



Published in final edited form as:

Transl Res. 2018 November ; 201: 136–153. doi:10.1016/j.trsl.2018.05.007.

Molecular Assessment of Circulating Exosomes towards Liquid Biopsy Diagnosis of Ewing Sarcoma Family of Tumors

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Abstract

Ewing Sarcoma was first described in 1921 in the Proceedings of the New York Pathological Society by an eminent American pathologist from Cornell named James R. Ewing as a “diffuse endothelioma of bone”. Since this initial description, more has been discovered regarding Ewing Sarcoma and in the 1980's both Ewing Sarcoma and peripheral primitive neuroectodermal tumors due to their similar features and shared identical genetic abnormality were grouped into a class of cancers entitled Ewing Sarcoma Family of Tumors (ESFT). Ewing Sarcoma is the second most common pediatric osseous malignancy followed by osteosarcoma, with highest incidence among 10 to 20-year olds. Ewing Sarcoma is consistently associated with chromosomal translocation and functional fusion of the *EWSR1* gene to any of several structurally related transcription factor genes of the E26 transformation-specific (ETS) family. These tumor-specific molecular rearrangements are useful for primary diagnosis, may provide prognostic information, and present potential therapeutic targets. Therefore, ways to rapidly and efficiently detect these defining genomic alterations is of clinical relevance. Within the past decade liquid biopsies, including extracellular vesicles (EVs), have emerged as a promising alternative and/or complimentary approach to standard tumor biopsies. It was recently reported that fusion mRNAs from tumor-specific chromosome translocations can be detected in Ewing Sarcoma cell-derived exosomes. Within this review, we overview the current advances in Ewing Sarcoma and the opportunities and challenges in exploiting circulating exosomes, primarily small bioactive EVs (30–180 nm), as developing sources of biomarkers for diagnosis and therapeutic response monitoring in children and young adult patients with ESFT.

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Keywords

Circulating Exosomes; Liquid Biopsy; Ewing Sarcoma Family of Tumors

Introduction

In Dr. Ewing's paper in 1921 (1), he reported a 14-year-old female who developed a tumor of the radius believed to be osteosarcoma, which, at that time, was already well known to clinicians and usually managed by amputation. For reasons unknown, this patient received therapies other than amputation. With no clinical improvement, she was then treated at Memorial Hospital with 12,760 mCi-hr of radium every two weeks for three doses, she obtained complete response by both examination and radiographic imaging. The responsiveness of this tumor to radiotherapy suggested to some clinicians that this was a distinct entity from osteosarcoma. Unfortunately, after tumor recurrence, a biopsy of the tumor was taken to settle differences in opinion regarding the tumor. Pathology confirmed that the tumor was unlike osteosarcoma, and rather, was described vaguely using the term "round cell sarcoma" by Dr. Ewing. He further noted that the tumor cells had the appearance of blood vessels of the bone and therefore characterized the tumor as an "endothelioma of bone".

ESFTs are a clinicopathologic spectrum of the same neoplastic entity, consisting of osseous Ewing Sarcoma, extrasosseous Ewing Sarcoma, peripheral primitive neuroectodermal tumor (PNET) and Askin tumor (Ewing Sarcoma arising from the chest wall). From a gross pathology standpoint, the ESFT have a gray-tannish appearance with infiltrative borders. Morphologically, ESFT are composed of sheets of small round blue cells with a high nuclear-to cytoplasmic ratio. While microscopically, these tumors are divided into three major histologic subtypes: classical Ewing Sarcoma, atypical Ewing Sarcoma (tumors arising from soft tissue) and peripheral primitive neuroectodermal tumor (PNET); with classical Ewing Sarcoma makes up the majority of cases. Strong cell-surface glycoprotein p30/32^{MIC2} (CD99) expression is characteristic of ESFT and diffuse membranous staining is present in 95%–100% of tumors (2). ESFTs are consistently associated with chromosomal translocation and functional fusion of the *EWSR1* gene to any of several structurally related transcription factor genes of the E26 transformation-specific (ETS) family (3). *EWS-Ets* fusions have been seen in 80-95% of cases with Ewing Sarcoma, atypical and PNET (Figure 1) (4). It was first thought that the ESFTs were each distinct biological entity. However, based on their immunohistochemical, cytogenetic, molecular uniformity, range of neural differentiation (PNET being most differentiated) and their shared/similar response to chemotherapeutic regimens, it was determined that they are all related and originate from unique mesenchymal stem cells capable of multilineage differentiation (5).

Diagnosis and Treatment of Ewing Sarcoma

ESFT is the second most common pediatric osseous malignancy (6) in children and young adults exceeded in prevalence only by osteosarcoma. The incidence of ESFT is approximately 200 new ESFT diagnoses annually in the United States and with the highest

incidence among 10-20 year olds (7–9). Caucasians are more affected than Asians and especially African-Americans (9). It is an aggressive sarcoma within the pediatric population, and often presents with a high rate of metastases (10). Although the overall survival (OS) for patients with localized disease now approaches 65-75%, the acute and long-term toxicities of therapy are substantial. Lymph node involvement and staging for sarcomas as defined by the American Joint Committee on Cancer are not relevant in ESFT, since the rarity of nodal involvement. The strongest prognostic factor across different treatment strategies is presence of metastasis at time of diagnosis. Currently the five year overall survival remains <30% for patients with initially metastatic disease (10). However, those with isolated pulmonary metastases have better clinical outcomes than those with metastases at other sites (3 year Event Free Survival [EFS], 29%–52%) (11–14). Despite the current standard intensive therapy regimens and improved local control therapy, 30-40% of ESFT patients experience recurrence. The 5-year survival for children with progressive (refractory) or recurrent pediatric ESFT remains stagnant at 20% (15).

The clinical presentation of ESFT are usually nonspecific, with pain (82%–88% of patients) and a mass/swelling (60% of patients) being the most common signs or symptoms (16, 17). For this reason, diagnosis is often done by first ruling out more common diseases. When a child or young adult is diagnosed with a suspected form of bone cancer, the standard diagnostic assay is obtainment of a tissue biopsy for gross pathologic evaluation, immunohistochemistry staining and cytogenetic evaluation in order to confirm diagnosis of ESFT. Although it is the gold standard, tissue biopsies are, in general, invasive, costly, and painful (18). These factors as well as others, make it difficult to perform repeated biopsies on a pediatric patient, thus it remains impractical to monitor tumor changes to therapy over time (19).

A majority of patients with localized disease on initial presentation have subclinical micrometastatic disease, thus the treatment of ESFT relies on a multidisciplinary approach which combines risk-adapted chemotherapy and focal therapy (surgery, radiation or both) to minimize risk of long-term sequelae and maximize the chance of cure in all ESFT patients. The standard frontline therapy for these patients primarily involves the use of neoadjuvant chemotherapy with the goal of eliminating micrometastases and reducing the size/volume of the primary tumor. Further, local control consists of surgical resection, radiation therapy (RT), or both. A benefit of surgical resection after neoadjuvant chemotherapy is in the opportunity to gather information concerning amount of necrosis of the tumor. In the French Society of Paediatric Oncology (EW88) study, event free survival at 5 years for patients with >95% necrosis was 75%, 70-95% necrosis was 48%, and <70% necrosis was 20% within the resected tumors (20). Radiation for local control is recommended for non-resectable ESFT tumors as well as when there is poor likelihood of adequate surgical margins with resection alone. Diagnosis and monitoring of patients with ESFT tumors consist of imaging techniques such as radiography, magnetic resonance imaging (MRI) and computed tomographic (CT) scans. On radiography, ESFT of bone reveals aggressive features, reflecting the high-grade nature of this malignant lesion (21). Coupled with CT, Positron emission tomography with 2-deoxy-2-[fluorine-18]fluoro-D-glucose (FDG PET) has been shown to demonstrate sensitivity and specificity in the staging of ESFT (22). In regards to osseous metastases, FDG PET has been shown to be far superior to bone scintigraphy, while

for primary bone lesions (18% not evident at bone scintigraphy when compared with FDG PET) (23). FDG PET is more useful in evaluation of tumor response to treatment, with its ability to depict molecular changes before morphologic abnormalities are evaluated on cross-sectional imaging. Macrometastasis, which is the presence of clinically apparent metastases, has prognostic significance, and staging of patients is based on imaging of the primary tumor and the sites of likely metastasis. Lab based studies include a complete blood count, baseline chemistries, erythrocyte sedimentation rate assay (increased in ~ 50% of patients) and serum lactate dehydrogenase measurement. Laboratory advancements and the improvements in tumor banking, have made way for an increase exploratory studies for biomarkers in ESFT. But, as to date there exists no readily available biomarkers for this disease.

Molecular Characterization of ESFT

The advent of cytogenetic evaluation of biopsied lesions demonstrate that ESFT tumors share a common karyotype abnormality. ESFT are characterized by specific rearrangements of one of the five alternative *Ets* family member genes, *i.e.*, *FLII*, *ERG*, *ETVI*, *EIAF*, and *FEV* with *EWSR1* (Figure 1) (24). This recipient gene encodes for a multifunctional protein that is involved in various cellular processes, including gene expression, cell signaling, and RNA processing and transport. In approximately 90% of these lesions, the disease-associated genomic alteration is a translocation between the long arms of chromosomes 11 and 22 (t[11 ;22] [q24;q12]) (25–27). Upon cloning of the specific breakpoints, it was shown to involve the *EWS* gene (*EWSR1*) on chromosome 22 and the *FLII* gene on chromosome 11. This genetic mutation is thought to lead to the oncogenic conversion of EWS by exchanging its RNA binding domain with different DNA binding domains, thus generating tumor-specific fusions proteins. Subsequent studies found that many alternative forms of these two gene translocations exist, corresponding to variations in the locations of the *EWS* and *FLII* breakpoints. The most common form, “Type 1”, accounts for approximately 51% of cases and consists of the first seven exons of EWS joined to exons 6-9 of FLU; while “Type 2”, accounts for approximately 27% of cases and also includes *FLII* exon 5(28, 29). The second most common cytogenetic aberration after *EWS-FLI1*, seen in only 5%–10% of ESFT, is the translocation t(21;22)(q22;q12), which is referred to as *EWS-ERG* (Figure 1) (30). The fusion protein derived from *EWS-ERG* is similar to that of the *EWS-FLII* gene product and thought to also be associated with the pathogenesis of the disease. Others have suggested, *EWS-FLII* alone cannot fully explain the pathogenesis of ESFT. Elzi and colleague suggested that *FLII-EWS*, a fusion gene reciprocal to *EWS-FLII*, is frequently expressed in Ewing Sarcoma and might also be required for Ewing sarcomagenesis (31). While the differences in the proliferation rates of ESFT had been previously thought to be assigned to distinct transcriptional activation potentials of EWS-FLI1 Type 1 and EWS-FLI1 Type 2 proteins (32), genomic expression studies have failed to demonstrate this relationship (33). A prospective evaluation of *EWS-Ets* transcript structure in more than 560 patients lacked statistical significance of prognostic benefit in patients with localized disease carrying Type 1 *EWS-FLII* expressing tumors. This evaluation did however, find that patients with non-type 1/non-type 2 EWS-FLI1 demonstrated a small but insignificant risk of progression or relapse than other fusion types (34).

Circulating Exosomes: Emerging Liquid Biopsy for Diagnosis of ESFT

Despite significant advancements in treating localized ESFT, the 5-year overall survival for patients with metastatic or relapsed/recurrent ESFT remains dismal. A possible contributor to these poor clinical outcomes is that, aside from clinical symptomatology and radiographic imaging, no suitable and readily available biomarker exists, that can specifically monitor disease progression (refractory) or detect recurrence in ESFT. Tissue biopsy is the gold standard, while it is highly invasive and not practical to collect tissue samples repeatedly on pediatric patients. Within the past decade liquid biopsies for the analysis of cell-free DNA, RNA, and soluble proteins have emerged as a much-heralded alternative and/or complimentary approach to the current standard tumor biopsies. They are often minimally invasive, quicker, and more easily repeatable because they collect needed information from a bio-fluid sample, such as blood, urine, saliva, or cerebrospinal fluid. Potentially revolutionary, liquid biopsy promises to dramatically improve disease diagnosis, surveillance, and survival prediction (35, 36). Despite the incredible promise, traditional liquid biopsies have not been able to entirely replace tissue biopsies (37). However, the discovery of small microvesicles termed exosomes, which are enriched in tumor-derived RNAs (including miRNAs) as well as an assortment of proteins, encapsulated within a lipid bilayer, are leading to way towards changing the liquid biopsy landscape.

We, and others, have shown that tumor cells constitutively release exosomes which are characteristic of the parental cell of origin (38–43). Virtually all tumor cells release exosomes, and the accumulation of these vesicles represent key features of tumor transformation to malignant status (44). The rate of exosomal release is significantly increased in most neoplastic cells and occurs continuously at all stages of tumor development (45). In addition, qualitative and quantitative changes in the cargo transported by these microvesicles are observed with tumor progression (46, 47). Extracellular shedding of exosomes occurs in other types of cells under specific physiological conditions and accumulation of exosomes from non-neoplastic cells is frequently observed *in vivo* (48). However, there are more tumor-derived exosomes accumulated in biologic fluids, including sera, malignant ascites, and pleural fluids (49–51). Cancer cell derived exosomes may facilitate the growth, invasion and metastasis of these malignant tumors due to their ability to provide autocrine, paracrine, and endocrine signaling, which promote tumorigenesis (52–56). Cancer cell derived exosomes may also contain oncogenic elements, which have the potential to initiate tumor pathway signaling and promote metastasis (39, 57). The constitutive release and enrichment of certain proteins and nucleic acids into exosomes prompts consideration of the use of these vesicles as diagnostic tools in cancer. While circulating tumor cells and cell-free DNA have taken prominent focus as predominant liquid biopsy-based biomarkers (58–61), exosomes offer a robust alternative since they are i) enriched with original cellular content (*e.g.*, proteins, mRNAs and microRNAs), ii) actively released from viable cells instead of shed or apoptotic cells, iii) relatively abundant (*e.g.*, 1×10^6 to 1×10^{11} particles per mL of blood), iv) very stable in bodily fluids (62–64), and v) representative of the heterogeneous tumor cell population.

To date, most efforts invested in exosome research have been focused on adult cancers and very limited progresses have been reported in childhood cancers. Recently, it has been

reported that fusion mRNAs from tumor-specific chromosome translocations can be detected in Ewing Sarcoma cell-derived exosomes. The first group, Miller and colleagues spiked purified Ewing Sarcoma derived exosomes into healthy human plasma and were able to isolate and detect these exosomes bearing the genetic information of Ewing Sarcoma (65). A second group, Tsugita *et. al.*, were able to detect the *EWS-FLII* mRNA in nanovesicles in four out of ten mice inoculated with the Ewing Sarcoma cell line A673 and three out of seven mice inoculated with the Ewing Sarcoma cell line TC135 (66). To advance our knowledge of tumor-derived exosomes and establish their utility as valuable tools for the diagnosis and therapeutic monitoring in ESFT patients, we recently used molecular approaches to profile the proteome and miRNA content of ESFT-derived exosomes. For example, global proteomic profiling via mass spectrometry identified approximately 1,200 exosomal associated proteins, including many with biomarker potential in ESFT. Two proteins identified are currently being used in conjunction with our microfluidic platforms (discussed below) to enrich for ESFT-associated exosomes from clinical samples using immuno-capture techniques. These approaches appear promising in that we are now able to routinely detect the *EWS-Ets* fusion transcripts in enriched exosomes from clinical samples (Samuel and Godwin, unpublished data). Such limited advancement in the studies of pediatric cancer-derived exosomes highlights the need of new enabling technologies to address current challenges in isolation and of exosomes in malignancies with reliable quality and substantial concentration and in molecular characterization of exosomal cargos in relation to their biological and pathological roles.

Reliable and efficient isolation of exosomes in malignancies is still a major challenge, especially within the pediatric population, due to: 1) size overlap with other EVs, including microparticles (200 nm - 1 μ M) and apoptotic bodies, and lack of specific exosomal markers limiting purity of isolated exosomes, which interfere the accuracy of subsequent molecular diagnosis seriously (67); and 2) low isolation efficiency and poor sensitivity of conventional exosomal detection assays requiring large amount of bio-fluid samples (68), which within the pediatric population may be limited due to sample volumes that can be safely collected. Currently, a variety of exosome isolation techniques have been developed utilizing different physical and chemical properties of exosomes, such as size, density, morphology, membrane rigidity, and membrane composition (69). These techniques can be roughly classified into five categories, based on the mechanisms and the platforms: ultracentrifugation-based protocols, size exclusion and filtration, affinity capture, exosome precipitation, and microfluidics-based technologies. It is noted that microfluidic technologies may exploit the same principles as the bench-top methods, such as size exclusion and immunocapture. Several reviews have summarized prior technologies for exosome isolation and analysis (70–75). In the following sections we will focus on the state-of-the-art progress of exosome technologies, especially the microfluidics-based technologies, and discuss our outlook on the future development of exosome-based liquid biopsy for diagnosis and prognosis of pediatric cancer, including ESFTs. To this end, we will first briefly summarize existing conventional bench-top techniques to benchmark the performance of the newly emerged microfluidic platforms.

Conventional Exosome Isolation Techniques

There are a number of exosome isolation methods that have been extensively adapted in standard biology and clinical laboratory settings. Differential ultracentrifugation is the gold standard and one of the most extensively used methods for exosome isolation (76). Ultracentrifugation-based methods usually consist of a serial steps starting with low-speed centrifugation for a short time to remove large particles like cell debris, followed by higher speed (e.g., 10,000× g) for extended time period to remove large vesicles, and finalized by pelleting and washing small exosomes by ultracentrifugation at >100,000× g. While this method suffers from low recovery efficiency (~5-20%) and poor purity, it shows that 81% of the worldwide researchers choose ultracentrifugation for their exosome isolation needs, according to a recent survey reported by Chris Gardiner in 2016 (77). This interesting observation could be attributed to several factors: 1) the approach is easy to use and robust, requiring very little technical expertise; 2) it requires minimal reagent and instrument cost when having the access to an ultracentrifuge; and 3) it affords flexibility to handle cell culture conditioned media and a range of bodily fluids, such as plasma, serum, urine, cerebrospinal fluid, and ascite fluids, with variable volumes from milliliters to liters. Variations on this method, such as adding in a sucrose gradient centrifugation step, lead to higher purity of extracted exosomes (78). In this case, one important factor that one should take into consideration is that the solution should be kept iso-osmotic throughout the gradient in order to preserve particle sizes.

Ultrafiltration and size-exclusion chromatography (SEC) isolate exosome and other EVs based on their sizes. Ultrafiltration uses nanoporous membranes with common cut-off pore sizes between 1-100 nm as the filter to extract exosomes of this size range (79). The recovery efficiency was found to depend on adherence interaction between exosomes surface proteins and the membranes. Therefore, the use of a certain type of hydrophilized membranes is recommended to enhance filtration efficiency. SEC resolves particles into bands of different sizes which are eluted out of the column sequentially. In contrast to ultrafiltration, SEC doesn't enrich separated exosomes. These size-exclusion methods are faster than ultracentrifugation and do not require highly sophisticated equipment. However, forced filtration and shearing force may cause membrane fusion and loss of integrity, which will potentially interfere the results of downstream analysis.

In addition to the density- and size-based methods, exosome precipitate methods have gained increasing popularity. Exosomes can be precipitated out from biofluids by adding certain precipitants, such as water-excluding polymers (e.g., polyethylene glycol, dextrans, or polyvinyls), to change the solubility or dispersibility of exosomes, and then collected by low-speed centrifugation (80). Several exosome precipitation kits based on this mechanism have been commercialized, such as ExoQuick (System Bioscience) and Total Exosome Isolation Reagent (ThermoFisher). The major advantage of these kits is that they are very convenient to use and only require routine laboratory equipment (e.g., microcentrifuge). Nevertheless, a major drawback of this method arises from co-precipitation of other less soluble contaminants, such as proteins and polymeric materials, limiting the yield and purity of isolated exosomes from complex biological samples. Thus, additional sample preparation procedures are required to improve the purify of extracted materials, such as pre-isolation

treatments to remove subcellular particles (e.g., lipoproteins) and/or post-isolation processing to remove the polymeric precipitants.

A promising route for harvesting highly purified exosomes is affinity-based capture that exploits specific antibody-antigen interactions or receptor-ligand binding. Immunomagnetic capture methods have been established, such as a commercial Exo-Flow Capture Kit (System Bioscience), to target either generic exosome markers (e.g., tetraspanin proteins CD9, CD63 and CD81) or specific proteins expressed on the membrane of exosomes for targeted and rapid isolation of exosomes. With this strategy, the selectivity of exosome isolation can be improved significantly, which confers a distinct advantage in some specific designs where enrichment of certain subpopulations of exosomes from biofluids is preferred. However, it is also important to note that this method needs extensive optimization for individual cases owing to the facts that exosomal expression of proteins is heterogeneous, targeted surface makers may be expressed in only a small fraction of exosomes, and the lack of clear understanding of exosome biology to define specific exosomal biomarkers (81). These limits could result in variable efficiency and specificity of exosome isolation, which interfere the accuracy of subsequent molecular profiling seriously. Meanwhile, immunoaffinity based methods are better suited for processing small-volume biofluid samples and can be expensive for large-scale preparation of exosomes (76). In addition to immunocapture, Wan et. al. recently reported a new lipid-affinity method for rapid enrichment of total EVs, which is a lipid nanoprobe composed of a lipid tail for EV membrane insertion and a biotin tag for collecting labelled EVs by NeutrAvidin-coated magnetic beads (82). This method permitted rapid isolation of EVs in 15 minutes, while yielding similar isolation efficiency and molecular composition as that of ultracentrifugation. Such isolation performance could facilitate the studies and application of exosomes, such as point-of-care cancer diagnostics.

The aforementioned techniques are the most commonly used exosome isolation methods and their performance was briefly summarized in Table 1. These method are coupled with conventional benchtop assays for downstream analysis, such as electron microscopy, Western blot, mass spectrometry, nanoparticle tracking analysis (NTA), and flow cytometry. The lack of standardized characterization methods for isolated exosomes and large differences in the types of bio-fluids make the cross-assay comparison difficult. Many studies on exosome isolation have been conducted in the past few years and it has been documented that isolation methods have significant impact on the observed molecular profiles of isolated exosomes as they result in vast difference in dependence on sample matrix, isolation yield, enrichment factor, exosome purity, and effects on subsequent extraction of proteins and RNAs (83–87). While there is still no consensus on the optimal exosome isolation method, the on-going efforts in systematic characterization of these methods and development of innovative techniques, along with rapid progress in exosome biology, would provide much better guidelines for users to select and optimize an isolation method that best fits their specific applications and needs (88–90).

Microfluidic Exosome Isolation Techniques

Unique microscale fluid behaviors and properties, the ability to integrate functional microelements, and the amenability to circuit-level design make microfluidics a powerful platform for developing innovative exosome isolation strategies. Compared to conventional bench-top methods, microfluidic platforms offer significant reductions in sample volume, reagent consumption and isolation time, while tremendously improving both isolation yield and exosome quality (72, 74). Not only do microfluidic technologies implement the working mechanisms used in conventional methods, but also enable us to create innovative approaches leveraging on the unique flow behaviors at the micrometer scale and the inherent advantages in device integration and scaling. Herein we will discuss the recently reported microfluidic isolation methods that are roughly grouped into two categories: 1) have the same principal mechanism of isolation as in the conventional methods; and 2) utilize unique microscale flow properties and/or innovative microfabricated structures to create new or substantially improved exosome isolation strategies.

The microfluidic technologies in the first group primarily adopted the immunoaffinity capture and the size-based isolation mechanisms. Specific capture of exosomes by antibodies immobilized on solid surfaces represents the most commonly used approach because of its simplicity, compatibility with microfabrication, and ability to target specific subpopulations. In 2010, Chen et al., reported the first microfluidic exosome isolation platform in which an anti-CD63 antibody-functionalized herringbone structure was used for immunocapture of exosomes from human sera for off-chip mRNA analysis (91). Similarly, an ExoChip device developed in the 96-well format also utilized the unstructured anti-CD63 antibody-functionalized surface of microchambers to capture exosomes, which were detected by non-specific fluorescent staining with a lipophilic membrane carbocyanine dye (DiO) (92). To further improve the capture efficiency and capacity, various nanomaterials or nanostructures have been investigated as nano-interfaces to immensely increase the binding surface area and capture probe density. We have developed a nano-IMEX chip (Figure 2a) in which a graphene oxide/polydopamine (GO/PDA) interface is incorporated by microfluidic layer-by-layer deposition of GO and polydopamine films which induces the formation of nanoporous structure of the PDA coating (93). Such nanostructured interface profoundly improves the immunocapture efficiency of exosomes to afford ultrahigh sensitivity for detection of exosomes in human plasma for cancer diagnosis. In these surface-based isolation chips, the capture efficiency is governed by the bulk-to-surface mass transfer and the capture capacity is largely determined by the pre-designed chip dimensions. In contrast, several microfluidic methods have been developed in which immunomagnetic microbeads are used for exosome capture (70, 94–97). The bead-based format offers several advantages, including near-solution binding kinetics, flexible capture capacity, and great scalability for large-volume samples. For instance, we have reported a simple and robust ExoSearch chip (Figure 2b) in which the sample and the suspension of immunomagnetic beads are co-flowed and passively mixed in a serpentine microchannel for rapid and efficient exosome capture (94). This ExoSearch chip features extremely simple and scalable design and the continuous-flow operation, thus providing highly expendable capture capacity for processing large quantities of biological fluids in a high throughput manner.

Size-based isolation of exosomes from other components in biological fluids is another useful approach because of its simplicity and potential for high-throughput sample processing. Wang and colleagues fabricated a porous nanowire-on-micropillar structure which is made of ciliated silicon micropillars (Figure 2c) (97). Exosomes with diameters between 40 and 100 nm were trapped onto these nanostructured micropillars and subsequently recovered *via* dissolution of the nanowires in PBS for 24 h. The double membrane based filtration approaches, driven by pressure or electrophoresis, have been studied extensively (99–101). In these cases, the first membrane was used to remove cells and other debris and the second one to collect exosomes. Woo et. Al. recently reported a “lab-on-a-disc” Exodisc system (Figure 2d), which integrates sample loading, double filtration, and exosomes recovery in a single centrifugal microfluidic disc chip (98). Driven by g-force of low-speed spinning, this Exodisc was found to remove >95% of protein contaminants, afford high recovery rate, and yield >100-fold higher concentration of exosomal mRNA than that produced with ultracentrifugation. Nevertheless, using the membranes of 20 and 600 nm pore sizes in the Exodiscs will likely lead to co-isolation of exosomes with larger EVs, including microvesicles and apoptotic bodies, which limits the exosome purity. Liu et. al. developed a modular microfluidic exosome total filtration unit, termed as ExoTIC (102). Several ExoTIC modules, each with a different membrane pore size (*e.g.* 200, 100, 80, 50, and 30 nm), can be connected in series to produce enriched exosomes at several specific, narrow size ranges. Not only does this tandem modular device increase the purity of isolated exosomes, but also enables well-defined fractionation of exosomes for exploring size-related molecular characteristics.

Beyond the conventional mechanisms, newly emerged microfluidic tools have expanded the spectrum of the properties of exosomes assessable for flow-mediated isolation, such as compressibility, surface charges, diffusivity, and dielectric properties of exosomes. Lee et. al. performed continuous and versatile sorting of exosomes (diameter <200 nm) from cell culture medium and other types of EVs of larger sizes from stored red blood cell products using an acoustic nanofilter system (103). To achieve this level of size resolution, interdigitated transducer (IDT) electrodes were used to produce a symmetric standing surface acoustic wave perpendicular to the flow direction, deflecting larger particles toward side outlets while concentrating small particles at the center outlet (Figure 3a). Wu et. al. further combined a cell-removal module with the exosome-isolation module to construct an integrated acoustofluidic chip which enables isolation of circulating exosomes from whole blood with a blood cell removal rate of >99.999% (104). Liu et. al. reported a viscoelasticity-based microfluidic system that separates exosomes from larger particles relying on the particle migration induced by size-dependent elastic lift forces in a viscoelastic medium (Figure 3b) (105). Satisfactory exosome purity (>90%) and recovery rate (>80%) were achieved, using a small amount of biocompatible poly(ethylene oxide) (PEO) as the additive in the media to control the viscoelastic forces exerted on exosomes. Different dielectric properties of particles and surrounding fluid also provides a useful route for particle separation and enrichment (106). Dielectric particles will experience a dielectrophoretic force (DEP) when subjected to a gradient electric field. Ibsen et. al. reported DEP-assisted enrichment and isolation of exosomes using an alternating current electrokinetic (ACE) microarray chip (Figure 3c) (107). The ACE microarray was used to

generate dielectrophoretic force to trap small exosomes in the high-field regions around the circular microelectrodes within 15 min, while larger cells or smaller molecules remain relatively unaffected by the DEP field and can be washed away. Using this device, the authors demonstrated rapid isolation and on-chip fluorescence detection of glioblastoma (GBM) exosomes from undiluted human plasma samples in less than 30 min.

Deterministic lateral displacement (DLD) is a continuous-flow particle sorting mechanism enabled by using an array of laterally staggered micro-/nano-pillars to manipulate the laminar flow at the (sub)-micrometer scales. The geometry and arrangement of micropillars govern the paths of flow streamlines and determine a critical cutoff particle diameter D_C . Particles with a diameter larger than D_C will be displaced laterally following a bumping mode throughout the array, while smaller particles travel along with the streamlines without lateral displacement on average; thus particles of various sizes can be sorted into different streams in a continuous-flow fashion. Recently Wunsch et. al. first adapted this mechanism to develop a nano-DLD platform for separation of colloids and exosomes as small as 20 nm, by scaling the pillar array structure down to the nanometer size range (Figure 3d) (108). A potential limitation of this technology arises from the need of expensive and sophisticated nanolithography for device fabrication and extremely small volume capacity of the nanodevice, both of which could hamper practical applications of this technology. A clever solution to address these limitations was recently reported which makes it possible to use a regular microfluidic DLD device with micrometer-sized pillar arrays (Figure 3e)(109). In this approach, bioparticles are captured on the surface of 1 μ m polymer beads and induces the change in size and/or electrostatic charges which cause the lateral displacement of the beads flowing through the DLD array. Interestingly, the degree of lateral shifts of the beads was found to be dependent on the amount of bioparticles bound to the beads, expanding the DLD chip from a separation device to a useful tool for label-free, quantitative measurement of the target bioparticles. Ko et. al. attempted to overcome the low throughput and susceptibility to clogging of nanofluidics for exosome isolation by developing an exosome track-etched magnetic nanopore (ExoTENPO) chip (110). This method used a commonly used nanopore membrane to form magnetic traps at the edges of pores for immunomagnetically labelled exosomes, creating a multiple-channel nanofluidic system for high-throughput, highly selective exosome isolation based on both size exclusion and surface protein expression. The authors demonstrated the potential applications of the ExoTENPO chip by isolating exosomes from murine and human plasma samples for downstream mRNA profiling by qPCR. Combined the mRNA profiling with a machine learning algorithm, this approach generated a set of optimized linear discriminators to identify samples from heterogeneous pancreatic cancer individuals, in both a murine model and in a clinical cohort. It is worth noting that most of these new exosome isolation strategies enabled by microfluidic technologies are contact free and label free, which ease the recovery of intact, active exosomes and increase the throughput of sample processing. While further optimization and improvement are needed, the emergence of these conceptually new exosome isolation approaches will open new opportunities to propel rapid progress in basic and translational exosome research towards ultimate clinical utilities.

Integrated Microfluidic Analysis of Exosomes towards Liquid Biopsy

Liquid biopsy analysis of tumors holds great promise to shift the paradigms in both cancer diagnosis and personalized therapy. As an emerging dimension of liquid biopsies of tumors, exosomes have attracted increasing importance during the progress towards precision medicine. Exosome secretion is a dynamic process, producing diverse populations with 5-fold differences in size and as much as a 10^4 -fold differences in concentration between healthy and disease states (81, 111–113). Such heterogeneity hinders the sensitive and specific analysis of exosomes in bio-fluids. The aforementioned isolation technologies can be employed in a stand-alone manner, functioning as preparative front ends that interface with conventional downstream detection and analysis. Meanwhile, microfluidic platforms also hold potential to leverage or even transform quantitative detection and comprehensive molecular characterization of exosomes by virtue of offering an attractive combination of high throughput and sensitivity with low reagent consumption and the potential for portability. Over the past few years, researchers have developed a number of integrated microfluidic platforms for analysis of exosome levels, quantification of disease-specific subpopulations, and omics-level characterization of exosomal proteins and RNAs (97, 100, 114, 115). Herein we will focus on the microfluidic devices for the point-of-care (POC) and clinical applications and discuss the studies on microfluidic analysis of disease-associated exosomal biomarkers in bodily fluids (*e.g.*, serum, plasma, and whole blood).

Vaidyanathan et. al., demonstrated a microfluidic device that produces a tunable alternating current electrohydrodynamic flow (nanoshearing) in microchannels to enhance the specificity and sensitivity of immunocapture and detection of exosomes (114). This multiplexed device permitted simultaneous colorimetric detection of multiple exosomal markers (HER2, PSA, and CD9) for breast cancer diagnosis. We have developed for the first time a cascading flow chip that enables analysis of intravesicular proteins in selected exosome subpopulations (116). This device integrates exosome immuno-isolation, enrichment, chemical lysis, protein immunoprecipitation, and sandwiched immunoassay assisted by chemifluorescence detection (Figure 4a). Using this technology, quantitative analysis of both type 1 insulin growth factor receptor (IGF-1R) and its phosphorylation status in plasma-derived circulating exosomes was demonstrated for detection of non-small-cell lung cancer patients from healthy individuals. To further improve the exosome detection sensitivity, we combined the GO/PDA nano-interfaced exosome capture chip with a sensitive and robust sandwich immunoassay targeting a combination of CD9, CD81 and EpCAM markers (93). This technology affords an extremely low limit of detection (LOD) of 50 exosomes per μL and the ability to detect circulating exosomes directly from minimal volume of human plasma for cancer detection. Shao et al. devised a microfluidic nuclear magnetic resonance system (pNMR) in which EVs bound with marker-specific magnetic nanoparticles resulted in faster decay of the NMR signal depending on the protein expression levels (117). The pNMR-based exosome sensing enabled diagnosis and monitoring treatment responses for glioblastoma patients by probing four GBM signature protein biomarkers (EGFR, EGFRvIII, PDPN, and IDH1 R132H), which offered >90% combined accuracy (AUC = 0.95). A nPLEX chip (Figure 4b), developed by the same group, consists of an array of nanohole lattice patterned on gold film-coated substrate of parallel

microfluidic channels for label-free transmission Surface Plasmon Resonance (SPR) sensing of immunocaptured exosomes (118). In this study, ascites samples from ovarian cancer patients were studied by probing several exosomal protein markers selected by proteomic profiling of exosomes from 10 ovarian cancer cell lines. Combined expression levels of EpCAM and CD24 markers in ascites-derived exosomes were found to improve ovarian cancer diagnosis with an accuracy of 97%. Jeong et. al., reported a portable magneto-electrochemical device (iMEX) as shown in Figure 4c (119). Exosomes are immunomagnetically captured from patient samples and profiled through electrochemical reaction in eight parallel channels. This assay was validated to discriminate 11 ovarian cancer patients from 5 healthy controls, by probing the expression levels of EpCAM and CD24. The iMEX system offered an affordable and miniaturized device that can be readily translated for on-site exosome detection.

In addition to the protein contents, exosomes contain numerous types of nucleic acids, including mRNAs, microRNAs, as well as both single and double-stranded DNA, which add to the significant value of exosomes for disease diagnosis, prognosis, and therapeutic treatment. Shao *et. al.* developed an integrated and multiplexed iMER platform to detection mRNA levels in tumor-derived exosomes (Figure 4d) (95). On-chip exosome capture, mRNA extraction and RT-qPCR analyses were carried out on a single chip tandemly. The iMER analysis of exosomal mRNAs in sera of GBM patients and healthy controls revealed that combined exosomal mRNA levels of *EPHA2*, *EGFR*, and *PDPN* have a diagnostic accuracy of 90% for GBM (AUC = 0.945). Furthermore, longitudinal measurements of mRNA levels of MGMT and APNG, two important enzymes involved in repairing DNA damaged by the drug temozolomide (TMZ), demonstrated the feasibility of using the iMEX-based exosome analysis to monitor the development of drug resistance during treatment. Recently, Reategui and colleagues adapted a nano-engineered herringbone microchip initially developed for CTC capture to exosome analysis (120). This ^{EV}HB-Chip features an engineered nano-interface coated on the herringbone grooves to maximize exosome interactions with antibody-coated surfaces, thus improving the limit of detection (LOD) to a level of 100 vesicles per μL and largely expedite RNA isolation from tumor-specific EVs to facilitate comprehensive mRNA profiling off-chip.

There is a surge of microfluidic platforms in the last five years that have been developed for integrated exosome analysis, as summarized in Table 2. Most of these systems employed immunoaffinity isolation of exosomes, owing to its compatibility with microfabrication, high isolation efficiency and importantly, unmatched specificity to target tumor-related exosomes in complex biological samples. Meanwhile, miniaturization and integration of molecular detection methods with sample preparation steps are beneficial, because the analytical performance for measuring exosomal biomarkers can be substantially improved, while reducing the sample loss, cross-contamination, and analysis time, in comparison to conventional bench-top assays. From a perspective of translation studies, rapid exosome isolation and reliable molecular assessment from smaller ‘real life’ clinical samples poses a key bottleneck to moving exosome-based liquid biopsy towards clinical utilities. The advantage of microfluidics in small sample requirement makes it an inherent suitable tool to overcome this challenge.

As discussed above, extensive studies have been conducted to investigate exosomal biomarkers for a variety of cancer types, ranging from pancreatic, ovarian, breast, lung, bladder and GBM, generating exciting evidences to support the promising potential of exosomes as a surrogate for tissue biopsy of tumors. However, these studies are all focused on adult cancers, and very limited efforts have been invested in childhood cancers. Compared to adult cancer, pediatric cancers present distinct challenges in the research, clinical trials and patient care. Pediatric cancers are often more aggressive and progressive (121) and the diagnosis mainly relies on either a surgical or needle biopsy to obtain sufficient tissue for the histological, molecular and cytogenetic workup. This highly invasive procedure is associated with potential risks of complications that can have a life-long impact on childhood patients, put economic burden on the family of a young patient, and has often no feasibility to repeat for accurate diagnosis and treatment monitoring to minimize the toxic side effects. Moreover, major ethical challenges arise, including clinical equipoise, when considering an invasive biopsy procedure for children (122, 123). Therefore, there is a pressing need in developing liquid biopsy-based biomarkers and tests to improve the disease management in pediatric cancer. Fortunately, the relatively slow progress in pediatric cancers has been recognized and childhood cancers have been listed as one of the ten research areas recommended for the Cancer Moonshot initiatives announced in 2016. These efforts will open tremendous opportunities to accelerating the assessment and translation of exosomes as liquid biopsy markers for pediatric cancers, including ESFTs. Ewing Sarcoma is an orphan disease that would greatly benefit from repetitive non-invasive tests, thus avoiding the risks and costs associated with repeated imaging and sedation and invasive biopsies in children. The lack of reliable blood biomarkers presents a serious obstacle to the treatment and management of Ewing Sarcoma, more importantly, recurrent or metastatic Ewing Sarcoma. Our laboratories are working closely to develop and validate our ESFT-exosome based assays, using pediatric clinical longitudinal samples, for early diagnosis, monitoring of disease burden and response to therapy, and detection of early recurrence of disease in children and adolescent with Ewing Sarcoma.

Conclusions:

Analysis of extracellular vesicles, primarily focused on exosomes, is a cornerstone of emerging liquid biopsy techniques, whose importance in diagnostics and precision medicine is only beginning to be fully understood. Efforts are underway to evaluate the clinical utility of circulating exosomes across diseases and clinical implications, especially in pediatric malignancies such as ESFT. But the development of technologies that can make routine analysis of liquid biopsy samples feasible is also critically important and presents a tremendous opportunity for microfluidic solutions. It is notable that most of the advancements highlighted here have occurred within the past 5 years, indicating that this field is still in its infancy. This body of literature lays a foundation for future progress by introducing new methods for exosome capture, detection, and analysis. These advancements also suggest directions for future work needed to realize the vision of exosome-based liquid biopsy applications.

ACKNOWLEDGMENTS

This work was supported by 1R21EB024101, 1R21CA186846, 1R33CA214333, and 1R21CA207816 from the NIH. Dr. Andrew K. Godwin is the Chancellors Distinguished Chair in Biomedical Sciences endowed Professor at University of Kansas Medical Center. All authors have read the journal's policy on disclosure of potential conflicts of interest. The authors declare no competing financial interest. All authors have read the journal's authorship agreement and the manuscript has been reviewed by and approved by all named authors.

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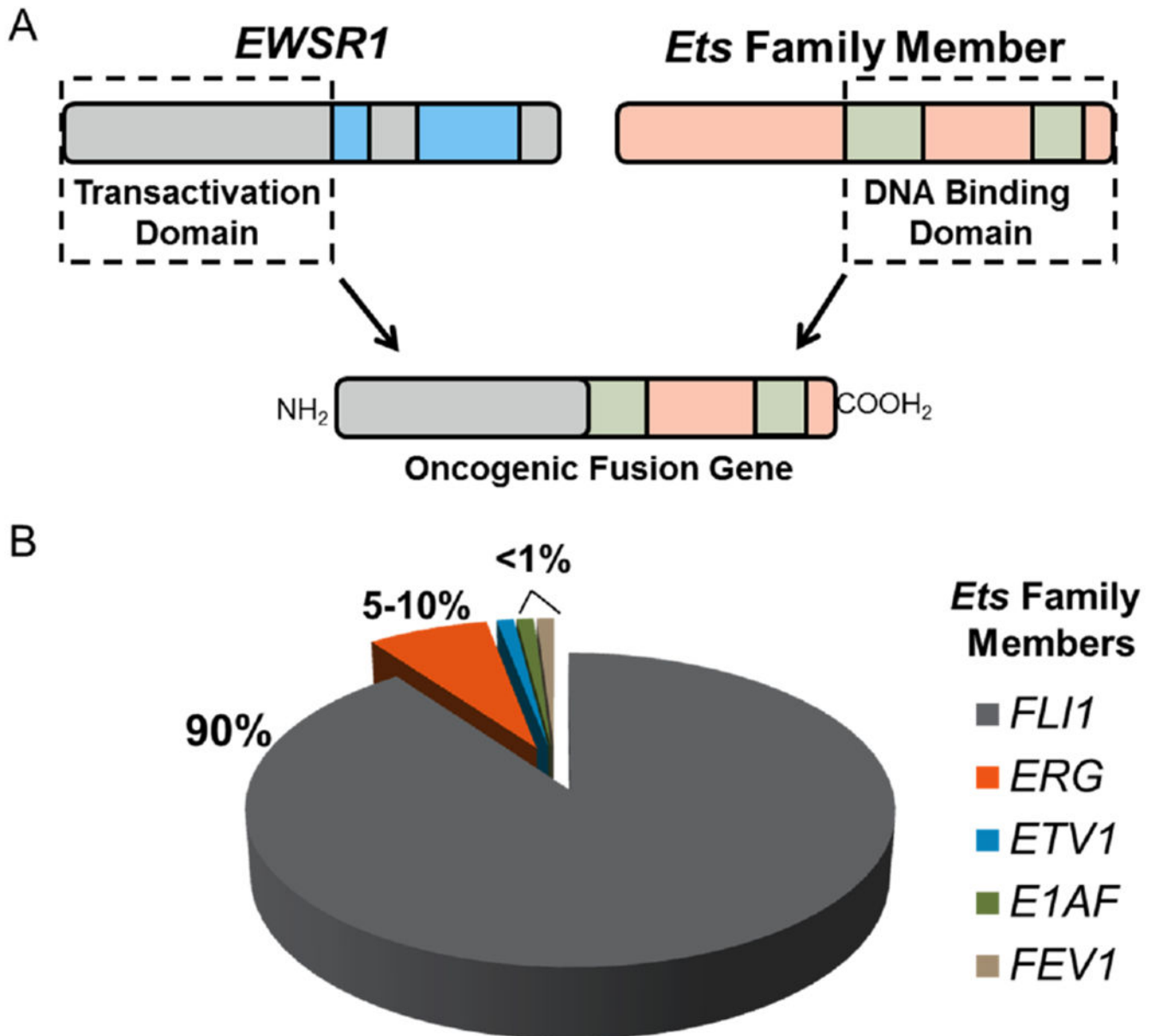


Figure 1. Visual schematic of the *EWS-Ets* fusion gene.

(A) The *EWS-Ets* rearrangement is caused by the fusion of the N-terminal transactivation domain of the *EWSR1* gene with the C-terminal DNA binding domain of a select member of the Ets gene family. (B) Approximately 90% or more of *ETW-Ets* fusions involve the *FLI1* gene. *ERG* is the next most common fusion partner accounting for between 5 and 10% of cases. Rarely, *EWS* has been observed to be paired with *ETV1*, *ETV5*, and *FEV1*, and these fusions account for less than 1% of total cases.

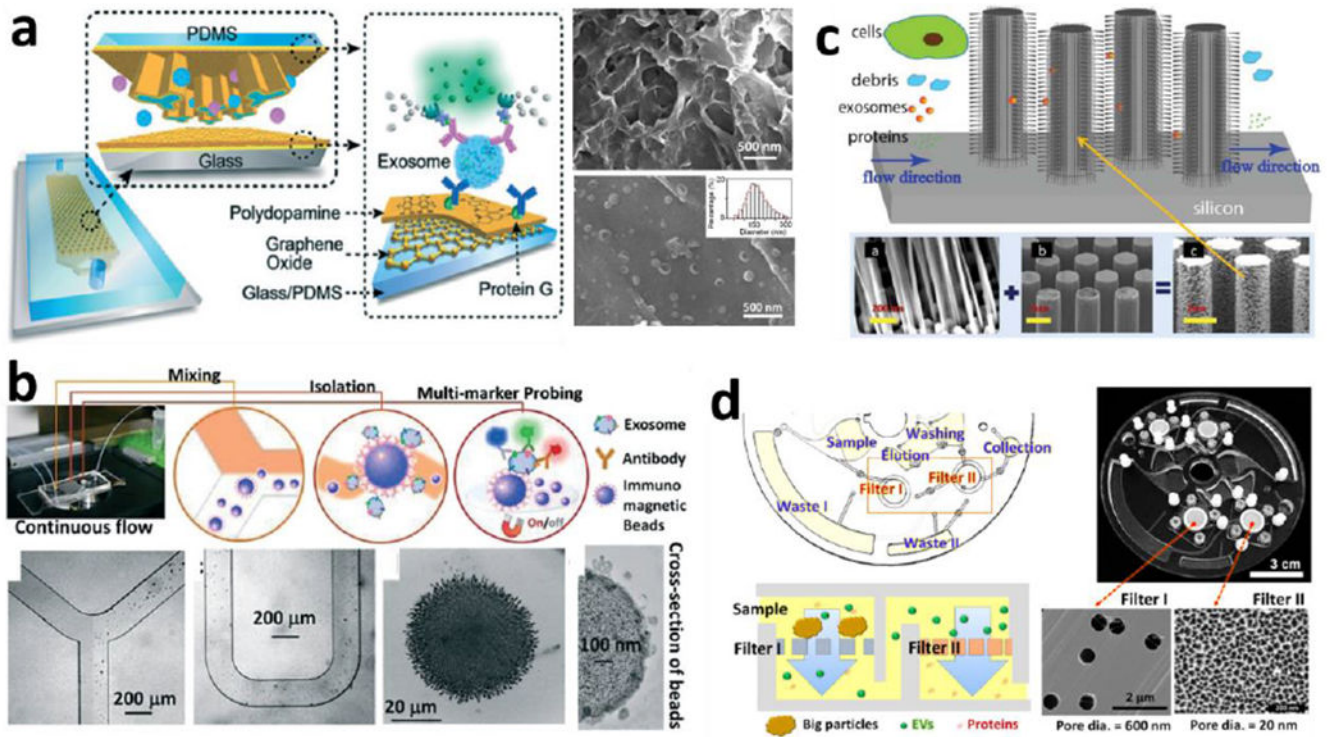


Figure 2. Microfluidic exosome isolation based on the immunocapture and size-exclusion mechanisms.

(a) Enhanced immunoaffinity capture of exosomes using a nanostructured GO/PDA interface in a nano-IMEX chip. Adapted with permission from reference (93). (b) Continuous flow mixing and immunomagnetic capture of exosomes using an ExoSearch chip. Adapted with permission from reference (94). (c) Trapping of exosome-like lipid vesicles on nanowire-on-micropillar arrays. Adapted with permission from reference (97). (d) Size-exclusion isolation of exosomes on an Exodisc platform. Adapted with permission from reference (98).

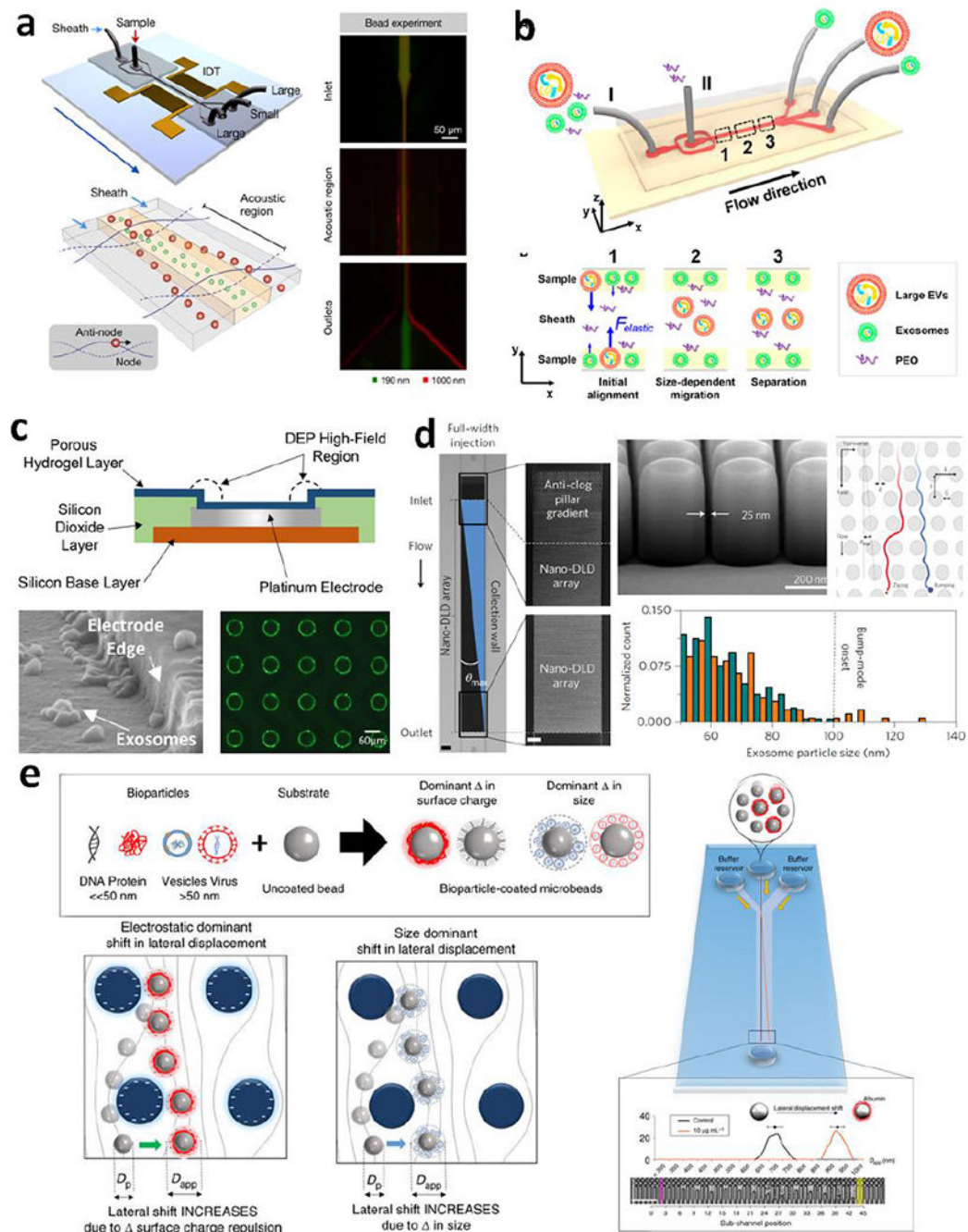


Figure 3. Hydrodynamic exosomes isolation in microchips based on various exosome properties. (a) Acoustic nanofilter system for exosomes isolation. Adapted with permission from Ref (103). (b) Isolation of exosomes by microfluidic viscoelastic flows. Adapted with permission from Ref (105). (c) Trapping of exosomes in ACE microarray chip. Adapted with permission from Ref (107). (d) Nano-DLD chips for size sorting of exosomes in laminar flow streams displaced by staggered nanopillars. Adapted with permission from Ref (108). (e) DLD fractionation of bioparticle-bead conjugates in an array of negatively charged micropillars based on the change of size and surface charge of micro-beads upon binding

with bioparticles, including proteins and exosomes. Adapted with permission from Ref (109).

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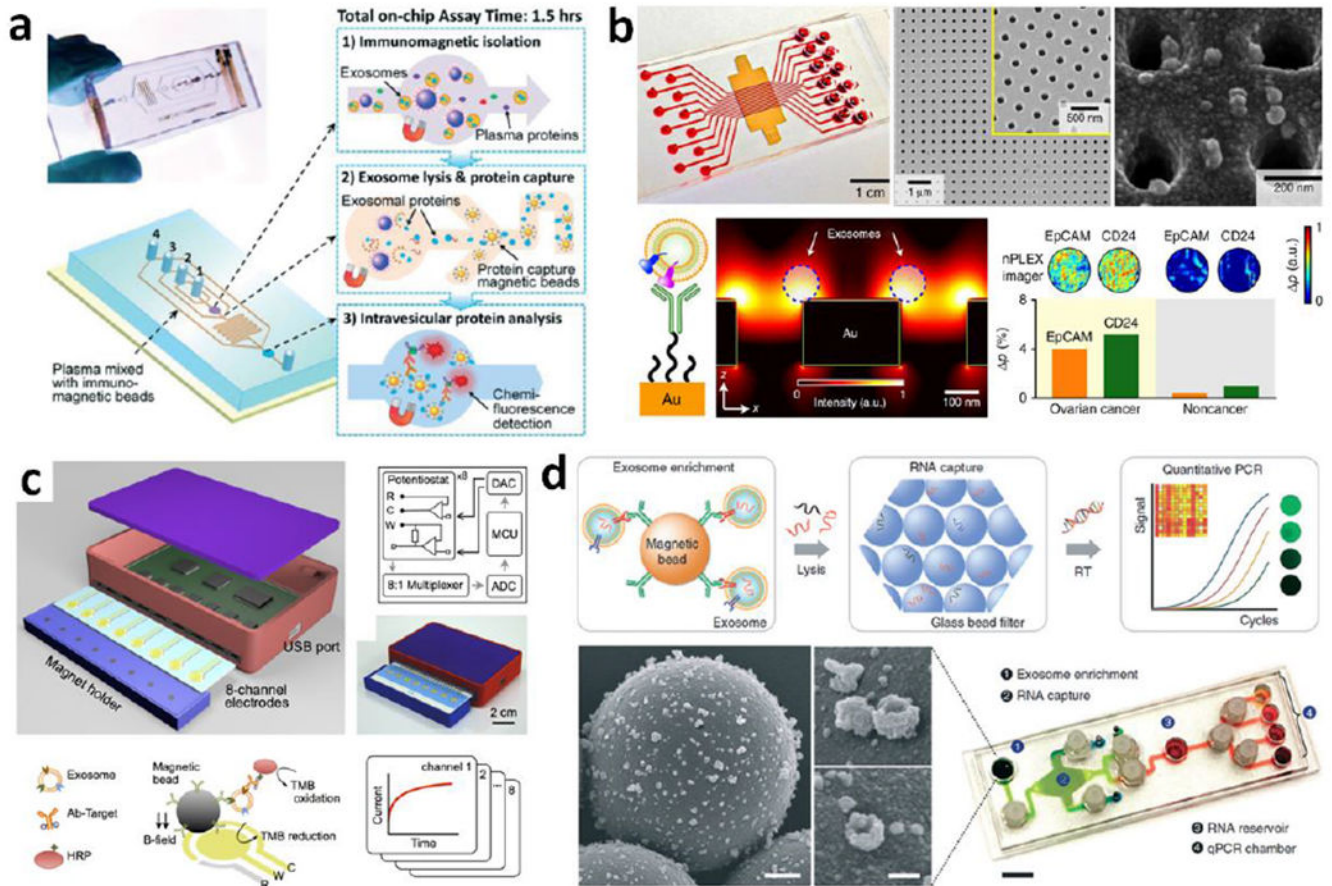


Figure 4. Examples of integrated microfluidic analysis of exosomes towards liquid biopsy. (a) Integrated platform for intravesicular protein analysis of ovarian cancer exosomes. Adapted with permission from Ref (116). (b) Molecular analysis of EpCAM and CD24 specific exosomes from ovarian cancer on nPLEX chip. Adapted with permission from Ref (118). (c) Portable magneto-electrochemical device (iMEX) for molecular diagnosis of ovarian cancer. Adapted with permission from Ref (119). (d) iMER chip for integrated and multiplexed quantification of exosomal mRNA levels. Adapted with permission from Ref (95).

Table 1.

A brief comparison of different conventional exosomes isolation methods.

Method	Time	Advantages	Disadvantages
Ultracentrifugation	5-10 hrs	+ Robust and low reagent cost + Isolation of total EVs + Large-scale production + Vesicle structure maintained	- Lengthy process - Low isolation efficiency - Co-isolation of contaminants
Sucrose density gradient	16-90 hrs	+ Vesicles divided into different populations	- Labor-intensive - Low yield - Long run time
Size exclusion/Ultrafiltration	2-4 hrs	+ Resolve vesicles of different sizes	- Impure factions: high pressure breaks larger vesicles into smaller ones
Polymer precipitation	0.5-12 hrs	+ No sophisticated equipment needed + Easy to use	- Low purity - Alters functionality of vesicles - Require pre-/post-treatment cleanup
Immunoaffinity	2-6 hrs	+ High purity + Specific molecular selection of exosome subpopulations + Small sample volume + Compatibility with robotic liquid handling for high-throughput sample preparation	- High cost - Antibody cross-reactivity - Lack of well-defined markers - Heterogeneity in surface marker expression limits isolation purity and reliability

Table 2.

Integrated microfluidic platforms for exosome analysis.

Microfluidic platform	Isolation method	Detection method	Targeted disease	Measured biomarkers	Clinical sample	Limit of Detection	Reference
ExoChip	Immunoaffinity (CD63)	Fluorescent staining (Dio)	Pancreatic cancer	Overall exosome counts	Serum	0.5 pM	Kanwar 2014 (92)
nano-IMEX	Immunoaffinity (CD81)	Enzymatic amplification of fluorescence	Ovarian cancer	EpCAM, CD9, CD81	Plasma	50 μL^{-1}	Zhang 2016 (93)
Exodisc	Double filtration	Colorimetric ELISA	Bladder cancer	CD9, CD81	Urine	N/A	Woo 2017 (98)
RInSE	Immunoaffinity beads labeled (EpCAM)	Flow cytometry	Breast cancer	CD81	Blood	N/A	Dudani 2015 (96)
μNMR	differential ultracentrifugation	Immunomagnetic tagging (CD63), μNMR detection	GBM	EGFRvIII, EGFR, PDPN, IDH1 R132H	Plasma	$10^4 \mu\text{L}^{-1}$	Shao 2012 (117)
mPLEX	Immunoaffinity (CD63)	Nanohole array SPR	Ovarian cancer	EpCAM, CD24	Ascites fluid	3000 exosomes (670 aM)	Im 2014 (124)
iMEX	Magnetic immunoaffinity (CD63)	Electrochemical sensing	Ovarian cancer	EpCAM, CD24, CA125, HER2, MUC18, EGFR	Plasma	$10^4 \mu\text{L}^{-1}$	Jeong 2016 (119)
ExoSearch	Immunoaffinity (CD9)	Multiplexed immunoassay	Ovarian cancer	CA125, CD24, EpCAM	Plasma	$750 \mu\text{L}^{-1}$	Zhao 2016 (94)
Integrated flow-through Chip	Immunomagnetic capture (EpCAM)	On-chip exosome lysis and ELISA	NSCLC	IGF-1R, phospho-IGF-1 R	Plasma	N/A	He 2014 (116)
mPES	Immunoaffinity (CD81)	Nano-plasmon enhanced scattering	Pancreatic cancer	EphA2	Plasma (1 μL)	N/A	Liang 2017 (125)
μMED	Magnetic immunoaffinity (CD81)	Fluorescence immunoassay	Concussion	GluR2	Mouse serum	$10^4 \mu\text{L}^{-1}$	Ko 2016 (73)
iMER	Magnetic immunoaffinity (EGFR and EGFRvIII)	On-chip exosome lysis and RT-qPCR	GBM	EPHA2, EGFR, PDPN, MGMT, APNG	Serum	N/A	Shao 2015 (95)
EV-HB-Chip	Immunoaffinity (EGFR, EGFRvIII, ephA2, podoplanin, PDGFR, M-CAM)	Off-chip exosome lysis and digital droplet PCR	GBM	mRNA profile (e.g., EGFR, EGFRvIII)	Serum, plasma	100 μL^{-1}	Reategui 2018 (120)
ExoTENPO	immunomagnetic nanopore trapping (CD63, CD9, CD81, EpCAM)	Off-chip RT-qPCR	Pancreatic cancer	mRNA profiles (CK18, CD63, ErbB3, KRAS)	Mouse plasma	N/A	Ko 2017 (110)