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CRITICAL REVIEW

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Integrated technologies for molecular profiling of genetic and modified biomarkers in extracellular vesicles

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Extracellular vesicles (EVs) are nanoscale membrane vesicles actively released by cells into a variety of biofluids. EVs carry myriad molecular cargoes; these include classical genetic biomarkers inherited from the parent cells as well as EV modifications by other entities (*e.g.*, small molecule drugs). Aided by these diverse cargoes, EVs enable long-distance intercellular communication and have been directly implicated in various disease pathologies. As such, EVs are being increasingly recognized as a source of valuable biomarkers for minimally-invasive disease diagnostics and prognostics. Despite the clinical potential, EV molecular profiling remains challenging, especially in clinical settings. Due to the nanoscale dimension of EVs as well as the abundance of contaminants in biofluids, conventional EV detection methods have limited resolution, require extensive sample processing and can lose rare biomarkers. To address these challenges, new micro- and nanotechnologies have been developed to discover EV biomarkers and empower clinical applications. In this review, we introduce EV biogenesis for different cargo incorporation, and discuss the use of various EV biomarkers for clinical applications. We also assess different chip-based integrated technologies developed to measure genetic and modified biomarkers in EVs. Finally, we highlight future opportunities in technology development to facilitate the clinical translation of various EV biomarkers.

Introduction

Extracellular vesicles (EVs) are nanoscale membrane-bound phospholipid vesicles released by all cells of the body,[1,2] especially by actively dividing tumor cells.[3,4] These vesicles abound in biofluids and carry various molecular cargoes. Previously thought to remove unwanted cellular components,[5] EVs are now being increasingly recognized as important vehicles that convey different molecular cargoes[6–11] for intercellular recognition and communication.[12–15] Specifically, EVs play diverse physiological and pathological roles. For example, EVs derived from immune cells have immunogenic properties as they bear major histocompatibility complex class I and II molecules to activate immune effector cells.[16–18] In addition, EVs released by tumor cells can promote cancer progression by mediating tumor growth, metastasis, angiogenesis and immune response.[12,16–20]

EVs are promising biomarkers for minimally-invasive clinical applications. They contain diverse cargoes – genetic and modified biomarkers – reflective of their parent cells and conditions. Specifically, EVs can inherit molecular cargoes from their parent cells;[14] these classical genetic biomarkers bear hereditary information and include nucleic acids and proteins. EVs also contain molecular modifications by binding with other entities; for example, EVs can associate with surrounding proteins[21–23] or carry drug-bound molecular targets.[24,25] As a stable source of biomarkers, EVs are found in high concentration in various biofluids, such as blood, urine, ascites and cerebrospinal fluid (CSF). Their lipid bilayer protects their molecular cargoes from degradation. Furthermore, the small size of EVs allows them to pass through the blood–brain barrier, thereby enabling them to report on the central nervous system using blood samples.[12,26] Collectively, EVs present a stable, robust and informative source of biomarkers for clinical assessment, particularly for monitoring of disease progression and treatment efficacy.

Despite their growing importance, EV analysis remains challenging, primarily because of the vesicles' nanoscale

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dimension. For example, EVs are highly heterogeneous in their composition and such heterogeneity evolves with changes in cellular states. Traditional EV analytical approaches (*e.g.*, immunoblotting and ELISA), however, have limited sensitivity and resolution to recognize these nanoscale features and compositional differences. Due to the small dimension of EVs and the abundance of many contaminants in complex biofluids, conventional methods typically require laborious purification and extensive enrichment of EVs from biofluids. These approaches not only severely limit the analytical performance (*e.g.*, sensitivity and throughput) but also risk losing informative biomarker signatures (*e.g.*, rare modification biomarkers).

To address these challenges, recent work has focused on the development of integrated technology platforms to facilitate EV biomarker discovery and translation. These technologies leverage complementary signal transduction capabilities and versatile molecular assays to measure different EV biomarkers in complex patient specimens. As such, these technologies not only facilitate the identification of new biomarker signatures but also empower the clinical translation of EVs for minimally-invasive diagnostics and monitoring. In this article, we introduce the biogenesis of EVs and discuss various EV biomarkers for clinical applications. Next, we review recent developments in integrated technologies for the analysis of different genetic and modified EV biomarkers. Finally, we provide an outlook

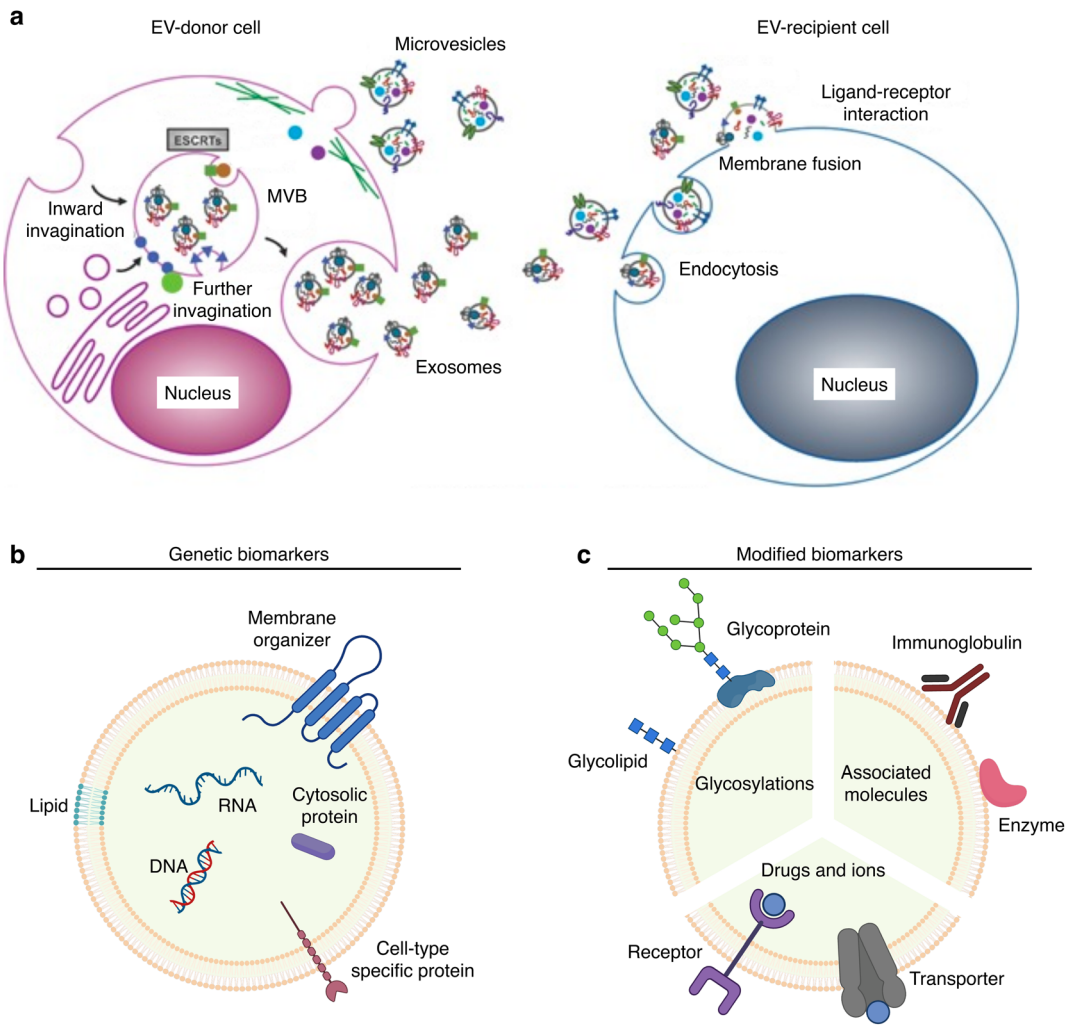

Fig. 1 EV biogenesis and molecular cargoes. (a) Biogenesis pathways. Microvesicles are produced by the direct outward budding of the cell membrane. A multi-step process produces exosomes. First, early endosome is created by the inward invagination of the cell membrane. Next, multivesicular bodies (MVBs) are produced by further invagination. Fusion of the MVBs with the cell membrane releases the intraluminal vesicles, which are termed exosomes. For cell-to-cell communication, EVs from donor cells are delivered to recipient cells through fusion or endocytosis, *via* ligand-receptor interaction. (b) Genetic biomarkers. The EV biogenesis causes EVs to inherit myriad molecular cargoes from their parent cells. These cargoes include surface proteins such as membrane organizers and cell-type specific proteins, cytosolic proteins, nucleic acids including DNA and RNA, and lipids. (c) Modified biomarkers. EV modifications can capture dynamic changes in the parent cells and/or their immediate environment. These modifications include diverse glycosylations on EV membrane such as glycolipids and glycoproteins, molecules associated with EV membrane such as immunoglobulins and enzymes, as well as drug-bound targets such as receptor proteins and transporter proteins. Panel a adapted from ref. 28 with permission from Wiley Periodicals, Inc., Copyright 2015.

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on future opportunities in technology development to facilitate EV clinical translation.

EV biogenesis

There are three categories of EVs classified based on their size, biogenesis and molecular composition — exosomes, microvesicles and apoptotic bodies.[14] Here, we focus on EVs released from living cells (*i.e.*, exosomes and microvesicles) and detail their biogenesis that leads to distinct biophysical and biochemical compositions[27] (Fig. 1a).

Microvesicles are 150–2000 nm vesicles produced from the direct outward budding of the cell membrane.[2] As a result, microvesicles have a membrane composition that is very similar to their parent cells; phosphatidylcholine and sphingomyelin make up the outer lipid bilayer while phosphatidylserine and phosphatidylethanolamine make up the inner lipid bilayer.[29] Correspondingly, microvesicles also have similar cytosolic constituents and surface protein compositions to their parent cells.

Exosomes are 50–200 nm vesicles produced by endocytosis.[1] First, the cell membrane invaginates inwards to form the early endosome. Further inward invagination of the endosomal membrane produces multivesicular bodies (MVBs) containing intraluminal vesicles.[30] Finally, the MVBs fuse with the cell membrane to release intraluminal vesicles into the extracellular space.[15,27] These intraluminal vesicles are known as exosomes. Exosomes contain cytosolic constituents of the parent cells due to the inward invagination of the endosomal membrane.[31] They also have similar membrane compositions as the parent cells as a result of the double invagination process. In addition to membrane constituents inherited from the parent cells, exosomes also carry unique proteins due to processes that control exosome biogenesis and release. The endosomal sorting complex required for transport (ESCRT) conveys specific proteins (*e.g.*, Alix and TSG101) into the intraluminal vesicles to mediate their protein content and exosome release rate.[27,31–34] Hence, ESCRT proteins are commonly used as exosome markers.[35] In addition, other exosome markers include: the tetraspanin proteins CD9, CD63 and CD81 which mediate endosomal sorting,[36,37] the Rab family of small GTPases which participate in sorting and membrane fusion,[38–40] and the enzyme sphingomyelinase, which is involved in exosome release.[41]

Microvesicles and exosomes, through their biogenesis, carry a myriad of molecular cargoes that reflect the contents of their parent cells.[14] These include proteins,[28,42] nucleic acids,[12,13,43,44] lipids[8,9] and modifications.[45] In a landmark study, Skog *et al.* demonstrated that an oncogenic mutation could be detected from exosomal nucleic acids isolated from glioblastoma (GBM) patients' serum samples, thereby enabling disease diagnosis through molecular measurement of exosomes from biofluids.[12] This work sparked extensive interest in the analyses of EV molecular cargoes for various clinical applications.

EV biomarkers for clinical applications

EVs contain diverse molecular cargoes reflective of their parent cells and conditions. For example, EVs can inherit molecules from their parent cells and serve as circulating surrogates. These classical biomarkers are genetic in nature (*e.g.*, nucleic acids and proteins) and reflect hereditary information as well as genetic and epigenetic control (Fig. 1b). On the other hand, EVs can also harbor molecular modifications. These modified biomarkers include post-translation modifications (*e.g.*, glycosylation), ion-bound proteins or drug-bound molecular targets[24,25] as well as associations with other molecular entities (*e.g.*, EVs can bind with surrounding proteins,[21–23] and tend to capture dynamic changes in the parent cells and/or their immediate environment (Fig. 1c). This very diversity of EV biomarkers – genetic and modified biomarkers – thus carry rich molecular information across different cell systems and can empower various clinical applications for personalized medicine (Table 1). In this section, we discuss the different types of EV biomarkers and their applications for disease diagnostics and longitudinal monitoring.

Genetic biomarkers

Nucleic acids. DNA is the permanent and heritable form of molecular information in cells. Changes in DNA sequence result in modifications in the RNA and protein products, which may trigger disease onset. Notably, DNA biomarkers have been reported in EVs. For example, mutated *BRAF* was detected in exosomal DNA from human primary melanoma cell lines with the *BRAF* mutation.[43] Amplified *c-Myc* DNA was also found in serum microvesicles from mice that were implanted with medulloblastoma cells carrying this mutation.[44] As validated by these studies, EV DNA can reflect the genetic status of the parent cells, enabling their use as biomarkers for disease diagnosis and prediction of treatment response.

As compared to DNA in EVs, RNA remains the predominant form of nucleic acids in vesicles. Cellular RNAs have a cytosolic distribution – this colocalization with the EV biogenesis pathway promotes RNA packaging into EVs. EV RNAs thus offer an attractive biomarker for disease diagnosis and monitoring. First, EV encapsulation protects RNA from degradation by extracellular RNases, allowing stable access to RNA biomarkers. Second, EV RNAs can reflect important changes in the parent cells' genetic and epigenetic status. For instance, exosomal O^6-methylguanine–DNA methyltransferase (MGMT) mRNA levels have been found to reflect MGMT promoter methylation status in parent GBM cell lines and primary tissues.[46] In addition, serum exosomal MGMT and alkylpurine–DNA–*N*-glycosylase (APNG) mRNA levels correlated to patient response to temozolomide. Hence, EV mRNA levels can reflect and monitor epigenetic modifications in cells for disease diagnosis and treatment monitoring.

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Table 1 EV biomarkers and their clinical applications

EV biomarker		Biomarker example	Clinical application	Disease example	Ref.
Genetic biomarkers	Nucleic acids	DNA mutations	Cancer diagnosis and treatment monitoring	Melanoma, medulloblastoma	43, 44
		RNA abundance (*e.g.*, miRNA, mRNA, lncRNA)	Disease diagnosis and treatment monitoring	GBM, Parkinson's disease	46, 47
		RNA splice variants	Disease diagnosis	Type 1 myotonic dystrophy, Duchenne muscular dystrophy	48
	Proteins	Protein mutations	Cancer diagnosis and treatment monitoring	GBM	49
		Protein abundance	Cancer diagnosis and treatment monitoring	GBM, ovarian, colorectal and gastric cancers and melanoma	49, 50
			Diagnosis of acute organ injuries	Myocardial infarction and acute kidney injury	51, 52
Modified biomarkers	Glycosylations	Unconjugated glycans, glycolipids, proteoglycans and glycoproteins	Diagnosis of congenital disorders, infections, immune system disorders, chronic inflammations and cancer	Gastric cancer, colorectal cancer, prostate cancer and autosomal dominant polycystic kidney disease	45, 53, 54
	Metal ions	Metal ions and associated proteins	Diagnosis and treatment of multiple diseases including iron deficiency anemia and neurodegenerative disorders	Early schizophrenia, cardiovascular dysfunction, musculoskeletal diseases, cancer, neurodegenerative disease, Fe deficiency diseases	55–57
	Drug interactions	Drug-bound EV receptors (*e.g.*, afatinib-bound EGFR)	Cancer diagnosis and treatment monitoring	Lung cancer	24
		Drug-bound EV enzymes and transporters	Disease diagnosis and treatment monitoring	Cardiovascular diseases, cancers, gout disease	58
	Associated molecules	EV-associated small molecules	Cancer diagnosis and classificaiton	Breast cancer	59
		EV-associated nucleic acids	Cancer diagnosis	Early pancreatic cancer	60
		EV-associated proteins (*e.g.*, auto-antigens, complementary proteins and coagulation factors)	Diagnosis of multiple diseases including inflammatory diseases, acute renal disease and diseases with coagulation-abnormalities	Systemic lupus erythematosus, rheumatoid arthritis, hemolytic-uremic syndrome, Scott syndrome, cancer, endotoxemia, viral and bacterial infections	61–64
		EV-associated protein aggregates (*e.g.*, amyloid β protein aggregates)	Diagnosis and prognosis of neurodegenerative diseases	Alzheimer's disease	23, 65, 66

Dysregulated levels of RNAs, such as mRNAs, microRNAs (miRNAs) and long non-coding RNAs (lncRNAs), lead to multiple diseases.[67,68] mRNA levels affect the levels of proteins synthesized during translation, while miRNAs and lncRNAs mediate transcription and translation.[69,70] In a clinical study, CSF exosomal RNAs comprising a set of miRNAs, mRNAs and lncRNAs could differentiate patients with Parkinson's disease from Alzheimer's disease (AD) and healthy controls.[47] In addition, RNA splice variants encoding different protein isoforms cause neurologic and neuromuscular diseases such as myotonic dystrophy and Duchenne muscular dystrophy. Differentially spliced extracellular RNA found in urine were able to distinguish myotonic dystrophy type 1 patients from controls without known muscular dystrophy and controls with muscular dystrophy (both Duchenne and Becker phenotypes).[48]

Proteins. Proteins carry out diverse cellular functions (*e.g.*, cellular signaling, intercellular communication) and hence dysregulation in protein functions and protein activities may cause diseases. Such dysregulation can be effectively captured by EVs. For example, EVs isolated from GBM patients have higher levels of extravesicular epidermal growth factor receptor (EGFR), EGFRvIII mutant, podoplanin (PDPN) and cytosolic isocitrate dehydrogenase 1 (IDH1) R132H mutant, and the levels of these proteins reflected response to treatment.[49] Diagnosis, prognosis and/or treatment monitoring using extravesicular proteins of EVs have also been shown for ovarian,[71] colorectal and gastric[72,73] cancers and melanoma.[50] Furthermore, EV proteins can be used to diagnose patients with acute organ injuries, such as myocardial infarction[51] and acute kidney injury.[52] These examples demonstrate the utility of EV proteins for cancer diagnosis and treatment monitoring, and acute organ injuries.

Modified biomarkers

Glycosylations. EV membranes are modified with diverse sugars. These glycan modifications can be classified into unconjugated glycans, glycolipids, proteoglycans and glycoproteins by their compositions. They are essential in multiple biological functions such as cell proliferation, differentiation and intercellular communication. Various diseases including congenital disorders of glycosylation,[53]

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infections, immune system disorders, chronic inflammations and cancer[74] can be indicated by altered glycan conjugations. Specifically, in EVs, glycan modifications can mediate vesicle biogenesis, intercellular recognition and vesicle uptake. As such, changes in EV glycan profiles can be seen as a remarkable alternative to conventional disease biomarkers (*e.g.*, changes in nucleic acids and proteins). For example, EV glycans characterized from gastric[45] and colorectal cancer[75] patients were able to differentiate disease prognoses, and the severity of prostate cancer is correlated to the amount of bisecting *N*-acetylglucosamines from patients.[76] In addition, the profiling of urinal EV glycans from patients with autosomal dominant polycystic kidney disease showed distinct signature changes as compared to that of control individuals.[54]

Metal ions. Metal ions are crucial components of numerous enzymes and proteins that drive vital cellular processes, including oxygen transport, antioxidant defense, energy metabolism, and immune function.[77] In cells, metal homeostasis is orchestrated by ion transport proteins and metal-binding proteins. Despite the crucial role of metals, their presence and significance in EVs remains poorly explored until recent discoveries.[55] Indeed, new studies have since shown that EVs carry both essential (*e.g.*, copper, iron, and zinc) and non-essential metals (*e.g.*, nickel, rubidium, and titanium)[78–80] as well as ion transport proteins and metal-binding proteins.[81–85] These studies suggest that EVs may play an important role in mediating metal efflux and/or maintaining metal homeostasis. As such, EV metals and EV metal-associated proteins have the potential to serve as reflective biomarkers for multiple diseases including iron deficiency anemia and neurodegenerative disorders. Indeed, Goetzl *et al.* reported abnormal levels of mitochondrial Ca^{2+} channel proteins in plasma neuron-derived EVs of patients with psychosis, which led to the diagnosis and treatment of early schizophrenia.[56] In addition, the recent discovery of the first functional ion channel in EVs further implies the dynamic nature of metal ions in EVs, potentiating these inorganic cargoes as attractive real-time biomarkers for disease diagnostics and therapeutic monitoring.[55]

Drug interactions. Besides metal-binding proteins and ion transport proteins, EVs can also contain drug-bound proteins including receptors, enzymes and transporters. For example, multimodal characterization of plasma samples from lung cancer patients demonstrated the presence of EVs containing afatinib-bound EGFR, and confirmed its correlation with cellular drug occupancy and treatment potency.[24] Drug-bound metabolizing enzymes and transporters in EVs, characterized in a direct or indirect manner, could be used to analyze the underlying drug absorption, distribution, metabolism and excretion.[58] Motivated by the findings on the degradation pathways of drug-bound targets, these activity-based EV biomarkers could reflect cellular drug effects, thereby providing a new avenue for minimally-invasive therapeutic evaluation, even for solid tumors.

Associated molecules. With a rich amount of surface modifications, EVs can bind to diverse extracellular molecules including small molecules, nucleic acids and proteins (*e.g.*, immunoglobulins, coagulation factors, enzymes). These biomarkers are thus associated with EVs; the organization of these bound biomarkers can indicate specific diseases such as inflammatory diseases, kidney diseases and neurodegenerative diseases. In inflammatory disease, pro-inflammatory immune complexes are formed by EVs and auto-antigens.[61] Such complexes can serve as clinical biomarkers for systemic lupus erythematosus (SLE)[86] and rheumatoid arthritis (RA).[87] During acute renal disease hemolytic-uremic syndrome (HUS), specific associations exist between platelet EVs and complement proteins C3 and C9.[88] Moreover, platelet EVs can actively interact with tissue factors in acute HUS.[62] In neurodegenerative diseases such as AD, EVs show different affinities with aggregated proteins.[65,66,89] Specifically, EVs show a preferential affinity to prefibrillar amyloid β (Aβ) protein aggregates over Aβ monomers; subtyping of EV-bound Aβ proteins in blood samples could thus be correlated with clinical positron emission tomography (PET) imaging data of brain Aβ plaques.[23] Moreover, studies have also found that the amount of EV binding with coagulation factors is dysregulated in various diseases such as Scott syndrome, cancer,[63] endotoxemia and viral and bacterial infections.[64] These examples demonstrate that EV–molecule interactions could serve as innovative biomarkers for clinical applications.

New technologies for molecular profiling of different EV biomarkers

The ability to detect and interpret multi-dimensional EV biomarkers – classical genetic biomarkers inherited from parent cells and modified biomarkers reflective of EV interactions with the environment – presents new opportunities for more accurate diagnostics and personalized medicine. Nevertheless, the rare amount and large diversity of these biomarkers pose significant challenges for their accurate and specific detection. For example, genetic biomarkers from distinct EV subpopulations demonstrate varied composition, density and distribution, and abundant biomarker analogues exist in free-floating forms in native biofluids. In addition, modified biomarkers demonstrate high spatial and temporal heterogeneities due to complex EV–environment interactions, and require specialized assay methodologies to preserve and reveal their presence.

Conventional analytical approaches, however, have limited performance to measure diverse EV biomarkers. For the analysis of EV genetic biomarkers (*e.g.*, nucleic acids and proteins), existing analytical methodologies require extensive EV enrichment and have limited scalability. These isolation techniques enrich EVs according to vesicular density, affinity and size, and include ultracentrifugation, sucrose-gradient

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centrifugation, co-precipitation and size-exclusion chromatography;[90] they are universally time-intensive and laborious, necessitate large sample volumes, and have low throughput. On the other hand, for the analysis of modification biomarkers (*e.g.*, drug-bound proteins), existing analytical methodologies have limited sensitivity and can only be applied for high-abundance targets. To address these challenges, several new technologies have been developed based on emerging micro- and nanotechnologies.[91] Such developments leverage size-matching detection principles and versatile molecular labeling assays to enable multiplexed EV biomarker detection. In this section, we review several on-chip sensing strategies to improve the detection of genetic and modified EV biomarkers (Table 2). Specifically, for measuring EV genetic biomarkers, integrated technologies focus on improving the detection performance and translatability. On the other hand, for detecting modified biomarkers, new technologies focus on leveraging versatile molecular assays to preserve and reveal previously undetectable signatures.

Detection of genetic biomarkers

EV nucleic acids. Despite the clinical potential of profiling EV nucleic acids, particularly RNAs, the small size of EVs means that a large amount of sample is required to obtain sufficient nucleic acids from EVs. In addition, conventional methods such as real-time quantitative polymerase chain reaction (RT-qPCR) are time-consuming and laborious. As a result, new technological advancements have been developed. These enable EV enrichment from complex biofluids and/or direct nucleic acid measurement to translate EV nucleic acids for clinical applications.

Earlier work by Shao *et al.* focused on capturing disease-specific EVs for subsequent RNA analysis.[46] The immunomagnetic exosome RNA (iMER) platform comprises three integrated components: enrichment of GBM-derived exosomes using magnetic microbeads functionalized with anti-EGFR antibodies, extraction of exosomal mRNA, and RT-qPCR measurement of mRNA. Magnetic enrichment is conducted by capturing the microbeads with a permanent magnet, which enables easy exosome purification from complex biofluids as they do not have ferromagnetic properties.[95,96] The integration of these three components on a microfluidic chip greatly improves measurement efficiency and labor costs, as well as decreases the sample amount required for analysis. Using the iMER platform, the expression levels of serum exosomal EPHA2 and EGFR mRNAs were found to be significantly higher in GBM patients as compared to healthy controls ($P < 0.05$), which enabled a high diagnostic accuracy of 90%. Moreover, exosomal MGMT mRNA levels correlated with the methylation status of the MGMT promoter in primary tissues. Response to TMZ treatment was also reflected by the changes in exosomal MGMT and APNG mRNA levels. On the whole, the iMER platform was able to quickly (~2 h) and accurately

Table 2 Integrated techniques for EV biomarker detection

EV biomarker		Platform	Mechanism	Native sample	Target EV biomarker	Ref.
Genetic biomarkers	Nucleic acids	iMER	Integrated magnetic enrichment of EVs, mRNA extraction and qPCR measurement	Plasma	Exosomal *GSTπ1*, *MGMT*, *APNG*, *ERCC1*, *ERCC2*, *MVP*, *ABCC3*, *CASP8* and *IGFBP2* mRNAs	46
		DNA–Au@MNPs electrochemical analysis	Magnetic enrichment of miRNAs on microelectrode and associated electronic reconfiguration	Unpurified serum and 50% blood	*miR-21*	92
	Proteins	μNMR	Immunomagnetic labeling of EV proteins for on-chip NMR detection	Plasma	Microvesicular CD63, EGFR and EGFRvIII	49
		EV-CLUE	Colloidal inkjet printing of nanostructured microreactors on microfluidic chips for EV protein profiling	Plasma	EV CD61 and MMP14	93
		TPEX	Immunolabeling of Au seed and associated EV templated growth of Au nanoshell for plasmon-quenched fluorescent measurements	Ascites	Exosomal CD63, CD24, EpCAM, and MUC1	72
Modified biomarkers	Glycosylation	iMAGE	Magnetic labeling of EV glycans and gradient aggregation for on-chip GMR measurement	Urine, serum and ascites	Jacalin, ConA, RCA120, PHA-E, STA, LEL, WGA, DSL, and LCA binded EV glycans	45
		EVLET	Combines vibrating membrane filtration and thermophoretic accumulation based on EV glycans	Plasma	ConA, WGA, RCA I, SBA and UEA I binded EV glycans	94
	Drug interaction	ExoSCOPE	Competitive target labeling and spatially controlled plasmonic sensing to reflect drug–target interactions in EVs	Plasma	Afatinib-EGFR interaction	24
	Associated molecules	APEX	Enzymatically amplified plasmonic sensing for co-localized profiling of Aβ proteins-associated EVs	Plasma	EV-associated Aβ42 aggregation	23

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diagnose GBM, indicate tissue epigenetic status and predict treatment efficacy by analyzing exosomal mRNAs from as little as ~100 μl of serum.

More recently, Tavallaie *et al.* developed an ultra-sensitive electrochemical method to directly measure miRNAs from biofluids.[92,97] This electrochemical method involves the use of magnetic nanoparticles as dispersible electrodes. By functionalizing gold-coated magnetic nanoparticles with probe DNA (DNA–Au@MNPs), target miRNAs can be pre-enriched *via* the electronic reconfiguration of DNA–Au@MNPs. The method involves three components: mixing the analytes with Au@MNPs functionalized with probe DNA labeled with methylene blue, magnetic enrichment of DNA–Au@MNPs on a gold microelectrode surface, and electronic reconfiguration of DNA–Au@MNPs for target miRNA detection (Fig. 2a). Fig. 2b shows a schematic illustration of the electrochemical setup, which comprises a gold foil working electrode fitted into a custom-made cell. To establish electronic reconfiguration, DNA–Au@MNPs were introduced into the electrochemical cell and assembled with a magnet placed underneath the setup to enrich the nanoparticles onto the gold microelectrode surface. Electronic reconfiguration was achieved through square-wave voltammetry, which causes target miRNA-hybridized DNA–Au@MNPs to migrate towards the electrode surface; the target hybridization increases the distance between the nanoparticles, causing decreased electron tunneling and reduced electrochemical response. The high sensitivity of the DNA–Au@MNPs method

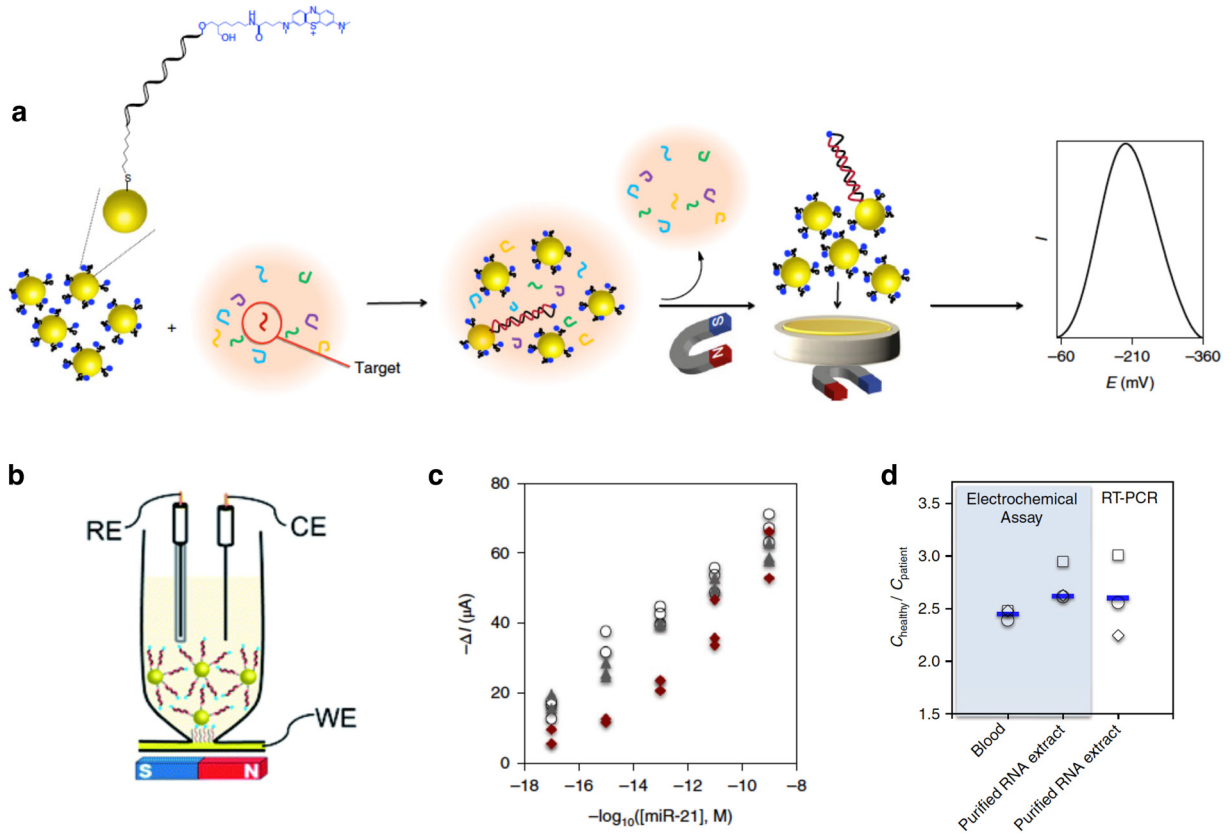

Fig. 2 DNA–gold-coated magnetic nanoparticles (DNA–Au@MNPs) system. (a) Overview of DNA–Au@MNPs method. Au@MNPs functionalized with probe DNA labeled with methylene blue are incubated with samples. Probe DNA hybridizes to complementary miRNA, and DNA–Au@MNPs are magnetically enriched onto the gold microelectrode surface. Square-wave voltammetry causes the electronic reconfiguration of the DNA–Au@MNPs mixture, and the level of target miRNA is measured by comparing the stable peak currents before and after sample incubation. (b) Schematic of the electrochemical setup. For electrochemical measurements, a gold foil working electrode (WE) was fitted into a custom-made glass electrochemical cell. Ag|AgCl, 3.0 M NaCl was used as the reference electrode (RE) and platinum wire as the counter electrode (CE). Au@MNPs were introduced into the electrochemical cell, then magnetically assembled with a magnet placed underneath the setup. (c) DNA–Au@MNPs detection of extremely low amounts of synthetic miR-21. Serially diluted miR-21 was spiked into phosphate buffered saline (PBS; white), healthy human serum (grey) and 50% healthy human blood (red). There were changes in the square wave current caused by hybridization of miR-21 targets. (d) DNA–Au@MNPs detection of miR-21 in blood from mice with human lung tumors. A sample of whole blood (100 μl) and total RNA extracted from whole blood (100 μl) were profiled with the DNA–Au@MNPs method, while total RNA extracted from whole blood (300 μl) was measured by conventional RT-qPCR. Mice with tumors were found to have a higher expression of miR-21. In addition, the DNA–Au@MNPs method was in agreement with and had lower variation than qRT-PCR. Each data point indicates the ratio of miR-21 levels from a mouse with tumor as compared to a mouse without tumor while blue lines indicate the average miR-21 ratio for mice with tumors compared to mice without tumors. Panel a, c and d reproduced from ref. 92 with permission from Nature Publishing Group, Copyright 2018. Panel b reproduced from ref. 97 with permission from The Royal Society of Chemistry, Copyright 2021.

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was demonstrated by the detection of extremely low levels (10 aM to 1 nM) of synthetic miR-21 from phosphate buffered saline (PBS), 100% human serum as well as 50% whole human blood (Fig. 2c). This is 10^7-fold more sensitive than conventional planar surface detection. DNA–Au@MNP profiling of whole blood revealed that miR-21 from mice with tumors was 2.45 ± 0.03 times that of control mice (Fig. 2d). This was in agreement with conventional RT-qPCR measurements, as well as DNA–Au@MNP profiling of RNA extracted from blood. Collectively, the sensor is ultra-sensitive and detects miRNAs quickly (~30 min) and directly from whole blood. These features enable the technology to profile miRNA and other biomolecules using minimal amounts of whole blood for accurate disease diagnosis.

EV proteins. For the detection of EV proteins, conventional technologies require extensive isolation and purification, to concentrate target molecules and remove the complex biological background of clinical specimens (*e.g.*, free proteins) before protein analysis techniques such as Western blotting and enzyme-linked immunosorbent assay (ELISA). To address these challenges, past research focused on specifically capturing EVs *via* surface proteins from complex biofluids for analysis. For example, a micro-nuclear magnetic resonance (μNMR) system was developed for sensitive detection of EV proteins.[49] The μNMR system was modified from a previous study on profiling circulating tumor cells[98,99] to optimize for EV size *via* microfluidics and analytical technology. EVs are specifically labeled with magnetic nanoparticles (MNPs) to make them superparamagnetic, thereby causing a faster decay of the ^{1}H NMR signal which indicates protein concentration. Due to the negligible ferromagnetic background of the biological samples, this system thus provides ultra-sensitive and accurate approach for revealing EV protein signatures present in biofluids for convenient disease diagnosis and treatment monitoring.

In addition to the specific isolation of EVs through biological interaction, biophysical approaches have also been employed to improve the performance of EV enrichment and protein detection, such as inertial-based[100] and geometry-based methods.[101] Zhang *et al.* developed a 3D nanopatterned microfludic chip, through colloidal inkjet printing, for detecting surface markers of plasma EVs[93] (Fig. 3a). The nanoengineered chip incorporates 3D nanostructuring of functional microreactors to improve the flow manipulation and molecular recognition (Fig. 3b). The nanopatterned microchips with enhanced surface area were applied for a dual quantitative-based and activity-based detection of matrix metalloproteinases on plasma EVs (Fig. 3c). The total EV concentration was determined by total count of CD63 and CD9 in parallel. With the optimized design, the nanopatterned chip achieved ultrasensitive EV

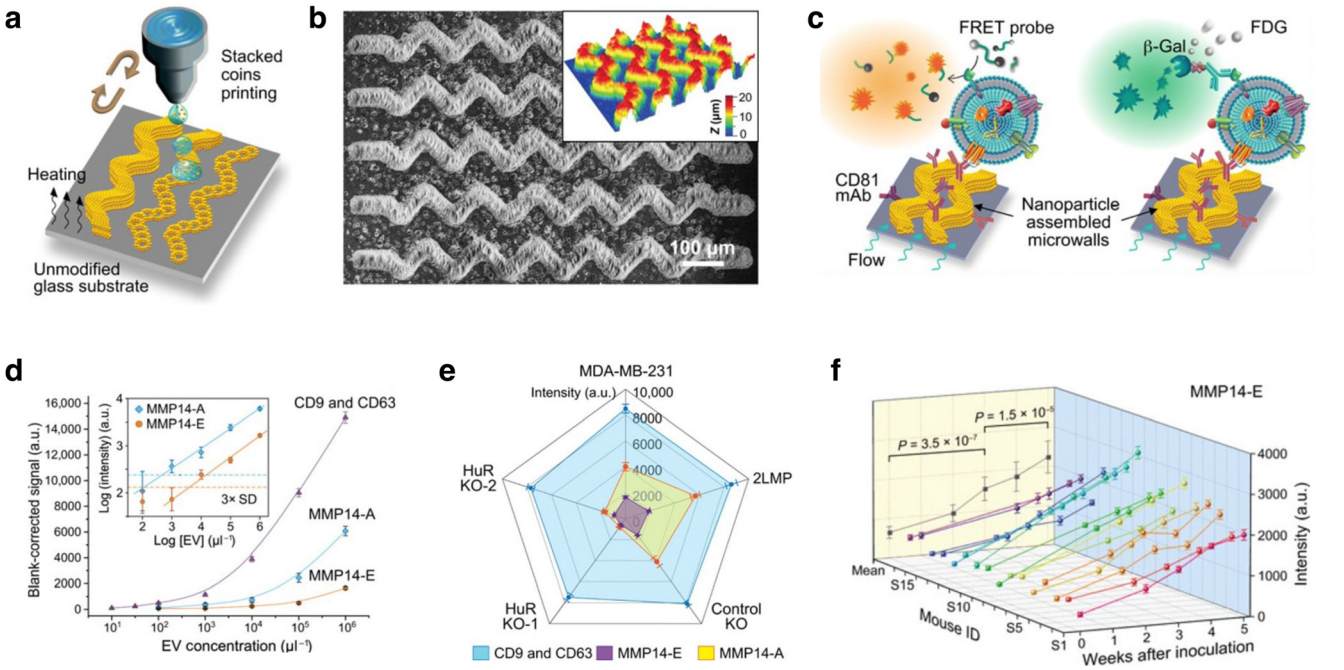

Fig. 3 Nanopatterned microchips for EV protein analysis (EV-CLUE). (a) Schematic of the colloidal inkjet printing technology for assembly of nanopatterned microwalls. (b) SEM image and corresponding optical profilometry plot (inset) of the microwalls. (c) Schematics for the activity-based and quantification-based EV analyses of matrix metalloproteinases (MMPs). (d) Sensitivity of the nanopatterned microchip for measuring the total EV concentration (determined by total CD9 and CD64, purple curve), MMP14 expression (orange curve) and MMP14 activity (blue curve). Inset shows the determination of the LODs. (e) Multiplexed EV analyses of the total EV concentration (blue), MMP14 expression (purple) and MMP14 activity (yellow) for molecular and functional phenotyping of EVs isolated from multiple cell lines. (f) Longitudinal monitoring of MMP14 expression levels of circulating EVs from individual mice (n = 16) using the nanopatterned chips. Reproduced from ref. 93 with permission from American Association for the Advancement of Science, Copyright 2020.

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quantification (limit of detection, LOD = ~16 EVs per μl) with combined CD63 and CD9 expression, and identified a small fraction of $MMP14^+$ EV over total EVs (Fig. 3d). The chip was then used for the measurement of MMP14 phenotypes of EVs isolated from multiple cell lines to compare with that from a lung metastatic subline (Fig. 3e) and was further applied for longitudinal monitoring of breast cancer evolution in mice (n = 16) over 5 weeks (Fig. 3f). The results demonstrate the potential of EV MMP14 as a biomarker of tumor invasion and metastasis. In addition, as this integrative EV phenotyping technology avoids conventional nanolithography procedures, it could facilitate and/or accelerate the applications of non-invasive liquid biopsies of EV biomarkers for early detection of tumor invasion and metastasis.

Studies have also targeted the nanoscale size of EVs for exosomal protein detections. For example, Wu *et al.* designed a dedicated plasmonic sensing platform to selectively analyze exosomal protein biomarkers, disregarding the interference from non-vesicle, free-floating proteins.[72] This sensing platform, termed templated plasmonics for exosomes (TPEX) labels gold nanoparticles (AuNP) onto both free and exosomal proteins. While the free-floating and free protein-bound AuNPs grow into larger spherical gold particles and lead to negligible spectral peak shift, the AuNP assembly on exosomes grow into shell-like gold nanostructure templating the size and shape of nanoscale vesicles. This structure triggers a strong peak shift in the infrared absorbance spectra and quenches the visible fluorescence signal of specific tags, enabling simultaneous biophysical and biomolecular analyses of EVs. The TPEX technology was further incorporated into a microfluidic device and integrated with a smartphone-based optical detector. The absorbance and fluorescence measurements could be implemented on the integrated platform with diverse sets of filters. Using the TPEX platform, the authors identified four types of EV protein biomarkers in a complex background of spiked human serum samples. Clinically, the TPEX platform was applied to analyze ascites samples from colorectal and gastric cancer patients. The profiles of four EV protein biomarkers were obtained and showed high accuracy in prognosis classification across both cancers as compared to conventional ELISA.

Detection of modified biomarkers

EV glycosylations. Mass spectroscopy and lectin microarray are conventionally applied to identify EV glycan signatures. Nevertheless, both approaches require extensive EV purification from complex biological samples and have limited clinical utility. For example, mass spectroscopy is expensive, processing-extensive, and requires large amounts of glycan samples, while lectin microarrays suffer from low sensitivity due to the limited binding affinity in solid-phase assays.

To reduce the interference from complex biological background, magnetic detection presents a promising approach for the measurement of EV glycosylation as it shows high sensitivity and signal-to-noise ratio even in native biofluids. Leveraging this unique attribute, Wang *et al.* recently developed a dedicated magnetic platform for dual-selective detection of EV glycans directly in native urine, serum and ascites samples.[45] Named integrated magnetic analysis of glycans in extracellular vesicles (iMAGE), the technology uses polycore magnetic particles (PMPs) to label EVs; the labeled EVs aggregate upon the addition of targeting lectins which cause multivalent binding and aggregation of the labeled EVs based on their glycan profiles. As more EV glycan-specific aggregates form, they are sedimented by an external magnetic gradient and depleted from the solution. The remaining PMPs in the supernatant are measured through a real-time giant magnetoresistance sensor, and can be directly correlated to EV glycan profiles. The iMAGE platform was applied to profile EV glycans secreted from five cancer cell lines, achieving three orders of magnitude higher sensitivity than conventional ELISA. Clinically, the authors profiled EV glycans in ascites samples collected from colorectal and gastric cancer patients, and identified glycan signatures indicative of poor disease prognosis.

Efficient enrichment of EVs provides another significant approach to enhance the sensitivity of glycan measurements. Li *et al.* reported a lectin-based thermophoretic assay (EVLET) combining vibrating membrane filtration (VMF) and thermophoretic amplification for fast, sensitive, selective and cost-effective EV glycan profiling in plasma samples from breast cancer patients[94] (Fig. 4a). In EVLET, EVs were specifically labeled with FITC-conjugated lectins and isolated from unbound lectins and free-floating glycoproteins/lipoproteins using a customized VMF. A thermophoretic assay was then applied to accumulate EVs for amplified fluorescence measurement of specific EV glycans. This integrated technology provides highly sensitive detection of EV modifications, where the LOD is two orders of magnitude lower than lectin-based ELISA (Fig. 4b). Leveraging a panel of lectins, EV glycans from five cell lines were profiled and showed distinguished expression patterns (Fig. 4c). Clinically, the glycan expression patterns were analyzed to classify triple-negative breast cancer (Fig. 4d) as well as to monitor therapeutic response after treatment (Fig. 4e). The EVLET platform provided sensitive quantification of EV modifications in native plasma samples in less than 100 min and at a low price of $15 per sample, showing good potential for practical *in vitro* cancer prognosis and treatment monitoring.

Drug interactions and EV-associated molecules. Drug–target interactions in EVs provide rich information to reflect drug dynamics, which can play a significant role in clinical cancer diagnostics, treatment monitoring and drug evaluation. Nevertheless, current EV analytical approaches such as mass spectrometry face difficulties to directly detect such fine features of molecular interactions without disrupting the EV modifications.[25] To measure the EV drug dynamics in a non-invasive manner, Pan *et al.* developed a

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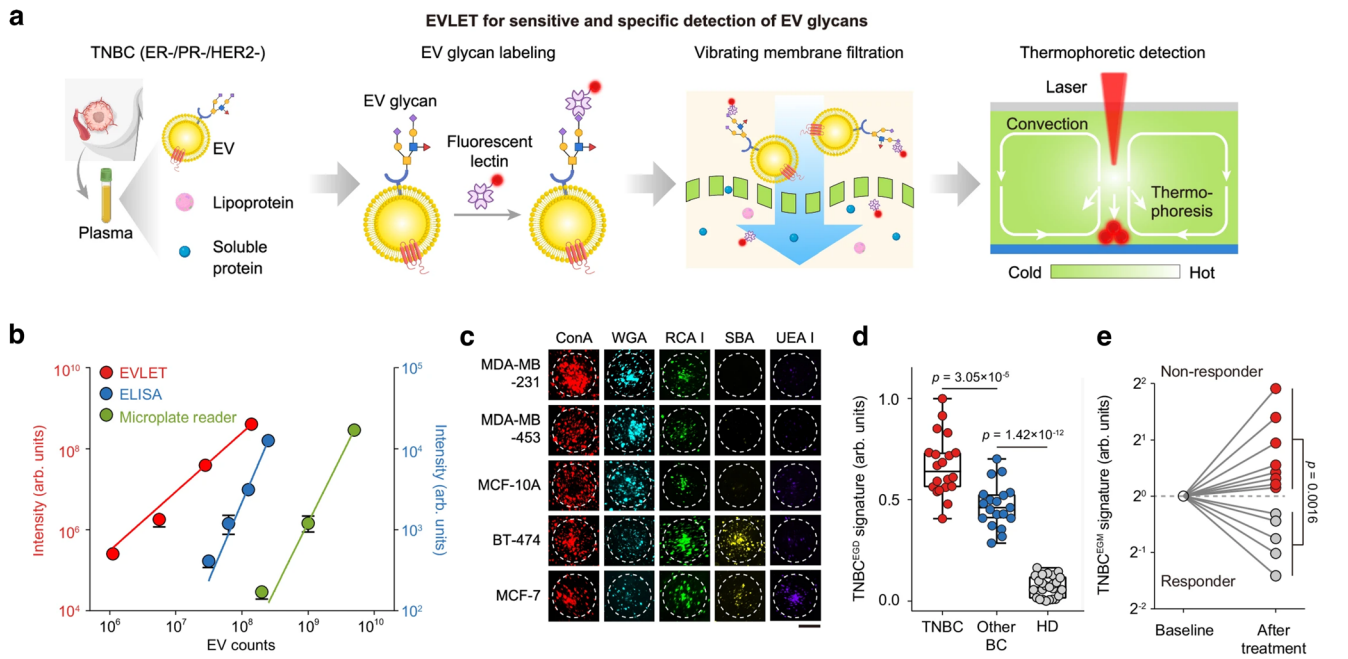

Fig. 4 Lectin-based thermophoretic assay for EV glycan profiling (EVLET). (a) Schematic of EVLET system for sensitive and specific detection of EV glycans. FITC-conjugated lectins were used to specifically label triple-negative breast cancer (TNBC)-associated EV glycans from patient plasma. After vibrating membrane filtration, the purified EVs were accumulated by thermophoresis for glycan analysis. (b) Sensitivity of EVLET (red, the left axis), ELISA (blue, the middle axis) and microplate reader (green, the right axis) for detecting EV glycans. (c) Fluorescence images showing multiplexed glycan profiling of EVs from different cell lines. (d) Weighted glycan signature for classification of TNBC, other breast cancer (BC) types and healthy donors (HD). (e) Weighted glycan signature for evaluation of therapeutic response. Reproduced from ref. 94 with permission from Nature Publishing Group, Copyright 2024.

nanotechnology platform to reflect drug–target engagement and protein expression changes in EVs.[24] Termed extracellular vesicle monitoring of small-molecule chemical occupancy and protein expression (ExoSCOPE), the platform utilizes competitive target labeling by bio-orthogonal small molecule probes and associated enzymatic amplification on a nanoplasmonic sensor to reflect drug–target interactions in EVs (EV drug occupancy). The enzymatic reactions were spatially patterned within the gap of plasmonic nanoring resonator to exploit the near-field enhancement of electromagnetic hotspots for sensitive detection. The ExoSCOPE platform demonstrated a LOD of ~1000 EVs, which is 10^4-fold better than conventional ELISA. Clinically, the authors analyzed multiplexed EV protein biomarkers from the plasma samples of 76 lung cancer patients and control subjects and achieved accurate disease classification. In addition, time-dependent variations in EV drug occupancy were evaluated for patients undergoing targeted treatment with EGFR inhibitors. The ExoSCOPE platform successfully distinguished clinical responders and non-responders, and achieved longitudinal treatment monitoring by analyzing the EV drug dynamics.

Finally, EVs also associate with external molecules under different physiological and pathological conditions.[102] Such extravesicular associations can thus reveal additional insights about the biophysical and/or biochemical properties of the EVs as well as their bound molecules, thereby providing a new avenue to distinguish subpopulations of circulating biomarkers. Conventional detection methods, however, do not have sufficient structural resolution to reveal such biomarker organizational states. To address these challenges, new technologies such as surface plasmon resonance (SPR) sensors have been investigated. Lim *et al.* developed a dedicated enzymatically amplified plasmonic sensing assay for multi-parametric profiling of EV molecular co-localization.[23] The assay, termed amplified plasmonic exosome (APEX), successfully detected Aβ proteins-associated EV organizations with high selectivity and sensitivity directly from native human plasma (Fig. 5a). APEX features a gold nanohole array to capture EVs through EV-specific antibodies. The immobilized EVs are then labeled by enzyme modified with anti-Aβ42 antibodies. Finally, the enzymatic reactions transform the soluble substrate into insoluble optical deposits *in situ* and trigger an amplified SPR signal (Fig. 5b). Using the dedicated opto-biological design, APEX demonstrated a high sensitivity (LOD ~ 200 EVs) compared to conventional methods (Fig. 5c) and provided multi-parametric profiling of EVs for co-localization measurements. Using the APEX microarray, the authors profiled diverse protein markers associated with neurodegenerative diseases (Fig. 5d). Clinically, the authors discovered that circulating exosomes show a preferential binding for aggregated Aβ42 and found that the exosome-bound Aβ42 measurements can be accurately correlated with paired clinical PET amyloid imaging results (R^2 = 0.9002), as compared to unbound (R^2 = 0.0193) and total Aβ42 (R^2 = 0.1471; Fig. 5e). The APEX

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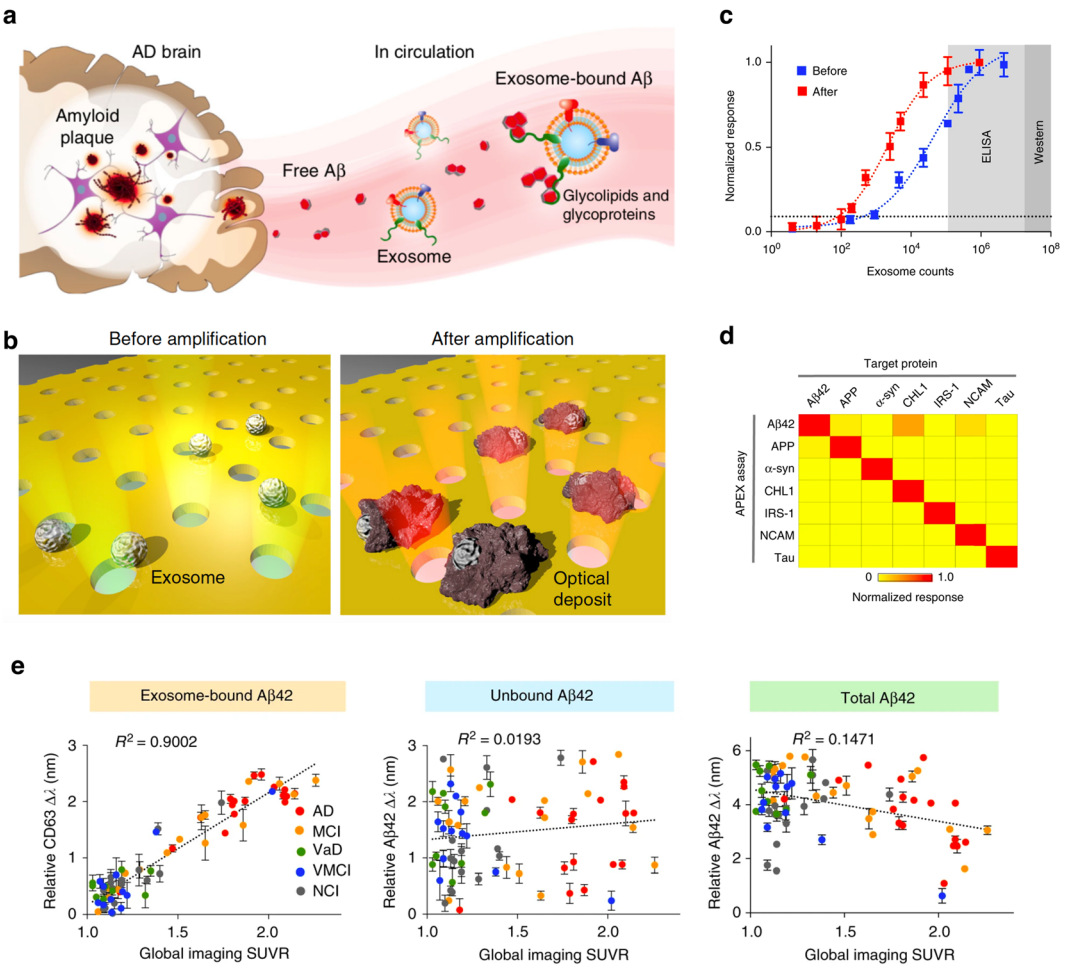

Fig. 5 Amplified plasmonic exosome (APEX). (a) Scheme of the association between exosomes and exosome-bound amyloid β (Aβ) proteins in circulation. (b) Scheme of the enzymatic reaction in APEX analysis. The exosomes were captured by the anti-CD63 coated gold nanopore SPR chip. With the labeling of anti-Aβ42 modified enzyme, free soluble substrates were transformed into insoluble optical deposits generating large SPR signals. (c) Comparison of sensitivities of APEX, ELISA and Western blotting to measure the levels of the CD63 marker. (d) Specific APEX analysis for profiling diverse neurodegeneration associated biomarkers. (e) Correlations of exosome-bound (left), unbound (middle), and total (right) Aβ42 with standardized uptake value ratio (SUVR) of global positron emission tomography (PET) brain imaging. The blood samples of patients with Alzheimer's disease (AD) and subjects with different levels of cognitive impairment were analyzed using APEX. The best correlation (R^2 = 0.9002) was observed between the exosome-bound Aβ42 with conventional PET results. Reproduced from ref. 23 with permission from Nature Publishing Group, Copyright 2019.

platform can thus analyze different subpopulations of circulating molecules (unbound or EV-associated) and reveal their correlations with physiological/pathological status, making it a powerful tool for early detection of diseases.

Outlook for future EV diagnostics

EVs are important vehicles for intercellular communication. They are stable, abundant, easily accessible and carry myriad molecular information. Indeed, EVs contain diverse molecular constituents (*e.g.*, nucleic acids, proteins and modifications); these cargoes can be genetically inherited from their parent cells or exist as modifications (*e.g.*, membrane-associated external molecules). EV genetic biomarkers have been widely shown to reflect hereditary changes (*e.g.*, DNA mutations and RNA dysregulation), while modified biomarkers are being increasingly recognized for their ability to capture dynamic physiological and pathological conditions in the parent cells and their environment. Because of these attractive features, EVs may empower the future of molecular diagnostics and prognostics. Further studies on the mechanism of EV biogenesis, the distribution of size, morphology and composition in various EV subpopulations, as well as the relationships between diseases and their corresponding EV molecular signatures could enhance the accuracy of EV-based diagnostics, to unveil informative signatures directly and safely through blood and other easily accessible bodily fluids (*e.g.*, urine, ascites and CSF).

Nevertheless, EV biomarker discovery and clinical translation remain challenging. EVs are nanoscale and heterogeneous, and circulate in complex biofluid

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environments; these properties bring forth distinct challenges for EV analysis. Traditional techniques not only require extensive sample processing to enrich EVs against the complex biological background of native clinical specimens, but also have limited analytical capability to reveal nanoscale EV features and compositional differences. To address these challenges, new micro- and nanotechnologies are in demand to accelerate the identification of new EV biomarkers and the translation of EV analysis for clinical applications; these new platforms incorporate interdisciplinary advancements in sensor development and assay integration to enable large-scale biomarker discovery as well as accessible clinical translation.

For the discovery of new biomarkers, molecular co-localization studies of individual EVs could significantly enhance our understanding of EV biogenesis and heterogeneity, offering important insights on EV composition and dynamics in mediating disease pathologies.[103,104] In this regard, single-EV profiling techniques with multidimensional analysis could uncover new EV biomarkers, including genetically-coded nucleic acids and proteins as well as environmentally-modified glycosylations, drugs, ions and EV-associated molecules. Indeed, technological advances are underway. To enable spatially-resolvable EV analysis, EVs are isolated on-chip through multiple mechanisms. For example, EV biophysical size selection has been demonstrated through inertial,[105] acoustic,[106] electric/dielectric[107] based microfluidic systems; likewise, bimolecular affinity capture of EVs can be achieved through antibody or aptamer-functionalized elements.[93] In addition to EV isolation, for single vesicle profiling, multiple analytical techniques have also been adapted to match to nanoscale EV dimensions. For example, cyclic immunofluorescence,[108] nanoscale cytometry[109] and total internal-reflection fluorescence imaging[110] have been applied for co-localization imaging and analysis of single vesicles. Likewise, droplet microfluidics facilitates digital quantification of cancer EVs with unprecedented accuracy down to individual vesicles,[111] and optofluidics implemented on mobile platforms could allow ultra-sensitive, rapid, multiplexed detection.[112] Finally, Raman tweezers microspectroscopy could capture single EVs and provide on-site biomolecular analysis.[113]

To assist the clinical translation of EVs for disease diagnostics and prognostics, researchers are developing new technologies for efficient EV isolation/enrichment as well as direct EV detection with reduced interference in complex native samples. These new techniques include acoustic filtering,[106,114] magnetic nanopore capture,[115] dual-mode chromatography,[116] thermophoretic enrichment[94] and microfluidic manipulation with 3D-nanopatterned chips.[93] Such enrichment technologies could be further integrated with multiparametric molecular sensing elements to establish synergistic on-chip microfluidic platforms for clinical EV analysis. Indeed, various micro- and nanotechnologies have been miniaturized with different molecular assays, including *in situ* enzymatic amplified nanoplasmonics,[24] nucleic acid sequencing,[117] plasmonic PCR[118] and μNMR,[119] to provide new opportunities for point-of-care clinical diagnostic applications. These integrated technologies achieve extensive and complementary functionalities, including isolation, culture, labeling, characterization and signal transduction; these advances not only improve the technologies' analytical capabilities but also streamline clinical sample processing, thereby empowering translational opportunities in various clinical applications. Finally, to promote clinical adoption, a rigorous validation process is required to benchmark various EV assays.[91] Specifically, a standard handling process including sampling, purification, isolation and storage is necessary for calibrating across different assays. By implementing these standard processings on emerging chip-based technologies, the integrated platforms will empower robust and informative clinical translation of EVs.

Data availability

No primary research results, software or code have been included and no new data were generated or analyzed as part of this review.

Author contributions

All authors contributed to the preparation of the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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