Commercial and Emerging Technologies for Cancer Diagnosis and Prognosis Based on Exosomal Biomarkers

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Exosomes are nano-sized extracellular vesicles excreted by mammalian cells that circulate freely in the bloodstream of living organisms. Exosomes have a lipid bilayer that encloses genetic material used in intracellular communication (e.g., double-stranded DNA, micro-RNAs, and messenger RNA). Recent evidence suggests that dysregulation of this genetic content within exosomes has a major role in tumor progression and in the surrounding microenvironment. Motivated by this discovery, we focused here on using exosomal biomarkers as a diagnostic and prognostic tool for cancer. In this review, we discuss recently discovered exosome-derived proteomic and genetic biomarkers used in cancer diagnosis and prognosis. Although several genetic biomarkers have been validated for their diagnostic values, proteomic biomarkers are still being actively pursued. We discuss both commercial technologies and emerging technologies for exosome isolation and analysis.

Keywords: *Circulating tumor biomarkers, Extracellular vesicles, Biological nanoparticles, Liquid biopsy, Biosensing.*

1. Significance of Circulating Tumor Exosomes in Cancer Diagnostics and Treatment

Cancer arises through a series of somatic mutations that allow cells to proliferate unchecked [1]. 'Cancer genes' typically block the normal "safety stops" in a cell that halt or retard uncontrolled proliferation [2]; the unregulated cancer cell clones then form tumors. The gold standard for cancer diagnosis has traditionally been tumor biopsy, which can provide tremendous insight into the morphology and gene expression patterns of tumors but is highly invasive, relying on the physical sampling of a tumor for pathologic analysis [3, 4]. Shortcomings of tissue biopsy include the possibility of bleeding, organ damage, and missing the intended target, which can lead to misdiagnosis and ineffective treatment [5], and the fact that it cannot be used for early detection, i.e., before a visible tumor forms. Other less invasive means of obtaining information on tumors for cancer diagnosis and treatment are actively being sought.

Some examples of less-invasive assessments include blood-based biomarkers such as circulating tumor cells (CTCs), circulating tumor DNA (CTDNA), and other circulating protein biomarkers [1]. In principle, the ability to detect cancer biomarkers in the blood (i.e., "liquid biopsy") can enhance the accuracy of diagnosis or the detection of residual disease after treatment [2]. Both CTCs and CTDNA have been intensely studied for these purposes, but to date the utility of CTCs is limited to certain cancer types and has very low sensitivity, and that of CTDNA is still limited by its ability to detect minimal residual disease, as well as being expensive and time-consuming [6]. Moreover, CTCs and CTDNA are both derived from dead cancer cells and are present in much smaller amounts than circulating cells or DNA from dead normal cells [7]. Circulating tumor exosomes (CTEs), on the other hand, reflect the genomic and transcriptomic contents of cancer cells and, because they are excreted constantly by viable cancer cells, are much more abundant than shed cells or DNA [3]. Exosomes are extracellular vesicles that store and

transport genomic and transcriptomic substances such as DNA and RNA [4, 5] and are 30–150 nm in diameter [8]. When cells become cancerous, both their exosome excretion rate and the amounts of surface proteins and internal genetic material increase. Analyzing exosome contents can yield direct insight into the state of the original cancerous cell [9]. Like CTCs, CTEs cannot be directly amplified and analyzed by deep sequencing or polymerase chain reaction (PCR); rather, the current state of the art in CTE analysis involves targeting the internal DNA/RNA [3, 8, 10].

Exosomes are excreted by many types of mammalian cells, including blood cells, endothelial cells, immunocytes, platelets and muscles. They form an intercellular communication network and are responsible for regulating the bioactivity of recipient cells through the transport of lipids, proteins, and nucleic acids while they circulate in the extracellular space. Exosomes have been implicated in immune responses, tumor progression, and neurodegenerative disorders, and substantial effort has been directed toward identifying exosomal biomarkers such as proteomic (surface proteins) and genetic biomarkers for early detection and post-treatment prognosis in cancer.

Exosomal biomarkers discovered to date include both genetic and surface-protein biomarkers for bladder, breast, colon, gastric, liver, lung, ovarian, pancreatic and prostate cancers as summarized in Table 1 [1-6, 8-30]. However, the diagnostic value of these potential biomarkers varies significantly. If they could be validated, circulating biomarkers would be particularly useful for the diagnosis of lung and pancreatic cancer, the former because of the risk of lethal tissue damage from traditional biopsy and the latter because of the absence of symptoms until late in the course of disease. That said, though, proteomic biomarkers are less useful for this purpose for two reasons—they seem to be less abundant on exosomes derived from healthy cells than from cancerous ones, and surface proteins are typically not uniquely associated with specific types of

cancer. Genetic biomarkers, on the other hand, usually are more reflective of the specific type of cancer and thus provide better clinical diagnostic value.

Table 1. *Exosome-derived genetic and proteomic biomarkers for cancer diagnosis*

Targeted Cancer	Genetic Biomarker	Surface Protein Biomarker	Application(s)	Ref
Bladder	lncRNA-PTENP1^{+} miR-146^{+ED} miR-375^{+} TP53 KDM6A^{+}	Apo B^{+}	Diagnosis Early diagnosis	[2] [11] [12]
Breast	miR-1246^{+} miR-21^{++} miR-378e miR-143	CD63 CD81 Hsp70 Alix	Diagnosis	[8] [9]
Colon	miR-125-3p^{++} miR320-L^{+ED} miR-193a^{+}	CD63 Alix TSG101 CD81 CD147	Diagnosis Early diagnosis	[1] [13] [14] [15]
Gastric	miR-451 lncRNA-UEGC1^{+} EGPC-3 TGPC-3	CA19-9 CA72-4 CA12-5	Diagnosis	[16] [17] [18]
Liver	Has-miR-122-5p^{M} Let-7a-5p^{M} miR-21-5p^{M} miR-199a-3p^{M} miR-18a^{+} miR-221^{+} miR-222^{+} miR-224^{+}	EpCAM CD144 CD63 CD9 CD81	Diagnosis Monitor HIV/HCV infected patients for liver failure	[19] [20] [21]
Lung	miR-126^{+T} miR-21^{+} miR-155^{+} T790M^{+} L858R miR-16	CD9	Diagnosis Therapy	[6] [5] [22] [23] [24] [25]
Ovarian	miR-32b^{+} miR-29a^{+} miR-30d^{+} miR-205^{+} miR-720^{+} SPINT2 NANOG	CD81 CD24^{+} Ca125 EpCAM EGFR MUC18 CLDN3	Diagnosis	[3] [26] [27]

Pancreatic	miR-1246^{+} miR-4644^{+} miR-3976^{+} miR-4306^{+} KRAS-G12D^{+} TP53-R273H^{+}	GPC1 CD446^{+P} Tspan8^{+P} EpCAM CD104^{+P}	Diagnosis Prognosis	[4] [28] [29]
Prostate	miR-1290^{+} miR-375^{+} TMPRSS2:ERG	CD73^{+}	Diagnosis	[10] [30]

^{T} Biomarker used for cancer therapy; $^{+}$ High potential to be used as a biomarker in clinical trials.

2. Commercially available approaches for exosome isolation

Several approaches for extracting exosomes from bodily fluids have been developed and are commercially available, each with its own specific protocol. The methods involved are summarized in Table 2 [1, 2, 4, 6, 8, 10, 19, 26, 27, 31-35] and include polymer/buffer-based precipitation, ultrafiltration, membrane affinity spin columns, ultracentrifugation, and immunological separation. These methods, and ongoing efforts to achieve higher purity, yield, efficiency, reliability, and reproducibility from them, are described in the sections that follow.

***Table 2**. Commercially available products for isolating exosomes from bodily fluids*

Commercial Products (Company)	**Technology**	**Bodily Fluid**	**Registry/ Immobilization**	**Typical sample volume**	**Recovery yield, %**	**Selectivity**	**Ref**
ExoQuick (System Biosciences)	Polymer-based precipitation	Serum, Plasma	No	100-250 µL	53	No	[1] [8] [10]
TEI Total Exosome Isolation Reagent (Thermo Fisher Scientific)	Polymer-based precipitation	Serum, Plasma Cell-free culture media	No	1-10 mL	50	No	[31]
miRCURY (EXIQON)	Buffer-based precipitation	Plasma, Serum, Cell culture Urine	No	0.5-1.4 mL 1-10 mL	>90	No	[19]
exoEasy (QIAGEN)	Membrane affinity spin columns	Serum, Plasma	No	0.2-4 mL	40	No	[2] [32]

Norgen Exosome Isolation Kit (BIOTEK corp.)	Membrane affinity spin columns	Plasma, Serum, Urine Cell culture Saliva	No	0.05-10 mL 0.25-30 mL 5-35 mL 2 mL	50-55	No	[19]
Ultracentrifugation (Biocompare, Alfa Wassermann, Beckman Coulter)	Low-*g* spin	Serum, Plasma, Cell culture	No	500 µL	20-60	No	[6] [4] [8] [26] [32]
Exocomplete Filterplate (Hitachi Chemical Diagnostics Inc.)	Filters of nano-membrane with pore sizes 0.1-0.8 µm	Urine, Serum, Plasma, Peritoneal fluid	Yes	400 µL (96 samples per plate)	75	No	[27] [33]
ExoPure™ (Biovision Incorp.)	ELISA-based Immunoplate	Plasma, Urine	Yes	50-100 µL	N/A	Yes	[34]
ExoTest (Galen laboratory supplies)	ELISA-based Immunoplate	Plasma, Urine, Serum, Cell culture	Yes	100 µL	N/A	Yes	[35]
DynaBeads (Thermo Fisher Scientific)	Immunologic separation with magnetic beads	Cell culture	No	100 µL	47	Yes	[8]

ELISA, enzyme-linked immunosorbent assay; N/A, not available

2.1. Polymer/buffer-based precipitation

Polymer- or buffer-based precipitation of exosomes involves mixing a buffer or polymer-containing solution with the biological fluid sample and subjecting the mixture to low-speed centrifugation. Several commercially available kits provide simple, fast, and high yield isolation of exosomes, as listed in Table 2 as ExoQuick, TEI, and miRCURY. In this precipitation technique, the physical forces on the exosomes are not intense, and thus the integrity of the outer membrane can be preserved. The pH of the mixture is held at close to physiological level, which in turn preserves the integrity of the inner cargo of the exosome. This precipitation technique does not separate exosomes based on size, thereby resulting in a non-uniform size distribution of extracellular vesicles with non-exosomal impurities.

2.2. Sieving with membrane affinity spin columns

In this process, a binding solution is mixed with the biological fluid sample in a spin column containing a semi-permeable silica membrane and subjected to low-speed centrifugation. The centrifuge forces the binding solution through the silica membrane, thereby isolating the exosomes from the solution. Several research-grade commercial kits are available for this purpose, including ExoEasy and Norgen. This process results in a relatively fast, high-purity extraction and preserves the integrity of the exosome for further analysis. However, this isolation process is also non-selective and requires prefiltration to remove larger extracellular vesicles (ideally through a 0.8-μm filter).

2.3. Ultracentrifugation

Ultracentrifugation, the most common technique used for exosome extraction, is a multi-step procedure that begins with low-speed centrifugation to remove cell debris, followed by a higher-speed centrifugation to remove larger extracellular vesicles, and a final step of precipitating the exosomes [4, 6, 8]. The ultracentrifugation procedure does not require additional chemicals or pretreatment of the biological fluid sample, and leads to lower contamination levels than polymer-based precipitation (ExoQuick and TEI) [31]. On the downside, ultracentrifugation is time-consuming, has a low recovery yield, risks damaging the outer membrane, and is non-selective.

2.4. Ultrafiltration

Isolation of exosomes by ultrafiltration is done by passing biological fluid samples through a membrane with pores 0.1–0.8 μm in diameter, which allows the exosomes to be separated from protein and other submicron particles larger than the pores. In commercial kits, the extracted

exosomes are immobilized by trapping them in microwells. One of the most common commercially available ultrafiltration kits, the ExoComplete Filterplate, provides a simple and reproducible procedure that has a high throughput and recovery yield. Shortcomings of this approach are non-uniform exosome sizes and the possibility of pore clogging, which can damage the exosome.

2.5. Immunologic separation

Immunologic separation techniques target exosomal surface proteins with antibodies for selective isolation. Popular commercially available kits based on this technique are the enzyme-linked immunosorbent assay (ELISA)-based ExoQuant and ExoTest immunoplates, which are functionalized with antibodies that attach to specific exosomal surface proteins. The advantages of this approach are low non-exosomal contamination and surface immobilization that allows further analysis of single exosomes. Another type of immunological isolation involves using magnetic nanoparticles linked to antibodies (e.g., Dynabeads), which results in similarly selective and high-purity output. The primary disadvantage of immunologic separation techniques is cost of expensive antibodies.

Exosomes derived from different cells acquire different sets of protein/lipids that represent the state of the originating cell. Thus, the method used to isolate exosomes must consider specificity, that is, to ensure that all extracted exosomes belong to a specific subtype with a shared origin. Contamination with non-exosomal particles produces incorrect results that do not reflect the biological activity of the exosome. This type of failed diagnosis must be avoided at all cost. Commercial technology for exosome isolation provides higher purity than methods are still at the development stage, however, not all provide the option of specificity (Table 2).

3. Commercially available approaches for exosome analysis

Harnessing the full potential of circulating tumor exosomes requires a selective isolation process with high purity. Once isolated, further characterization is required to provide the information needed for clinical diagnosis. Exosomes have been characterized by a variety of features, from their size to their genetic content. Size characterization can be done with scanning electron microscopy and atomic force microscopy [36], which allow visualization of single exosomes. Nanoparticle tracking analysis provides an alternative, flow-through approach for single exosome sizing and counting. However, simple variations in size do not reveal information on the complexity of the candidate biomarkers within the exosomes and by itself is of minimal diagnostic value. Unlocking the wealth of information stored in exosomes requires methods to analyze their genetic contents, surface protein biomarkers, or both (e.g., Table 3) [4, 5, 8-10, 27, 34, 37, 38]. Enzyme-linked immunosorbent assay (ELISA) -based immunofluorescence, immunoblot imaging, and immunofluorescence flow cytometry are all used to analyze surface proteins on exosomes. ELISA-based immunofluorescence captures and analyzes specific surface biomarkers by sandwiching exosomes between two complementary antibodies, one attached to a functionalized assay and the other to a fluorescent or catalyst label, with quantitative analysis done through fluorescence or colorimetric response. Although this technique allows quantitative detection, it is time-consuming, has insufficient detection limits , and lacks single exosome counting capability [33]. Another form of surface-protein biomarker–based analysis, chemiluminescence immunoblot imaging, is similar to ELISA-based immunofluorescence imaging but does not immobilize the exosome at a specific region [37, 38]. Immunofluorescence flow cytometry involves labeling exosomes with fluorescent markers so that light is first absorbed and then emitted in a different wavelength. A unique feature of this approach is the ability to count

thousands of exosomes rapidly. A known disadvantage of methods that target surface-protein biomarkers are their low diagnostic value relative to genetic biomarkers, because many surface biomarkers are also present on exosomes excreted by normal cells [36, 39]. They also have insufficient detection limit and resolution for counting single exosomes.

The current state of the art genetic profiling of circulating tumor exosomes relies mainly on next-generation sequencing (NGS) [4] or quantitative real-time polymerase chain reaction (Qrt-PCR) analysis of dsDNA mutations or deregulation of mRNA and miRNA relative to exosomes in normal cells [10, 18, 20, 34]. Since the source of exosomes is typically unknown when extracted from blood or serum, genetic profiling alone cannot provide sufficient information to identify the precise location of tumor. However, if the patient is already diagnosed, genetic profiling can provide a powerful tool for monitoring the disease progression and treatment response. The limitations of current genetic profiling is insufficient sensitivity, large blood volume required, and high cost.

Table 3. *Commercially available approaches for exosome analysis of genetic or proteomic biomarkers*

Technology (company)	Technology	Type of Biomarker	Protein Selective	Diagnostic Value	Multiplex Capability	Dynamic Range Detection Limit (Molecule)	Ref
Exo 1000 (Exosome Diagnostics)	NGS	Genetic	—	+	—	300 –10^6/ml 10/ml (Exosomal mRNA, miRNA or dsDNA)	[5]
Qrt-PCR (AH Diagnostics, Biocompare, Biomeme)	PCR	Genetic	—	+	—	0.102- 1.35ng/ml 0.102 ng/ml (mRNA, miRNA or dsDNA)	[4] [8] [9] [10] [27]
ExoQuant™ (Biovision Incorp.)	ELISA-based immunofluorescence capture	Proteomic	+	—	+	1.78x10^6 – 1.14x10^8/µl 0.38x10^5/µl (Exosome)	[34]
AmerSham ImageQuant 800 Using ECL prime Amersham blotting detection reagent (GE Healthcare)	Immunoblot imaging	Proteomic	+	—	+	40pg/µl-40ng/µl 40pg/µl (Exosomal lysates)	[37]

Immunofluorescence fllow cytometry (Biocompare, Beckman Coulter	Fluorescence-based imaging	Proteomic	+	—	+	$1.37x10^5 – 4.57x10^7$/µl $1.37x10^5$/µl (Exosome)	[38]

ELISA, enzyme-linked immunosorbent assay; NGS, next generation sequencing; PCR, polymerase chain reaction; dsDNA, double-stranded DNA; miRNA, micro-RNA; mRNA, messenger RNA

4. Emerging optical technologies for exosome isolation and analysis

The most common strategies for exosome detection and analysis involve complicated, time-consuming procedures of limited accuracy (Tables 2 and 3). Simpler and faster methods, such as those based on optical technologies, have been explored for exosome analysis. Many optical methods have been developed for this purpose, among them photonic crystal and micro-resonators, fluorescence techniques, Raman and surface-enhanced Raman scattering (SERS), surface plasmon resonance (SPR), localized surface plasmon resonance (LSPR), and etc (Table 4). [3, 26, 38, 40-61, 79]. Both labeled and label-free methods can be designed to detect proteomic or genetic biomarkers.

Table 4. *Emerging optical exosome analysis techniques*

Detection Technology	Extraction Method	Diagnostic Application	Targeted Biomarker	Label-free	Detection Limit/ Dynamic Range (exosomes/ RNA molar concentration)	Potential Clinical Use	Ref
Photonic crystal and micro-resonator devices							
3D plasmonic photonic crystal (ppc) biosensor	UC	Fibroblast L cells	EpCAM	Yes	10/µL 10–$1×10^4$/µL (Exosome)	—	[40]
Microfluidic photonic crystal biosensor	UC	Parasite infection	CD63	Yes	$2.18 × 10^6$/µL N/A (Exosome)	Diagnosis	[41]
Frequency-Locked Microtoroid Optical Resonators	—	lymphatic cancer	CD81	yes	Single exosome	Diagnosis	[42]
Fluorescence							

Magnetic beads assisted on chip immunocapture in microfluidic device	UC	Breast cancer	HER2, EpCAM	No	N/A N/A	Diagnosis	[43]
Microfluidic ExoSearch chip	ExoSearch chip	Ovarian cancer	CA-125, EpCAM, CD24	No	7.5×10^2/μL 7.5×10^2–2.7×10^4/μL (Exosome)	Diagnosis	[3]
Self-assembled 3D herringbone nanopatterned microfluidic chip	UC	Ovarian cancer	CD24, EpCAM FRα protein	No	10 /μL 10–10^3/μL (Exosome)	Diagnosis	[44]
Single Molecule Localization Microscopy Imaging using blinking Si Quantum Dots (Super resolution optical imaging)	ExoQuick-TC	Breast cancer	CD63	No	Single exosome	Metastasis	[45]
Copper-mediated signal amplification by cholesterol-modified magnetic bead capture	exoEasy Maxi Kit	Liver cancer	CD63	No	4.8×10^4 /μL 7.5×10^4–1.5×10^7/μL (Exosome)	Prognosis	[46]
Microfluidic integrated immunomagnetic isolation and protein analysis	—	NSCLC	IGF-1R	No	7.16×10^4 N/A (Exosomal Protein)	—	[47]
Digital qualification of exosomes by droplet microfluidics (droplet digital ExoElisa)	UC	Breast Cancer	CD63 GPC-1	No	10/μL 10–10^5/μL (Exosome)	Diagnosis	[38]
Exosome miRNA detection using Molecular Beacon (MB)	TEI, ExoQuick-TC, UC	Breast cancer	miRNA-21	Yes	2×10^7 /μL N/A (Exosome)	Diagnosis	[48]
	TEI	Breast cancer	miR-21 miR-375 miR-27a	No	6×10^{10} /μL N/A (Exosome)		[49]
Raman Spectroscopy							
Optical tweezers assisted	UC	Mesenchymal stromal cells & ovarian cancer	CD9	Yes	Single exosome	—	[50]
	UC TEI	Lung cancer, hepatocar-	CD 9 CD 63	No	Single exosome	—	[51]

		Cinoma, ovarian Carcer, acute T-cell leukae-Mia, acute myeloblastic Leukaemia, prostate cancer					
Surface Enhanced Raman Scattering (SERS)							
SERS switch with DSN-assisted recycling amplification	UC & miRNeasy kit	NSCLC	miRNA-21	No	5 fM 5 fM–20 pM (miRNA)	Diagnosis	[52]
Hybridization of miRNA and LNA on plasmonic head-flocked gold nanopillars	UC, UF exosome & TEI Kit	Breast cancer	miR-21, miR-222, miR-200c	No	1 aM 1 aM–100 nM (miRNA)	Early Diagnosis	[53]
Plasmonic nanowire interstice sensor	UC & miRNeasy kit	Prostate cancer	miR141, miR375	No	100 aM 100 aM–100 pM (miRNA)	Diagnosis	[54]
Magnetic nanobeads assisted SERS nanoprobes	ExoQuick-TC	Breast cancer	HER2	No	268 aM N/A (Exosome)	Diagnosis	[55]
Apta-immunocomplex assay on magnetic substrates	ExoQuick-TC	Breast cancer	HER2	No	32/μL N/A (Exosome)	Diagnosis	[56]
		Colorectal cancer	CEA		73/μL N/A (Exosome)		
		Prostate cancer	PSMA		203/μL N/A (Exosome)		
Silver film coated nanobowl SERS substrate	UC	Ovarian cancer	—	Yes	Single exosome	Study exosomes biological functions	[57]
Surface Plasmon Resonance (SPR)							
Real-time detection on SPR sensor chip	TEI	Breast cancer	HER2	Yes	8.28×10^3/μL 8.28×10^3–3.31×10^4/μL (Exosome)	Diagnosis	[58]
Nanohole-based iNPS (intravesicular nanoplasmonic system)	UC	Ovarian cancer	EpCAM, CD63 AKT1, HSP90, HSP70, TSG101	No	10^4/μL N/A (Exosome)	Diagnosis	[59]

nanohole array based nano-plasmonic exosome (nPLEX) sensor	UC	Ovarian cancer	CD63, CD24, EpCAM	Yes	670 aM N/A (Exosome)	Early Diagnosis & Prognosis	[26]
Colloidal gold nanoplasmonics assisted SPR sensor chip	UC	Multiple myeloma in bone marrow	HSPGs	Yes	60 pM N/A (Exosome)	—	[60]
Localized Surface Plasmon Resonance (LSPR)							
Self-assembly gold nanoislands -based biosensor	UC, TEI, ExoQuick	Lung cancer, neuro-blastoma	CD9	Yes	0.194 µg/mL 0.194–100 µg/mL (Exosome)	Diagnosis & Prognosis	[61]
Nanoplasmonic pillars for digitized exosome detection	ExoQuick-TC	Breast cancer	CD63	Yes	1×10^5 /mL NA (Single exosome)	Diagnosis	[62]
Bright field imaging							
Gold nanoparticle-based lateral flow immunoassay	UC	Melanoma	CD9 CD63	No	8.54×10^5 /µL 8.54×10^5–7.5×10^7 /µL (Exosome)	Diagnosis	[63]

CEA, carcinoembryonic antigen; DSN, duplex-specific nuclease; HSPGs, heparan sulfate proteoglycans; LNA, locked nucleic acid; PSMA, prostate-specific membrane antigen; TEI, total exosome isolation kit; UC, ultracentrifugation; UF, ultrafiltration; NSCLC, non-small cell lung cancer; 1200 exosomes = 268 aM

4.1. Photonic crystal and micro-resonator sensors

Photonic crystal is a popular plasmonic sensing platform that has been utilized for the detection of exosomes. In a recent study, 3D photonic crystal sensors are fabricated using nanoimprint lithography for exosome detection [40]. The 3D photonic crystal sensor developed with point defect cavities allows spacing in between the nanostructures that are comparable to the size of exosomes. This allows the exosomes to spread all around the 3D photonic crystals surface that can operate at low concentration of exosome solution. Another approach for exosome sensing is through surface functionalization of photonic crystal surface [41]. This approach provides a cheap and disposable sensor capable of selective sensing with improved spectral sensitivity and quick assay time. Functionalized microtoroid optical resonators is also a frequency-based technique that

provides a selective exosome analysis. Furthermore, the frequency shift can also determine the size and mass of the adsorbed exosome [42].

4.2. Fluorescence

Fluorescence imaging is a commonly used technique for exosome detection. Considerable work has been done on microfluidic chip environment with on-chip immunomagnetic capture for selective isolation and specific analysis of fluorescence tagged exosome immunoassay [3, 43, 46, 47]. Among these fluorescence-based systems, ExoSearch chip which utilize continuous flow mixing with single step multiplexed detection of exosomes from clinical samples is very promising as a point-of-care diagnostic tool [3]. The major limitations of these conventional platforms namely, effective mass transfer, surface binding capability and near surface flow resistance hinders the detection of exosomes at low concentrations which is crucial in early diagnosis. This is successfully addressed by microfluidic system with self-assembled 3D herringbone patterns enabling detection of extremely low counts of exosomes with few microliters of sample volume [48]. Apart from proteomic biomarkers, genetic biomarkers such as microRNAs are favorable as they show high specificity in determining cancerous exosomes. Detection and quantification of miRNA levels in cancer cell exosomes by a nano-sized oligonucleotide probe, molecular beacon (MB) with a fluorophore and a quencher at each end is another prominent way for making probes with high specificity and low background fluorescence [48, 49]. Measured fluorescence signal from the hybridization reaction of miRNA to MB corresponds to the exosome concentration in sample. Recent studies used various techniques to increase the delivery of MBs in to exosome for enhancing the hybridization signals. Permeabilization using pore-forming bacterial toxin is one

such method which is successfully implemented for the influx of MBs in to exosomes. Such techniques for detecting exosomes allow quick and simple early diagnosis of various diseases.

4.3. Raman Spectroscopy

Raman spectroscopy is one of the powerful characterization techniques which provides detailed information on material composition, chemical structure, crystal orientation and molecular interactions based upon the molecular vibrational modes. Combination of Raman spectroscopy with optical tweezers is a promising route to analyze individual nanoparticles that are undetectable with conventional microscopy. Recently, Laser tweezers Raman spectroscopy (LTRS) has been studied for exploring the chemical composition of cancerous and non-cancerous exosomes [51]. Multi spectral optical tweezers (MS-OTS) is an extension of this technique which incorporates fluorescence with Raman Spectroscopy for multiplex quantification [50]. Although this technology has the potential for distinguishing exosomes from different cell lines depend on spectral variation. However, Raman spectroscopy suffers from weak signals and difficulty in evaluating complex mixtures at low concentrations.

4.4. Surface Enhanced Raman Scattering (SERS)

Surface enhanced Raman scattering (SERS) provides enhanced Raman “molecular fingerprint” in complex biological environments and offers excellent multiplexing ability due to its narrow spectral bandwidth. A unique way for quantitative detection of exosomal miRNA by SERS switch method involves duplex-specific nuclease (DSN)-assisted signal amplification which operates at low concentrations. The technique utilizes hybridization of miRNA with capture probes enriched SERS nanoparticles (Nps). DSN is used to cleave off the hybridization to release the SERS Nps.

This process gets recycled multiple times for signal enhancement [52]. Several reports describe detection of exosomes with magnetic beads assisted SERS probes. Magnetic beads coated with aptamers used as the capturing substrate and SERS nanoparticles as the signal source, which uses the multiplexing capabilities of Raman reporters [56]. Overall, these SERS-based strategies are simple, expedient and have high sensitivity relative to other existing exosome detection methods.

4.5. Surface Plasmon Resonance

Surface plasmon resonance (SPR) has been studied extensively for quantitative and qualitative optical bio-sensing technologies which uses local refractive index changes on the sensor surface. Recent studies show the feasibility of exosome membrane protein analysis through SPR- based nanoplasmonic sensors [59, 60]. nPLEX sensors consist of optical transmission through periodic nanohole arrays which are matched to the size of exosome is a nominal way of employing surface plasmon resonance for quantitative profiling of exosomal proteins. Spectral and/or intensity variation is monitored for each functionalization step as a confirmation of surface modification and capturing exosomes in real-time [26]. A next-generation nanoplasmonic sensor, intravesicular nanoplasmonic system (iNPS) involving lysis of exosomes to expose all proteins is used to detect both transmembrane and intravesicular proteins simultaneously. The immuno-captured proteins are labeled with gold nanoparticles for further signal amplification by plasmonic coupling. This technique overcomes the incompatibility of older methods with intravesicular proteins [59]. SPR based techniques provide a powerful detection method which is in its research and development stage. Further validation and technical modifications should be explored for this approach to be implemented in clinical practice.

4.6. Localized Surface Plasmon Resonance

Common techniques for detecting and characterizing proteins such as ELISA and SPR are not well-suited for the size and complexity of exosomes. Localized surface plasmon resonance (LSPR) sensing techniques is considered as an ideal platform for achieving exosome detection at low concentration. Most research focus on changing the shape of plasmonic nanostructures for enhancing the LSPR sensor performance characterized either by refractive index bulk sensitivity or figure of merit defined by the ratio of bulk sensitivity and full width at half maximum of the resonance peak. LSPR based approach can easily achieve single-exosome detection by matching sensor dimension to the size of the individual exosome [40, 41, 61, 62]. LSPR imaging mechanism involving patterned gold nanosensors that are size-matched to a single exosome improved the limit of detection down to a single exosome. The sensors built on top of quartz nanopillars, allow smaller proteins/molecules to be distinguished from exosomes and thereby reduce the nonspecific binding. This approach can be used to detect single-exosome in femtomolar concentration levels [62].

4.7 Microfluidic plasmonic detection on nanoporous gold disk arrays

Our group has developed a unique, high-performance plasmonic nanoarray consisting of nanoporous gold disks (NPGDs). We created an array of single NPGDs with a tunable diameter from 200 to 500 nm, 75 nm thick, and with interconnected internal pores (7 to 15 nm) by using hybrid fabrication that combined lithographic patterning and atomic dealloying (Figure 1a-d) [64, 65]. In addition to having a greatly enlarged surface area that allows ~10X binding sites, a striking feature of NPGD is that high-density “hot spots” are distributed across the entire particle, in drastic contrast to other plasmonic nanoparticles that have primarily dipolar “edge” resonance. As a rule of thumb, target binding to hot spots generates significant LSPR shifts; those that bind to “dark

spots" are unlikely to be detected. As a result, NPGD nanoarrays have superior sensitivity to target binding and fewer "blind spots." The 3-dimensional porous network throughout the NPGD in a 45º angle is illustrated in Figure 1(e), with a finite-difference-time-domain computed image of electrical field distribution shown in the inset.

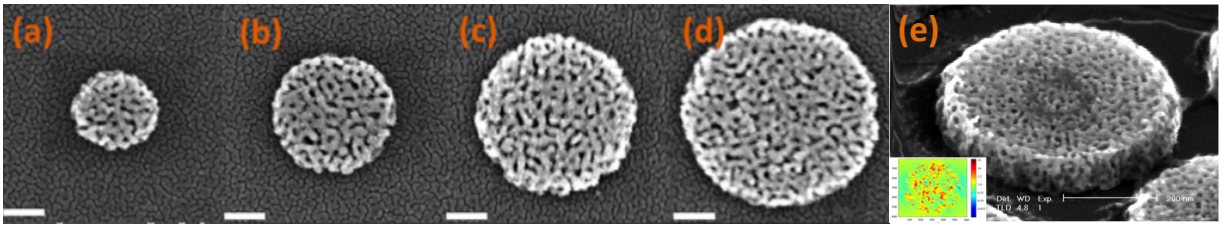

Fig. 1*. Scanning electron microscopy (SEM) images of nanoporous gold disks (NPGDs) of various diameters: (a) 200, (b) 300, (c) 400, (d) 500 nm. (e) NPGD viewed from 45º to show its 3D porous network.*

We have developed several fabrication techniques to produce NPGD arrays in a scalable fashion. We used nanosphere lithography to fabricate wafer-scale NPGD arrays on silicon and glass substrates (Fig. 2a). Nanosphere lithography can produce highly regular NPGD arrays with hexagonal configuration and tunable center-to-center distance that is much smaller than the disk diameter (Fig. 2b). Alternatively, electron beam lithography has also been used to fabricate NPGD arrays in square configurations with precise diameter and center-to-center distance; an array with 100-nm disk diameter and 100-nm spacing is shown in Figure 2c, and another array with 200-nm disk diameter and 100-nm spacing is shown in Figure 2d.

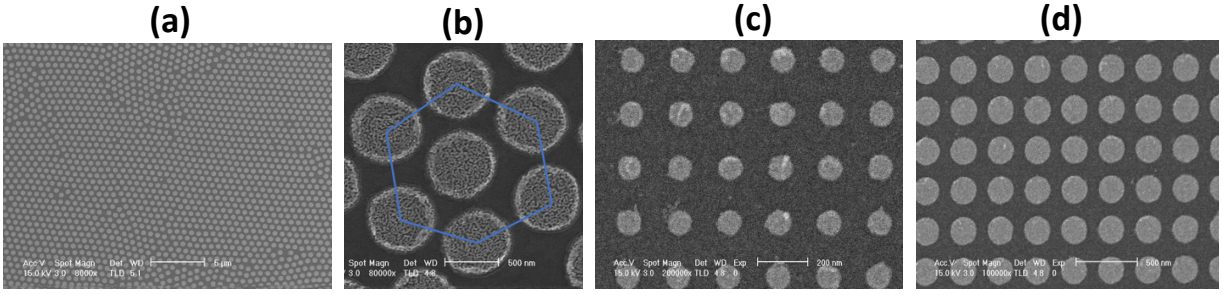

Fig. 2*. Nanoporous gold disk (NPGD) nanoarrays in various configurations. (a) Large-scale NPGD array fabricated by nanosphere lithography; (b) hexagonal nanoarray configuration by nanosphere lithography; (c) square nanoarray with 100-nm disk diameter and 100-nm edge-to-edge spacing; (d) square nanoarray with 200-nm disk diameter and 100-nm edge-to-edge spacing.*

We have used NPGD disk arrays to implement several molecular sensors for high-sensitivity and high-specificity sensing of various target molecules such as DNA [42, 66], malachite green [67], creatinine [68, 69], rhodamine 6G [70], urea [70], dopamine [70], glutamate [70], cyanine 3 [42, 66], hydrocarbons [71], urine acetaminophen [69], telomerase activity [72], among others, with single molecule limit of detection and concentrations in the nM to pM range (ppb-ppt), as well as cellular targets such as bacterial cells and spores with single-unit sensitivity [73, 74]. These results indicate the universal high sensitivity and label-free fingerprinting nature of our plasmonic sensor and the robustness and reproducibility of the NPGD nanoarray as high-performance plasmonic substrates. In addition to biosensing, NPGD has been shown to provide plasmon-enhanced heterogeneous catalysis [75], and is a highly effective photothermal platform [76-79].

4.7.1. Functionalizing the nanoarray surface for specific enrichment and detection of circulating tumor exosomes

To enhance exosome detection, we have functionalized the NPGD nanoarray surface with antibodies that can recognize upregulated surface antigens on cancer exosomes such as CD9, CD63, and CD81. Briefly, a thiol-poly(ethylene-glycol) (PEG)-biotin self-assembled monolayer is first coated onto the nanoarray surface by incubating overnight at 5 mM. Neutravidin is then introduced, followed by the biotin-antibody. To ensure the control of highly precise surface functionalization, the *in situ* LSPR shift ($\Delta\lambda$) is monitored during the entire process flow including the binding sequence of neutravidin, anti-CD63, and two types of exosomes as shown in Figure 3. The amount of LSPR shift was found to correlate well with the exosome concentration, and the sensor could readily detect exosomes at 10^8/mL concentration. The results suggest that anti-CD63

has a much higher capturing efficiency of cancer exosome (H460) over non-cancer exosome (HETA-1).

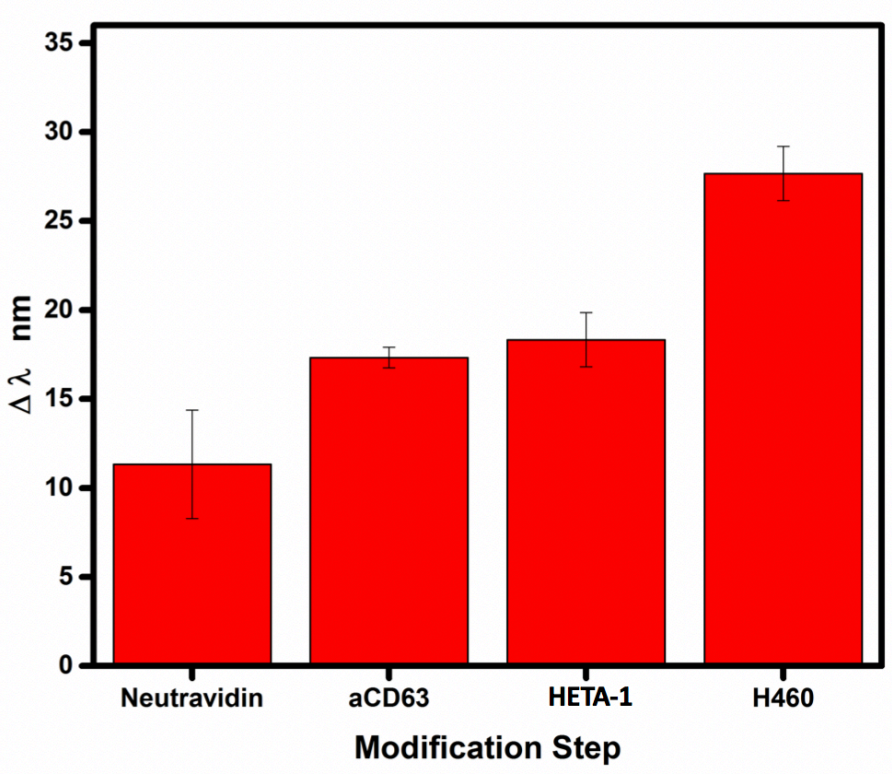

Fig. 3*. Plasmonic sensing of two types of exosomes: LSPR shift vs. successive binding.*

5. Emerging non-optical approaches to exosome isolation with the potential for commercialization

Other non-optical approaches for isolating and quantifying exosomes that have the potential for commercialization include electrochemical, electromechanical, electric field-induced, and electromagnetic techniques. These techniques are summarized in Table 5 [80] and described briefly in the sections that follow.

Table 5*. Emerging non-optical approaches for exosome analysis*

Detection Technology	Extraction Method	Diagnostic Application	Biomarker	Label-Free	Detection Limit/ Dynamic Range	Potential Clinical Use	Refs
Electrochemical							
Differential pulse voltammetry	UC	Breast cancer	CD81	Yes	379 EVs/mL – 10 EVs/mL	Early Diagnosis	[80]
Quantum dot-based enhanced stripping voltammetry	UC Magnetic isolation	Breast and colon cancer	CD9 CD63	No	100 EVs/µL – 10^2 EVs/µL	Diagnosis	[81]
DNA nanotetrahedron-assisted (DNA-based nanostructure)	UC	Hepatocellular liver cancer	Aptamer	Yes	3.96×10^5 EVs/mL 3.96×10^5 – 10^6 EVs/mL	Diagnosis	[82]
Electro-Mechanical							
Nanomechanical sandwich assay (cantilever deflection)	UC	Breast cancer	CD63, CD24, EGFR, Glypican-1	Yes	2×10^2 EVs/mL N/A	Diagnosis	[83]
Electro-Magnetic							
Integrated magneto–electrochemical sensor for exosome analysis (iMEX)	Immuno-magnetic isolation	Ovarian cancer	CD63, EpCAM, CD24 CA125	No	3×10^4 EVs/mL $3\times 10^4 – 10^5$ EVs/mL	Diagnosis	[84]
Electric-field induced							
Electric field-induced release and measurement	Electric-field induction	Lung cancer	CD63	No	NA	Diagnosis	[85]

EV, extracellular vesicles; UC, ultracentrifugation; NA, not applicable

5.1. Electrochemical

Electrochemical sensors are based on a redox reaction that produces an electrical signal proportional to the concentration of the analyte at a working electrode. The methods for measuring potential differences between electrodes or generated current are called potentiometry and amperometry. Voltammetry is a commonly used subcategory of amperometry that measures the current as the applied potential varies. The main advantage of the electrochemical sensors is their high sensitivity as well as their simplicity [86-88]. They can easily be miniaturized, which makes them highly applicable for personalized medicine and clinical settings. In one report, breast cancer exosomes at a concentration of 10^2 exosomes per ml were detected by binding CD81-containing exosomes to immuno-modified gold electrodes via differential pulse voltammetry and

electrochemical impedance spectroscopy [80]. To enhance the sensitivity of this approach, another group implemented quantum dots–enabled signal enhancement in anodic stripping voltammetry quantification to detect tumor exosomes in serum at a concentration of 10^5 exosomes per ml [81]. Another novel method of DNA nanotetrahedron-assisted aptamers for capturing exosomes on gold electrodes [82].

5.2. Electro-Mechanical

Another innovative approach to quantify exosomes is based on measuring electro-mechanical properties of a micro-cantilevers. The measurement involves a sandwich technique in which multiplexed cantilever array sensors compare the expression level of exosomal-surface antigens to distinguish, in real time, tumorigenic from nontumorigenic exosomes [83]. In this method, the nanomechanical bending is scaled proportionally with the concentration of exosomes in the samples. The technique is simple and offers an inexpensive opportunity to develop other exosome isolation techniques for early diagnosis of disease.

5.3. Electro-magnetic

In a novel application of immunomagnetic separation, a portable, integrated magnetic-electrochemical exosome (iMEX) sensor was developed in which magnetic beads are used to immunomagnetically capture and label exosomes, which are then profiled through electrochemical sensing. This approach offers several practical advantages: (i) cell-specific exosomes can be isolated directly from complex media without need for extensive filtration or centrifugation; (ii) the detection sensitivity is high owing to magnetic enrichment and enzymatic amplification; (iii) by means of the electrical detection scheme, the sensors can be miniaturized and expanded for

parallel measurements [84]. The parallel nature of iMEX detection recently enabled simultaneous measurements of four putative cancer markers (CD63, EpCAM, CD24, and CA125). These experiments demonstrated the iMEX's clinical potential for on-the-spot detection of exosomes and other extracellular vesicles.

5.4. Electric field-induced

An electric field, particularly one with a non-uniform profile, can stimulate vesicle deformation in biological samples and direct the flow of the released biomolecules. Therefore, an assay called electric field-induced release and measurement (EFIRM) was developed to quantify the contents ("cargo") of exosomes [85]. After exosome capture by anti-CD63 antibody-labeled magnetic beads, the exosome membrane is disrupted by low-voltage electric cyclic square waves, leading to the release of the inner cargo. Among these cargo contents are specific RNAs or proteins which are then hybridized to DNA primers or antibodies on an electrode surface. This process allows a quantification process of captured exosomes on changes in the electrical current. This technology has the unique advantage of quantifying exosome cargo without using chemical lysis, which might interfere with analytical procedures.

6. Conclusion

Analysis of circulating tumor exosomes is a form of liquid biopsy that can provide insights on the state of a cell without the need for invasive procedures. However, technical challenges remain in acquiring and translating the results into early diagnosis and detection of residual cancer, the greatest among them being the isolation of exosomes from body fluids, which requires various slow and complicated steps for purification. Although exosomes can be isolated by several

different methods, not all are selective. Once exosomes are isolated, analyses are done with devices that are not yet fully capable of analyzing both proteomic and genetic biomarkers. The primary obstacles for most approaches are insufficient detection limits, no single exosome counting capability, and small dynamic range, all of which have led to low sensitivity. New technology that can detect both surface proteins and genetic biomarkers would provide unprecedented capabilities for early and residual cancer detection.

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