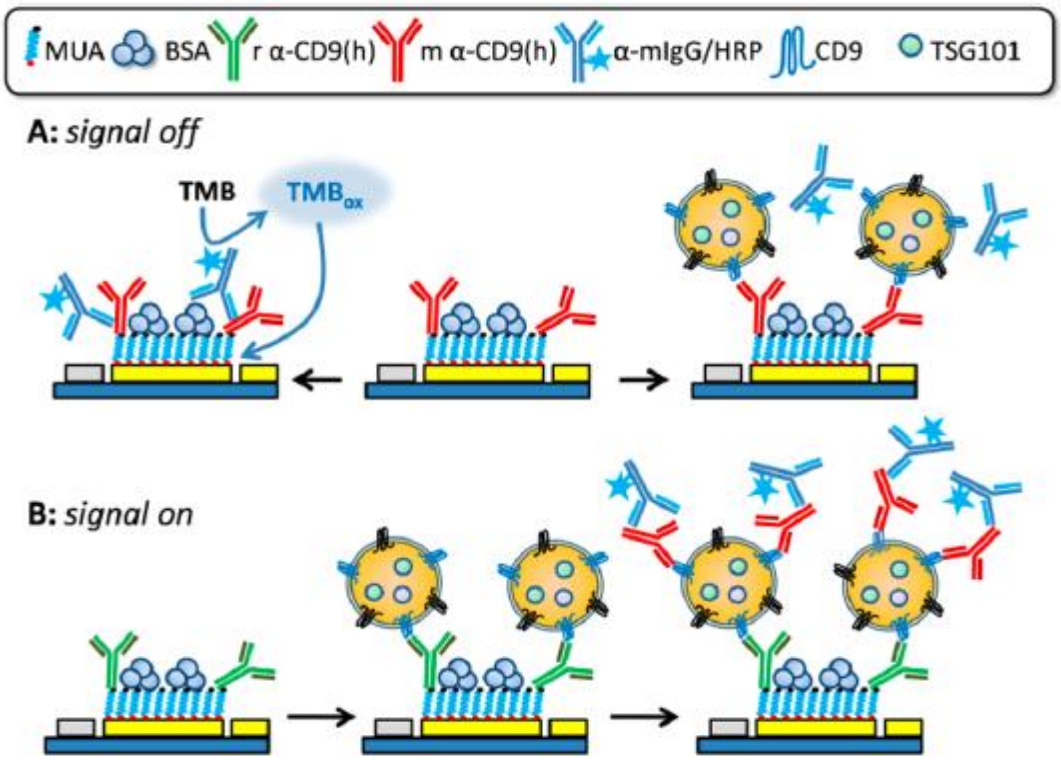

Figure 6. "Signal-Off" and "Signal-On" Strategies. Reprinted with permission from reference [28]. Copyright 2016 American Chemical Society.

In recent years, metal-organic framework (MOFs) have became new electroactive materials because of their flexible porosity, high surface area, chemical ductility and durable sensing characteristics. In particular, Zr-MOF has a high affinity for phosphate group and has been applied to

enrich a phosphate biomolecule by the formation of a Zr-O-P bond. Sun et al. proposed an electrochemical biosensor for the analysis of GBM-derived exosomes without the use of labels and enzymes (Figure 7) [29]. This method was based on the interaction of the Zr-O cluster and the internal phospholipid bilayer of exosome. In this work, a peptide ligand was assembled onto the gold electrode for the specific capture of GBMs exsome by bindding to EGFR and EGFRvIII. Exosome was directly determined by measuring the electrochemical signal inside Zr-MOF without the use of identification and amplification elements. The exosomes at the concentration of 9.5×10^3 to 1.9×10^7 particles/μL have be determined. The LOD was found to be 7.83×10^3 particles/μL.

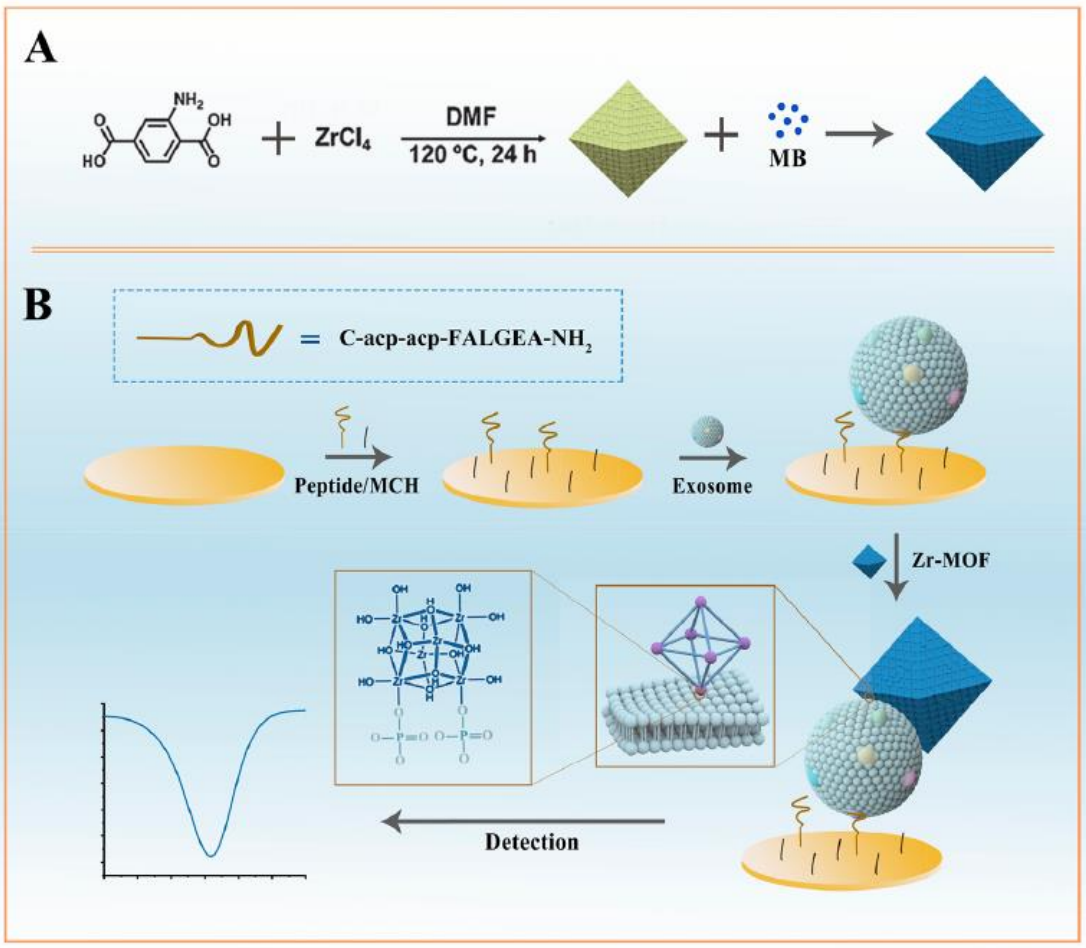

Figure 7. Schematic diagram of (A) the fabrication process of MB@UiO-66-based nanoprobe and (B) the principle of the electrochemical biosensor for the detection of GBM-derived exosomes. Reprinted with permission from reference [29]. Copyright 2020 American Chemical Society.

4. MAGNETOELECTRIC DETECTION

Exosomes from complicated body fluids can be seperated and enriched by a magnet via the interaction between surface markers and transmembrane members of CD63 and CD81 [30]. Magnetoelectric detection has a good application prospect in sensitive, specific, and portable analysis of disease biomarkers, including exosomes. Magnetic beads modified with anti-CD63 antibodies have

been used to extract exosomes for magnetoelectric detection on modification-free electrodes. Once attached onto the electrode, the exosome-binding beads were analyzed by the HRP-conjugated antibodies.

Dong et al. reported the indirect electrochemical detection of exosomes derived from human prostate cancer cells (Figure 8) [31]. This method is mainly divided into two parts. In part A, aptamer and its complementary M1, M2 and M3 DNA were modified on the surface of magnetic beads. Exosome can bind with the aptamer to release M1, M2 and M3, thus transforming the detection of exosome into the determination of DNA. Theoretically, an exosome can release three DNA strands, thus effectively improving the detection sensitivity. In part B, differential pulse voltammetry (DPV) was used to detect M1, M2 and M3 released from the magnetic beads. The signal amplification was realized by Exo III, further improving the detection sensitivity. The DPV peak current increased linearly with the logarithm of the number of exosomes (1000 ~ 120000) with a LOD of 70 particles/μL.

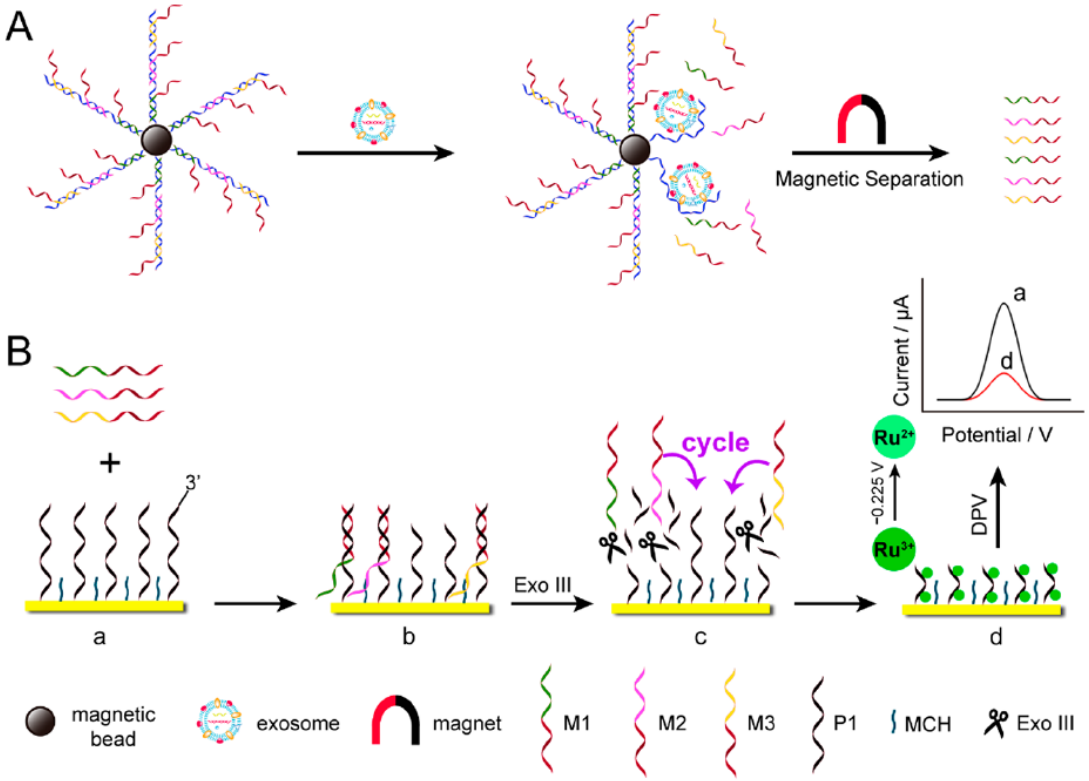

Figure 8. Detection of tumor exosomes. Reprinted with permission from reference [31]. Copyright 2018 American Chemical Society.

Boriachek's group have pulished several works in the electrochemical detection of exosomes [32-34]. They first reported the analysis of clinical exosomes using screen-printed electrodes [32]. The magnetic beads were functionalized with tetraspanin CD63 antibodies. The magnetic beads were dispersed into the extracted samples to capture a large number of exosomes. Next, the specific exosomes of breast and colon cancer were quantified using CdSeQDs biotinylated HER-2 and FAM 134B antibodies, respectively. This method successfully tested exosomes in breast and colon cancer cell line with a LOD of 100 particles/μL. In 2019, they reported the separation and direct visual, colorimetric, and electrochemical detection of exosomes (Figure 9) [34]. The Au-NPFe_2O_3NC was

originally functionalized with CD9 or CD63 antibody and used as the dispersive capture agent for BeWo (choriocarcinoma) cell medium. After magnetic collection and purification, the exosome-bound Au-NPFe_2O_3NC was transferred to the surface of the screen-printed electrode modified with PLAP antibodies. The Au-NPFe_2O_3NC with peroxide-like activity could catalyze the oxidation of TMB in the presence of H_2O_2.

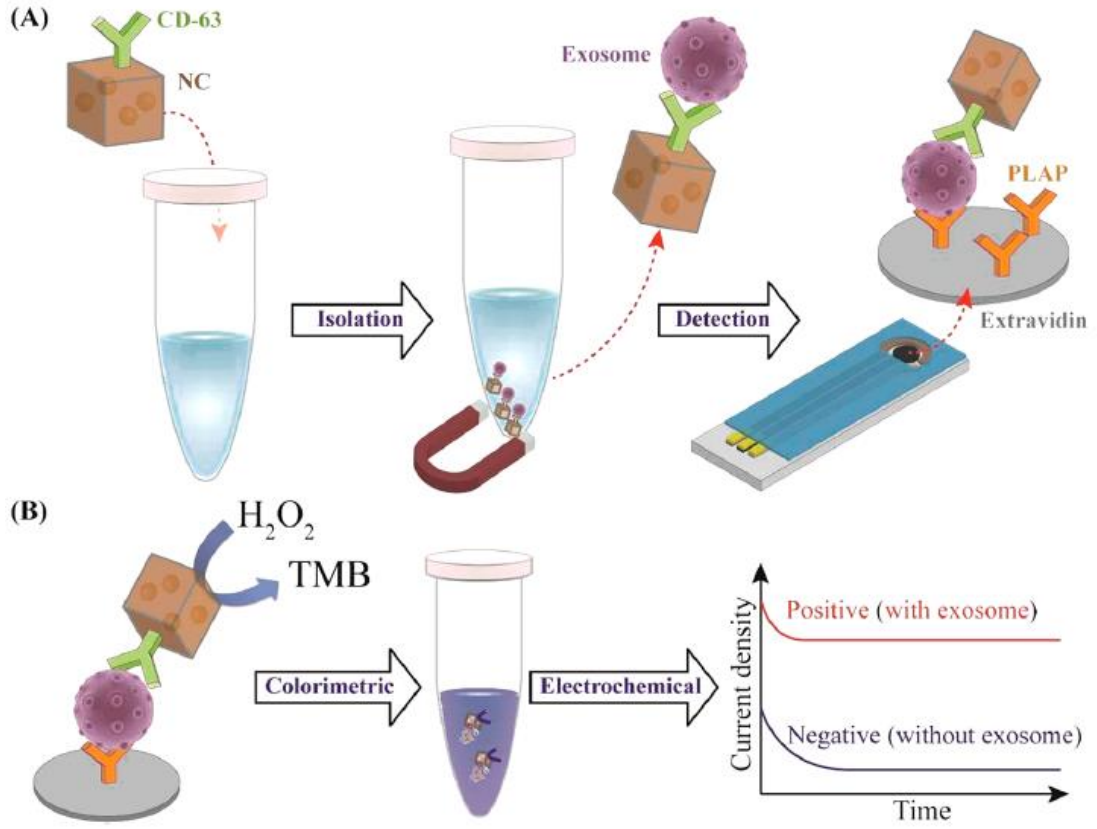

Figure 9. Schematic representation of the assay for direct exosome isolation and detection from cell culture media. Reprinted with permission from reference [34]. Copyright 2019 American Chemical Society.

Cao et al. developed a "principle-of-proof" electrochemical biosensor for signal-amplified detection of exosomes with HepG2-derived exosome as the model [35]. The target exosome was enriched by the anti-CD63 functional magnetic beads, then recognized by the CD63 aptamer. Then, a catalytic molecular machine based on the cascade toehold-mediated strand displacement reaction was initiated. The biosensor showed a linear range from 1×10^5 to 5×10^7 particles/mL and achieved a LOD of 1.72×10^4 particles/mL.

iMEX (integrated magnetic-electrochemical exosomes) is an excellent exosome detection method that combines two orthogonal modes, magnetic selection and electrochemical detection. Jeong et al. reported an iMEX platform which integrated magnetic seperation and electrochemistry (Figure 10) [36]. Firstly, magnetic beads were modified with antibodies that can specifically recognize the proteins on the exosomes, which are used to selectively capture exosomes in body fluids such as serum, plasma and urine. Then, the second antibodies labeled with HRP were added into the signal unit. Finally, the electrochemical signal amplification was realized by the redox of TMB catalyzed by

HRP. The iMEX platform shows the advantage of highly specific interaction between antigen and antibody. The use of magnetic beads greatly simplified the detection procedure. The sample matrix and excessive reagents can be removed by washing, and the captured exosomes are concentrated on the sensor surface. This method was capable of determining exosomes with a LOD of 1×10^5 exsomes.

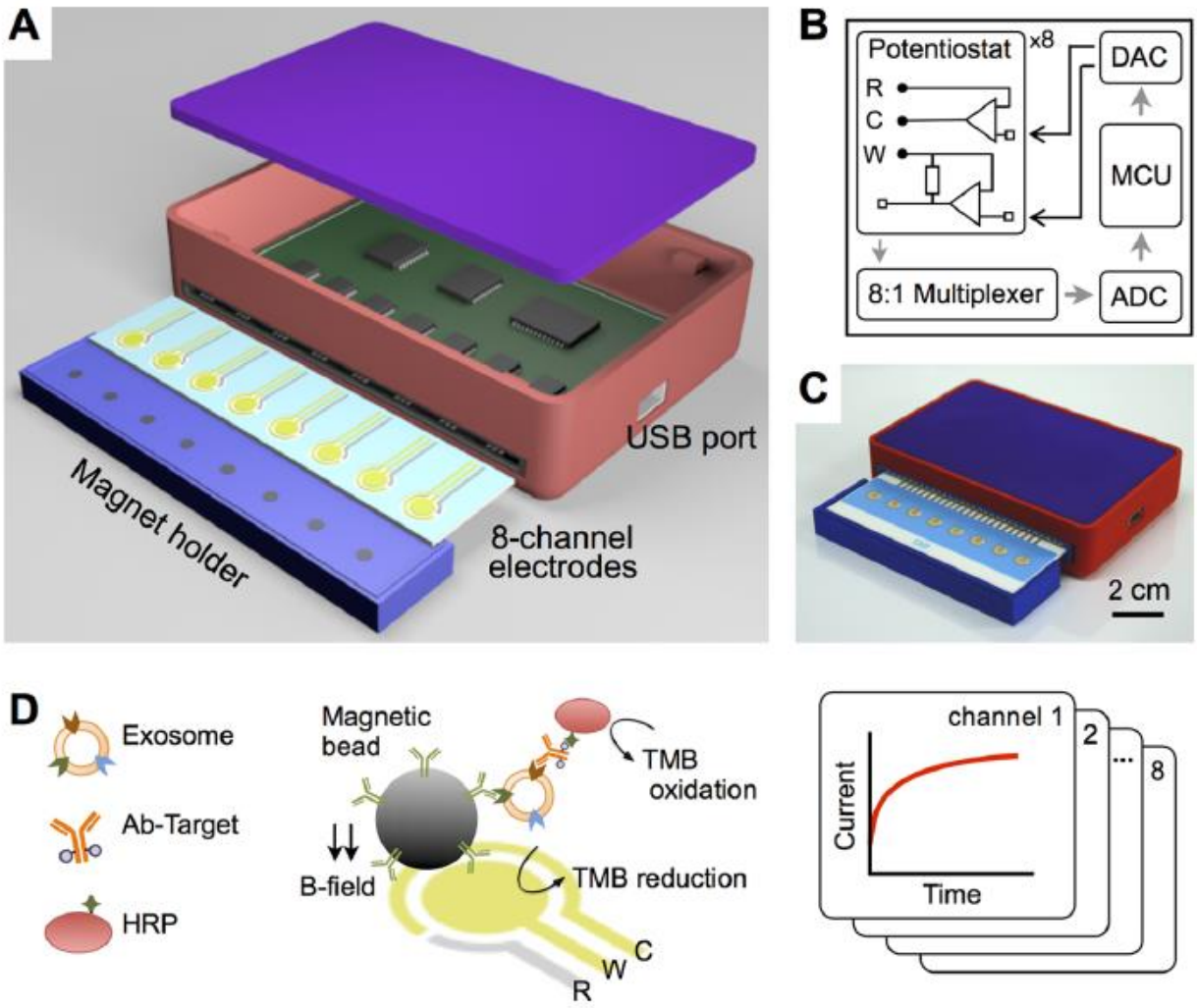

Figure 10. Integrated magnetic-electrochemical exosome (iMEX) platform. Copyright 2019 American Chemical Society [36]. Copyright 2016 American Chemical Society.

The latest development of microfluidic technology provides a huge potential for building integrated platform for analysis of exosome on a microchip. Xu et al. proposed a microfluidic analysis platform with integrated chip separation and novel signal transduction strategy (Figure 11) [37]. After capture by Y-shaped micropillars, a label-free electrochemical aptasensor was designed to determine exosomes. CD63-positive exosomes opened the ssDNA hairpin probes to form the hemin/G-quadruplex complexes, which served as the DNAzyme and NADH oxidase for signal amplification. The method achieved the detection of exosomes down to 4.39×10^3 particles/mL. The sensing platform responses within 3.5 h and the small-volume of sample was 30 μL. A satisfactory result was obtained for clinical analysis of serums from liver cancer patients and healthy controls. The positive

correlation between exosome and tumorigenesis indicated that the microarray has potential application value in clinical application and tumor diagnosis.

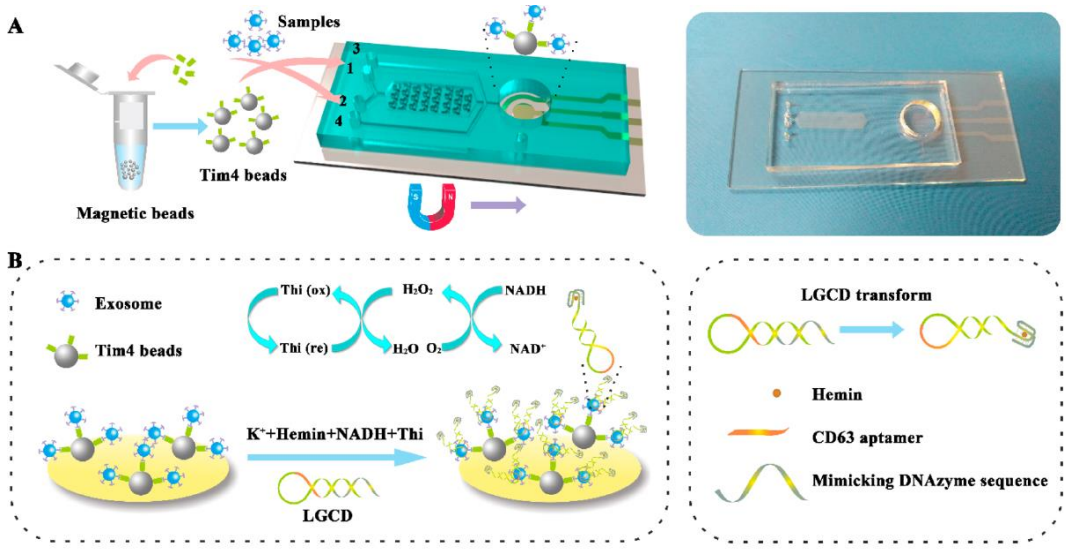

Figure 11. Integrated exosomes isolation and analysis platform: (A) schematic diagram of the exoPCD-chip and (B) schematic of the electrochemical sensor on the surface of ITO electrode. Copyright 2019 American Chemical Society [37]. Copyright 2018 American Chemical Society.

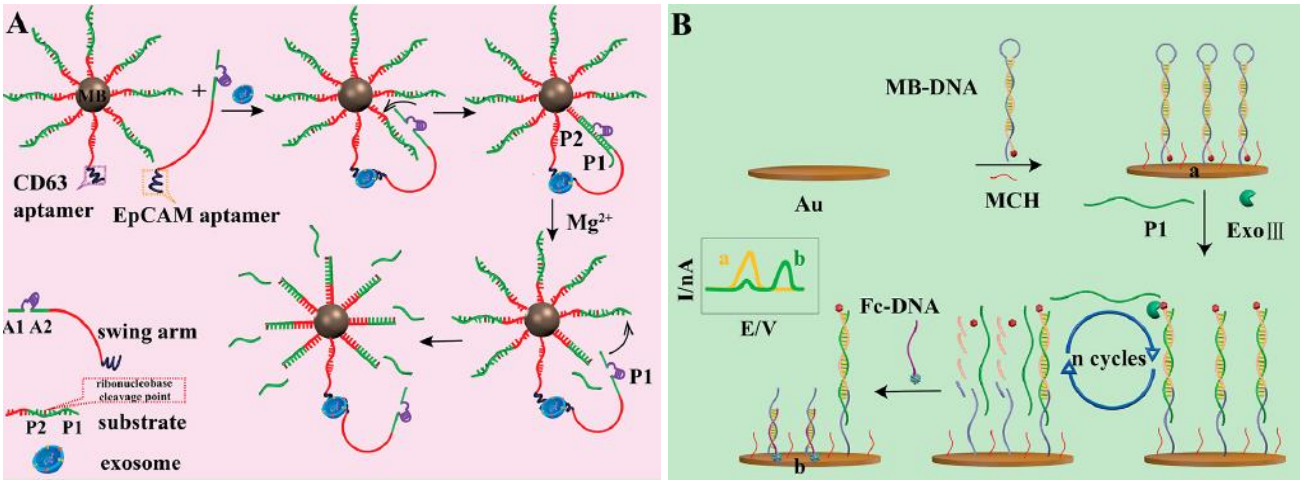

Figure 12. Schematic illustration for the detection of exosomes through (A) 3D DNA walker amplification and (B) Exo III-assisted electrochemical ratiometric assay. Copyright 2019 American Chemical Society [39]. Copyright 2019 American Chemical Society.

Ratiometric electrochemical biosensors are a new type of sensing techniques with strong anti-interfence ability, high reliability, and low LOD as well as background [38]. The aptamers of CD63 and EpCAM were used as the probes to capture and identify exosomes secreted by MCF-7 cells by Zhao and co-workers (Figure 12) [39]. High-density of oligonucleotides were assembled on magnetic

beads as three-dimensional DNA walkers for signal amplification. Exosomes secreted by MCF-7 cells were detected by CD63 and EpCAM ligands. The movement of three-dimensional DNA walkers magnified the recognition process. A LOD of 1.3×10^4 particles/mL was achieved with good selectivity.

Table 1 Analytical performances of electrochemical methods for detection of tumor-derived exosomes.

Detection Model	Receptors	Signal Label	Linear range (particles/mL)	Detection limit (particles/mL)	Ref.
Direct detection	Antibody	–	$(0.78\sim3.50)\times10^9$	2.8×10^8	[15]
	Aptamer	–	$10^5\sim10^{12}$	2.09×10^4	[17]
	Anti-CD63	–	$3.3\times10^4\sim3.3\times10^9$	3.3×10^4	[18]
	Antibody	–	$10^5\sim10^5$	1.9×10^5	[19]
	Anti-CD81	–	$10^2\sim10^9$	77	[14]
Sandwich-like detection	CD63 aptamer	HRP	$1.12\times10^5\sim1.12\times10^{11}$	9.6×10^4	[25]
	EGFR aptamer	HRP	$2\times10^4\sim2\times10^9$	2.9×10^4	[26]
	Anti-CD9	HRP	$2\times10^5\sim2\times10^9$	2×10^5	[28]
	Peptide	MB@UiO-66	7.83×10^6	$9.5\times10^6\sim1.9\times10^{10}$	[29]
	Antibody	antibody	$4.7\times10^8\sim3\times10^{12}$	4.7×10^8	[33]
Magnetoelectric detection	Anti-CD63	Au-NPFe_2O_3 NC	$10^3\sim10^7$	10^3	[34]
	Antibody	HRP	$3\times10^4\sim3\times10^8$	3×10^4	[36]
	Tim4	DNAzyme	$7.61\times10^4\sim7.61\times10^8$	4.39×10^3	[37]
	CD63 aptamer	redox probe	$10^5\sim10^{10}$	1.3×10^4	[39]

5. CONCLUSION

Many existing methods cannot specifically quantify tumor-derived exosomes from a large number of exosomes. Therefore, it is of great significance to develop a simple, sensitive and rapid method to distinguish tumor derived exosomes from total exosomes. Although some new electrochemical techniques have been proposed with high convenience and sensitivity, there are still some limitations. For example, the use of antibodies to identify common transmembrane proteins is not economical and lack of specificity. It is believed that the clinical application of bioassays of exosomes will be further developed with the development of detection and seperation techniques.

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