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Multiparametric Biosensors for Characterizing Extracellular Vesicle Subpopulations

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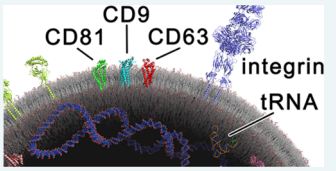
ABSTRACT: Extracellular vesicles (EVs) are an important intercellular communication conduit for cells that have applications in precision therapy and targeted drug delivery. Small EVs, or exosomes, are a 30−150 nm phospholipid-encased subpopulation of EVs that are particularly difficult to characterize due to their small size and because they are difficult to isolate using conventional methods. In this review, we discuss some recent advances in exosome isolation, purification, and sensing platforms using microfluidics, acoustics, and size exclusion chromatography. We discuss some of the challenges and unanswered questions with respect to understanding exosome size heterogeneity and how modern biosensor technology can be applied to exosome isolation. In addition, we discuss how some advancements in sensing platforms such as colorimetric, fluorescent, electronic, surface plasmon resonance (SPR), and Raman spectroscopy may be applied to exosome detection in multiparametric systems. The application of cryogenic electron tomography and microscopy to understanding exosome ultrastructure will become vital as this field progresses. In conclusion, we speculate on some future needs in the exosome research field and how these technologies could be applied.



KEYWORDS: *extracellular vesicles, exosome, tangential flow filtration, surface plasmon resonance (SPR), microfluidics, sensors, cryogenic electron tomography and microscopy*

Exosomes are a subpopulation of small extracellular vesicles (EV), which are collectively composed of various membrane-bound structures including apoptotic bodies, microvesicles, and ectosomes. They are a new type of targeted therapeutics that is naturally produced by cells and that can be exploited to develop new cell-specific medical interventions. Although the generic term EV is preferred by the International Society for Extracellular Vesicles (ISEV) when discussing these submicron vesicles, we will use the term exosome to refer to this smaller, and much more difficult to characterize, population of EVs. Following ISEV recommendations, a clear definition of exosome should be provided at each use[1] since there is some disagreement as to the proper nomenclature. In this manuscript, we will refer to exosomes as the smallest EVs, between 30 and 150 nm that are formed in multivesicular bodies and then actively secreted by almost all cell types into the extracellular space under physiological and pathological conditions.[2,3] They are endogenous communication vectors, participating in intercellular communication and transfer of cellular cargo directly into the cytosol of recipient cells. Exosomes contain bioactive molecules such as proteins and RNA, mainly miRNA that can modulate a range of functions in recipient cells.[4,5] Because exosomes are nanosized particles and express cell surface molecules, they have inherent target-specific homing and the ability to extravasate into tissues. Exosomes transmit biological signals from donor cells to recipient cells (even crossing the blood−brain barrier), including signals to distant targets through peripheral circulation. However, due to their size, exosomes are difficult to isolate, characterize, and manipulate in biological processes.

Recent research has uncovered important pathways of exosome biogenesis, regulation of their release, heterogeneity of their morphology, and the biomarkers that characterize exosomes produced during disease. Many recent studies have shown that most exosomes express several surface biomarkers such as cluster of differentiation 81 (CD81), CD9, and CD63 and contain various forms of nucleic and ribonucleic acid (DNA and RNA).[6] Yet, there remains a lot we do not know about exosome structure and function. Although many studies treat exosomes as an identifiable cluster of nanosized vesicles, mounting evidence suggests that, even at the nanoscale, there are likely subpopulations of exosomes, each with a different cargo. In particular, elucidation of exosomes has been hampered by two very difficult technical challenges: (1) sufficiently robust separation techniques to differentially isolate heterogeneous exosome subpopulations, and (2) the inability to characterize exosome ultrastructure at the nanoscale with sufficient resolution to determine intravesicular morphology. There are several excellent reviews of exosomes that have

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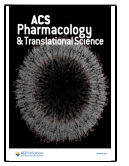

comprehensively discussed current methods of isolation and characterization.[7] In this review, our focus is to examine techniques that could potentially be applied to multiparametric systems that could identify subpopulations of exosomes. We will examine some of the existing technologies used to isolate and characterize exosomes with the view that multiparametric approaches, ones that both isolate and characterize exosomes, are likely on the horizon of exosome breakthroughs. Furthermore, we will endeavor to discuss these technologies with respect to the challenge of exosome size—the nature of the challenge and how it can be overcome.

1. GENERATION OF EXOSOMES

Exosomes are produced via a sophisticated and tightly controlled intercompartmental exchange process that requires deformation, fission, fusion, and vesicle formation as has been extensively reviewed by others.[8] The process begins when the plasma membrane invaginates, forming early sorting endosomes (ESE) and then later sorting endosomes (LSE). As LSE mature, they undergo inward budding and formation of multivesicular bodies (MVBs) through the endosomal sorting complex required for transport (ESCRT) or an ESCRT-independent process, which results in MVBs with a very complex intravesicular structure,[9] specific phospholipid composition,[10] and cargo such as ubiquitin.[11] MVBs migrate to the plasma membrane and are released into the extracellular space. Much of this early work was done in the reticulocyte system[10] where phospholipids were used to track the fusion and fission of these structures. How MVBs fuse with the plasma membrane is still unclear, although there is evidence that MVBs that contain high amounts of cholesterol are more likely to release exosomes[12] and a recent study using Fourier transform infrared (FT-IR) spectroscopy indicated that fatty acid content may influence protein content in some exosomes.[13] Throughout this process, multimolecular complexes are formed on the vesicle membranes to allow for membrane fusion and to overcome the energy barriers of plasma membrane merging to achieve vesicle reconfiguration.[14] Many of the markers that we use to detect exosomes are, in fact, important components of these multimolecular complexes. For example, tumor susceptibility gene 101 (TSG101) in ESCRT recognizes disulfide bonds and induces endosomal membrane depression.[11] MVBs can fuse with endolysosomes that contain degraded proteins or waste products, shuttling them to the plasma membrane, and therefore all EVs were once thought to be a cellular waste disposal system.[10,15] The process of cargo packing at these various stages is an active area of investigation, and some recent work in insects suggests that proteins involved in nucleocytoplasmic trafficking such as exportin 6 may be involved,[16] which may explain why exosomes contain so many different forms of RNA.[17]

Exosomes can potentially carry several types of cargo including proteins, lipids, nucleic acids, and metabolites (Figure 1). Protein cargo often includes proteins involved in their biogenesis such as TSG101, programmed cell death 6 interacting protein (PDCD6IP or ALIX), GTPase proteins, heat shock proteins (HSP70 and HSP90), and tetraspanins (CD9, CD81, and CD63). Nucleic acids include mRNAs, long noncoding RNAs (lncRNAs), microRNAs (miRNAs), transfer RNA (tRNA), and mitochondrial DNA (mtDNA). Metabolic analysis shows that exosomes may contain active enzymes and generate metabolites even after release from cells.[18]

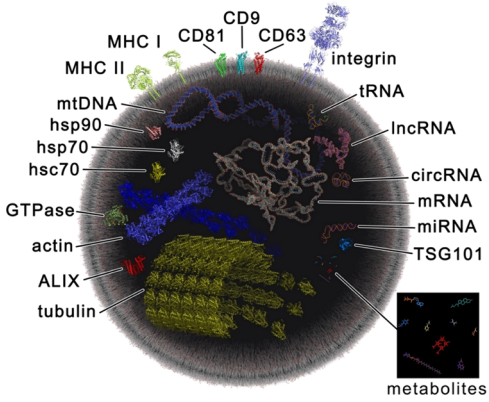

Figure 1. All-atom-scale model of a human exosome. The cross section of a 100 nm particle is shown containing some of the most common markers. Numerous types of nucleic acid molecules are represented including circular RNA (circRNA), long noncoding RNA (lncRNA), messenger RNA (mRNA), transfer RNA (tRNA), mitochondrial DNA (mtDNA), and microRNA (miRNA). Cytoskeletal proteins are commonly detected in exosomes. In this image, we are representing two fibers, actin (blue) and tubulin (yellow). Three types of chaperone proteins are shown, namely, hsp70, hsp90, and hsc70. Membrane proteins include MHC I and II as well as CD81, CD9, CD63, and integrin. Other markers including GTPase, ALIX, and TSG101 are shown. Exosomes are a rich source of metabolites (inset box), which include amino acids, cofactors, cholesterols, and many other lipid varieties. This image was generated using multiple software as previously described.[20] Accession numbers were obtained from the protein databank.

Due to their complex cargo profiles, exosomes (along with other types of extracellular vesicles) play an important role as messengers in cell-to-cell communication and participate in regulatory processes, especially cancer.[19] Exosomes have the capability to selectively enter cells and deliver a molecular cargo by bypassing the endolysosomal pathway, thereby protecting their cargo from degradative pathways. The tissue-specific binding properties, efficient intracellular delivery of content, and favorable size of exosomes make them attractive tools as novel carriers for drug and exogenic nucleic acid delivery in cancer therapy, regenerative medicine, and immunotherapy.

Once an exosome reaches its target cell, its internalization and intracellular fate depends on the pathway used to internalize the exosome. At least five uptake mechanisms may be involved in the cellular internalization of exosomes, including a clathrin-dependent pathway, micropinocytosis, lipid-raft, membrane fusion, and caveolin-dependent endocytosis. The contribution of an individual pathway to the internalization of exosomes likely varies among the cell type, cell cycle, culture medium, and the origin of the exosomes.

2. EV SUBPOPULATIONS COME IN ALL SHAPES AND SIZES

Even when isolated from a single cell line and using a single technique, exosomes have different shapes and sizes that can only be characterized using cryogenic electron microscopic (cryoEM) analysis. Other methods, such as flow cytometry, can perhaps differentiate large differences in size based on forward scatter and size light scatter, but they do not have the resolution to distinguish between different morphologies. Detailed cryoEM analysis of exosomes isolated from a single cell type indicates that although the majority of these exosomes

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Table 1. Advantages and Disadvantages of Conventional Exosome Isolation Methods Based on Size, Density, and Protein Biomarkers

	working principle	advantages	disadvantages
differential centrifugation	size-based separation under sequential alternative low and high centrifugation speeds	low cost, simple operation, suitable for large sample volumes	potential damage to exosomes, time-consuming, low yield, specific equipment required
precipitation	size- and density-based separation by adding polymer that alters exosome density in solution	high capacity, simple operation	low purity, cleanup steps required, time-consuming, presence of contaminants
ultrafiltration	size-based separation by trapping exosomes in nanoscale membranes	simple operation, capable of operating with a small volume of sample, fast procedure	membrane clogging, possible damage to exosomes caused by stress, moderate purity
immunoaffinity	use of antibodies to capture exosomes	high purity and selectivity, isolation of specific exosome subpopulations	nonspecific binding, low processing volume and yield, exosomes are attached to beads requiring elution steps

appear to be spherical, there are multimembranous, long tubule-like, ovoid, and filamentous structures that would be difficult to detect by any other approach than cryoEM. However, these diverse exosomes likely contain diverse cargo due to the physical capacity of their internal space (Figure 1). It is reasonable to assume that only large spherical exosomes can accommodate the relatively large tubulin polymers, whereas smaller and irregularly shaped exosomes can carry RNA or metabolites.

For example, we have used all atom-molecular models (similar to the model in Figure 1) to estimate that the ratio of volume to outer surface area for a 30 nm exosome is 1.6, whereas a 100 nm exosome would have a ratio of 12.3. Simply put, the smaller exosome would have 8 times more lipid cargo as a part of its membrane than its 100 nm counterpart in a sample of equivalent mass. Smaller exosomes (less than 55 nm) would also be incapable of accommodating larger molecular cargo such as a tubulin fiber, itself 28 nm in diameter, or larger nucleic acid molecules like mRNA, lncRNA, and mtDNA (Figure 1). The curvature of the outer surface of smaller exosomes is significantly greater, impeding complex multireceptor/co-receptor/ligand interactions with other surfaces that could play a role in exosome entry. Exosome filtration by the liver and tissue access would also be greatly affected by size. Undoubtedly, size-dependent characteristics have a tremendous impact on exosome function. Thus, effort must be placed on the development of approaches that can subfraction purified exosome samples according to particle size. This is explored further in Section 6. At present, we are unable to isolate these exosome subpopulations to test this hypothesis. Therefore, isolation methods that can enrich these subpopulations are needed.

3. ISOLATION TECHNIQUES

The potential of exosomes in diagnostic and therapeutic applications is vast, but there are also many challenges associated with working with these nanostructures. Their submicron size and very wide size range (between 30 and 150 nm), as well as their coexistence with other small particles in bodily fluids make exosomes extremely difficult to isolate, manipulate, and sort for characterization through imaging and other techniques. Differential centrifugation protocols include a high-speed ultracentrifugation step that does not distinguish exosomes from vesicles of nonendosomal origin such as structural vesicles that are derived from outward plasma membrane budding or nonvesicular structures such as the newly identified exomeres.[21,22] Many experts in the field have argued that the ultracentrifugation and density gradient isolation protocols produce exosomes that are heavily contaminated with nonexosomal subpopulations.[17]

Current separation techniques to isolate exosome subpopulations from body fluids such as blood, urine, and cerebral spinal fluid and in cell culture supernatants generally rely on three physical properties of exosomes: size, density, and protein biomarkers.[23] Within the context of each of these properties, several methods can be applied (Table 1) and each of these methods is associated with certain advantages and disadvantages. Recently, advances in nanoelectronics and sensing platforms have allowed for less invasive mechanobiological approaches based on inertial lift force, viscoelastic flow, and acoustic waves,[23] which presumably preserve the ultrastructural integrity of exosomes during processing.

Scalable methods to produce and purify exosomes from cells are lacking, which is a significant limitation to the biological understanding of exosomes. Bioreactors have been used for the generation of clinical-scale quantities of therapeutic cells and recently they have also been adapted for large-scale exosome production. In particular, hollow fiber bioreactor technology involves seeding cells into cylindrical hollow fibers through which media flows continuously resulting in the production of 4-fold more exosomes than from a traditional 2D flask.[24,25]

Any technologies applied to the culture of cells prior to the isolation and characterization of exosomes must be used with caution since they can significantly impact the outcome of exosome production and cargo composition. Cells can be seeded in any cell culture system under a variety of culture conditions (in the absence and presence of inflammatory mediators) to produce exosome-rich conditioned media (CM). Exosomes from CM can be concentrated and purified using tangential flow filtration (TFF), a scalable concentration and buffer exchange strategy used during large-scale manufacturing of biologics and viruses. Exosomes isolated using TFF can be compared side-by-side to ultracentrifugation (UC), the current gold standard of exosome isolation.[26] TFF isolates exosomes according to their size, whereas differential UC relies on both vesicle size and sedimentation properties. Traditionally ultracentrifugation involves two main variations: modifying centrifugal forces and using density gradients. CM from cells is first subjected to a centrifugal force of 3000−10,000g to remove contamination from cell debris followed by 10,000−20,000g spin to remove organelles and nonexosomal vesicles, then a last centrifugation step of 10,000−120,000g to obtain a final pellet of the exosomes. Exosomes can be further purified from other vesicles via flotation using density gradients made of sucrose cushions or commercially available reagents, such as iodixanol. Although UC can be used for small-scale operations,

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Table 2. Microfluidic EV Isolation Methods

isolation method	advantages	disadvantages
filtration	minute volume of samples (μL, nL, pL, fL), adjustable microchannel size (nm to μm), simple assembly and operation	complicated and time-consuming fabrication process, clogging
inertial lift force	rapid processing with controlled pressure and speed	co-isolation of small particles
viscoelastic flow	high yield with easy automation and integration	addition of reagents
acoustic waves	high purity and efficiency	requires external force, potential damage to exosomes

it has significant limitations for larger volumes of conditioned media. Another frequently used method of isolation is immunoaffinity capture, which uses antibodies that recognize specific epitopes expressed on exosomes to bind and precipitate them.

It is unknown how many naturally occurring exosome subpopulations are released by host cells, as well as how they vary in cargo content and how this diversity in cargo composition may lead to differing functions for each population. At least two exosomal subpopulations have been confirmed and analyzed,[27,28] they are classified as large and small exosomes, with sizes ranging from 90 to 120 nm and from 60 to 80 nm, respectively. An in-depth particle composition analysis revealed significant differences between and within each subclass of exosomes.[28] For example, the expression of classic exosome markers, such as CD9, CD63, and CD81, varied significantly in distribution between the two identified subclasses, indicating a heterogeneity within themselves and suggesting the presence of many other unidentified subpopulations. A selective packaging of proteins, glycans, lipids, and nucleic acids in exosomes is believed to form distinct subsets.[28] Considering that this heterogeneity in cargo composition between each subpopulation promotes a specific response on target cells, selective packaging may cause different functionalities, properties, and biodistribution of these nano-size particles. A better understanding of the biology of exosomes will allow insights into cellular communication, biogenetic mechanisms, molecular composition, functionality, and biodistribution. However, due to the tight relationship between exosomal heterogeneity and size, the advancement in our knowledge of exosome biology depends significantly on the development of innovative sensing techniques and isolation methods capable of dividing the smallest exosome subpopulations.

These methods isolate exosomes as a whole population and are incapable of further subdividing exosomes into smaller size ranges. In addition, these common methods tend to be time-consuming, user-dependent, and result in low isolation yields (Table 1). Therefore, microfluidics-based methods are emerging as promising techniques for exosome isolation.[29] Microfluidics allows for the rapid separation of particles in minute volumes and can isolate particles with high yield and purity. Some of the microfluidics techniques that have been applied to exosome isolation use filtration, inertial force, viscoelastic flow, and acoustic waves as methods of isolation (Table 2), but each approach must be carefully chosen because each has advantages and disadvantages.[23]

Microfluidics is the flow of a minute volume of liquid under controlled pressure and speed, usually pushing liquids through microchannels ranging in size from 100 nm to 500 μm. This approach also allows for microfluidic mixing which has recently been used for the design of vaccines. Microfluidic analysis of exosomes is normally modular, meaning that they often have several functionalities built into their process such as immunoaffinity capture and various separation strategies. Much of this research has been driven by the cancer field because exosome isolation and characterization is believed to be the new frontier in noninvasive cancer diagnosis.[30] However, other applications such as vaccine development and drug delivery to the brain[31,32] are also becoming active areas of investigation. Many recent reviews have been published on the use of microfluidics in exosome isolation[30,33,34] and therefore we will only briefly touch on a few important features of microfluidic isolation of exosomes. First, microfluidic approaches are particularly attractive because they provide a rapid, portable, and highly selective approach for exosome isolation and do not normally require specialized equipment (Table 2). Fabrication of microfluidic devices, however, is time-consuming, expensive, and requires highly specialized equipment that must be maintained in clean room fabrication facilities. This has made microfluidic approaches less accessible to biologists and clinicians.

Despite fabrication challenges, microfluidic systems have the potential to integrate different mechanical parameters into one practical, inexpensive, and efficient device. The centrifugal microfluidic disc with functionalized membranes proposed by Zhao et al.[35] is an example of such a multiparametric system that can isolate exosome from whole blood samples. The disc is composed of engraved units that communicate with each other by capillary channels and consists of two polycarbonate (PC) layers with the bottom layer engraved with six hydrophilically modified functional units (a loading chamber, a chamber for whole blood separation, a mixing chamber, an input for exosome loading buffer, an exosome isolating buffer chamber, and a commutator unit). There are two functional membranes positioned before and after the mixing chamber, where the first membrane is used to filter blood cells and larger particles, and the second membrane is used to enrich the exosomes by adsorbing the negative charge on their surfaces. The microfluidic disk[35] was capable of isolating and purifying exosomes from blood samples with high yields and reasonable concentrations.

The design of the microfluidic chambers can have a significant effect on the mixing and interaction of the exosomes with functionalized surfaces. Under some circumstances, this approach can increase sensitivity when the number or concentration of exosomes in a sample is very small—like in a tumor tissue. For this reason, some designs have used self-assembled 3D herringbone nanopatterns where a PDMS chip containing patterned herringbone channel arrays is positioned between clean glass slides. A solution of silica colloids can be injected into the microchannels where co-assembly of colloids

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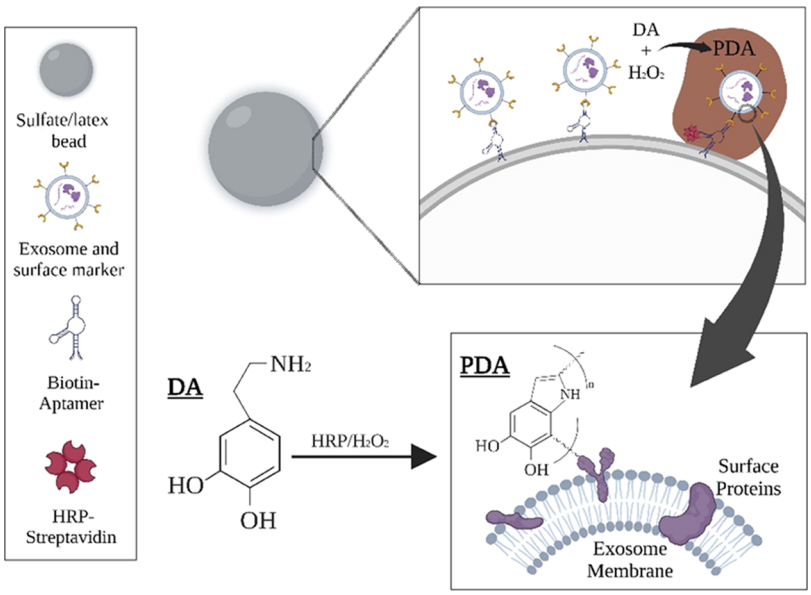

Figure 2. Schematic illustration of the proposed ExoAptaSensor with HRP-accelerated dopamine polymerization and deposition for exosome detection. Exosomes anchored on sulfate/latex beads were captured by biotin-conjugated aptamer specific to CD63, followed by incubation with streptavidin-conjugated HRP for colorimetric reaction to generate brown-black-colored polydopamine. The oxidation and polymerization processes were accelerated under HRP catalysis and hydrogen peroxide (H_2O_2) as the oxidant.[46]

with different sizes is promoted. The packed device is then dried and peeled off the patterning chip and can be used to detect low levels of exosomes associated in tumors.[36] This device allows for greater sensitivity because the nanopatterns promote microscale mass transfer, increasing surface area and probe density to improve particle−surface interactions for exosome binding.[37]

4. DETECTION TECHNIQUES

Although the isolation techniques discussed above are essential to understanding exosome biology, a truly innovative future multiparametric device must also be able to detect exosomes—and thereby monitor its own success at isolating these populations. In recent years, a series of strategies have been developed for sensitive and multiplexed detection of exosome biomarkers, such as proteins and nucleic acids. Western blot analysis,[37] enzyme-linked immunosorbent assay (ELISA),[38] and mass spectrometry have been used in these multiparametric approaches on the same device.[39] However, since these approaches rely heavily on the starting purity of the exosome source, their success is sample-dependent, and processing can be time-consuming. Therefore, there is an increasing need for more sensitive and reproducible methods to detect exosomes, especially in complex mixtures such as urine, saliva, and liquid biopsies. Numerous biosensors have been developed for the sensitive detection of exosomes, such as colorimetric, electrochemical, fluorescent, surface plasmon resonance (SPR), and surface-enhanced Raman scattering (SERS) biosensors, which we will discuss below.

5.1. Colorimetric Biosensor. The colorimetric method is one of the most user-friendly approaches in biosensing and has been used in clinical point-of-care (POC) applications due to its easy operation, convenient readout, and cost-effectiveness. Colorimetric biosensors can be easily and instantly observed with the naked eye through a color change that yields a "yes/no" answer or semiquantitative result without any additional analytical instrumentation.[40] However, these traditional colorimetric methods usually employ organic chromogens such as 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS) or 3,3′,5,5′-tetramethyl benzidine (TMB) as a colorimetric substrate, and their sensitivity is often limited because the extinction coefficients of the organic chromogens are low. Since these strategies also depend on the alteration of optical density of one color, they require specialized equipment to detect these slight variations and provide quantifiable readouts.[41] To improve the sensitivity of detection, various signal amplification strategies can be employed, including those based on the use of nanomaterials.

Colorimetric biosensors can be categorized into different groups based on the properties of nanomaterials. For example, nanomaterials with innate optical properties (such as colloidal AuNPs) and catalytic properties (such as Fe_3O_4 NPs)[42] can be used when fabricating these biosensors. The biosensor can also be optimized based on the targets, such as nucleic acids and proteins, that they are designed to detect.[43] Finally, biosensors can be categorized based on the formats of detection: paper-based or solution-based.[40] The colorimetric biosensors platform has potential utility as a high-performance screening tool in point of care in clinics because it can be detected rapidly without complicated sensing equipment, making it low cost.[44]

Many sensor platforms for exosomes are based on antibodies that can bind specifically to biomarkers such as CD81 and CD63 on the surface of the exosome. However, antibodies have limitations (low affinity, instability, and expensive to produce), and therefore these sensors have similar limitations. A new way of designing specific binding surfaces for exosomes is the use of aptamers, short DNA or RNA oligonucleotides with affinities for exosome biomarkers. Unlike antibodies, aptamers are stable, easy to synthesize (especially in scale-up manufacturing), can bind nonprotein targets, have high affinity, are easily modifiable, and have controllable specificity.[45] One very large advantage of aptamers is that they are more readily absorbed by the tissues and therefore can penetrate areas that antibodies cannot. Recently, a colorimetric aptasensor has been

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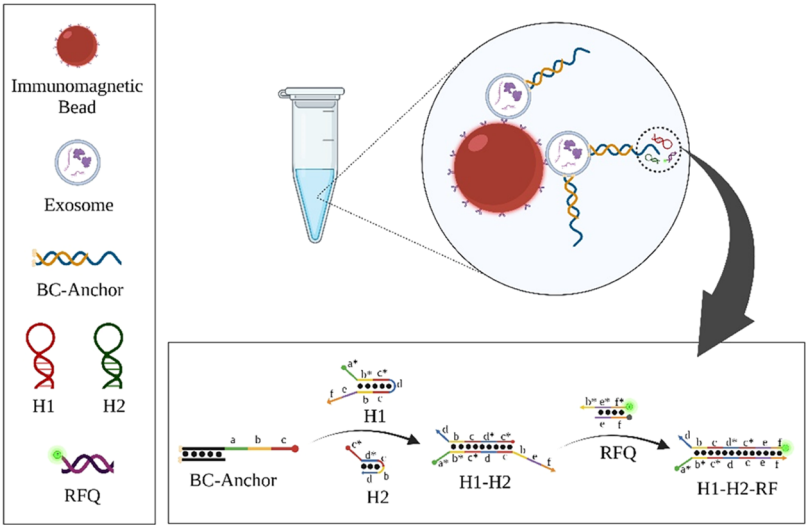

Figure 3. Schematic illustration of the proposed method for the quantitative evaluation of exosome based on magnetic separation and enzyme-free signal amplification. Anti-CD63 antibodies were immobilized on carboxylic acid-functionalized magnetic beads (MBs). The exosomes sample was mixed with anti-CD63 MBs in a microtube, where the exosomes were captured on the surface of anti-CD63 MBs through antibody−antigen reactions. Subsequently, the BC-anchors were introduced and spontaneously inserted into the lipid bilayer membrane of exosomes captured on anti-CD63 MBs. Region a of the BC-anchor can function as a toehold to trigger interactions with the exposed region a of H1. Next, the newly exposed region c of H1 is free to hybridize with the toehold region c* of H2 to form the H1−H2 duplex. The exposed toehold region of the H1−H2 duplex continues to hybridize with region b* of RFQ. The hybridization triggers the branch migration reaction to displace the RQ quencher, which restores the RF fluorescence signal in the obtained H1−H2−RF complex (adapted from Wang et al.).[50]

developed for exosome detection (ExoAptaSensor) which uses horseradish peroxidase (HRP) accelerated dopamine (DA) polymerization and local polydopamine (PDA) deposition.[46] A biotinylated aptamer specific for CD63 (Figure 2) and streptavidin-conjugated HRP was used to bind to the exosome, followed by rapid addition of freshly prepared DA solution (colorless). HRP accelerated the colorimetric reaction leading to the formation of a colored product (PDA) after polymerization. The color intensity correlated to the presence of CD63 (and therefore exosomes) and the limit of detection was 7.7×10^3 particle/mL such that the signal intensity improved by 3−5 orders of magnitude from conventional dot-blot methods.[46] Overall, this approach was successful at detecting small concentrations of exosomes but further studies are needed to determine whether it is capable of detecting exosomes in a clinical sample. If this approach is able to detect exosomes in body fluids such as urine or saliva, it could be adapted to a point-of-care device for cancer early detection and diagnosis.

5.2. Fluorescent Biosensor. Compared to colorimetric detection, fluorescence-based sensors are much more sensitive since they can measure photons of light emitted when a material is excited at a specific wavelength. The high accuracy and sensitivity of fluorescence imaging technology have made it a widely used method in exosome detection systems. In recent years, the fluorescence biosensors that have been developed for exosome detection have been based on three system formats: paper-based, solution-based, and microfluidics-based.[47]

Since specificity can sometimes be an issue, paper-based sensing platforms often require that samples are pre-conditioned in some way such as microfluidic pre-concentration or heating. Streptavidin agarose resin-based systems, coupled with ELISA with anti-CD63 capture antibodies, can detect exosomes in cell culture supernatants, even showing some specificity when FBS-depleted media is used.[48] Surasak et al. built a proof-of-concept paper-based ELISA that used a fluorescently conjugated anti-CD9 antibody and a chromatography filter paper laminated surface to detect exosomes from cell culture of human ovarian cancer cells using a fluorescent microscope.[49] Although this device could detect some fluorescence in very highly concentrated samples of exosomes (10^{10} exosomes/mL), there was considerable variability and other components in the culture media appeared to interfere with sensitivity.[49]

Solution-based fluorescent biosensors are generally based on the formation or release of fluorescent nanoparticles or fluorophores. A fluorescence assay can be developed based on a combination of immunomagnetic separation and a two-step signal amplification strategy for direct isolation and subsequent detection of exosomes. Immunomagnetic beads can be used to capture and enrich exosomes via common biomarkers such as CD9 or CD63. In one such system, bivalent cholesterol (BC) anchors were spontaneously inserted into the lipid bilayer of bead-captured exosomes, amplifying the signal and creating a binding site to trigger interactions with DNA oligonucleotides (H1 or H2; Figure 3). The enzyme-free DNA circuits bound to the BC anchor hybridize, creating the fluorescent signal. This system can detect exosomes ranging from 5.5×10^3 to 1.1×10^7 particles/μL with a limit of detection of 1.29×10^3 particles/μL but required a fairly pure sample of exosomes and the entire system relied on the specificity of the antibody to CD63.[50]

The microfluidics-based fluorescence detection method is increasingly used in exosome detection approaches because of its sensitivity and adaptability to multiparametric systems. A simple, low-cost, microfluidic-based platform was developed to isolate cirEVs enriched in exosomes directly from blood serum allowing simultaneous capture and quantification of exosomes in a single microfluidics device by Kanwar et al.[51] To capture specific exosomes, they employed "ExoChip", a microfluidic device fabricated in polydimethylsiloxane (PDMS) and immunolabelled with antibodies specific for CD63. The

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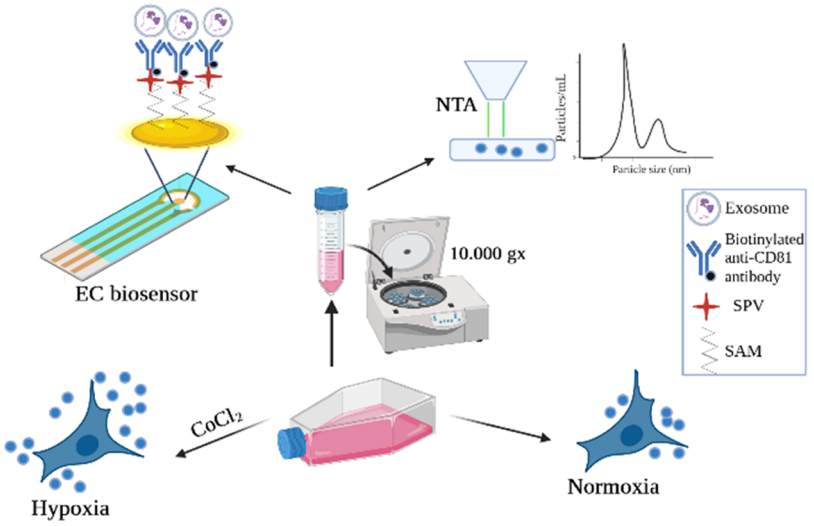

Figure 4. Experimental steps followed for exosome detection using a label-free electrochemical (EC) biosensor. MCF-7 cells were exposed to either $CoCl_2$-induced hypoxic or normoxic conditions. Isolation of EVs was done via ultracentrifugation. Characterization and quantification of EVs were done via NTA and EV biosensors that are designed to capture CD81 EV biomarker via biotinylated anti-CD81 antibody immobilized through streptavidin−biotin interaction on SAM-modified Au SPE surface (adapted from Kilic et al.).[53]

immunolabelled exosomes were stained with a fluorescent carbocyanine dye (DiO) and quantified using a standard plate reader. Although this type of approach is sensitive, it once again relies on the identification of a surface biomarker on exosomes and is biased only to that subpopulation that expresses that particular biomarker. Moreover, this approach does not discriminate the exosomes according to size, allowing for the simultaneous analysis of the bulk distribution of exosome populations.

5.3. Electrochemical Biosensor. An electrochemical biosensor is an analytical device that has a recognition element (e.g., antibody, aptamer) that can specifically bind to an exosomal biomarker. Once bound, the electrochemical signal altered by binding of the exosomes can be used to quantify its presence relative to an electrical signal (e.g., current, voltage, impedance, etc.). Electrochemical assays have a wide range of advantages in the exosome detection field because of the small sample volume needed, low cost, simplicity, and ability to detect trace amounts of biomolecules. Electrochemical integrated with magnetic enrichment have profiled protein expression and shown that cancer cell lines release exosomes that closely resemble the parent cells.[52] These types of approaches once again use typical exosome surface markers such as CD63 to enrich the exosomes which does not account for different-size populations and biases the analysis to $CD63^+$ exosomes. However, this approach allows for rapid characterization of plasma samples from cancer patients, making it a potentially powerful diagnostic tool.

A label-free electrochemical (EC) biosensor can detect EVs released from a breast cancer cell line (MCF-7) due to $CoCl_2$-induced hypoxia.[53] The biosensor can monitor changes in electrochemical signals due to bio-recognition reaction between anti-CD81 antibody and CD81 present on the lipid membrane of breast cancer EVs. Figure 4 summarizes the method by which MCF-7 cells were exposed to either $CoCl_2$-induced hypoxic or normoxic conditions and then isolated via ultracentrifugation. Characterization and quantification of EVs were done via nanoparticle tracking analysis (NTA) and EVs biosensors that are designed to capture CD81 EVs biomarker via biotinylated anti-CD81 antibody immobilized through streptavidin (SPV)-biotin interaction on thiol terminated self-assembly monolayer (SAM) modified Au SPE surfaces.[53] The detection limit (LOD) of this device was 77 EVs/mL with a dynamic detection range of 10^2−10^9 EVs/mL which was a large improvement over some of the fluorescent and colorimetric systems discussed above. Due to its sensitivity, this biosensor could detect EVs in serum samples but could also be adapted to detect exosomes in tissues, such as tumor microenvironments for chemotherapeutic drug testing. Although this approach can detect bulk EV populations, it cannot resolve subpopulations, and once again relies on antibody specificity to one exosome biomarker, CD81.

5.4. Surface Plasmon Resonance (SPR) Biosensor. Surface plasmon resonance is a label-free, real-time analysis technique that can detect molecular interactions on a surface and measures the resonant oscillation of electrons stimulated by the incident light at the interface between a negative and a positive dielectric constant material. The oscillating is extremely sensitive to small changes in the refractive index within the dielectric near the sensing interface, where various binding events and interactions can be detected. Hence, this technique is extremely sensitive to biological binding events occurring within 200 nm (wave depth) of the gold layer, which closely matches the dimension of exosomes and is therefore well suited for the study of exosomes.

Liu et al. developed a localized surface plasmon resonance (LSPR) sensor chip that could detect small amounts of exosomal biomarkers.[54] Self-assembly silver nanoparticles decorated on gold nano-islands (Ag@AuNIs) sensor chip were used to provide site-specific bio-conjunction of biotinylated antibodies (anti-CD9 ABs, anti-CD63 ABs, anti-EGFRvIII (epidermal growth factor receptor variant III) ABs, anti-MCT4 ABs) for the detection of exosomal surface biomarkers. GM-derived exosomes were isolated from the blood serum of glioblastoma multiform (GBM) mice using an EGFRvIII-based immunocapture method. The biotinylated antibody functionalized (BAF) Ag@AuNIs LSPR biosensor sensitively detected CD63, an exosome marker, and monocarboxylate transporter 4 (MCT4), a GBM progression biomarker, in malignant GMs-derived exosomes in the

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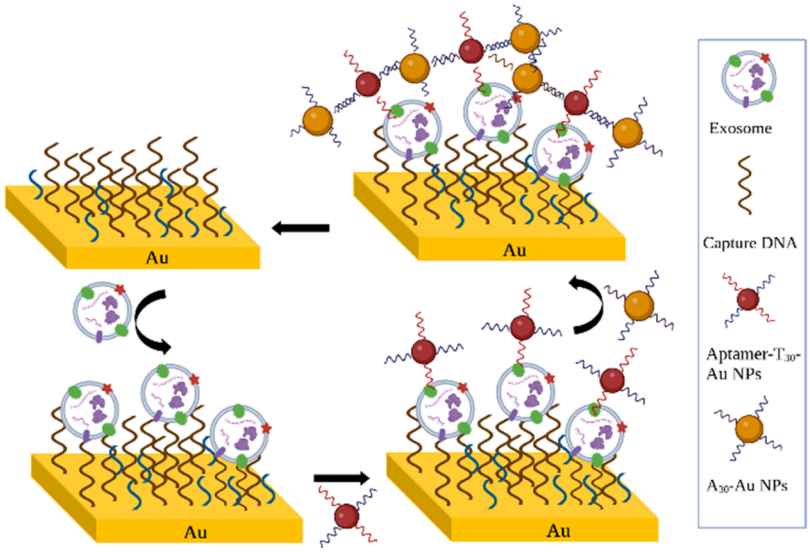

Figure 5. Schematic illustration of dual AuNP-assisted signal amplification for detection of exosomes. First, the Au film was functionalized with capture DNA and the target exosomes were detected by direct measurement. Next, aptamer/T_{30}-linked AuNPs were added, and target exosomes were detected by a single AuNP-amplified SPR aptasensor. Finally, A_{30}-coated AuNPs could be captured on the aptamer/T_{30}-linked AuNPs through the hybridization of two complementary sequences of T_{30} and A_{30}. The target exosomes were detected by dual AuNP-amplified SPR aptasensor (adapted from Wang et al.).[55]

dynamic range of 4 × 10 to 50 μg/mL with LOD of 0.4 ng/mL.

Qing Wang et al. also fabricated a sensitive aptasensor for exosomes detection using SPR with dual gold nanoparticle (AuNP)-assisted signal amplification (Figure 5). Dual nanoparticle amplification was achieved by controlled hybridization attachment of AuNPs resulting from electronic coupling between the Au film and AuNPs. Nonspecific adsorption of AuNPs onto the SPR chip surface was suppressed by blocking the Au film surface with 11-Mercapto-1-undecanol (MCU), allowing for the regeneration of the SPR sensor. This method was highly sensitive with a LOD of 5 × 10^3 exosomes/mL, which showed a 10^4-fold improvement in LOD compared to commercial ELISA.[55]

5.5. Surface-Enhanced Raman Scattering (SERS). Raman spectroscopy has also been used to characterize exosomes. However, this is a very inefficient process and only one in 10^{6-8} photons is scattered when performing the analysis. Therefore, a high sample concentration is required in combination with high laser power and long signal integration times. The Raman signal can be strongly enhanced up to 10^{14-15} times using SERS.[56] SERS is based on the enhancement of the incident and scattered electromagnetic field by plasmon excitation on metal surfaces, typically composed of gold, silver, or copper, to basic metals, such as iron (Fe), cobalt (Co), and nickel (Ni), and some semiconductor materials, as well as their nanocomposites.[50] As it has single molecule sensitivity, SERS is increasingly applied to the characterization of biological samples. Many types of SERS substrates have been developed to obtain plasmon enhancement and record Raman spectra from cellular components down to the single biomolecule level.[57] In recent years, SERS has been increasing as a tool for exosome detection. The detection can be divided into two categories: label-free exosome detection and exosome detection with SERS tags. Since the detection sensitivity relies directly on the interaction between substrates and optical properties, for both mentioned categories, the noble metallic structure is especially essential for efficient SERS substrates.[58]

6. NEXT STAGE OF EXOSOME BIOSENSORS AND FUTURE PERSPECTIVE

The unique role of exosomes as intercellular transport vehicles has placed them as a leading target of therapeutic development. While we continue to make advances in our understanding of these small vesicles, some areas of innovation are required for this field to make significant advances.

The unique spectrum of exosome size puts these particles at a range comparable to extremely small viruses. Structural studies of such small particles require cryogenic electron tomography and microscopy methods that allow the study of viral matrixes, capsids, and membrane spike proteins. To date, these approaches have been unable to detect ultra-structures in exosomes; thus, we are limited to analyzing individual molecular components through mass spectrometry, nucleic acid sequencing, and NMR. As a result, we are unable to determine if exosome surface markers are associated with unique cargo or if there is a relationship between cargo and size. We know that exosomes display unique extracellular markers such as LAMP-2B, CD81, and other transmembrane structures;[59] however, the power of current electron microscopy instruments has not resolved their structure or interactions on the membrane. Emerging modern microscopes that can achieve molecular or atom-scale resolution will help us decipher key structural characteristics of the exosome. NMR and X-ray crystallography require pure molecular samples and thus are inadequate for elucidating structural characteristics of exosomes. NMR or X-ray crystallography, while powerful analysis tools of protein structure, are inadequate at examining larger and more complex exosomes. One approach that has not received much attention are *in silico* modeling approaches such as molecular modeling and machine learning. Although bioinformatics and in silico analysis of endogenous RNA have been used to examine cargo, these approaches have not been used to model overall 3D structure of the exosome itself. In silico all-atom exosome modeling can be instrumental in determining the possible cargo/size relationships within the

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exosome subpopulations—as we have done in Figure 1. However, these approaches require access to fast computer cores and until that technology moves forward, it will be some time before it is effectively applied to exosome research.

7. CONCLUSIONS

As mentioned above, exosome isolation approaches include differential ultracentrifugation, size exclusion chromatography, tangential flow filtration, immunoaffinity purification, and microfluidics capture.[60] These techniques are generally time-consuming and require dedicated equipment to process samples. The methodologies that are fast, require minimal equipment, and produce analysis-ready samples for biochemical and electron microscopy characterization are an important frontier of exosome research. The discovery of innovative nanotechnologies and employing renewable nontoxic materials would make access equitable across the scientific community.

Numerous studies have isolated exosomes from cell lines and resolved exosome and EV populations according to size.[61,62] Mounting evidence indicates that exosome size can fluctuate drastically depending on the cell type and its stimuli[61] with diameters ranging from 20 to 160 nm. In more complex samples, such as serum and saliva, there are countless cell types contributing to the final exosome population, thus generating undefined, broader size ranges, intrinsically difficult to fractionate into specific subpopulations. Although some researchers have used flow cytometry to isolate and gate exosomes,[63] they exist at the lower-resolution limit of this technology resulting in artifact generation and poor resolution.

Exosome heterogeneity derives from a complex and intertwined relationship between size, cargo composition, and biogenetic mechanisms. The natural occurrence of at least two subpopulations of exosomes released from host cells has been confirmed, as well as cargo differences between and within these subclasses.[27,28] Selective packaging of contents in EVs is believed to form distinct subsets.[28] This heterogeneity in cargo composition dictates the response on target cells and thus cause different functionalities of each subpopulation of exosomes. This is just a glimpse into the vast potential and complexity of these nano-size vesicles. Advancements in the knowledge of EV biology will provide insights not only into cellular communication but also into biogenesis mechanisms, molecular composition, biodistribution, and functionality. Better understanding of exosome biology is essential to harness the pharmaceutical potential of exosomes and to develop innovative new sensing platforms. Future exploitation of exosomes as dynamic biomaterials will require the development of engineered exosomes capable of delivering specific content to targeted cells. A better understanding of EV biology combined with the development of refined isolation and characterization techniques will most certainly revolutionize diagnostic and therapeutic applications.

Upcoming technologies in the study of exosomes need to address these critical challenges. First, new technology needs to generate approaches to separate exosome subpopulations with resolution as fine as 10−20 nm. The fact that exosomes are variable in both size and shape is crucial to understanding their function, but we are currently unable to separate these subpopulations and new technology will need to be developed to address this need. Second, separated subpopulations could be immobilized on a surface to allow for high-power electron microscopy and other biochemical analyses including RNA sequencing and mass spectroscopy. Importantly, future equipment should not rely on expensive platforms providing equitable access to the technology and a sustainable avenue for research in this field.

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Notes

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