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Optical, electrochemical and electrical (nano)biosensors for detection of exosomes: a comprehensive overview

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Abstract

Exosomes are small vesicles involved in many physiological activities of cells in the human body. Exosomes from cancer cells have great potential to be applied in clinical diagnosis, early cancer detection and target identification for molecular therapy. While this field is gaining increasing interests from both academia and industry, barriers such as supersensitive detection techniques and high efficiency isolation methods remain. In the clinical settings, there is an urgent need for rapid analysis, reliable detection and point-of-care testing. With these challenges to be addressed, this article aims to review recent developments and technical breakthrough in the field of optical, electrochemical and electrical biosensors for exosomes detection in the field of cancer and other diseases and demonstrated how nanobiosensors could enhance the performance of conventional sensors. Working strategies, limit of detection, advantages and shortcomings of the studies are summarized. New trends, challenges and future perspectives of exosome-driven point-of-care testing (POCT) in liquid biopsy have been discussed.

Keywords: Exosomes, cancer biomarker, optical, electrochemical, electrical, biosensors, liquid biopsy.

1- Introduction

Exosomes, as extracellular vesicles (EVs) are of the most incredible discoveries of the past decades in both biology and medicine. So far, a large amount of literature has demonstrated that cells can communicate with adjacent cells and neighboring cells with the help of the secreted EVs (1). Exosomes can be secreted by all prokaryotic and eukaryotic organisms (2). EVs can generally be subdivided into smaller EVs (exosomes) derived from membrane invagination and microvesicles produced by exocytosis (3). Both have a very rich range of contents, including many types of proteins and nucleus acids, which can be used as specific biomarkers in medical diagnostics. Van Niel reported that exosomes play essential roles in antitumoral immune responses as a novel vehicle, while microvesicles were initially seen as useless cell fragments, called 'platelet dust' until recently have they been found to be involved in cell to cell communication (4). Nevertheless, currently the main classification method to differentiate various types of EVs is based on their origins. Exosomes are formed within the endosomal network and are released into the extracellular matrix upon fusion of multi-vesicular bodies (MVBs) with the plasma membrane **(Figure 1)**.

Based on their secretion and working mechanism, exosomes can play an important role in medicine. They can be used to differentiate stem cells into specific cell types and vice versa. They can be also evacuated to be used as carriers for drug and gene therapy (5-7). In diagnostics, they have been largely used as biomarkers or a package of biomarkers for detection of diseases (8-10). In this review, recent advances in exosome isolation and detection methods are discussed. Detection approaches focusing on optical, electrochemical and electrical are explained with each modality explained in details. Such advances form the basis for a next generation point-of-care testing of cancer and other diseases.

2- Exosome biology, role and function

The term exosomes were first proposed in 1983 when they were discovered in the sheep reticulocyte. It now refers to the disc-like vesicles with a diameter of 40-150 nm (11, 12). It has been demonstrated that a variety of cells can secrete exosomes under normal and pathological conditions for a long time (13). Exosomes are involved in many physiological activities of cells in the human body. Lowry and his colleagues proposed that these small vesicles greatly contribute to cell invasion, metastasis, and apoptosis and even affect drug resistance and immune system (14). Moreover, exosomes have a lipid bimolecular structure, which is rich in cholesterol and sphingomyelin. There are other proteins on exosomal surface including four main transmembrane proteins (CD63, CD81, CD9, CD82) enriched on their membrane and are considered as ideal markers for their characterization (15).

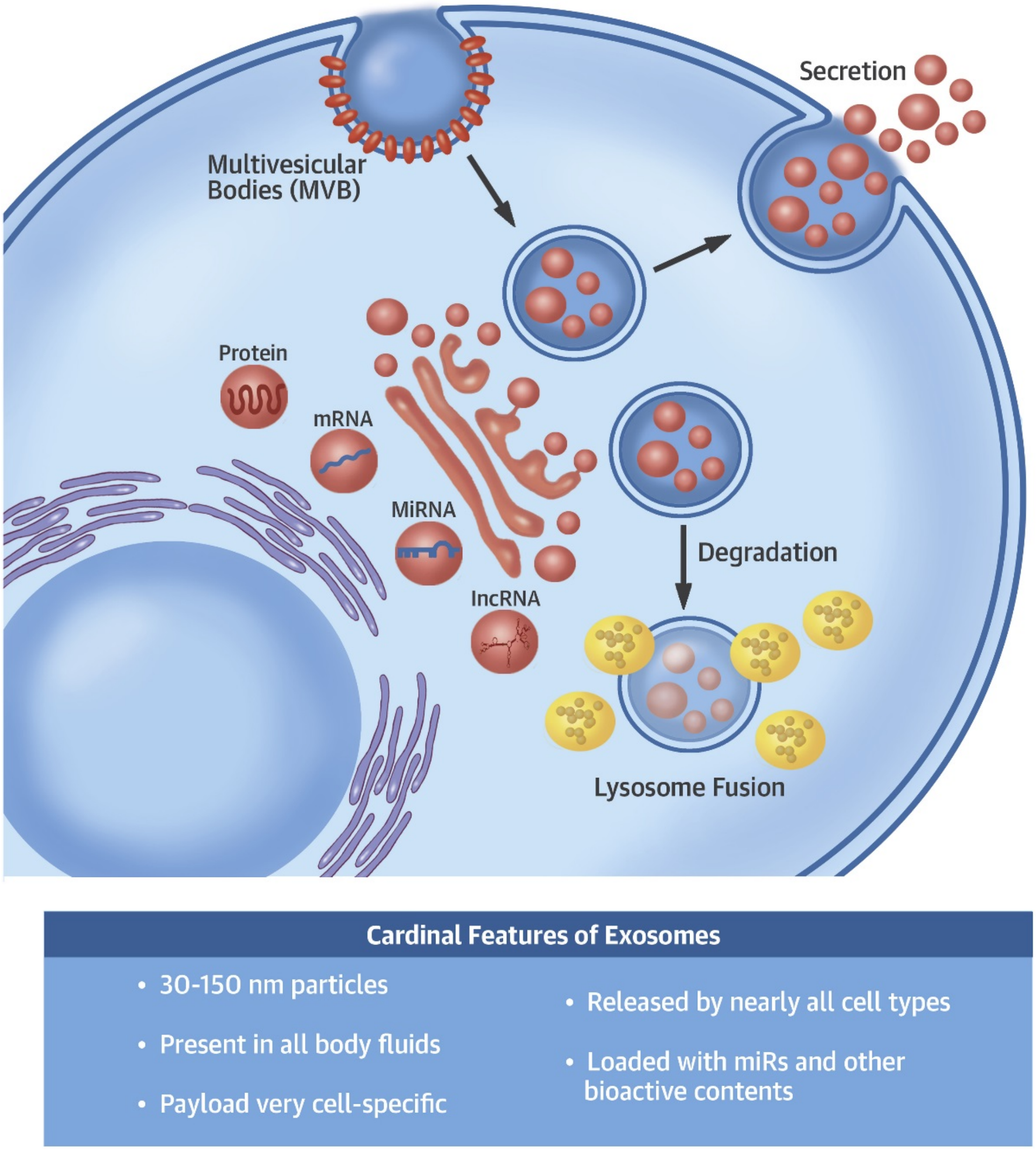

Figure 1. The biogenesis and features of exosomes (reprinted from (16))

Many studies have proved that exosomes have been circulating in body fluids such as blood, urine, and saliva (17). Exosomes contain different molecules such as proteins, miRNAs, mRNAs among others, and some of them have already been identified as disease biomarkers. Take lung cancer detection as an example, Jakobsen et al. stated that exosomal surface are highly clustered with numerical CD317 proteins and epidermal growth factor receptors (EGFR), which are regarded as important markers for the diagnosis of non-small cell lung cancer (NSCLC) (18). Therefore, exosomes can be an effective source of biomarkers for diseases. In the case of cancer diagnosis, biomarker-containing exosomes from body fluids could facilitate noninvasive or less-invasive screen of cancers through liquid biopsies. These exosomal biomarkers can also be identified for the assessment of cancer progression and monitoring of pre- and post- treatments (19).

3- Common exosomes isolation and detection methods

3-1- Common exosomes isolation methods

According to the distinct physical and biochemical properties of exosomes, several separation methods for isolating exosomes from human tissue fluids have been established in recent researches (20). One of these methods is density gradient centrifugation based on the density differences of vesicles. It has received extensive attentions and application so far (21). Another widely used separation technique is chromatography. This method is based on the difference in particle size between exosomes and hybrid protein, resulting in the elution sequence of exosomes first and hybrid protein particles later (22). Moreover, by utilizing the existing specific surface proteins on the exosomes, such as CD9, CD63 or CD81, modified immunomagnetic beads with antibody conjugated can also be used for capturing these antigens on exosome surfaces for the isolation of target exosomes (23).

In addition to the above methods, many new isolation methods have been reported. For example, Lim et al. described a novel methodology with the application of antibody-conjugated magnetic nanowires, which enlarged the capture efficiency to approximately three times compared to magnetic beads (24). Moreover, membrane-mediated exosomes separation is another technique to improve magnetic separation efficiency. Combined with streptavidin-modified iron oxide nanoparticles (SA-IONPs), Zhang and his colleagues claimed that the MVs are rapidly isolated by magnetically activated sorting from the supernatant of their donor cells (25). In another interesting example, Kabe and his colleagues reported a new technique called ExoCounter which can capture exosomes via nano-sized magnetic beads. These beads combined with numerous antibodies against exosomes surface antigens are coated onto an optical disc. It has been evidenced from this study that by the disc counting amount, HER2-positive exosomes were obviously soared in patients with breast cancer compared with healthy groups (26). These approaches are great attempts in addition to traditional magnetic bead capture to significantly improve the efficiency of isolation for EVs including exosomes.

3-2- Conventional quantitative and qualitative detection of exosomes

Exosomes need to be well characterised before being investigated as biomarkers for disease diagnosis. Based on their unique size, specific markers on the surface, lipid profiles, or even genomic profiles, various approaches and protocols have emerged for the assessment of exosomes in recent decades. In this regard, these approaches are playing an essential role in the characterisation of exosomes. For example, a typical method called nanoparticle tracking analysis (NTA) has been widely exerted in the field of exosomes detection. NTA offers the capacity to count Brownian motion of the exosome particles by light scattering. Though NTA is currently considered as one of the most commonly-used detection approaches for quantifying exosomes, currently there is no golden standard for setting the measurement conditions such as the selections of camera level and threshold values (27). Similarly, this principle also applies in dynamic light scattering (DLS) measurement. This method can transmit the information of dynamic particle size through fluctuations in scattered light. In addition, electron microscopy (EM), as an effective and convenience classical method, has been proved to be a credible mean to measure particle diameter and vesicle morphologies (28). Nevertheless, this type of technique is not suitable for particle concentration measurement. Besides, Western Blotting can be utilised to verify the presence of biomarker proteins in EVs. Additionally, one of the most widely applied detection methods is flow cytometry. Owing to the presence of various fluorescent proteins which come from the cell itself or fluorescently labelled antibody, this methodology can quantify multiple protein markers present in EVs, after immobilization on microbeads, with the help of multiple fluorescence channels (29). Conventional flow cytometry is suitable for identifying larger vesicles but not nano-sized vesicles. High-sensitivity flow cytometer (HSFCM) has now been developed to enhance the limitation of detection from around 500 nm to as low as 40 nm, in which case the detection of exosomes from other EV populations, e.g. microvesicles, becomes possible. Furthermore, multi-parameter quantitative detection of extracellular vesicles with single-particle level resolution has also been successfully achieved (30, 31). Apart from those conventional lab-based techniques, a number of new methods based on optical, electrochemical and electrical detection principles have emerged in the past few years. One of the most important ways for exosome detection/quantification are biosensors and nanobiosensors which are explained in the next section.

4- Biosensors and nanobiosensors for exosome detection

Over the past decades, biosensors as fast, reliable and precise methods to detect and/or quantify an analyte, opened their own ways into medical and biological markets, worldwide (32-34). For example, the most important commercial biosensors are portable glucometers (electrochemical biosensor) and paper-based pregnancy tests (optical biosensor). Biosensors generally are analytical devices designed to detect and/or quantify biomarkers precisely. They use biological receptor or interaction that bring a very high specificity towards the distinct target biomarker over non-specific molecules that can be found in medical samples (33, 35, 36). A transducer, as the most important part of a biosensor, will transform biological signals into measurable electrical or visual signals or signs. In fact, the biosensors are divided into main categories based on their transducer types including optical, electrochemical, electrical, mechanical or thermal. Each biosensor type has its own pros and cons and researchers or developers choose them based on their needs and designs (37-39).

In recent years, with the advancement of nanotechnology, most of the developed biosensors are taking advantages of using different types of nanomaterials in their detecting system in order to enhance the sensitivity and accuracy of the detection and/or quantification (34, 40, 41). These are simply called nanobiosensors and they are forming the most share of biosensors today and they comprises nanomaterials with various types of source materials, shapes, sizes and compositions. These innovations are increasing significantly and offer vast enhancement in function of the biosensors (40, 42).

Exosomes can be detected or quantified using these biosensors and nanobiosensors. Due to their novelty and many advantages for medical sciences discussed earlier, exosome biosensing has begun over the past few years. Optical, electrochemical and electrical biosensors have been used for exosome detection (43, 44). Most of the researches have used nanomaterials to improve accuracy and sensitivity considering the low concentrations of exosomes (45, 46). In some cases, additional strategies such as chip- and microfluidics based devices have been incorporated to enhance performance (2, 43). Each of these modalities and their applications for exosome biosensing will be discussed in the following parts of this review. The setup design, working mechanism, material makeup, advantages or disadvantages will be discussed.

5- Optical biosensors/nanobiosensors for exosome detection

A few decades ago, liquid biopsies are of continuous interest in clinical medicine because of the promising detection prospect for tumour analysis, disease assessment and early diagnosis (19). Exosomes detection, together with detections of circulating tumour cells (CTCs), cell-free DNAs (cfDNAs), is now considered as one of the most popular areas in liquid biopsy analysis. This field however still lacks effective and reliable quantitative methods of detection. Novel concepts of various detecting methods should be validated, and major technical breakthroughs should be made before moving exosome-driven liquid biopsy forward for the ultimate clinical application and detection/monitoring of diseases. Therefore, in this aspect, exploring detection modalities suitable for high-throughput screening, low limit of detection (LOD), real-time analysis and small sample volume will play an essential role in this field (47). At present, optical methods displayed outstanding accuracy and stability in measuring biological targets. Many optical techniques such as fluorescence, Raman scattering, surface plasmon resonance (SPR), and colourimetry have been applied to measure exosomal proteins or miRNA. **Figure 2** shows an example of each category with recent advances summarised below.

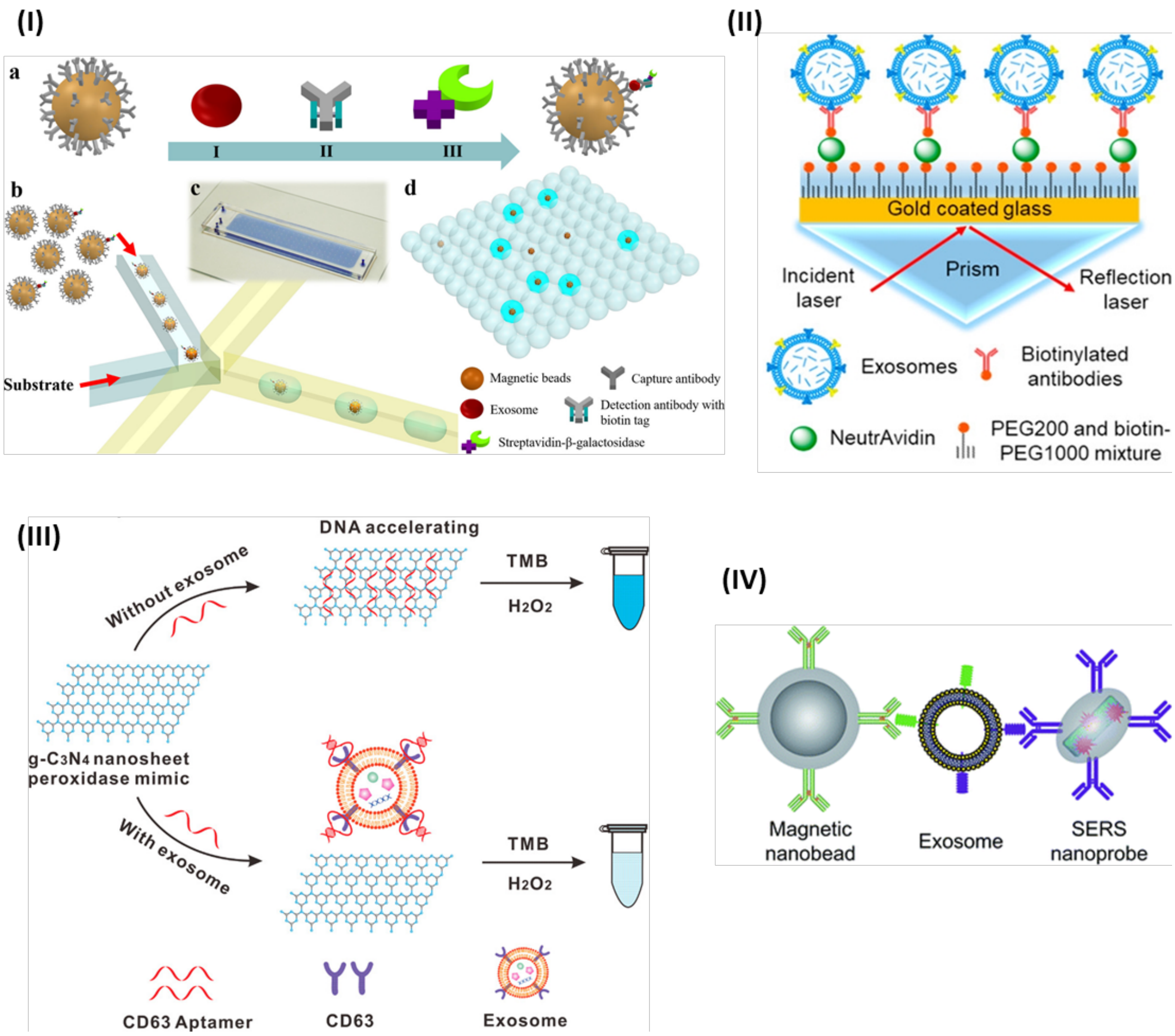

Figure 2. Examples of optical biosensors for the detection of exosomes. (I) Fluorescent biosensors. Demonstration of a droplet digital ExoELISA for exosomes quantification (48). (II) Surface Plasmon Resonance (SPR) biosensors. Illustration of SPR principle of exosomes detection (49). (III) Colourimetric biosensors. Novel colourimetric detection of exosomes based on the intrinsic peroxidase-like activity of g-C3N4 NSs (50). (IV) Surface-Enhanced Raman Scattering (SERS) biosensors. Illustration of the SERS-based exosomes detection method (51).

5.1 Fluorescence-based biosensor

Over the past several years, fluorescence-based methods have been commonly used as readout approaches in developing biosensors because of their high sensitivity. Based on fluorescence principle, a number of laboratory-based techniques for exosome detection, such as flow cytometry, real-time polymerase chain reaction (rt-PCR), fluorescence spectroscopy have been greatly developed (52). However, in clinical settings, due to the lack of professional testers and space constraints, it remains a critical challenge to achieve the goal of the rapid and point-of-care detection (53). More portable and smart fluorescence-based biosensors have been reported for exosomes detection in recent years. **Table 1** summarizes the recent advances in these biosensors with the focus on the detection targets, detection limits and exosome isolation method. For example, Liu *et al*. demonstrated a fluorescence-based immunosorbent assay using several droplet microfluidics for the digital qualification of target GPC+ exosomes from breast cancer patients with a limit of detection of 10 exosomes per micro litre (54). Researchers have also paid attention to the direct interaction between signal probes and exosomal biomarkers to design detection kits. In order to overcome present technological bottlenecks, Huang and his colleagues built a dual-signal amplification platform to achieve leukemia-derived exosomes detection. This work involved steps including aptamer recognition, magnetic enrichment, and rolling cycle amplification to achieve the enlargement of the detection signal. With the continuous accumulation of fluorescence signals, the lowest detection limit reached a level of 1×10^2 particles/μL (56).

In addition to the enlargement of biomarker signals, researchers have also been contributing to design compact and rapid detection kit. Recently, Ibsen *et al*. displayed an alternating current electrokinetic (ACE) microarray chip-based device to achieve fast isolation through differences in dielectric properties between exosomes and undiluted human plasma. As reported, it only takes 30 min to achieve the whole glioblastoma exosomes separation process and fluorescence analysis on the chip (57).

In the clinical diagnosis, high-throughput analysis is one of the advantages for a clinically oriented technology. Unlike the PCR based methods, oligonucleotide probes or molecular beacon technique reported by Lee *et al*. utilized PCR-free techniques for exosomal microRNAs detection, and has sharply reduced the cost of human resources and time costs (58). Moreover, it is noteworthy that this small oligonucleotide probe-based approach enables simultaneous identification of multiple miRNAs in one run, which provides the potential for various biomarkers detection using various fluorophores. It is, therefore, no wonder that signal amplification, chip design, and high-throughput analysis add additional features of fluorescence detection techniques can facilitate robust disease diagnosis.

Table 1. Summary of fluorescence-based biosensors for the detection of exosomes.

Category	Specific target	Source of Exo/Disease	Exosome isolation method	Detection method	Detection limit	Reference
Protein	Human liver cancer cells (Hep G2)	Liver cancer	Standard ultracentrifugation method	Upconversion LRET between UCNPs and Au NRs	1×10^3 exosomes/μL	(59)
Protein	COLO-1	Ovarian cancer patients	Standard ultracentrifugation method	Fluorescence	50 exosomes/μL	(60)
Protein	GPC-1	Breast cancer cell line MDA-MB-231; Breast cancer patients	Ultracentrifugation	Fluorescence (droplet digital ExoELISA)	10 exosomes/μL	(48)
Protein	Tyrosine-protein-kinase-like 7 (PTK7)	CCRF-CEM cells	ExoQuick- TC™ solution	Total-internal-reflection-fluorescence (TIRF)	5.2×10^3 exosomes/μL	(61)
Protein	CD63 and putative markers (EpCAM)	Prostate cancer cell lines (MCF-10A)	Ultracentrifugation	ExoAPP assay	1.6×10^2 exosomes/μL	(62)
DNA nuclear	HER2 and EpCAM	Breast cancer	Ultracentrifugation	Fluorescence microscopy	/	(63)
Protein	Glypican-1 and CD63	Pancreatic ductal adenocarcinoma patient samples	Ultracentrifugation	Fluorescence	/	(64)
Protein	CD63 protein and internal TSG101 protein	U87-EGFRvIII cell lines	Ultracentrifugation	ACE (Alternative current eletrokinetic) microassay chip (immunofluorescence)	$1\text{-}10 \times 10^6$ exosomes/μL	(57)
Protein	EpCAM	Breast cancer cell lines (MCF-7)	Exosome-specific dual-patterned immunofiltration (ExoDIF) devices centrifuge	Fluorescence (Alexa Fluor 647)	/	(65)

Protein	CD63	Melanoma cells line (B16), breast cancer cell line (MCF-7), human ovarian carcinoma cell line (OVCAR-3), and human liver cancer cell line (Hep G2)	Ultracentrifugation	Fluorescence (Cy3)	1.4×10^3 exosomes/mL	(66)
Protein	Aβ42 oligomers	Alzheimer's disease patient	ExoQuick™ exosome precipitation solution	Fluorescence	1.2×10^7 exosomes/μL	(67)
DNA	LZH8	Liver cancer cell lines (Hep G2)	Ultracentrifugation method	Fluorescence	/	(68)
RNA	Glypican-1 mRNA	Pancreatic cancer cell lines	Ultracentrifugation method	Fluorescence	$0.18\text{-}3 \times 10^3$ exosomes/μL	(69)
Nucleic acid	miRNA-21	Breast cancer cell lines	Ultracentrifugation	Fluorescence	1.8×10^4 exosomes/μL	(70)
Protein	CD63	Human breast cancer cell lines (MDA-MB-231)	ExoEasy Maxi Kit	Fluorescence	5.0×10^4 exosomes/μL	(71)
Protein	GPC-1	Panc-1 cell lines	Ultracentrifugation; ultrafiltration (100 kDa)	Rapid isothermal nucleic acid detection assay (RIDA)	/	(72)
Protein	CD147	Human colorectal cancer cell (HCT15)	Ultracentrifugation	Two-colour fluorescence	1.2×10^5 exosomes/μL	(30)
DNA	CD63 and nucleolin	Human leukemia cell line (HL-60)	Ultracentrifugation	Fluorescence	1×10^2 exosomes/μL	(56)
Protein	CD63	Hep G2 cells	ExoEasy Maxi Kit	Fluorescence	4.8×10^4 exosomes/μL	(73)
Nucleic acid	miR-21, miR-375, and miR-27a	Breast cancer cell line (MCF-7)	Ultracentrifugation	Fluorescence (Cy3)	$6 \text{ x } 10^7$ exosomes/μL	(58)
Nucleic acid	microRNA-1246	Breast cancer cell line (MCF-7)	Ultracentrifugation	Fluorescence	2×10^7 exosomes/μL	(74)

Protein	CD147	HER-2 breast cancer patients	Affinity magnetic nanobeads (magnetic separation)	Fluorescence (ExoCounter)	23.2 ng/mL	(26)
Protein	CD63, EpCAM, HER2, and MUC1	Hela (cervical cancer cells), chondrocytes (benign cells), MCF-7 (breast cancer cells), SKOV3 (ovarian cancer cells) and HepG2 (liver cancer cells) cancer cell lines	/	Fluorescence	0.24 μg/mL	(75)
Protein	CD63	Colon cancer cell line (LS180)	Centrifugation	Fluorescence	2.5×10^{1} exosomes/μL	(76)
RNA	Hsa-miRNA-21 (mir-21)	Human cervical carcinoma cell line (HeLa)	ExoQuick-TC™ System	TIRF imaging platform	378 copies/μL	(77)

5.2 SPR biosensor

SPR is a highly sensitive and real-time optical detection method. It is a label-free technology and does not require tedious sample handling steps, making it very attractive for exosomal protein analysis. **Table 2** lists the SPR biosensors reported for exosomes detection. Conventional SPR biosensors have been used in exosomes concentration detection in solution and clinical samples. For instance, the Biacore 2000 SPR instrument was used to measure the CD63 expression of exosomes derived from the human mast cell line HMC-1.2, and Biacore 3000 SPR instrument was used to detect six surface proteins (CD63, CD9, CD24, CD44, EpCAM, and HER2) of exosomes isolated from culture medium of breast cancer cells and from plasma samples of healthy controls (78). Efforts have been made to develop custom-built SPR platforms for exosomes detection, such as HER2+ exosomes from breast cancer patients (79). Nanotechnology facilitates the development of more sensitive SPR biosensors, that is SPR-based nanosensors, or nanoplasmonic biosensors, which have gained great attention in recent years due to the sensitiveness in detecting the binding of exceedingly small numbers of molecules (80). These plasmonic biosensors rely either on surface plasmon polarisation or on localized surface plasmons on continuous or nanostructured noble metal (usually gold) surfaces to detect molecular-binding events. Interestingly, the distance of measurement of the gold surface can be extended to a small distance above the SPR surface ~200 nm, which is well suitable for measuring particles of dimension < 200 nm (81). Nanoplasmonic techniques have been indeed reported to detect exosomes/extracellular vesicles in biological samples (2). Recently, Liu *et al*. developed an intensity-modulated nanoplasmonic biosensing assay to detect exosomal proteins (nPLEX) for ovarian cancer diagnosis. The compact SPR biosensor showed higher detection sensitivity than ELISA and similar sensing accuracy as ELISA (54). Such simple, integrated, and user-friendly sensing platforms, serving as an *in vitro* diagnostic test for cancer, are highly needed with low-cost, reliability and adaptability for rapid bioanalytical measurements in clinical settings.

Table 2. Summary of SPR sensors for the detection of exosomes.

Category	Specific target	Source of Exo/Disease	Exosome isolation method	Detection method	Detection limit	Reference
Protein	ICAM-1	Vascular endothelial cells from patients with coronary heart disease	Ultracentrifugation	SPR	/	(82)
Protein	CD63	Human mast cell line (HMC-1.2)	Ultracentrifugation	Dual-Wavelength SPR	/	(78)
Protein	HER2	HER2(+) BT474 and HER2(-) MDA-MB-231 cells and breast cancer patients	Centrifugation (Total Exosome isolation reagent)	SPR	2,070 exosomes/ µL	(79)
Protein	EpCAM	Fibroblast L cells	Centrifugation	LSPR (localized surface plasmon resonance)	$10\text{-}10^{8}$ exosomes/ µL	(83)
Protein	CD9, CD41b, MET	Human hepatocellular carcinoma cell lines	Ultracentrifugation	SPRi (surface plasmon resonance imaging)	/	(84)
Protein	CD9	Human lung cancer cells (A549), human neuroblastoma cells (SH-SY5Y)	Ultracentrifugation	LSPR	0.194 µg/mL	(85)
Protein	CD81	Mesenchymal stem cells	Centrifugation	Magnetic nanoparticle-enhanced SPR	0.76-3 µg/mL	(86)
Protein	EGFR, PD-L1	Nonsmall cell lung cancer cells (NSCLC A549), NSCLC patients	Centrifuge + total exosome isolation kit	SPR	2×10^{7} exosomes/ µL	(49)
Protein	Ganglioside GM1	Plasma	qEV Columns	SPRi	1 µg/mL	(87)
Protein	HSP90, HSP70, TSG101 and CD63, EpCAM, EGFR	Ovarian cancer cell lines (OVCAR3, OV420, CaOV3)	Ultracentrifugation	Nanohole-based SPR (Termed iNPS (intravesicular nano-plasmonic system))	10^{4} EV/mL	(88)

5.3 Colourimetric biosensor

Colourimetric biosensors as exosomes detection techniques are of significant prospective for POCT field. **Table 3** is a summary of recent colourimetric biosensors developed for the detection of exosomes. As **Figure 2III** displayed, a novel hybrid nanozyme was designed for sensitive colourimetric detection of exosomes. According to the differential expression of CD63 in breast cancer cell line (MCF-7) and control cell line (MCF-10A), the catalytic activity of the nanosheet can be improved by coupling g-C3N4 NSs with ssDNA. This work reveals the high potential of ssDNA-NSs hybrids in the clinical diagnosis of fluid biopsies (50). Based on the concentration of coloured compounds in solution, the colourimetric assay can address complex blood serum sample matrices *via* colour rendering principle, and it can be instantly observed with the naked eye to generate 'yes/no' answer without additional analysis equipment (36).

In colourimetric biosensors, the detection sites of exosomes can be divided into nucleic acid sites and protein sites. Recently, a novel isothermal amplification technology named recombinase polymerase amplification (RPA) has been defined as an effective method to explore the molecular detection of nucleic acid. Daher *et al* further stated that RPA is becoming the molecular tool of choice for rapid, specific and cost-effective identification of pathogens, and that POC bioassay and automated fluid platforms are key elements of this technology (89). Interestingly, Liu *et al*. have established a system through using a gold nanoparticle-based colourimetric assay to detect RPA-TMA reaction compound which combined RPA and transcription-mediated amplification (TMA). With a low limit of detection achieved (10^2 particles/mL), this work can be applied to quantify plasma LMP1+ and EGFR+ TEX levels in nasopharyngeal carcinoma patients and used in early diagnosis for liquid biopsy (54).

In the past decades, compared to the detection of nucleic acid sites, the colourimetric determination of protein sites has attracted more attention from researchers owing to the diversity of those biomarkers. For example, a novel fast colourimetric assay has been reported for distinguishing proteins by naked eyes, which utilised commonly used colloidal gold nanoplasma or nanoparticle-protein corona interaction. Indeed, the minimum detection limit for this method has reached to 5 ng/μL protein contaminants (90). However, this assay can only detect EVs rather than exosomes population. Besides, recent advances in capture techniques for exosomes population largely contributed to the improvement of colourimetric assays. Chen and his colleagues presented a three-dimensional (3D) scaffold chip device, which was composed of ZnO nanowires. These interconnected nanowires were designed with macropores under a high flow rate environment to enhance the immunocapture ability of exosomes particles (91). Then, the quantity of exosomes is prone to readout *via* UV–vis spectrometry or microplate reader.

Nevertheless, it is still very hard to achieve cost-effective detection of exosomes through most of the reported colourimetric approaches, especially for the clinical purpose. In this example, Yang *et al*. dramatically demonstrated a low-cost pH paper-based detection assay for exosomes *via* HRP-mediated promotion of mussel-inspired surface chemistry. In this study, the reagent-free functionalization of urease molecules is a key element which can raise the pH value (52). Therefore, the cheap and widely used commercial pH paper can be regarded as analysis indicator, which can dramatically reduce the cost of the assay.

Table 3 Summary of colourimetric sensors for the detection of exosomes.

Category	Specific target	Source of Exo/Disease	Exosome isolation method	Detection method	Detection limit	Reference
Protein	CD63	Breast cancer cells (MCF-7)	Ultracentrifugation	Colourimetric aptasensor	5.2×10^5 exosomes/μL	(92)
Protein	CD63	Breast cancer cells (MCF-7)	ZnO nanowires/ultracentrifugation	Colourimetric assay	2.2×10^4 exosomes/μL	(91)
Protein	CD63, EpCAM, PDGF, PSMA, PTK7	HeLa, PC-3, CEM, and Ramos cells	Ultracentrifugation	Colourimetric assay	/	(93)
Protein	LMP1, EGFR	Nasopharyngeal carcinoma (NPC) cells and plasma from patients with NPC	Magnetic separation	Gold nanoparticle (AuNP)-based colourimetric assay	1×10^2 exosomes/mL	(54)
Protein	CD63	Breast cancer cells (MCF-7)	Ultracentrifugation	Colourimetric pH-responsive bioassay	4.46×10^3 exosomes/μL	(52)
Protein	N/A	Human serum	Differential centrifugation (DC)	Nanoplasmonic colourimetric assay (Au NPs)	35 fM (2.1×10^4 exosomes/μL)	(90)
Protein	CD63	Human serum	ExoQuick™ Exosome Precipitation Solution	AuNP-based colourimetric assay	3.4×10^6 events/μL	(94)
Protein	CD63	Breast cancer cells (MCF-7)	Ultracentrifugation	UV−visible absorption spectrophotometer	13.52×10^5 exosomes/μL	(50)
Protein	EpCAM	PC3 cells lines and plasma	Ultracentrifugation + AE Magnetic Beads	AE Chromatography	3.58×10^3 exosomes/μL	(95)
Protein	CD63	Human liver cancer cell lines (Hep G2)	Ultracentrifugation	Luminescence resonance energy transfer (LRET)	1.1×10^3 exosomes/μL	(59)

5.4 SERS biosensor

Raman scattering or Surface-enhanced Raman scattering (SERS) is another popular optical method which was widely accepted by POCT researchers because of its potential to remarkably enhance signals from those negligible or low amount biomarkers on the exosomal surface. During the SERS-based strategy, Raman spectroscopy is used for analysing the chemical composition of exosomes. In comparison to SPR or fluorescence, this type of biosensor can identify distinctive spectral signals in the complicated and changeable biological environment (96). Now, this biosensor has been applied in exosomes concentration detection in laboratory and clinical samples, as summarised in **Table 4**. For instance, Zong *et al*. proposed a novel concept called sandwich-type immunocomplex, which was designed by SERS nanoprobes, exosomes, and magnetic nanobeads. Accordingly, the immunocomplex can be precipitated through the presence of a magnet field, and the SERS signals, therefore, can be captured *via* nanoprobes (51). Gold nanomaterials are the most popular signal enhancing materials in SERS detection field. For example, Kwizera *et al*. proposed gold nanorods to be applied in the detection of surface-specific proteins on exosomes using SERS biosensors. Each device can process over 80 purified samples with small exosomes concentration (2×10^6 exosomes/mL) in 2 hours (17). It is noteworthy that this method reduces the running time, cost and raises efficiency. Another study by Ma *et al*. reported the preparation of Au@R6G@AgAu nanoparticles (R6G attachment on the gold nanoparticles, then encapsulation in AgAu alloy shell nanoparticles named as ARANPs) with an inter small nanogap as stable SERS probe. This sensing system was successfully proved to notably improve the sensitivity. Furthermore, it can detect as little as 5 µL of sample volume from patients of recurrence in non-small-cell lung cancer (NSCLC) (97). This advanced technique is expected to make great progress in point-of-care diagnosis.

Table 4. Summary of SERS sensors for the detection of exosomes.

Category	Specific target	Source of Exo/Disease	Exosome isolation method	Detection method	Detection limit	Reference
Protein	HER2	Breast cancer cells (SKBR3)	Immunomagnetic separation	SERS tags (gold core-silver shell nanorods, Au@Ag NRs)	1200 exosomes/μL	(51)
Protein	HER-2, CEA and PSMA	Breast, prostate and colorectal cancer cell lines (SKBR3, LNCaP and T84, respectively), patients with breast, colorectal and prostate cancers	ExoQuick-TC™ exosome precipitation kit	SERS tags (AuNPs)	32, 73, and 203 exosomes/μL (for the SKBR3, T84, and LNCaP exosome)	(98)
Protein	CD9	Human liver carcinoma cells (Hep G2)	Immunomagnetic separation	SERS tags (AuNS@4-MBA@Au AuNSs)	27 exosomes/μL	(99)
Protein	GPC-1	PANC-1 and HPDE6c7 cells and serum from pancreatic cancer patients	Ultracentrifugation	PDA encapsulated antibody-reporter-Ag@Au multilayer (PEARL) SERS tags	1 exosome/2 μL	(100)
Nuclear acid-microRNA	miRNA-21	Non-small-cell lung cancer (NSCLC, A549)	Ultracentrifugation	SERS tags (Au@R6G@AgAu)	6.59×10^{-3} molecules /exosome	(97)
Nuclear acid-microRNA	microRNA-10b	Plasma from pancreatic ductal adenocarcinoma (PDAC), chronic pancreatitis (CP) and normal controls (NC)	Magnetic separation	SERS tags (Fe3O4@Ag-DNA-Au@Ag@DTNB)	1 aM	(101)
Protein	N/A	Prostate cancer cell lines (LNCaP and PC3)	Size exclusion chromatography	Raman spectroscopy	/	(102)
Protein	HER2	Breast cancer cells (MDA-MB-231, MDA-MB-468 and SKBR3) and HER2-positive breast cancer patients	Ultracentrifugation	SERS tags (AuNRs coated with QSY21)	2×10^3 exosomes/μL	(17)

6- Electrical biosensor/nanobiosensors for exosome detection

Electrical biosensors are one of the promising tools for the detection of molecular reactions by measuring solely the currents and/or voltages. These biosensors hold promise as a diagnostic tool for point-of-care and on-site applications due to their small size, low cost, and low power (103, 104). According to previous studies, exosomes can be electrically detected owing to their ability to store electrical energy when electrically polarized. The administration of voltage leads to the formation of an interface effective layer due to the interaction of the exosome surface with its surrounding media (105). The major challenge facing exosome-based detection methods is the difficulty of their isolation from a heterogeneous medium due to their small size and low buoyant density. Accordingly, the development of new extraction techniques and devices that (i) lower the mechanical damage to exosomes (ii) do not rely on antibody affinity binding (iii) do not require multiple steps (iv) reduce the time between sample collection and isolation and (v) do not impair the biological activity of protein biomarkers and exosomal RNAs are of crucial importance (104, 106). Figure 3 is presenting some examples of electrical biosensors for exosome detection.

In a recent study, Ibsen *et al.* developed an altering current electrokinetic (ACE) microarray chip device for rapid isolation and detection of exosomes from undiluted human plasma (106). ACE microarrays are capable of generating a dielectrophoretic (DEP) separation force upon the application of alternating current (107). The resulting DEP high-field regions around the circular microelectrode edges can, in turn, attract the nanoparticles and other nanoscale entities in the sample. The ACE microchip developed by Ibsen *et al.* was successfully able to collect the plasma exosomes (derived from glioblastoma cells) along with their associated surface markers and exosomal RNAs. The generated DEP high-field regions at the microelectrode edges preferentially captured bionanoparticles of a defined size range including EVs, exosomes and circulating DNA nanoparticles. Larger particles, cells, and smaller biomolecules could be removed from the chip during a washing step. The entire process including exosome separation and on-chip fluorescence analysis was completed in three simple and rapid steps leading to the efficient isolation of glioblastoma exosomes in less than 30 min. Meanwhile, this microchip enabled further on-chip immunofluorescence detection of protein biomarkers including external CD63 and internal TSG101 proteins and provided viable mRNA (glioblastoma-specific mutated EGFRvIII mRNA and the housekeeping β-actin mRNA) for RT-PCR (106).

Among the different electrical biosensing systems, Field Effect Transistor (FET)-type sensors have gained increasing attention (108). In general, FET sensors measure the open circuit potential variations occurring at the electrode interface. Meanwhile, the reactions occurred at the surface of gate electrode are evaluated by providing a non-Faradic electrochemical measurement without the need for redox marker (109). FET-type biosensors are composed of two elements including a recognition element and an electrically conductive channel. The targets, in this system, are detected via the electrostatic charge they carry in relation with the surrounding solution. The recognition element which traps the target upon contact can be selected from the entities either biologically- or be chemically-engineered. FETs have become promising candidates for point-of-care and on-site applications owing to their attractive properties including high sensitivity, small size, label-free detection, rapid response time, low cost due to integrated circuit technology, portable instrumentation, easy operation and applicable with small amount of sample (108, 109). According to the previous studies, FET biosensors employed for on-site detection of biological samples fall into 4 major groups including planar, graphene-based, carbon nano-tube (CNT)-based and nanowire (104). Various studies have been conducted using FET technology to sensitively detect micro-vesicles and exosomes (110-112). Multiplex detection of exosomal surface markers leads to the specific diagnosis of exosomes from other serum proteins. FET-type biosensors provide the potentiality of multiplexing, unique sensitivity, label-free detection and kinetic measurements over traditional detection methods (104). Recently, Pulikkathodi *et al.* have reported a FET biosensing system using AlGaN/GaN high electron mobility transistor (HEMT) for the detection and enumeration of extracellular vesicles (EV), in particular exosomes, derived from human embryonic kidney (HEK-293T) cells in a physiological salt environment without the need to be diluted (112). The utilisation of AlGaN/GaN heterostructures as sensors has shown a great potential due to the presence of a 2-dimensional electron gas (2-DEG) at the heterointerface (107). The AlGaN/GaN-based HEMT sensors have demonstrated a high chemical stability and an extremely high sensitivity due to the proximity of the conducting channel to the surface (113). According to Pulikkathodi *et al.* when the sample solution containing a high concentration of salt (~150 mM) is administered to the AlGaN/GaN HEMT surface in which a pulsed gate voltage is applied, the electrical double layer (EDL) is re-distributed at the solid-liquid interfaces generating a solution capacitance. The generated capacitance controls the potential drop across the AlGaN transistor and, therefore, a drain current signal is produced. It is worth noting that, the biological interactions (receptor-ligand binding) on the surface of sensor also lead to alteration of the solution capacitance which, in turn, results in the generation of the drain current signal. As a result, the developed anti-CD63 functionalized sensor demonstrated a high sensitivity with a wide dynamic range of (10^7–10^{10} EVs/mL) and a detection limit of 10^7 EVs/ml which is two orders lower than the normal concentration of EVs in human cell line (HEK-293T). Meanwhile, the successful capture of exosomes on the biosensor was confirmed by fluorescence microscopy. The major advantages of this system include that the detection of EVs can be done in solutions with high ionic strength, small volumes of the starting sample (~5 µL), short sample incubation time of ~5 min in label-free manner, and the removal of additional reagents and wash/block steps (112).

Detection of exosome-encapsulated miRNAs has become a promising strategy for non-invasive diagnosis of a wide variety of cancers (114-116). A sufficient concentration of RNA extracted from body fluid exosomes is required to be detected by the conventional methods, such as reverse transcription polymerase chain reaction (RT-PCR). A typical standard process for the extraction of exosomal RNA requires large starting sample volume, multiple time-consuming steps, multiple instruments and various chemical kits and washes. To overcome the aforementioned limitations, Taller *et al.* recently presented a novel microfluidic chip by combining a surface acoustic wave (SAW) exosome-lysis chip with a separate chip detecting the extracted RNA using an ion-exchange nanomembrane platform. In this device, the isolated exosomes from pancreatic cancer cell medium were lysed using Rayleigh waves generated by the application of AC through an inter-digitated electrode transducer, on the surface of a piezoelectric crystal. The SAW on the surface of piezoelectric crystal scatter into the liquid droplet or film, producing an acoustic pressure which leads to an effective turbulent mixing. Meanwhile the electric component of the SAW generates an electric Maxwell pressure at the interface of solid and liquid. Accordingly, exosome lysis was accomplished taking advantage of the effects of acoustic radiation force and the dielectrophoretic force on small particles. Thus, the SAW-based lysis does not interfere with the RNA detection process as compared with the conventional chemical or surfactant lysates. The RNA detection sensor in the presented work comprises an anion-exchange nanoporous membrane sandwiched between two reservoirs of fluid in which the anions are driven through the membrane pores when an electric current is applied. The drive of anions through the pores leads to a voltage drop which can be measured across the membrane. As a result, this integrated microchip demonstrated a lysis rate of 38% and a detection limit of 2 pM. Furthermore, this system remarkably reduced the total analysis time to ~1.5 h (~30 min for lysing and ~1 h for detection) and the required starting sample volume to ~100 μL, making it ideal for the studies on mouse models and for the analysis of fine needle aspiration (FNA) samples from clinical patients (116).

In 2017, Chae *et al.* reported the development of an oxygen-plasma-treated reduced graphene oxide (rGO) electrical biosensor for the detection of exosomal Aβ peptides extracted from the apparent Alzheimer's disease (AD) patients. In this study, the rGO thin films, being employed as the biological sensing interfaces, were deposited on the wafer-level SiO2 insulating substrates. In order to modify the rGO's functionality, they were treated with oxygen plasma and the optimal condition for the rGO treatment was obtained by optimizing the duration of exposure and the radiofrequency power of the plasma. The atomic force micrographs revealed that the oxygen plasma treatment considerably contributed to the covalent immobilization of antibodies on the rGO surface. According to the measured electrical characteristics and topographic analysis, it was found that the oxygen-plasma treatment of rGO enhanced the sensor's surface functionality including sensing performance with a 3.33 fold steeper slope for the curve representing the target specific interactions as compared to the untreated sensor. Meanwhile, the molecular activity of oxygen-plasma-treated rGO surfaces remained at 46-51% of the initial value after the duration of 6 h in ambient condition (117).

In 2012, a semiconductor-based potentiometric sensor array was reported by Goda *et al.* to detect exosomal miRNA following RT-PCR. Semiconductor-based electrical sensors work by detecting the target's innate charge after hybridization on the surface of biosensor. Furthermore, this type of sensor is capable of getting miniaturized using integrated circuit technology that makes it ideal for the assessment of miRNA expression in a microelectrode array platform without requiring an optical assistance. As presented by Goda *et al*. the exosoms derived from HEK293 serum-free medium containing miR-143 and miR-146a were subjected to simultaneous process of digestion and RT-PCR. The PCR amplicons, maintaining the miRNA original sequences were then hybridized to the DNA probes on the surface of the microarray sensor. In order to create a functionalized electrode in the form of a nanometer-scaled film, a self-assembled monolayer (SAM) was formed by co-immobilizing 50-SH-(CH2)6-DNA and sulfobetaine-3-undecanethiol (SB). The hybridization events, in this type of biosensor, lead to changes in the interface potential which can be transformed into a potentiometric signal in an electrometer. Compared to the conventional methods, the presented biosensor had a simple analytical set-up and sample handling and achieved a LOD of >20 pM with a dynamic range of two orders of magnitude and a sensitivity of -6.5 mV per decade at the range of at the range of 2–200 pM. It is worth to note that label-free systems generally suffer from low signal-to-noise ratio (S/N) due to the non-specific adsorption of molecular species on the detection surface, however the proposed system by Goda *et al.* showed a high

S/N ratio because of the application of SB SAM that leads to an excellent anti-fouling property against biomolecules (118).

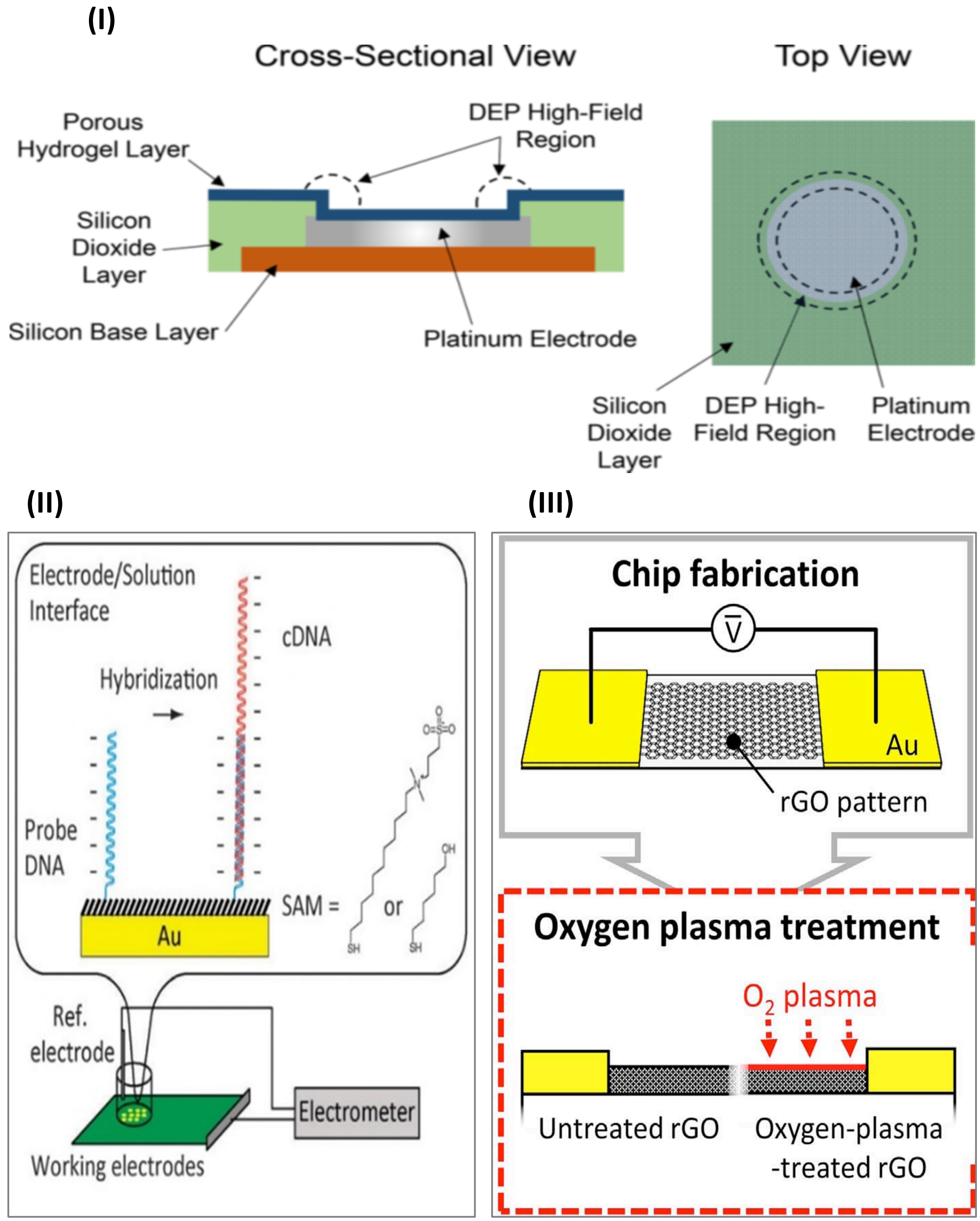

Figure 3. Examples of Electrical biosensors of exosome detection. (I) microelectrode array chip for electrical detection of the glioblastoma exosomes using platinum electrode on a microarray chip device (57); (II) electrical detection of exosomal microRNAs using a

microelectrode array in semiconductor-based potentiometry (118); (III) reduced graphene oxide-based electrical biosensors for detection of exosomal Aβ protein (117)

Few studies have reported a novel type of electrical system employing an electric field for exosome lysis and diagnosis (119-121). An electric field, in particular a non-uniform electric field, can potentially stimulate polarization or redistribution of lipid vesicles incorporating a biomolecule and, therefore, lead to their deformation. This deformation occurs either by rupturing the membrane or by disrupting the tertiary structure of the exosomal bilayer leading to a temporary formation of pores and release of the harbored biomolecules. In addition, the electric field can control the flow of the released biomolecules (119). Recently, Wei *et al.* reported the development of a new method based on the electric-field-induced release and measurement (EFIRM) for simultaneous exosomal content release, as a result of exosome disruption, and on-site detection of the released exosomal RNA/protein. The exosome deformation was carried out by applying a special cyclic square wave electrical field (csw E-field). The csw E-field, in comparison to the direct current electric field, generates voltages with the magnitude of several hundred mV which is not disruptive to most biomolecules. The released biomolecules were detected by an amperometric electrochemical sensor using an array of 16 bare gold electrode chips and workstation, each array unit containing a counter electrode, a working electrode and reference electrode. The analytical performance of the developed EFIRM was demonstrated by detecting the exosomal house keeping mRNA (GAPDH) and the transfected hCD63-GFP fusion protein which was subsequently expressed as an exosomal surface protein. This study further confirmed the hypothesis that the tumor-shed exosomes travel through the vascular system of the host and can further traffic into saliva, using an *in vivo* mouse tumor model. As a result, this study demonstrated for the first time the application of EFIRM for the detection of tumor-shed exosomes in saliva. In addition, the entire process was completed in approximately 3 h including a lysis process of approximately 200 s, using a starting raw sample of ~10 µL. The proposed system exhibited a high specificity and it was demonstrated that the EFIRM sensitivity for the detection of both mRNA and GFP protein is comparable to that of conventional methods (120).

The discussed research papers in electrical detection of the exosomes are summarized in the **Table 5**. As it is clear, number of electrical methods are less than two other main types of biosensors; electrochemical and optical. In addition, the electrical methods have been used for detection of different range of disease, while the electrochemical methods were used for cancers mostly.

Table 5: Summary of electrical biosensors for the detection of exosomes.

Target	Disease	Sample	Exosome isolation method	Detection method	Linear detection range	Detection limit	Assay time	Highlights	Refer ence
CD63 and TSG101	Glioblastoma	Spiked PBS and normal human plasma	ACE microarray chip	On-chip fluorescence analysis			<30 min	Florescent tagged antibodies	(106)
CD63		Spiked PBS	Differential centrifugation	HEMT-based FET	10^7–10^{10} EVs/mL	10^7 EVs/mL	5 min		(112)
Exosomal miR-550	Pancreatic cancer	Spiked PBS		Ion-exchange nanomembrane sensor	2-decade dynamic range	2 pM	1.5 h		(116)
Exosomal Aβ peptides	Alzheimer's disease	Spiked PBS	ExoQuick precipitation and centrifugation	Oxygen-plasma-treated rGO ES					(117)
Exosomal miR-143 and miR-146a		Real time RT-PCR product	Centrifugation	Semiconductor-based potentiometric sensor array	-	>20 pM		SAM to functionalize the sensor	(118)
CD63	Lung cancer	Serum	Magnetic bead-based exosome extraction	EFIRM			3 h		(120)

7- Electrochemical biosensors/nanobiosensors for exosome detection

Electrochemical biosensors and nanobiosensors are the most attended types of biosensors due to their advantages including but not limited to cost- and time-effective procedure, higher sensitivity, as well as needing lower sample quantity (122-124). Because of these incredible properties, and due to the very small size and quantity of the exosomes, electrochemical biosensors can be a good way to detect/quantify exosomes in medical samples (45, 125, 126). In addition, electrochemical methods can be integrated with different types of platform especially those for sample manipulation devices such as microfluidics chips that can be a great advantage for exosome detection (127, 128). There are different electrochemical detection techniques (including Voltammetric, Amperometric, Impedimetric, Potentiometric) that can be used in different detection strategies for exosome detection which are reviewed in this section. Figure 4 is representing some examples of electrochemical methods for detection of exosomes.

7.1. Voltammetric methods

Voltammetric methods, in which current is measured as a function of electrode potential, are the most common used in electrochemical biosensors because of their technical advantages over other electrochemical methods. They can be used for the quantitative determination of ions and molecules with less limitations than other techniques (124, 129). They have been used for exosome detection which are reviewed as follows.

Boriachek et al. have recently presented an electrochemical nano-immunosensor based on anodic stripping voltammetric (SWASV) for recognition of breast and colon tumor-specific exosomes using quantum dots (QDs) as a signal amplifier. In this work, the magnetic beads were firstly functionalized with a generic tetraspanin CD63 antibody. Next, biotinylated- FAM134B (colon cancer) and HER-2 (breast cancer) monoclonal antibodies were functionalized with streptavidin-coated CdSe QDs. Using inorganic colloid tracers such as QD nanoparticles in combination with SWASV readout increases the sensitivity of this immunoassay, as well as the use of magnetic beads-based exosome separation method that can remove non-specific molecules and vesicles in the detecting sample. Therefore, the presented method showed a detection limit of 100 exosomes/μL with the relative standard deviation (RSD %) of <5.5% in cancer cell lines (130).

In another nano-immunosensor for exosome detection, Yadav and et al. used a sandwich strategy assay to design and construct a cost-effective and simple proof-of-concept electrochemical biosensor for directly quantifying breast cancer cell-derived exosomes in bulk exosome populations using Differential pulse voltammetry (DPV) method. The surface of screen-printed electrode was modified by avidin and biotinylated tetraspanin biomarker (e.g., CD9) antibodies were immobilized onto the ExtrAvidin-modified electrode surface via avidin/biotin interaction. The human epidermal growth factor receptor 2 (HER2)-positive exosomes derived from breast cancer cells were sandwiched between CD9 antibodies and HER2 antibodies. The suggested electrochemical biosensor presented a limit of detection 4.7×10^5exosomes/μL with an RSD of <4.9% (n= 3) that was far better compared to ELISA (10^7 exosome). In addition, unlike the conventional assay methods, this assay was done within 2 hours with 5μL of the sample. The results indicate that this approach is fast and sensitive enough to distinguish HER2 (+) exosomes in the real samples (131).

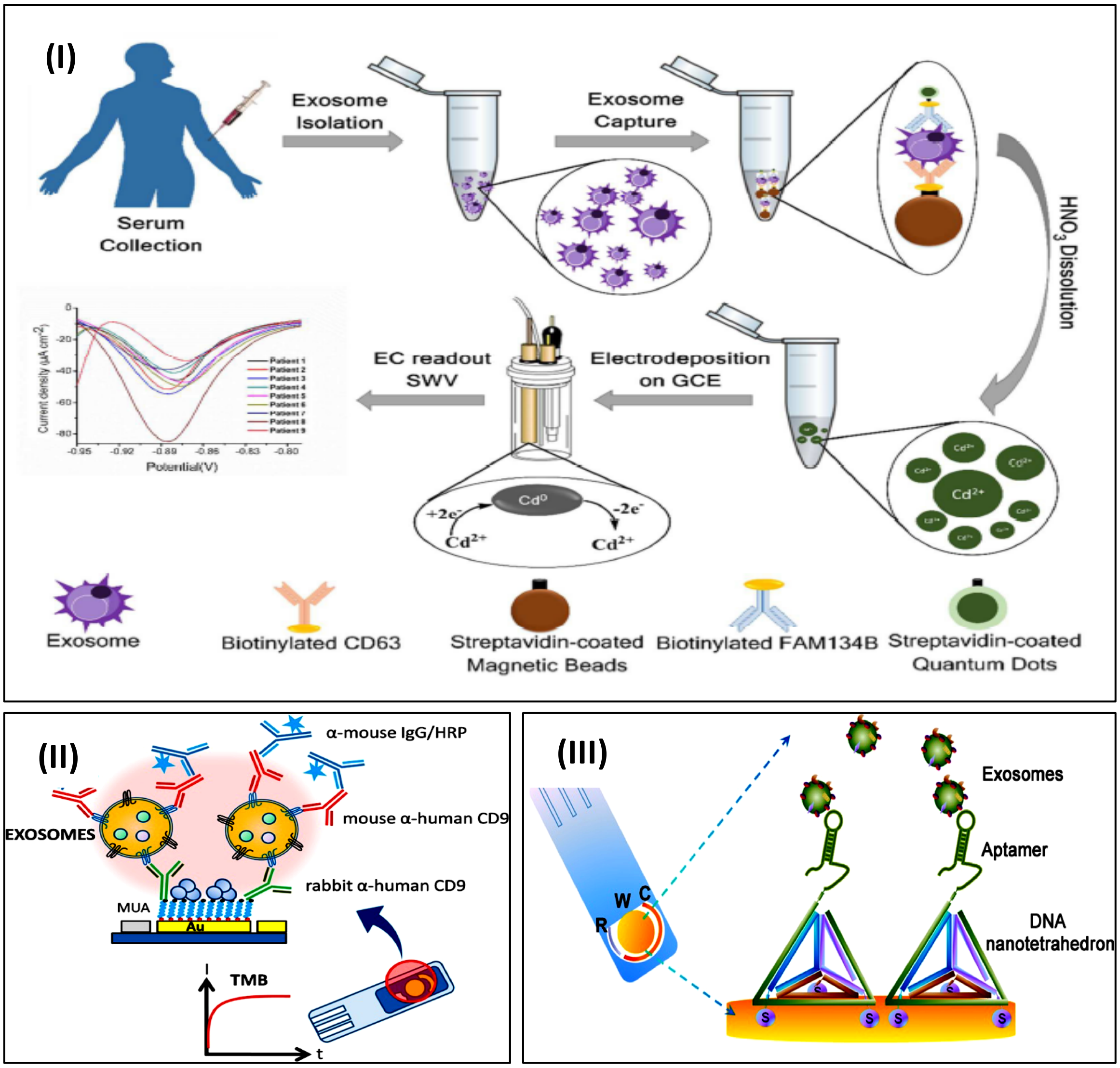

Figure 4. Examples of electrochemical biosensors of exosome detection. (I) Magnetic electrochemical detection of CD63 exosomes for detection of breast and colon cancers using quantum dots as signal amplifier (55). (II) Electrochemical amperometric biosensor with enzymatic readout of the CD9 exosomes by using different antibodies (132). (III) Use of DNA nanotetrahedron nanostructures conjugated with aptamer as electrode modification to detect hepatocellular exosomes (133)

Some voltammetric assays have used aptamers instead of antibodies to detect exosomes. Aptamers with their distinct shapes which is formed based on their sequences, can detect their specific target with very specific affinity. They are mostly based on DNA or RNA and even peptide sequences and comparing to antibodies, they are less-expensive and less-complicated to work with and also can be used for detection of variety of molecules ranging from proteins to ions. Biosensors in which aptasensor have been used as a biorecognition are often called aptasensors and they have been used for detection of exosome even more that antibody-based biosensors (32, 134-137).

In a voltammetric aptasensor, Zhou et al. designed a multiplexed electrochemical method (using Linear sweep voltammetry (LSV) technique) using gold (Au)-modified circular Au electrodes as a platform and silver nanoparticles (AgNPs) and copper nanoparticles (CuNPs) as labels for recognition and characterization of specific protein markers expressed on exosomes and microsomes of prostate cancer patients. In this work, for effective capture of epithelial exosomes or microsomes, the surface of the sensor was modified with thiolated anti- epithelial cell adhesion molecule (EpCAM) aptamers. AgNPs and CuNPs were used to simultaneously detect EpCAM and prostate-specific membrane antigen (PSMA), respectively. The electrochemical readout was done by direct electro-oxidation of the labeled metal nanoparticles (MNPs) because their oxidation potentials are in the potential range of the Au electrodes and are well separated, providing multi-marker identification, peak currents dose with the increase of exosomes or microsomes concentrations in samples were noticeably enhanced. The charge intensity for the detection of AgNPs–anti-EpCAM displays a seven-to-eightfold of decrease over that of the detection of CuNPs–anti-PSMA. This electrochemical biosensor demonstrated a limit of detection (LOD) of 50 exosomes/sensor. Compared to the conventional methods, their electrochemical assay is reported to be simple, rapid, cheap and needs low volume of sample (138).

In a study by Dong et al., a highly sensitive electrochemical aptasensor (using DPV electrochemical method) was developed for evaluation of tumor exosomes based on aptamer recognition-induced multi-DNA release and cyclic enzymatic amplification. In this work, the biosensor was designed based on a dual signal amplified strategy including the release of multiple DNAs and Exo III-assisted target recycling amplification. The prostate-specific membrane antigen (PSMA) aptamers-modified magnetic beads were used for capturing tumor exosomes derived from LNCaP cells. A linear range of 1000-120000 particles/μL and a detection limit down to70 particles/μL have been reported for this biosensor which is lower than the LODs of most currently available methods which can be because of magnetic separation method, use of aptamers, and dual signal amplification strategy especially enzyme assisted target amplification (139).

In 2017, the strengths of advanced aptamer technology, DNA-based nanostructure, and portable electrochemical devices (using Square wave voltammetry (SWV) electrochemical method) have been used to design and construct a nanotetrahedron (NTH)-assisted aptasensor for direct identification of hepatocellular exosomes by Wang et al (133). The NTHs were created by self-assembly of four single-stranded sequences; aptamers were made into one of the NTH sequences, while the other three ends with thiol groups. Aptamer-containing NTHs were immobilized via three thiol groups onto the screen-printed gold electrode surface. The oriented immobilization of aptamers remarkably enhances the availability of an artificial nucleobase-containing aptamer to suspended exosomes. This aptasensor achieved a detection limit of 2.09×10^4/mL. In comparison with the single-stranded aptamer-functionalized aptasensor, the NTH-assisted aptasensor can detect exosomes with 100-fold higher sensitivity (133).

7.2. Amperometric methods

Amperometry is simply measuring the current of electrode in which most of the amperometric electrochemical biosensors are based on a redox activity of an enzyme (mainly horseradish peroxidase (HRP)). Chronoamperometry is a derivative of amperometric methods in which currents are caused by the potential steps in different times (32, 140, 141). Enzymes have catalytic activity and specific binding capacity; also, they can act as an accelerator in chemical reactions (123). Thus, the use of this macromolecular biological catalysts leads to improving the sensitivity of biosensing systems (123). In enzyme-based biosensing devices that ate extensively used to quantitative analysis a variety of substrates including exosomes, the efficiency depends on the modifications of the surface electrode, type of enzyme, substrate, and the use of a mediator (142). HRP as secondary detection reagents has the most application in designing enzyme-based biosensor (142). Among different substrate for this enzyme, the TMB/H_2O_2 component is by far the most optimal choice (142). Enzymes have some disadvantages such as the limited stability, tend to lose activity after a relatively short time, the dependency of functions on environmental factors (ionic strength, pH, temperature), and expensive of source and extracting, isolating, purifying processes (123).

In 2016, Doldán et al. constructed an amperometric electrochemical biosensor based on sandwich assay for detecting exosomes on gold electrodes functionalized with α-CD9 antibodies.

The CD9 is a transmembrane protein that is present in many copies in the surface of the exosomes and functions as a suitable exosome biomarker. In this research, the rabbit antihuman CD9 antibodies were immobilized onto gold electrode and monoclonal antibodies were used against CD9 for recognition of captured exosomes. After adding samples, target exosomes were sandwiched between the captured rabbit anti-human CD9 antibodies and detector mouse anti-human CD9 antibodies. The signal was amplified by binding the multiple detector antibodies to the surface of each captured vesicle. An HRP-conjugated α- mouse IgG antibody was finally applied, and the detection was done by amperometric measurements, which was based on enzymatic reaction performance and electrochemical reduction of 3,3′,5,5′-tetramethyl benzidine (TMB) on the gold electrode surface. The designed sensor acts with 1.5 µL sample volume and can recognize as low as 200 exosomes per microliter, with a limit of detection of 2×10^2 particles/µL and a linear range nearly 4 orders of magnitude. The electrochemical immunoassay is specific and readily discriminates between exosomes and other extracellular vesicles (i.e., microvesicles) in samples containing 1000-fold excess of the latter. Capability of sensing exosomes in real samples (diluted serum) was assayed successfully. Using amperometric method and also signal amplification method can be the cause of the high sensitivity as well as the high selectivity using different antibodies (132).

In another amperometric detection method, Park Xu et al. reported a urine-based electrochemical platform for detection of kidney transplant rejection. In order to facilitate urine EVs (uEVs) assay, they constructed a compact and portable integrated kidney exosome analysis (iKEA) device for amperometric electrochemical readout. The Jurkat T cells were used as a model cell line cell-derived EVs was collected from culture media for detection of CD63 tetraspanin with separation using magnetic beads (diameter = 2.7 µm) labeled with a secondary antibody against the target marker for enriching T-cell-specific EVs. The collected EVs were then labeled with horseradish peroxidase (HRP) enzyme via a secondary antibody. In the next step, mixing magnetically modified- beads with 3,3′,5,5′-tetramethylbenzidine (TMB) (a chromogenic electron mediator) were dropped onto the screen-printed electrode surface prior to chronoamperometry electrochemical analysis. With simultaneous use of magnetic enrichment and enzymatic signal amplification, this portable electrochemical biosensor showed high sensitivity ($\sim 10^4$) in evaluating EVs by chronoamperometry analysis (143).

In 2016, Jeong et al. combining magnetic enrichment, the sandwich assay strategy, and enzymatic amplification suggested an integrated magneto−electrochemical amperometric biosensor for fast and on-site exosome screening. This miniaturized integrated magnetic−electrochemical exosome (iMEX) system was designed with eight independent channels which are simultaneously used for the high-throughput measurements. The magnetic beads were coated with antibodies against CD63 to capture exosome and targeting antibodies with HRP enzyme were employed. The final electrochemical current signal was generated when HRP enzyme was interacted with TMB/H_2O_2 as its substrate. This electrochemical biosensor can simultaneously detect the profile of multiple protein markers using only 10 µL of samples and with a sensitivity of $<10^5$ vesicles within an hour which is better than conventional methods in assay sensitivity and speed. The detection limit 3×10^4 exosomes was obtained and also, the clinical potential of iMEX system was assayed by profiling EVs collected from ovarian cancer patients and showed capacities for fast, high-throughput, and on-spot analysis, the iMEX could be an effective detection modality and accelerate the exosome analysis toward routine clinical testing (144).

7.3. Impedimetric methods

In impedimetric methods the impedance of a system is measured over a range of frequencies. The most important impedimetric method in electrochemical biosensing field is Electrochemical Impedance Spectroscopy (EIS). EIS can be performed as a label-free detection methods which is an excessive advantage over other electrochemical techniques (145, 146).

In 2018, a label-free electrochemical sensor via EIS technique has been fabricated to measure the nanoscale EVs secretion levels of hypoxic cells in breast cancer by Kilic et al. (147). MCF-7 cell line was employed as a model breast cancer cell line and hypoxic condition was created using $CoCl_2$ exposure. Biotinylated anti-CD81was immobilized through streptavidin-biotin interaction onto screen printed gold electrode surface. Using such simple platform and label-free strategy, this immunosensor achieved a linear range of 10^2–10^9 EVs/ml with a detection limit of 77 EVs/mL. The results indicate that the designed biosensor has excellent potential not only for identification of EVs from blood samples but also for integration with platforms that mimic tumor microenvironment for chemotherapeutic drug testing. Also, the biosensor function was compared to enzyme-linked immunosorbent assays (ELISA) and nanoparticle tracking analysis (NTA) and showed higher sensitivity and sensitivity and lower limit of detection compared to two other techniques (147). In fact, this sensor presented an appropriate function such as a superior limit of detection and high sensitivity without using labeling procedures, expensive microfabrication methods, and application of the nanoparticles and also has the potential to be integrated with cell culture platforms to monitor changes in EVs secretion by oxygen tension.

In another study in this filed, Li et al. introduced a highly sensitive EIS biosensor for detection both external (tetraspanin) and internal (syntenin) exosome-specific markers. The syntenin-1 is a protein that is overexpressed in multiple human cancers and often appears to scale with disease progression. The exosome detection limits obtained were 1.9×10^5 particles/mL and 3–5 picomolar (equivalent to 320 aM) for tetraspanin and syntenin, respectively. This sensing strategy can be used for determining exosome concentration without any necessity to use NTA and also provide some advantages to intact sample preparation and quantification (148).

The mentioned progresses in electrochemical detection of exosomes are described briefly in the **Table 6**. As it can be concluded from the summarizing table, most of the publications were aimed to detect cancers based on quantification of exosomes, where it can be used for detection of other kind of disease in future. Another interesting fact is that most of the exosome isolation techniques are centrifugation-based and there is no evidence to use any other separation methods in electrochemical-based methods. It is expected that additional separation or more precise isolation can be achieved by microfluidics devices. Additionally, it can be seen that there are varieties of strategies with different nanoparticles/nanostructures that led to wide range of detection limits.

Table 6: Summary of electrochemical biosensors for the detection of exosomes.

Target	Disease	Sample	Exosome isolation method	Detection method	Linear detection range	Detection limit	Assay time	Highlights	Reference
FAM134B (colon cancer) /HER-2 (breast cancer)	Colon and breast cancer	Serum	Centrifugation	SWASV	10^2-10^7 exosomes/µL	100 exosomes/µL		CdSe QDs as signal amplifier	(130)
EpCAM and PSMA	Prostate cancer		Centrifugation	LSV		50 exosomes/sensor		AgNPs and CuNPs as labels	(138)
HER-2	Breast cancer	Serum	Centrifugation	DPV	3×10^7 to 4.7×10^5 exosomes/µL	4.7×10^5 exosomes/µL	2 h	-	(131)
α-CD9		Plasma	Centrifugation	Amperometric	2×10^2 to 1×10^6 exosomes/µL	200 exosomes/µL		HRP enzyme	(132)
CD81	Breast cancer	Blood	Centrifugation	EIS	10^2 to10^9 EVs/ml	77 EVs/mL		$CoCl_2$ exposure	(147)
PSMA	Prostate cancer	Fetal bovine serum	Centrifugation	DPV	1000-120000 particles/µL	70 particles/µL		Cyclic enzymatic amplification	(139)
Hepatocarcinoma cells (HepG2) exosomes	Liver cancer		Ultracentrifugation	SWV	10^5 to 10^{12} exosomes/mL	2.09×10^4/ exosomes/mL		Nanotetrahedron-assisted aptasensor	(133)
CD 63			Centrifugation	SWV	1×10^6 to 1×10^9	1×10^6 particles/mL			(127)

					particles/mL				
CD 63	Liver cancer	Serum		SWV	7.61×10^{4} to 7.61×10^{8} particles/mL	4.39×10^{3} particles/mL	3.5 h	G-quadruplex as a signal reporter	(128)
CD3	Kidney transplant rejection	Urine	Centrifugation	Chronoamperometry	10^{4} EVs	~1.6×10^{4} EVs	2h	HRP conjugated CD63 for signal generation	(143)
CD 63	Ovarian cancer	Plasma	Centrifugation	Chronoamperometry	10^{4} to 10^{8} exosomes	3×10^{4} exosomes	1 h	Magnetic enrichment and HRP enzyme	(144)
Tetraspanin and syntenin exosome markers	-	Serum	Centrifugation	EIS	-	3-5 pM syntenin; 1.9×10^{5} particles mL^{-1} tetraspanin	-	-	(148)

HER-2: human epidermal growth factor receptor 2, SWASV: square-wave anodic stripping voltammetry, CdSeQDs: CdSe quantum dots, EpCAM: epithelial cell adhesion molecule, PSMA: prostate-specific membrane antigen, LSV: Linear sweep voltammetry, AgNPs: Silver nanoparticles, CuNPs: copper nanoparticles, DPV: Differential pulse voltammetry, HRP: horseradish peroxidase, EIS: Electrochemical Impedance Spectroscopy, EVs: extracellular vesicles, SWV: Square wave voltammetry, EVs: Extracellular vesicles

8. New trends and challenges

As summarised in the review, protein profiles are the main exosomal biomarkers for cancer diagnosis in optical modality. There are not too many studies on targeting DNA or RNA profiles in exosomes. There are even little studies focusing on their lipid profiles. More exciting investigations on these subcategories would be foreseen in the near future. Indeed, improving sensitivity is still one of the challenges for developing robust detection assay, especially for clinical set-up. As explained in previous parts, many studies in exosome biosensors fields reported enhanced sensitivity by using nanoparticles and nanostructures such as QDs (55), metal NPs (AuNPs and CuNPs) (138), DNA Nanostructures (133), Au@Ag NRs (51), magnetic nanoparticles (26), and magnetic nanowires (24).

For various optical detection methods, the poor refractive index is becoming one of the largest barriers in the exosome detection field. What encourages us is that many efforts have already been put into the enlargement of biomarker signals and enhancement of detection robustness. Just to mention an example, Daaboul and his colleagues developed a novel method based on Single Particle Interferometric Reflectance Imaging Sensor (SP-IRIS). This interferometric imaging concept has been verified by firstly capturing exosomes-size particles from a small volume (20 µL) of human cerebrospinal fluid (hCSF) and then measuring the signals (149). Additionally, unlike the SPR described above, localised surface plasmon resonance (LSPR), a phenomenon occurring on metal nanostructures, is deemed as the next generation of plasma-based techniques.

Microfluidics devices are new trends for handling, manipulating and detecting samples containing biomarkers in molecular biology and medicine. Application of a microfluidics sample preparation and manipulation in a detection device such as biosensors has increased over the past decade. This integration of sample preparation and detection can bring some advantages such as enhancing the speed and accuracy of the biosensors to reach a high-throughput analyzing device, as well as decreasing the human work force mistakes (150, 151).

Klinghammer et al. positively presented a microfluidic-channel-integrated sensor for label-free detection of biomolecules (**Figure 5**). Vertically united and closely combined gold nanorods form around 1 cm^2 array in a limited area, which can amplify the optical signal to achieve stable biosensing measurement （152）. In electrochemical detection methods, Zhou and his coworkers have recently introduced an electrochemical aptasensor using an aptamer that binds to CD63 on a gold electrode surface and integrated into a microfluidic system. The interaction of the aptamer-modified electrode with target exosomes led to the displacement of the antisense strands and decreased redox signal. The miniaturization by photolithography and incorporated into microfluidic devices provide exosome detection from a small sample volume. The suggested biosensor represented a detection limit of 106 particles/mL that it is100 times lower in comparison with commercial immunoassay, even without performing handling or processing steps such as labeling or washing thanks to microfluidics-integrated method (127) **(Figure 6 I)**.

Xu et al. in 2018 designed a magnetic-based microfluidic device for on-chip isolation and rapid and simple detection of tumor-derived exosomes with Y-shaped micropillars mixing pattern with two sample inlet for magnetic bead-based exosomes capture. In this proposed structure, exosomes capture efficiency was promoted by creating anisotropic flow without any surface modification. To quantify the exosomes, a two-stage microfluidic platform (ExoPCD-chip) was designed so it contains a CD63 aptamer and a mimicking DNAzyme sequence. In fact, the single-stranded DNA forms a hairpin structure and the G-rich mimicking DNAzyme sequence in the strand will be caged in the stem-loop structure. When the target CD63-positive exosomes interact with LGCD, the single-stranded DNA hairpin opens and form a small G-quadruplex- contained hairpin configuration as a signal reporter. The hemin/G-quadruplex simultaneously acts as the NADH oxidase and HRP-mimicking DNAzyme with increase in the electrochemical signal current. This label-free and immobilization-free electrochemical aptasensor can detect CD63 positive exosomes as low as 4.39×10^3 particles/mL with a linear range 7.61×10^4 to 7.61×10^8 and rapid response time within 3.5 h in a small-volume sample (30 μL). Also, the ExoPCD-chip function was assayed with serum of liver cancer patients and also can differentiate liver cancer patients from healthy controls while a ELISA method cannot do so (128) (Figure 6II).

Another new trend in exosomes analysis, yet challenging, is single vesicle analysis, for providing high-resolution images with information on both the structure and the composition, or biophysical parameters to reach statistical power (78). In future researches, exosomes will be continuously used in clinical diagnosis and prognosis, and additionally to drug delivery studies, molecular therapy, clinical broad-spectrum cancer screening, and early detection of brain diseases such as Alzheimer's disease (67). These research directions indicate that the demand for sensitive and specific detection of exosomes will continue to grow.

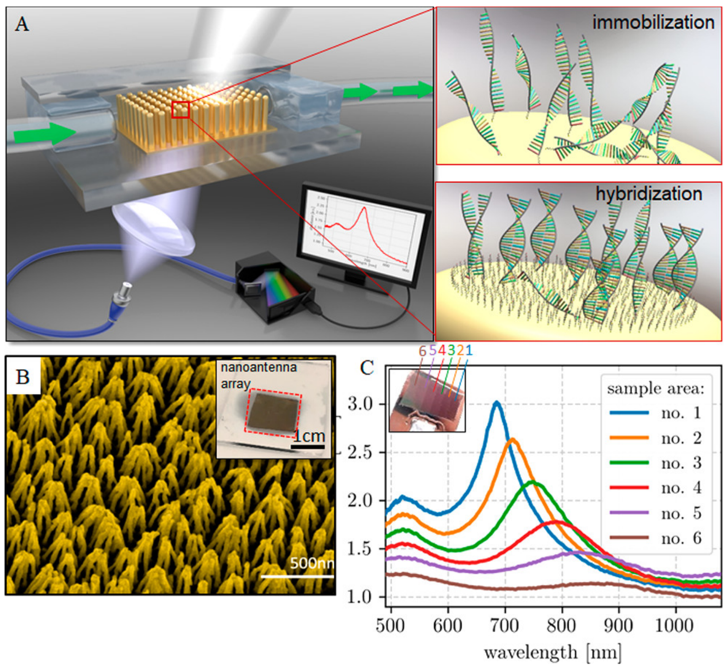

Figure 5. Illustration of a microfluidic-channel-integrated localized surface plasmon resonances (LSPR) sensor for the label-free detection of biomolecules (152).

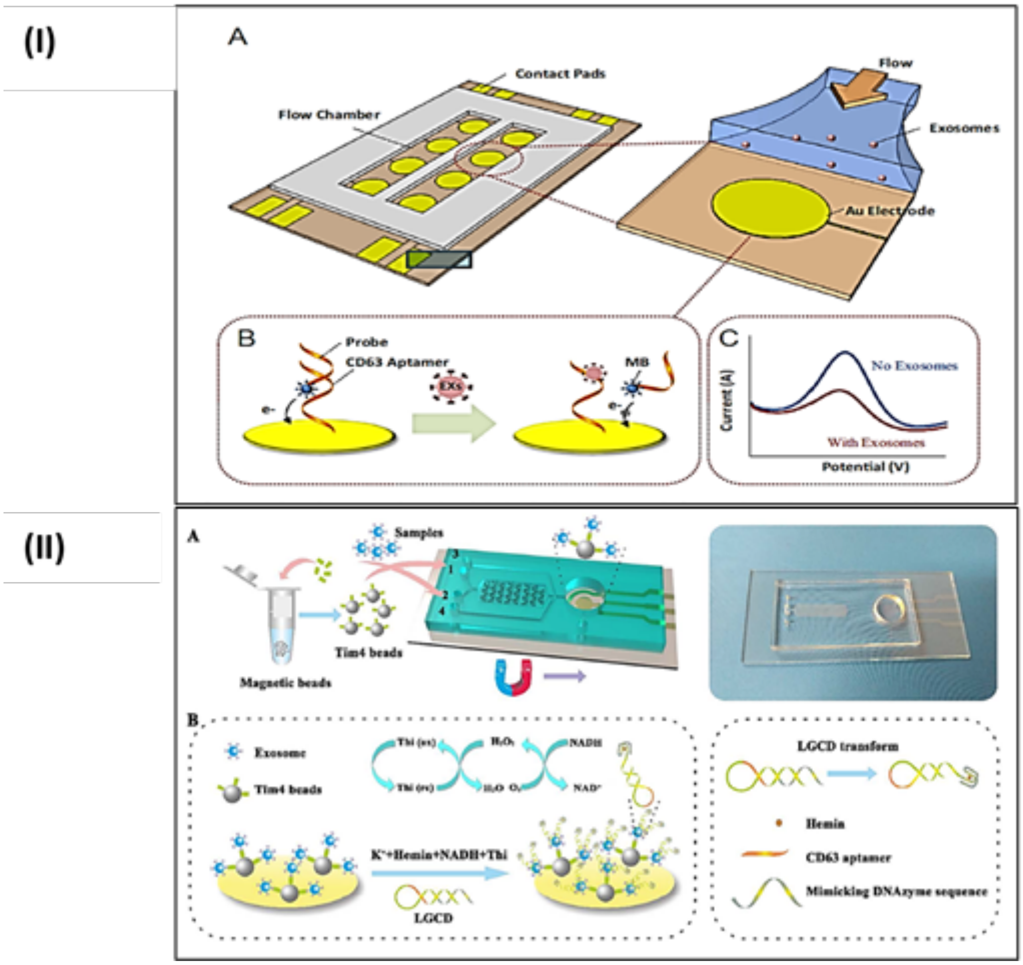

Figure 6. Recent advancements of microfluidics-integrated electrochemical detection of exosomes. (I) electrochemical aptasensors on a microfluidics chip for detection of CD63 exosomes (127) (II) a two-stage microfluidic platform (ExoPCD-chip) which integrates onchip isolation and in situ electrochemical aptasensor of CD63 exosomes (128).

9. Conclusions

In summary, we reviewed recent encouraging results in the detection of cancer-derived exosomes by optical, electrical and electrochemical biosensors. Each modality were categorized based on the principle of operation. Aspects such as detection of various exosomal biomarkers, limit of detection, detection time were discussed. Notably, in early cancer diagnosis, sensitivity and portability are major factors that researchers are constantly pursuing. Interestingly, with the integration of optical physics and polymer material chemistry into the development of biosensors, including the appearance of nanoparticles, nano-probes, and magnetic nano-beads, the sensitivity of biosensors has been excitingly improved in these several years. These techniques not only show advantages such as shortened detection time and enhanced sensitivity in the diagnosis and prognosis, but also have become a promising alternative to conventional techniques. Electrochemical biosensors are currently more for the detection of cancers based on quantification of cancer-derived exosomes, but more applications in other kinds of diseases will be expected in near future. In comparison, the electrical biosensors have been used for detection of different range of diseases. Nanomaterials and nanocomposites are playing an essential role in designing and fabricating robust and sensitive biosensors for disease diagnosis via exosome detection and they are expected to be used more in future researches and commercial products in this field. In addition, some new approaches such as microfluidics are needed for the development of next generation novel biosensors mostly for sample preparation and preconcentration. Researchers will not only focus on the protein markers or DNA/RNA marker of exosomes but also profiling the lipid components as continuous endeavor to discover new exosomal markers for disease detection. There is some progress in portable exosome detection devices that were reported here, and more are likely to appear in the future hopefully with higher sensitivity and lower detection limit. In a word, biosensors and nanobiosensors are believed to be highly promising in the field of exosomes detection towards POC liquid biopsy.

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